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Filogenia, filogeografia e comportamento dos pequenos ciprinídeos do género *Chondrostoma* Agassiz, 1832 (Actinopterygii: Cyprinidae)

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Esta tese é dedicada

aos meus pais Manuel e Maria do Carmo
ao meu irmão André
ao Vítor
à Ana

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Resumo

Os peixes anteriormente incluídos no género *Chondrostoma* (Cyprinidae: Leuciscinae) distribuem-se pelo Sul e Centro da Europa, desde o Oceano Atlântico até ao Mar Cáspio e desde o Mediterrâneo até ao Báltico. Estes peixes encontram-se ainda presentes na Ásia Menor, Cáucaso e Mesopotâmia. O número de espécies incluídas neste grupo tem variado de acordo com os autores, devido ao uso de diferentes critérios de diagnose (i.e. morfológicos e osteológicos ou moleculares). Os estudos anteriores sobre a filogenia molecular deste grupo tinham originado politomias não resolvidas, embora alguns clados monofiléticos e estatisticamente robustos tenham sido identificados: *toxostoma, lemmingii, polylepis, arcasii, nasus, soetta* e *genei*.

A presente tese propôs-se atingir um conjunto de objectivos numa multiplicidade de escalas. Numa escala macroevolutiva, propôs-se resolver com mais taxa e fragmentos de um número maior de genes a filogenia dos peixes tradicionalmente incluídos no género *Chondrostoma*. Ao mesmo tempo, procurou-se contribuir para a compreensão da diversificação deste grupo na Península Ibérica.

Entre os principais resultados destacam-se os seguintes.

Elaborou-se uma filogenia estatisticamente robusta dos peixes que constituíam o género *Chondrostoma*, que mostrou que as politomias anteriormente obtidas resultavam de amostragem insuficiente quanto ao número de taxa e/ou quantidade de fragmentos de ADN.

O mapeamento dos caracteres morfológicos na filogenia obtida mostrou que caracteres anteriormente considerados como diagnósticos do género eram homoplásicos, pelo que se impôs uma revisão do género *Chondrostoma*. Desta revisão resultou a restrição do género *Chondrostoma* às espécies do grupo *nasus* e a criação dos novos géneros: *Pseudochondrostoma, Parachondrostoma, Iberochondrostoma, Achondrostoma* e *Protochondrostoma* (que correspondem respectivamente às linhagens *polylepis, toxostoma, lemmingii, arcasii* e *genei*).

Os eventos cladogenéticos que deram origem à formação destas linhagens parecem ter tido lugar há cerca de 11 milhões de anos, excluindo portanto a hipótese da difusão e

diversificação do grupo ter ocorrido durante a fase oligohalina do Mediterrâneo (Lago Mare) no fim do Messiniano. Fica assim excluída a aplicação para estes peixes do modelo de dispersão proposto por Bianco.

Três dos seis géneros definidos neste trabalho são endémicos da Península Ibérica e um quarto género tem a maioria das espécies na península com uma pequena extensão em França. Estes dados e as cronologias estimadas para a diversificação dentro destes géneros sugerem que grande parte da radiação do grupo se deu na Península Ibérica muito antes do final do Miocénico parecendo, no que se refere a este grupo, que os contactos entre a península e o resto da Europa em períodos mais recentes foram muito reduzidos, limitando-se a trocas entre o nordeste de Espanha e a França.

Na península os géneros *Achondrostoma*, *Iberochondrostoma* e *Parachondrostoma* têm distribuições disjuntas, mas adjacentes, que sugerem que processos de vicariância podem ter tido um papel fundamental na sua diferenciação. *Pseudochondrostoma* apresenta grande número de semelhanças (e.g. boca ínfera e dotada de estojo córneo, tamanho comparativamente grande, migrações pré-reprodutoras) com os géneros *Parachondrostoma* e *Chondrostoma*. Discute-se a possibilidade destas semelhanças resultarem de convergência ou de episódios de hibridação antigos.

A análise filogenética do género *Achondrostoma* permitiu mostrar que este género inclui duas linhagens separadas já desde o Miocénico. *A. arcasii* revelou-se polifilético incluindo peixes das duas linhagens acima referidas, sendo urgente uma revisão da sua taxonomia. Esta informação filogenética combinada com dados morfológicos permitiu identificar uma nova espécie no sudoeste da área de distribuição do género *Achondrostoma*, endémica do distrito de Lisboa e considerada Criticamente Em Perigo (*Achondrostoma occidentale*).

Por o nome se encontrar indisponível foi necessário renomear *Achondrostoma macrolepidotum*, para *A. oligolepis*.

A análise da filogeografia profunda do género *Iberochondrostoma* levou à proposta de um modelo de especiação em que uma grande espécie central, *Iberochondrostoma lemmingii*, originou na sua periferia e em diferentes períodos geológicos, diversas espécies de distribuição mais restrita. Este modelo de especiação do tipo peripátrico, suportado por dados de ADN nuclear e mitocondrial, parece consistente com a história geológica da Península Ibérica no Terciário.

Procedeu-se à análise filogeográfica das populações de uma das espécies deste género, *I. lusitanicum*, tendo-se identificado ESUs distintas que impõem a descrição, o mais breve possível, de uma nova espécie e fornecem informações importantes para o delineamento de estratégias de conservação desta espécie Criticamente Em Perigo.

O estudo do comportamento reprodutor de *I. lusitanicum* bem como o estudo do comportamento agonístico de *Pseudochondrostoma polylepis* forneceram elementos etológicos relevantes para a conservação destes ciprinídeos. Do mesmo modo, o estudo da expansão de *Alburnus alburnus* na Península Ibérica, que se tem acelerado de forma muito acentuada nos últimos anos, traz igualmente informações importantes para o delineamento de estratégias de conservação dos ciprinídeos nativos, cujas potenciais interacções ecológicas com esta exótica se encontram totalmente inexploradas.

O desenvolvimento de *primers* que permitem amplificar eficazmente um fragmento de mais de 900 bases do gene nuclear da beta-actina foi fundamental em quase todos os estudos genéticos referidos acima. Para além de contribuir para os estudos filogenéticos e filogeográficos já referidos, permitiu esclarecer a natureza do ancestral paterno de *Squalius alburnoides* uma espécie hibridogenética que resultou de cruzamentos entre fêmeas de *Squalius pyrenaicus* e machos filogeneticamente muito próximos, mas distintos, de *Anaecypris hispanica.*

O facto de peixes dos géneros *Alburnus* e *Squalius* hibridarem facilmente e a proximidade filogenética entre *Alburnus*, *Anaecypris* e uma das linhagens que integra *S. alburnoides* leva a considerar com grande preocupação a expansão de *Alburnus alburnus* cujo grande potencial de hibridação com peixes do género *Squalius* é conhecido e que pode hibridar e descaracterizar várias espécies endémicas da península.

Finalmente desenvolveu-se e validou-se um novo método que permite atribuir as diferentes bases presentes nos cromatogramas de ADN diplóide ou poliplóide a cada uma das cadeias constitutivas tirando partido de artefactos da sequenciação induzidos na vizinhança de *indels* em heterozigotia. Este método é útil tanto na análise de múltiplos SNP's no mesmo fragmento, como na identificação das sequências de ADN presentes em híbridos e na distinção de vários tipos de poliplóides.

Summary

Fishes formerly included in the genus *Chondrostoma* (Cyprinidae: Leuciscinae) are distributed through South and Central Europe, from the Atlantic to the Caspian and from the Mediterranean to the Baltic. They are also found in Asia Minor, the Caucasus and Mesopotamia.

The number of species included in the group has varied according with the authors due to the use of different diagnostic criteria (i.e. morphological and osteological or molecular). Previous molecular phylogenetic studies of this group yielded unresolved polytomies although some monophyletic and statistically well supported clades were identified: *toxostoma*, *lemmingii*, *polylepis*, *arcasii*, *nasus*, *soetta* e *genei*.

The present thesis aimed to achieve several objectives at various levels. At a macroevolutionary scale this study attempted to solve the phylogeny of the fish traditionally included in *Chondrostoma* with a broader sample of taxa and more DNA fragments. At the same time the study aimed to clarify the diversification of this group in the Iberian Peninsula.

The main results can be summarized as follows.

A statistically robust phylogeny of the fish formerly included in the genus *Chondrostoma* was obtained. The former polytomies were solved which indicates that they were likely due to insufficient taxon sampling or scarcity of molecular data.

Mapping of morphological characters on the inferred phylogeny showed that several traits considered to be diagnostic of the genus were homoplasic. Thus a revison of the genus *Chondrostoma* was undertaken. This revision restricted the genus *Chondrostoma* to the *nasus* lineage and gave rise to the new genera *Pseudochondrostoma*, *Parachondrostoma*, *Iberochondrostoma*, *Achondrostoma* and *Protochondrostoma* (corresponding to the lineages *polylepis*, *toxostoma*, *lemmingii*, *arcasii* and *genei*, respectively).

The cladogenetic events that gave rise to these lineages seem to have occurred 11 million years ago, excluding the hypothesis of diffusion and diversification during the oligohaline Lago Mare phase of the Mediterranean, near the end of the Messinian. Thus the model of dispersal proposed by Bianco does not hold for this group of fish.

Three of the six genera defined in this work are endemic of the Iberian Peninsula and another one has the majority of its species in the peninsula, with a little extension to France. These data and the chronology estimated for the diversification inside these genera suggest that most of the radiation in this group occurred in the Iberian Peninsula long before the end of the Miocene. It seems that in this group the contacts between the peninsula and the rest of Europe were, in recent times, very scarce, and limited to connections between northeast Spain and France.

In the Iberian Peninsula the genera *Achondrostoma*, *Iberochondrostoma* and *Parachondrostoma* have disjunct but adjacent distribution areas which suggests that vicariant processes may have played a fundamental role in their differentiation. *Pseudochondrostoma* shows several resemblances with the genera *Parachondrostoma* and *Chondrostoma* (e.g. inferior mouth with a horny blade, comparably large size and pre-reproductive migrations) with the genera *Parachondrostoma* and *Chondrostoma.* The hypotheses of these resemblances being the result of convergent evolution or ancient hybridization events are discussed.

The phylogenetic analysis of the genus *Achondrostoma* showed that this genus includes two lineages separated since the Miocene. *A. arcasii* is polyphyletic and includes fish from the two lineages referred above, thus a revision of its taxonomy is urgently needed. This phylogenetic information, combined with morphological data, allowed the identification of a new species in the southwest of the distribution area of the genus *Achondrostoma*, *Achondrostoma occidentale*. This new species is endemic of the Lisbon district and it is considered Critically In Danger.

As the name *macrolepidotum* was unavailable it was necessary to rename *Achondrostoma macrolepidotum* to *A. oligolepis*.

The analysis of the deep phylogeography of the genus *Iberochondrostoma* resulted in the proposal of a model of speciation in which a large central species, *Iberochondrostoma lemmingii*, originated in its periphery and at different geological times, diverse species with small distribution areas. This peripatric speciation model is supported by mitochondrial and nuclear DNA and it seems consistent with the geological history of the Iberian Peninsula in the Tertiary.

The phylogeographic analysis of the populations of *Iberochondrostoma lusitanicum* allowed the identification of distinct ESUs, which in turn impose the description of a new species, as soon as possible, as well as revealing important information to the conservation strategies of this Critically in Danger species.

The studies on the reproductive behaviour of *I lusitanicum*, and on the agonistic behaviour of *Pseudochondrostoma polylepis* revealed some ethological patterns relevant to the conservation of the species. It was also the case with the study on the expansion of *Alburnus alburnus* in the Iberian Peninsula, a species which has spread very quickly in recent years.

The development of primers that allowed the amplification of a fragment of more than 900 base pairs of the nuclear beta actin gene was fundamental to almost all genetic studies referred above. Beside its contribution to these studies, it allowed the recognition of the paternal ancestor of *Squalius alburnoides* an hybridogenetic species which resulted from crossings between *S. pyrenaicus* females and males phylogenetically very close to but distinct from *Anaecypris hispanica*.

The fact that species of the genera *Alburnus* and *Squalius* hybridize easily and the phylogenetic proximity between *Alburnus*, *Anaecypris* and one of the lineages that integrates *S. alburnoides*, brings great concerns about the expansion of *Alburnus alburnus*. The high potential of hybridization recorded between this last species and fishes of the genus *Squalius* is well known and may result in the genetic descaracterization of several species endemic to the Iberian Peninsula.

Finally, a new method was developed and validated, that allows the attribution of different bases present in the DNA chromatograms of diploid or polyploidy fishes to each one of the constitutive chains, taking advantage of artefacts of the sequencing process induced in the vicinity of heterozygous indels. This method is useful in the analysis of multiple SNP's in the same fragment, in the identification of DNA sequences present in hybrids and in the distinction of several kinds of polyploids.

Résumé

Les poissons traditionnellement inclus dans le genre *Chondrostoma* (Cyprinidae : Leuciscinae) se trouvent au Sud et au Centre d'Europe entre l'Atlantique et la mer Caspienne et la Méditerranée et la mer Baltique. Ces poissons peuvent aussi se trouver en Asie, Caucase et Mésopotamie. Le nombre d'espèces inclues dans ce groupe varie entre différents auteurs en raison d'un usage de différents critères diagnostiques (morphologiques et ostéologiques ou moléculaires). Études précédentes de la phylogénie moléculaire de ce groupe ont produit des polytomies non résolues, ayant cependant identifié un certain nombre de clades monophylétiques statistiquement bien soutenus: *toxostoma*, *lemmingii*, *polylepis*, *arcasii*, *nasus*, *genei* et *soetta*. La présente thèse vise plusieurs objectifs à divers niveaux. À une échelle macro-évolutionnaire, cette étude essaye de résoudre la phylogénie des poissons traditionnellement inclus dans le genre *Chondrostoma* recourant à un plus large échantillon de taxa et de gènes. En même temps cette étude a cherche à clarifier la diversification de ce groupe dans la Péninsule Ibérique.

Parmi les principaux résultats peuvent être relèves les suivants.

La phylogénie statistiquement robuste des poissons traditionnellement inclus dans le genre *Chondrostoma* qui a été élaborée montre que les polytomies anciennement obtenues advenaient de prélèvements insuffisant quant au nombre de taxa et/ou quantité de fragments de DNA La cartographie de caractères morphologiques sur la phylogénie obtenue a indiqué que certains des traits considérés diagnostiques du genre étaient en fait homoplasiques. Une révisons du genre *Chondrostoma* a du ainsi été entreprise.

De cette révision est advenue la limitation du genre *Chondrostoma* aux espèces du groupe nasus et la création des nouveaux genres *Pseudochondrostoma*, *Parachondrostoma*, *Iberochondrostoma*, *Achondrostoma* et *Protochondrostoma* (correspondant respectivement aux lignages *polylepis*, *toxostoma*, *lemmingii*, *arcasii* et *genei*). Les événements cladogénétiques à l'origine de ces lignages semblent s'être produits il y a environ 11 millions d'années, excluant pourtant l'hypothèse de diffusion et diversification du groupe durant la

phase oligohaline de la Méditerranée, á la fin du Messénien. L'application du modèle de dispersion proposé par Bianco est ainsi exclue pour ce groupe de poissons.

Trois des six genres déterminés dans ce travail sont endémiques de la Péninsule Ibérique et un quatrième a la majorité de ses espèces dans la Péninsule, avec une petite extension en France. Ces données, ainsi que la chronologie estimée pour la diversification dans ce genre, suggèrent que la plupart du rayonnement de ce groupe s'est passé dans la Péninsule Ibérique longtemps avant la fin du Miocène.

En ce qui concerne ce groupe, il semble que les contacts entre la Péninsule et le reste de l'Europe dans les temps plus proches, ont été très rares et limités aux connexions entre le nord-est de l'Espagne et la France. Dans la Péninsule Ibérique les genres Achondrostoma, Iberochondrostoma et Parachondrostoma ont des distributions disjointes, mais adjacentes, qui suggèrent que des cas de vicariance peuvent avoir joué un rôle fondamental dans leur différenciation. Pseudochondrostoma présente un grand nombre de ressemblances (par ex. bouche inférieure avec une trousse cornée, grande taille, migrations pré-reproductrices) avec les genres *Parachondrostoma* et *Chondrostoma.* L'hypothèse affirmant que ces ressemblances peuvent advenir d'évolution convergente ou d'anciens événements d'hybridation est discutée.

L'analyse phylogénétique du genre *Achondrostoma* a montré que ce genre inclut deux lignages séparés depuis le Miocène. *Achondrostoma arcasii* s'est révélé polyphylétique, incluant les poissons des deux lignages référés ci-dessus, étant pourtant urgent une révision de sa taxonomie. Cette information phylogénétique, combinée avec des données morphologiques, a permit l'identification d'une nouvelle espèce dans le sud-ouest du secteur de distribution du genre *Achondrostoma*, endémique du quartier de Lisbonne et considérée critiquement en danger (*Achondrostoma occidentale*). Il a été nécessaire de renommer. *Achondrostoma macrolepidotus* passant á *A. oligolepis*.

L'analyse de la phylogéographie profonde du genre *Iberochondrostoma* à mené á la proposition d'un modèle de spéciation dans lequel une grande espèce centrale, *Iberochondrostoma lemmingii*, à été á l'origine, au cours de différents temps géologiques, de plusieures espèces avec une distribution plus réstricte dans sa périphérie. Ce modèle de spéciation du type péripatrique, soutenu par des données d'ADN mitochondrial et nucléaire, semble conforme à l'histoire géologique de la Péninsule Ibérique pendant le Tertiaire. L'analyse phylogéographique des populations d'une des espèces de ce genre, I. lusitanicum, á été effectuée, menant á l'identification d'ESUs distincts, imposant une rapide description d'une nouvelle espèce, et fournissant d'importantes informations dans le développement de stratégies pour la conservation de cette espèce Critiquement En Danger.

L'étude du comportement reproducteur de *Iberochondrostoma lusitanicum* et du comportement agonistique de *Pseudochondrostoma polylepis* fournit d'importants éléments éthologiques pour la conservation des cyprinidés. De la même façon, l'étude de l'expansion d'*Alburnus alburnus* dans la Péninsule Ibérique, qui s'est accélérée dans les dernières années apporte d'importantes informations dans le développement de stratégies pour la conservation des cyprinidés natifs, dont les potentielles interactions écologiques avec cette exotique se trouvent complètement inexplorées. Le développement de primers qui permettent l'amplification efficace d'un fragment de plus de 900 bases du gène de la beta actine á été fondamental à presque toutes les études génétiques référées ci-dessus. Par ailleurs, il a permis de clarifier le caractère de l'ancestral paternel de *Squalius alburnoides* une espèce hybridogénétique qui a surgit des croisements entre des femelles de *Squalius pyrenaicus* et des mâles phylogénétiquement très proches mais distincts d'*Anaecypris hispanica*. Le fait que les poissons des genres *Alburnus* et de *Squalius* hybrident facilement et la proximité phylogénétique entre *Alburnus*, *Anaecypris* et un des lignages qui intègre *S. alburnoides*, amène à de grandes inquiétudes en ce qui concerne l'expansion d'*Alburnus alburnus* dont le haut potentiel d'hybridation avec des poissons du genre *Squalius* est connu, pouvant hybrider et décaractériser plusieures espèces endémiques de la Péninsule.

Finalement, une nouvelle méthode a été développée et validée, permettant attribuer les différentes bases présentes dans les chromatogrammes d'ADN diploïde ou polyploïde à chacune des chaînes constituantes, tirant profit des artefacts d'objets de séquençage induits en proximité d'indels en hétérozygotie. Cette méthode est utile autant dans l'analyse de SNP's multiples dans le même fragment, comme dans l'identification des séquences d'ADN présentes chez des hybride et dans la distinction de plusieurs genres de polyploïdes.

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Parte I

Introdução geral

Capítulo 1

Introdução

No recentemente publicado Livro Vermelho dos Vertebrados de Portugal (Cabral et al., 2005) os peixes de água doce representam o grupo de organismos com maior incidência de estatutos de ameaça à sua conservação. No que respeita aos ciprinídeos, a Península Ibérica apresenta uma taxa elevada de endemismos de distribuição muito reduzida e localizada, muitos deles pertencentes ao género *Chondrostoma*. Das 35 espécies anteriormente incluídas no género *Chondrostoma* Agassiz, 1832, 14 são endémicas da Península Ibérica, e nos últimos anos, o número de espécies reconhecidas não tem parado de aumentar (ver Robalo et al., 2007, capítulo 6). Quase todas estas espécies estão sujeitas a uma ou mais formas de ameaça à sua conservação. Assim, um dos temas unificadores dos trabalhos que constituem esta tese foi a preocupação em que o melhor conhecimento da genética das populações e do seu comportamento reprodutor dêem um contributo útil para o delineamento de estratégias mais eficazes de conservação desta parcela tão valiosa do nosso património biológico. Ao mesmo tempo, a presente tese não se limitou a uma perspectiva localista e procurou, no plano da análise filogenética e paleobiogeográfica, integrar o conhecimento da ictiofauna ibérica numa perspectiva mais ampla. Foi preocupação da autora procurar avaliar as implicações que o estudo deste grupo de espécies acarreta para a nossa compreensão da evolução dos ciprinídeos na Europa e para a sua taxonomia.

1.1 Breve perspectiva sobre a família Cyprinidae: afinidades, evolução e diversidade

A família Cyprinidae é a maior família de peixes de água doce (Nelson, 2006) compreendendo cerca de 2420 espécies distribuídas por 220 géneros. É uma das seis famílias da ordem dos Cypriniformes e estima-se que contenha aproximadamente 321 géneros e 3268 espécies com um máximo de diversidade no Sudeste Asiático. De acordo com o mesmo autor esta ordem encontra-se englobada na série Otophysi, pertencendo à super-ordem Ostariophysi, Sub-divisão Ostarioclupeomorpha (= Otocephala), à divisão Teleostei, à sub-classe Neopterygii e à classe Actinopterygii.

Os Ostariophysi compreendem 5 ordens, 68 famílias, 1075 géneros e cerca de 7931 espécies. Estão contidas nesta super-ordem cerca de 68% das espécies de peixes de água doce e cerca de 28% do total de espécies de peixes conhecidas. Ocupam praticamente todos os continentes com excepção da Antártida, Groenlândia e Nova Zelândia (Nelson, 2006). Nelson (2006) reconhece, entre outras, as seguintes características para os Ostariophysi: bexiga natatória presente (excepto num único género, *Gonorynchus*) e normalmente dividida numa câmara anterior mais pequena (que se encontra total ou parcialmente coberta por uma túnica peritoneal prateada) e noutra posterior maior (que se pode encontrar parcialmente reduzida ou mesmo ausente nalguns grupos); presença de pequenas projecções córneas unicelulares chamadas "unculi" em várias partes do corpo; presença de tubérculos multicelulares córneos durante a época de reprodução com uma cobertura de queratina bem desenvolvida (tubérculos nupciais); maxilar superior projectável. A maioria dos peixes deste grupo possui uma reacção específica perante sinais químicos de alarme, facto documentado pela primeira vez por Karl von Frish em 1938. Os peixes deste grupo, quando feridos, libertam de células presentes na pele, uma feromona ("Schreckstoff") que é detectada especificamente pelos conspecíficos e com menor afinidade por peixes filogeneticamente próximos. A detecção desta substância libertada por um peixe ferido provoca reacções de medo, fuga e eventuais manobras anti-predatórias (Pfeiffer, 1977).

Do ponto de vista taxonómico, a posição dos Ostariophysi tem mudado ao longo do tempo. Começaram por ser vistos como Euteleósteos basais, sendo incluídos na subdivisão Euteleostei e na divisão Teleostei (Nelson, 1994). Segundo Nelson (2006), na literatura mais antiga o termo Ostariophysi tinha o seu uso restrito aos Otophysi (hoje uma das suas séries). Estudos taxonómicos recentes concluíram que os Ostariophysi constituem o grupo irmão dos

Clupeomorpha estando, por isso, ambos incluídos na sub-divisão Ostarioclupeomorpha (= Otocephala) (Nelson, 2006). Este parentesco entre Ostariophysi e Clupeomorpha tinha sido já sugerido por alguns autores no passado, tendo por base dados morfológicos (e.g. Monod, 1968; Gosline, 1971) e dados paleontológicos e moleculares (Lê et al., 1993) mas manteve-se pouco reconhecido até ao trabalho de Lecointre e Nelson (1996) onde se re-analisou criticamente o problema com base na integração da evidência morfológica e molecular. Arratia (1997) confirmou estas conclusões com base na análise de fósseis. Mais recentemente vários artigos usando diferentes conjuntos de marcadores moleculares confirmaram este parentesco (e.g. Inoue et al., 2001; Zaragüeta-Bagils et al., 2002; Inoue et al., 2003; Saitoh et al., 2003). Para uma revisão sobre a literatura dos vários tipos de dados e ainda uma lista das sinapomorfias morfológicas propostas ao longo do tempo como representativas desta relação de parentesco ver Zaragüeta-Bagils et al. (2002).

Os Ostariophysi representam mais de 25% das espécies de peixes teleósteos e cerca de 80% de todos os peixes de água doce (Fink e Fink, 1981, 1996; Nelson, 1994; Stiassny et al., 2004). Englobam as séries Anotophysi e Otophysi. A primeira série inclui apenas a ordem Gonorynchiformes considerada por alguns autores (e.g. Greenwood et al., 1966, Rosen e Greenwood, 1970) como o grupo irmão dos Otophysi. Os Otophysi incluem as ordens Cypriniformes, Characiformes, Siluriformes e Gymnotiformes (Nelson, 2006).

No que respeita à biologia molecular, o uso de sequências mitocondriais completas (mitogenómica) na compreensão dos grandes padrões da filogenia dos teleósteos abre enormes perspectivas e o número de espécies sequenciadas aumenta de dia para dia. Para uma breve história da mitogenómica e um resumo das possibilidades desta abordagem ver Miya et al. (2003).

É interessante notar que tanto os Ostariophysi como os Clupeomorpha apresentam especializações anatómicas que atingem um elevado grau de elaboração e que apesar de se basearem em processos algo diferentes se caracterizam por aumentarem muito substancialmente a capacidade auditiva destes animais (e.g. Lagler et al., 1977; Evans e Claiborne, 2006). Nos ciprinídeos e muitos outros Ostariophysi, um conjunto de vértebras modificadas que constituem o chamado aparelho de Weber, transmitem as vibrações que fazem ressonância na bexiga gasosa, conduzindo-as ao ouvido interno. Nos clupeídeos, exemplos típicos de Clupeomorpha, existem projecções tubulares da bexiga gasosa que se estendem até ao ouvido interno, facilitando assim a transmissão das vibrações, funcionando como uma alternativa ao aparelho de Weber (Lagler et al., 1977). A descoberta que estes dois grupos, com sistemas auditivos tão especializados, são estreitamente aparentados levanta várias questões fascinantes do ponto de vista evolutivo. Que pré-adaptações permitiram a evolução de sistemas de transmissão de som da bexiga gasosa ao ouvido estruturalmente diferentes mas com funções semelhantes? Até que ponto as capacidades auditivas dos Ostarioclupeomorpha (= Otocephala) terão contribuído para o seu enorme sucesso à escala evolutiva, tanto nos meios dulçaquícolas como no meio marinho?

Em termos de número de espécies, os ciprinídeos constituem a segunda família de teleósteos. A sua distribuição abrange a América do Norte (do Norte do Canadá ao Sul do México), a África e a Eurásia. Nelson (2006) refere como características desta família, as seguintes: dentes faríngeos distribuídos por uma a três fiadas (e nunca mais de oito dentes em cada); lábios normalmente finos sem dobras ou papilas; boca com ou sem barbilhos; maxilar superior contornado apenas pela pré-maxila; o maxilar superior é normalmente projectável; algumas espécies apresentam raios espinhosos na barbatana dorsal.

Ao longo do tempo várias nomenclaturas foram sendo usadas no que respeita às subfamílias consideradas válidas dentro da família Cyprinidae (e.g. Howes, 1991; Nelson, 1994; Nelson, 2006). Para a generalidade dos autores esta família é considerada difícil do ponto de vista taxonómico. Howes (1991) reconhece 7 subfamílias (Cyprininae, Gobioninae, Acheilognathinae, Leuciscinae, Cultrinae, Alburninae, e Rasborinae). Usando dados morfológicos, Chen et al. (1984), Cavender (1991) e Cavender e Coburn (1992) concordam com uma classificação em duas grandes linhagens: Cyprinini (incluindo as subfamílias Tincinae, Barbinae, Cyprininae e Labeoninae) e Leuciscini (incluindo as subfamílias Danioninae, Leuciscinae, Cultrinae, Xenocyprinae, Gobioninae e Acheilognathinae) embora com pequenas variações nas subfamílias reconhecidas em cada um dos casos.

Nelson (2006), na sua recente obra *Fishes of the World*, segue a nomenclatura adoptada por Arai e Kato (2003) reconhecendo 10 subfamílias: Acheilognathinae, Cultrinae, Cyprininae, Barbinae (*Barbus*), Labeoninae, Squaliobarbinae, Xenocyprinae, Gobioninae, Rasborinae (= Danioninae) e Leuciscinae. Saitoh et al. (2006), num trabalho de mitogenómica em que apresenta 59 genomas mitocondriais completos de Cypriniformes, não reconhece como pertencendo à família Cyprinidae a subfamília Squaliobarbinae, reconhecendo outras não consideradas por Arai e Kato (2003) como Schizothoracinae, Phoxininae, Tincinae e Psilorhyncinae.

De acordo com Howes (1991) não são conhecidas sinapomorfias que garantam a monofilia dos Cyprinidae enquanto entidade taxonómica e apesar da monofilia de algumas das subfamílias estar bem documentada, as relações entre elas não estão ainda bem esclarecidas (Nelson, 2006).

As complexas relações de parentesco entre as várias subfamílias foram objecto de várias investigações baseadas em dados moleculares que no entanto até há pouco se tinham revelado pouco esclarecedoras (Briolay et al., 1998; Gilles et al., 1998, 2001; Zardoya e Doadrio, 1998, 1999; Cunha et al., 2002). Em todos estes trabalhos a posição relativa dos géneros *Tinca* e *Gobio* foi impossível de determinar, sendo considerados alternadamente como pertencendo a subfamílias independentes, como pertencendo à subfamília Leuciscinae, ou, no caso de *Tinca, incertae sedis* (i.e. um grupo taxonómico em que as suas relações filogenéticas principais são desconhecidas ou indefinidas) (Cunha et al., 2002). Várias razões foram apontadas pelos autores como tendo contribuído para estes resultados por vezes inconclusivos, como a sub-amostragem de espécies norte americanas e/ou asiáticas (Briolay et al., 1998; Gilles et al., 1998; Cunha et al., 2002), a ausência de linhagens monofiléticas (e.g. Cunha et al., 2002) e/ou a saturação do marcador molecular (Briolay et al., 1998). A incongruência entre dados moleculares e morfológicos pode dever-se também à saturação do marcador molecular (Briolay et al., 1998) ou à inadequação dos caracteres morfológicos escolhidos (e.g. presença ou ausência de barbilhos) (Cunha et al., 2002).

Os estudos moleculares de Zardoya e Doadrio (1998, 1999) evidenciaram a existência de duas grandes subfamílias nos ciprinídeos, Cyprininae e Leuciscinae, que numa larga medida corroboram as análises morfológicas realizadas por Chen et al. (1984), Howes (1991) Cavender (1991) e Cavender e Coburn (1992).

Recentemente, graças à acumulação de informação mitogenómica foi possível apresentar uma primeira resolução desta questão (Saitoh et al., 2006). Este trabalho confirmou a monofilia dos Cypriniformes e da família Cyprinidae. A filogenia obtida encontra-se representada na figura 1.1. Para uma comparação das filogenias obtidas por outros autores (com base na morfologia e também em dados moleculares) ver também figura 1.2.

Assim, mesmo que a delimitação de subfamílias ou a classificação de alguns géneros suscite ainda problemas que é necessário investigar, o reconhecimento das grandes linhas evolutivas referidas acima representa um importante marco para a compreensão da filogenia da família.

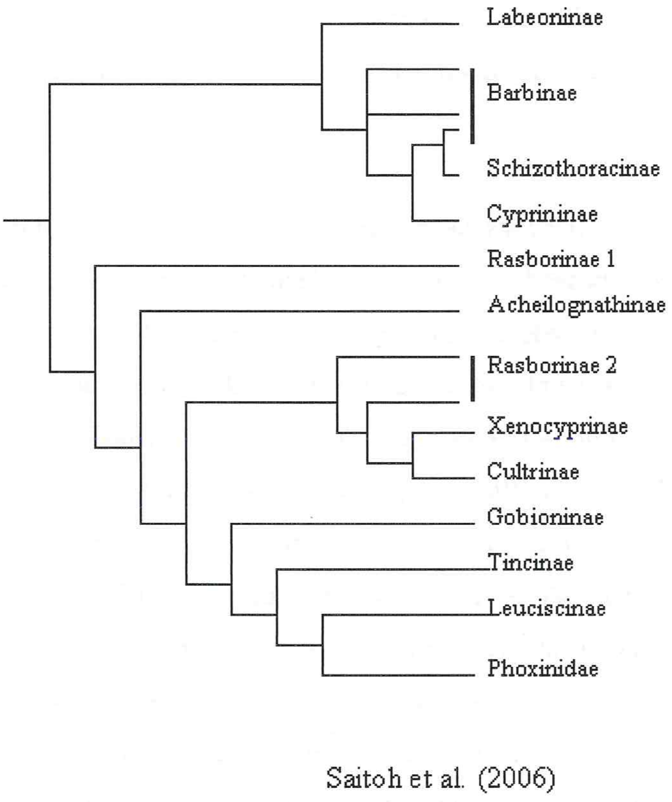
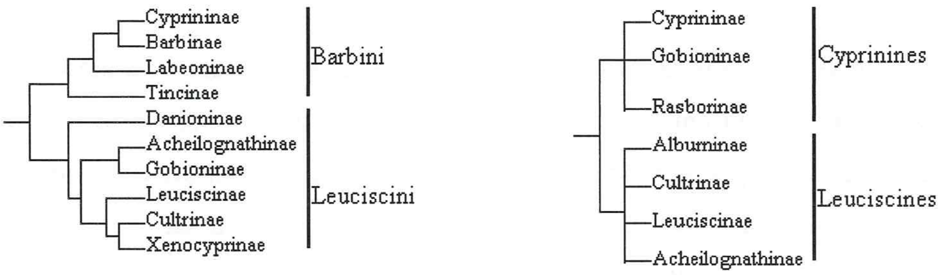


Fig. 1.1 Filogenia da família Cyprinidae proposta por Saitoh et al. (2006). Árvore adaptada do mesmo trabalho.

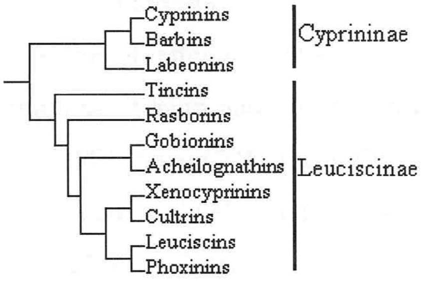
O género *Chondrostoma* pertence à subfamília Leuciscinae (Zardoya e Doadrio, 1999; Durand et al., 2003; Nelson, 2006). Esta subfamília encontra-se distribuída pela Eurásia (com excepção da Índia e do Sudeste da Ásia), pela América do Norte e pelo Japão (Nelson, 2006) e inclui, entre outros, os seguintes géneros: *Abramis*, *Alburnoides*, *Alburnus*, *Algansea*, *Anaecypris*, *Aspius*, *Blicca*, *Cyprinella*, *Chalchalburnus*, *Chondrostoma*, *Delminichthys*, *Dionda*, *Elopichthys*, *Gila*, *Hybopsis*, *Leuciscus*, *Luciobrama*, *Luciocyprinus*, *Lythrurus*, *Notemigonus*, *Notropis*, *Oreoleuciscus*, *Pelecus*, *Pseudophoxinus*, *Rhinichthys*, *Rutilus*, *Scardinius*, *Squalius*, *Tribolodon*, *Vimba* e *Yuriria*.

No que respeita aos habitats que ocupam, as espécies da família Cyprinidae são quase exclusivamente dulçaquícolas, ocorrendo muito raramente em águas salobras. Ocupam praticamente todo o tipo de cursos de água doce, de todas as dimensões e regimes de corrente, estando presentes mesmo em águas subterrâneas. Podem sobreviver em intervalos de temperatura que vão desde os 0 aos 40 ºC, apresentando contudo grandes variações no tempo de que necessitam para se aclimatar (e.g. Howes, 1991).



Chen et al. (1984)

Howes (1991)



Cavender e Coburn (1992)

Fig. 1.2 Filogenias da família Cyprinidae propostas por vários autores com base em dados morfológicos e moleculares. Árvores adaptadas de Saitoh et al. (2006).

Um aspecto extremamente interessante do ponto de vista da sua biologia da reprodução e do seu comportamento reside no facto desta família ser rica em exemplos de formação de híbridos. Scribner et al. (2001) demonstraram que nos peixes de água doce, os híbridos intergenéricos são raramente encontrados em outras famílias (num total de 139 híbridos analisados, 45 de 47 híbridos intergenéricos pertenciam à família Cyprinidae). Yakovlev et al. (2000) encontraram, só na Europa, 62 tipos de híbridos naturais, intra e intergenéricos. Os híbridos são particularmente frequentes na subfamília Leuciscinae (Tsai e Zeisel, 1969; Wheeler, 1978; Witkowski e Blachuta, 1980; Blachuta e Witkowski, 1984; Crivelli e Dupont, 1987; Kammerad e Wuestemann, 1989; Philippart e Berrebi, 1990; Howes, 1991; Gante et al., 2004; Ünver e Erk'Akan, 2005, entre muitos outros, para revisões ver

Scribner et al., 2000 e Yakovlev et al., 2000). Aparentemente os híbridos de primeira geração (F1) são geralmente férteis (Yakovlev et al., 2000), podem ocorrer mais frequentemente do que inicialmente previsto (pelo menos nos Leuciscinae, Freyhof et al., 2005), estando já descritos para a Europa casos nos géneros *Squalius* (Durand et al., 2000), *Telestes* (Salzburger et al., 2003), *Barbus* (Tsigenopoulos et al., 2002) e *Chondrostoma* (Costedoat et al., 2005).

No que se refere à origem dos ciprinídeos, parece estar-se ainda longe de um consenso. Uma origem em Gondwana (Howes, 1991) parece improvável uma vez que este super continente integrava a América do Sul, a África, a Austrália e a Antártida, além de outras placas menores. Os ciprinídeos encontram-se ausentes da América do Sul e da Austrália (e.g. Nelson, 2006), e mesmo de Madagáscar (que também fazia parte de Gondwana). Na Índia, que se destacou de Gondwana e experimentou uma ampla deslocação até entrar em contacto com a Laurásia, os fósseis de ciprinídeos parecem só surgir depois deste contacto, quando anuros e outros vertebrados dispersaram também da Ásia para a Índia (Cione e Prasad, 2002; Briggs, 2005). A falta de certezas sobre a origem dos ciprinídeos não poderia surgir com mais clareza do que quando se compararam obras do mesmo autor não muito espaçadas no tempo. Enquanto Briggs em 1995 apoiava uma origem dos ciprinídeos no Sudeste asiático, baseando-se no facto de aí se encontrar a maior diversidade de linhagens de ciprinídeos, o mesmo autor, 10 anos depois, propõe uma hipótese radicalmente distinta. De acordo com esta nova hipótese, os grandes acontecimentos cladogénicos que definiram as principais linhagens dos Ostariophysi seriam muito mais antigos do que se supunha anteriormente, tendo-se os grandes grupos originado na América do Sul bem antes do termo do Mesozóico, quando existiam ainda conexões terrestres com África. De acordo com esta hipótese, os ancestrais dos cipriniformes teriam dispersado da América do Sul para África e daí eventualmente para a Europa e posteriormente para a Ásia, embora não exista qualquer suporte paleontológico para esta via (Briggs, 2005).

Aparentemente, não se tem ainda informação suficientemente coerente, quer do ponto de vista paleontológico, quer do ponto de vista molecular, para se avançar com uma hipótese solidamente suportada sobre este problema. A extrema diversidade de caraciformes na América do Sul e de cipriniformes no Sudeste Asiático, a extinção dos caraciformes que já viveram na Europa até ao Miocénico a par com a coexistência de caraciformes arcaicos em África com cipriniformes aparentemente modernos, a presença de siluriformes na região holártica, nas regiões neotropical e paleotropical e a ausência de Ostariophysi em partes substanciais do que foi Gondwana, constituem um mosaico cuja interpretação não parece de todo clara na situação actual.

O problema torna-se ainda mais complicado dado que a identificação de "centros de origem" é feita por vários autores com critérios diversos. Enquanto para alguns a identificação de um centro de origem estaria associada à presença dos grupos mais arcaicos de uma linhagem (e.g. Hennig, 1966; Brundin, 1975), para outros "as marcas" do centro de origem seriam a grande diversidade de grupos derivados presentes na área (e.g. Briggs, 1979). Reconhecendo a impossibilidade de dar uma resposta conclusiva à questão anterior, a da origem dos ciprinídeos podemos, no entanto, fixar-nos num certo número de pontos sólidos relativamente à sua paleobiogeografia:

1 - a máxima diversidade de ciprinídeos encontra-se no Sudeste Asiático onde está representada a maioria das linhagens (e.g. Howes; 1991; Briggs, 1995, 2005; Nelson, 2006);

2 - os ciprinídeos norte americanos parecem na sua grande marioria ser derivados de ciprinídeos asiáticos (e.g. Briggs, 1995, 2005);

3 - os ciprinídeos europeus parecem derivar de ciprinídeos asiáticos que atingiram a Europa em uma ou mais vagas de colonização após o desaparecimento do estreito de Turgai que separava os dois continentes no Oligocénico (Briggs, 1995);

4 - em áreas restritas da Europa, como a Península Ibérica, alguns ciprinídeos, como os do género *Luciobarbus*, podem ter penetrado a partir do Norte de África no Miocénico final (Doadrio, 1990; Tsigenopoulos et al., 2003);

5 - os ciprinídeos africanos podem ter derivado de ciprinídeos asiáticos após a conexão dos dois continentes no Miocénico. De facto, no que se refere a África, apenas uma pequena fracção das grandes linhagens de ciprinídeos está representada e com uma diversidade não muito alta. Para mais, os ciprinídeos africanos pertencem normalmente a grupos que estão igualmente representados na Ásia ou no Médio Oriente (Briggs, 1995; Durand et al., 2002). Finalmente, os fósseis mais antigos de ciprinídeos conhecidos em África procedem do Quénia e foram datados como tendo vivido há 16-18 milhões de anos (Van Couvering, 1977).

Todos estes factos são compatíveis com a hipótese de que a fauna actual de ciprinídeos africanos provém da Ásia e pode ter-se originado no Miocénico, quando as conexões terrestres entre os dois continentes se estabeleceram devido à colisão da África com a Arábia (Briggs, 1979, 1995).

De acordo com a compilação de dados de vários autores realizada por Briggs no seu livro *Global Biogeography* de 1995, o registo fóssil suporta as conclusões expressas acima. A presença de fósseis de ciprinídeos está documentada na Índia no Eocénico (há cerca de 56.5 milhões de anos), no Oeste da Europa e na América do Norte no Oligocénico (há cerca de 35.4 milhões de anos) e finalmente em África no Miocénico, há cerca de 23.3 milhões de anos. Para mais informações paleontológicas ver http://www.wahre-staerke.com/.

1. 2 Modelos de colonização da Europa e das penínsulas meridionais europeias

De acordo com Banarescu (1992), a fauna que dominou os cursos de água da Europa no fim do Mesosóico, Paleocénico e Eocénico seria constituída por linhagens neste momento ausentes do continente europeu, algumas sem qualquer parentesco com grupos actuais, outras apresentando relações com as faunas presentes em África e na América do Norte.

Devido à sua fraca capacidade de dispersão entre cursos de água e intolerância à salinidade, os peixes de água doce encontram-se restritos a rios e lagos, estando a sua distribuição, na maioria dos casos, fortemente relacionada com a sua história biogeográfica. As espécies de ciprinídeos europeias apresentam padrões de distribuição extremamente interessantes, que têm despertado a curiosidade de vários autores. As penínsulas meridionais (Ibérica, Itálica e Balcânica) apresentam um elevado número de espécies e endemismos com pequenas áreas de distribuição, pertencentes a um número relativamente reduzido de géneros, especialmente na Península Ibérica. Pelo contrário, na Europa Central esta família encontra-se representada por um baixo número de espécies (embora incluídas num número considerável de géneros) e apresentando habitualmente amplas áreas de distribuição (e.g. Zardoya e Doadrio, 1999; Durand et al., 2003). Este padrão de distribuição das espécies europeias levou à formulação de várias hipótese para a colonização da Europa e das penínsulas meridionais.

De acordo com o registo fóssil, os primeiros ciprinídeos surgiram na Europa no Oligocénico (há cerca de 35.4 milhões de anos), depois do Mar de Turgai (que separava a Europa da Ásia) ter secado (e.g. Briggs, 1995).

No que respeita aos processos de colonização da Europa e das penínsulas meridionais pelos ciprinídeos têm-se confrontado dois modelos principais: a) colonização antiga das penínsulas desde o Oligocénico ao Pliocénico ("*northern-dispersal hypothesis*", originalmente proposta por Banarescu, 1973); b) colonização recente, durante a fase "Lago Mare" no Messiniano (Miocénico final) ("*southern sea-dispersal hypothesis*", originalmente proposta por Bianco, 1990).

Segundo Banarescu (1973, 1992; ver também Almaça, 1976) a colonização das penínsulas do Sul da Europa seria bastante antiga (desde o fim do Oligocénico até ao fim do Pliocénico, 35-1.7 milhões de anos atrás) e resultado de colonizações graduais das águas doces da região mediterrânica através de capturas de rios da Europa Central. A maior parte destes processos de colonização teriam tido lugar no Oligocénico e parte inicial do Miocénico, uma vez que os avanços da orogenia alpina (responsável pela formação de várias cadeias montanhosas que se estendem de oeste para leste, entre as quais os Pirinéus e os Alpes) tornariam cada vez mais difícil o contacto de cursos de água do centro e do Sul da Europa. Esta colonização poderia ter tido início na Sibéria e depois ter atravessado a Europa Central, através de conexões entre rios e lagos (Popov et al., 2004), durante o Oligocénio. Existem fósseis de *Paleotinca* e *Paleorutilus* encontrados em França e datados do Chattiano (Böhme e Ilg, 2003). De acordo com este modelo de dispersão apenas algumas linhagens teriam sido capazes de colonizar as penínsulas, particularmente a Península Ibérica.

A segunda hipótese, originalmente descrita por Bianco (1990) pressupõe uma colonização mais tardia, durante a fase "Lago Mare" do Mediterrâneo no Messiniano (há 5.5 milhões de anos atrás). Esta fase teve a sua origem geológica no desaparecimento do Mar de Tethys (há cerca de 20 milhões de anos), devido à colisão entre as placas Euro-Asiática e Africana na região da Arábia. Esta colisão, que pôs termo ao Mar de Tethys, deu origem ao Mediterrâneo como um mar isolado do Indo-Pacífico e em comunicação com o Atlântico.

O encerramento do Mar de Tethys e a orogenia alpina levaram à formação de um grande lago (Paratethys) separado do Mediterrâneo, e situado a norte deste, para onde desaguavam a maior parte dos rios europeus (Briggs, 1995; Popov et al., 2004). O fecho dos estreitos que ligavam o Mediterrâneo ao Atlântico (Norte-Bético e Sul-Rifenho) isolou este mar desencadeando a maior crise geológica e ecológica da sua história, conhecida pela crise de salinidade Messiniana (Briggs, 1995). Há 5.5 milhões de anos, como ocorre ainda hoje, a região mediterrânica apresentava um deficit hídrico. A precipitação é menor do que a evaporação e o nível do mar só é mantido graças às águas que flúem do Atlântico. O corte da comunicação entre o Atlântico e o Mediterrâneo levou à secagem deste. O Mediterrâneo ficou assim reduzido a pequenas lagunas hipersalinas e grandes áreas de territórios emersos para onde os rios drenavam e que poderiam formar alguns lagos junto às desembocaduras. Esta crise encontra-se geologicamente documentada em evaporitos e pensa-se que terá tido uma duração de cerca de 500 000 anos (Hsü et al., 1977; Briggs, 1995; Krijgsman et al., 1999). Na fase final deste período caracterizado pela hipersalinidade e seca de grandes áreas do Mediterrâneo (associadas à extinção de grande parte da sua fauna marinha) o grande lago Paratethys ligou-se ao Mediterrâneo (pensa-se que durante cerca de 0.1 milhões de anos) enchendo-o de água doce, o que resultaria na formação de lagos de água salobra (fase "Lago Mare" do Mediterrâneo, Briggs, 1995). De acordo com Bianco (1990), esta fase permitiria aos peixes de água doce oriundos do Paratethys colonizar vários rios europeus, entrando pelos seus estuários que desaguavam no Mediterrâneo. Segundo o mesmo autor isto explicaria a distribuição circum-Mediterrânica de várias espécies, assim como o elevado nível de endemismos registados na Península Ibérica e Sul da Grécia.

As condições marinhas do Mediterrâneo só se reestabeleceram depois da abertura do Estreito de Gibraltar (há cerca de 5.3 milhões de anos atrás), tendo a água salgada atingido as áreas correspondentes ao Paratethys (Mar Negro e Mar Cáspio) e causado uma extinção massiva das espécies de água doce endémicas desta região (Hsü et al., 1977; Krijgsman et al., 1999).

Banarescu e Coad (1991) tentaram compatibilizar as duas hipóteses vistas como mutuamente exclusivas até então: 1) os ciprinídeos europeus originaram-se na Ásia; 2) posteriormente dispersaram por duas rotas de colonização. Os taxa da Europa Central dispersaram pela rota do norte (proposta por Banarescu, 1973, 1992 e descrita atrás). Os peixes agora endémicos das áreas mediterrânicas teriam dispersado pela rota de colonização do Sul, através do Mediterrâneo na sua fase oligohalina no fim do Miocénico.

Esta hipótese levanta ainda um conjunto de problemas: a colonização da Europa pode ter ocorrido em mais do que uma vaga e ter seguido rotas diferentes. Por exemplo, a rota tradicionalmente assumida através da Sibéria para a Rússia europeia poderá não ser a única: conexões mais meridionais do Médio Oriente para o Sudeste europeu parecem apoiadas por dados moleculares (e.g. Durand et al., 2002). O Paratethys que passou por uma fase oligohalina, pode ter tido um papel muito mais relevante do que se assume habitualmente na dispersão entre a Ásia e a Europa (e.g. Durand et al., 2002). Finalmente, a passagem de ciprinídeos por uma via meridional através do Norte de África pode ter levado à dispersão de peixes do subgénero *Luciobarbus* para a Península Ibérica quando havia uma conexão terrestre entre a península e os territórios situados a norte do Rife, de modo que não é necessário invocar a hipótese do Lago Mare para explicar as afinidades entre os barbos norte africanos e ibéricos (Machordom e Doadrio, 2001; Doadrio et al., 2002). Embora os primeiros fósseis de *Luciobarbus* na península sejam do Miocénico final (Doadrio e Casado, 1989), e pudessem à primeira vista sugerir uma dispersão na fase Lago Mare, é importante lembrar que pouco tempo antes os estreitos Sul-Rifenho e Norte-Bético, que até então ligavam o Mediterrâneo ao Atlântico, tinham deixado de existir, criando assim uma conexão terrestre entre o Norte de África e o Sul da Península Ibérica. Aliás, foi precisamente o fecho destes estreitos e a formação de uma conexão terrestre entre a África e a Ibéria que separaram o Mediterrâneo e o Atlântico e desencadearam a crise Messiniana.

Este tema será retomado na discussão (capítulo 13) já que os resultados da presente tese têm incidências importantes na análise deste problema.

1.3 O impacto das glaciações

No Pleistocénico (que teve início há cerca de 1.64 milhões de anos) a distribuição dos organismos aquáticos, nomeadamente dos peixes de água doce, foi completamente dominada pelas glaciações, que tiveram como consequências óbvias, o aumento das áreas ocupadas por glaciares e uma diminuição do nível do mar (Banarescu, 1992). Embora ainda exista alguma incerteza sobre as condições climáticas do Pleistocénico, sabe-se que além das quatro principais glaciações existiram pelo menos mais uma dúzia de grandes dimensões e muitas mais pequenas (Crowley e North, 1991).

Na Europa Central, durante os máximos glaciares, o gelo avançou em duas direcções, deixando apenas uma estreita faixa não gelada que atravessava a Alemanha e se estendia para leste. Enquanto o glaciar Escandinavo se estendia para sul, os glaciares Alpinos associados às cadeias de montanhas que se estendem de oeste para leste avançavam para norte, de modo que o espaço livre de gelo entre as duas frentes glaciares se tornava mínimo (Banarescu, 1992; Briggs, 1995).

Banarescu (1992) dividiu as espécies de água fria da Europa Central afectadas pelos movimentos glaciares em duas categorias. As *"northern ice cap marginal species"* originaram-se no extremo norte e foram-se movimentando para sul, afastando-se da frente gelada. A maioria destas espécies voltou para a sua área original de distribuição depois das glaciações. Algumas espécies ainda vivem na Europa Central em habitats montanhosos e/ou caracterizados por águas especialmente frias. Por outro lado pensa-se que as *"southern ice cap marginal species"* estariam presentes nos Alpes e outras cadeias montanhosas da Europa central durante os períodos pré-glaciares e ter-se-iam deslocado para sul durante as glaciações.

Os glaciares eliminaram toda a vida nas áreas que ocuparam, alteraram o clima, destruíram bacias e criaram lagos. O seu degelo, libertou grandes quantidades de água que formaram grandes rios e lagos. O nível do mar voltou a subir, cobrindo alguns rios e lagos principalmente nas margens das plataformas continentais. Os organismos aquáticos parecem ter sobrevivido nas áreas mais a sul da Europa enquanto na Europa Central (entre a calote Escandinava e a calote Alpina) parece ter acontecido uma extinção em massa. A França, o extremo Oeste da Alemanha, a Bélgica e as terras baixas do médio e baixo Danúbio estão entre as regiões da Europa Central menos afectadas durante as glaciações (Banarescu, 1992). Nestas regiões alguma da fauna local teve a oportunidade de sobreviver. As penínsulas do Sul da Europa (Península Ibérica, Itália e Balcãs), o Noroeste de África, o Cáucaso e a Anatólia foram considerados refúgios para a fauna e flora da Europa Central no fim do Pliocénico e posteriormente centros de dispersão para os mesmos organismos depois das glaciações (Hewitt, 1999). A distribuição actual dos animais e plantas demonstra que, na maioria dos casos, a sua dispersão ocorreu a partir de um destes centros (Banarescu, 1992). Esta hipótese é compatível com os dados de diversidade genética obtidos para diversas espécies (Alexandrino et al., 2000 e referências aí citadas) em que as populações com origem nos refúgios a sul apresentam elevados níveis de diversidade genética, por oposição às populações das áreas colonizadas a norte, que apresentam níveis comparativamente mais baixos de diversidade genética. Para os peixes exclusivamente de água doce estas informações sobre outros organismos devem ser consideradas com cuidado, uma vez que a sua dispersão se encontra reduzida às capturas de rios e/ou aos contactos entre fozes.

No que respeita aos peixes de água doce, as espécies que se encontram presentes actualmente no Centro e Norte da Europa correspondem a espécies que sobreviveram localmente (e.g. Volckaert et al., 2002), espécies que sobreviveram em refúgios no Sul da Europa a norte das cadeias montanhosas (e.g. Nesbo et al., 1999) ou a espécies que ocupavam as penínsulas mediterrânicas durante as glaciações e que posteriormente dispersaram (e.g. via Danúbio) (e.g. Slechtova et al., 2004). Para mais exemplos ver também Perdices et al. (2003), Janko et al. (2005) e Culling et al. (2006).

Embora a Península Ibérica tenha sido considerada como um refúgio para a fauna não estritamente aquática durante as glaciações, à excepção de algumas áreas no Norte e Centro da península (Ribeiro et al., 1987; Dias, 1997; Vieira e Cordeiro, 1998) o Pleistocénico não se encontra muito bem documentado (Alexandrino et al., 2000). Para além disso, e como já se referiu, no caso dos peixes de água doce primários o papel da península como refúgio foi certamente muito mais limitado do que para animais e plantas terrestres. Com descidas no nível do mar que podem ter atingido mais de 120 metros nos máximos glaciares é provável que os rios formassem deltas extensos no que é hoje a plataforma continental, permitindo a migração de peixes de água doce entre rios próximos (Dias, 1997). Neste quadro, seriam concebíveis movimentos, por exemplo entre rios do Sul de França e do Nordeste de Espanha, mas o alcance destes contactos Pleistocénicos entre a península e a França deve ter sido relativamente limitado. Na Península Balcânica como já se referiu, os movimentos de peixes para a Europa Central eram facilitados pela presença de afluentes importantes do Danúbio (como o Rio Strymon), tornando possível a utilização dos Balcãs como refúgios. No caso da Península Ibérica, a grande dimensão do maciço pirenaico e a ausência de ligações fluviais entre Espanha e França devem ter reduzido em muito o seu papel como refúgio para os peixes de água doce.

1.4 Os estudos moleculares de ciprinídeos e a sua contribuição para a paleobiogeografia da ictiofauna dulçaquícola da Europa

No que respeita a análises com base em dados moleculares, existe alguma bibliografia já disponível sobre as relações entre os ciprinídeos da Eurásia (e.g. Briolay et al., 1998; Gilles et al., 1998; Zardoya e Doadrio, 1999; Gilles et al., 2001; Cunha et al., 2002 e Liu e Chen, 2003). Alguns géneros específicos foram já sujeitos a análises filogenéticas / filogeográficas das suas espécies e/ou populações (e.g., Durand et al., 1999; Machordom e Doadrio, 2001; Doadrio e Carmona, 2003a,b; Salzburger et al., 2003; Sanjur et al., 2003; Tsigenopoulos et al., 2003; Doadrio e Carmona, 2004; Ketmaier et al., 2004; Freyhof et al., 2006). Alguns trabalhos foram realizados sobre espécies de regiões específicas (e.g. Zardoya e Doadrio, 1998; Zardoya et al., 1999; Hrbek et al., 2004).

Alguns destes trabalhos tiveram como objectivo, com base no uso de relógios moleculares, testar as hipóteses de colonização da Europa e das penínsulas meridionais acima descritas. Estes relógios moleculares, calibrados com acontecimentos geológicos bem datados como a abertura do Estreito de Korinthos no fim do Pliocénico (há 2.5 milhões de anos) ou do Estreito de Gibraltar (há 5 milhões de anos) (Zardoya e Doadrio, 1999), podem ser de grande utilidade na compreensão dos tempos e rotas de colonização das espécies. A grande maioria dos trabalhos com ciprinídeos europeus tem recorrido ao uso do gene mitocondrial que codifica o citocromo *b* (Zardoya e Doadrio, 1999; Durand et al., 2003; Sanjur et al., 2003; Doadrio e Carmona, 2003b, 2004), cuja calibração proposta por Dowling et al. (2002), com base em dados paleontológicos e comparação de sequências é de 0.53% por linhagem por milhão de anos, ou seja, cerca de aproximadamente 1% de divergência entre linhagens por milhão de anos.

Quando se tenta sintetizar os resultados desta grande diversidade de estudos o quadro que emerge está longe de ser simples. Arriscando um breve esboço de síntese pode afirmar-se o seguinte:

1 - a paleobiogeografia dos Leuciscini e dos Cyprinini (*sensu* Zardoya e Doadrio, 1998, 1999) apresenta discordâncias entre as duas linhagens o que pode significar que a sua dispersão a partir da Ásia se deu em momentos diferentes, podendo ter originado rotas distintas de penetração na Europa. Enquanto os Leuciscinae das penínsulas mediterrânicas pertencem a linhagens estreitamente relacionadas com as da Europa Central, existe uma linhagem de *Barbus* (subgénero *Luciobarbus*) que parece ter dispersado da Ásia através do Norte de África (onde quase não há Leuciscini) e daí para territórios mais a norte, nomeadamente a Península Ibérica (Zardoya e Doadrio, 1999; Tsigenopoulos et al., 2003);

2 - existem afinidades muito acentuadas entre rios e largos importantes da Península Balcânica e a Europa Central, com partilha de grande número de géneros e espécies. Como já se referiu, há afluentes do Danúbio que penetram profundamente nos Balcãs de modo que estes e as áreas da parte terminal do Danúbio e à volta do Mar Negro podem ter formado um complexo de refúgios glaciares interrelacionados;

3 - existe uma região de forte endemismo na região da Dalmácia que pode ter preservado espécies que tiveram uma dispersão muito mais ampla na Europa antes das glaciações (Freyhof et al., 2006);

4- demonstrou-se, como já se referiu acima, a existência de espécies que sobreviveram *in loco* às glaciações a norte dos Alpes, mas no essencial, a fauna actual da Europa Central parece ter uma relação bastante reduzida com a fauna que lá existia até ao Pleistocénico. De facto, muita da uniformidade da fauna da Europa a norte dos Alpes parece ter resultado da dispersão a partir dos refúgios já referidos no Danúbio inferior e seus afluentes e na região do Mar Negro após a última glaciação, já que o degelo deve ter criado condições de muito baixa salinidade no Báltico e na região do Mar do Norte, permitindo que certas espécies de peixes dulçaquícolas se estendam da Rússia às ilhas Britânicas;

5 - a Península Ibérica, no que se refere aos Leuciscinae, parece ter recebido um número muito restrito de linhagens, que aí chegaram provavelmente em fases muito precoces da orogenia pirenaica. Este pequeno número de linhagens sofreu uma intensa diversificação local, mas parece ter tido uma contribuição muito diminuta para a formação da ictiofauna do resto da Europa.

Uma das linhagens que conseguiu chegar à Península Ibérica corresponde a peixes que até há pouco se incluíam no género *Chondrostoma* e que são objecto de diversas investigações apresentadas nesta tese. Uma vez que vários autores pretenderam utilizar os peixes desta linhagem para tentar testar diferentes modelos biogeográficos revê-se em seguida brevemente essa literatura.

No que respeita à dispersão e diversificação dos *Chondrostoma* europeus, mesmo usando o mesmo relógio molecular, diferentes autores encontraram resultados discrepantes, que apoiam diferentes teorias de colonização. Durand et al. (2003) encontrou evidências de dois eventos de colonização para este género: a) no Messiniano (aproximadamente há 5,5 milhões de anos atrás) um ancestral de *Chondrostoma* com origem no Parathetys teria atingido os principais rios do Norte do Mediterrâneo (na Península Ibérica, Sul de França, Itália e Grécia) apoiando assim a hipótese “Lago Mare” (Bianco, 1990); b) em tempos mais recentes (há cerca de 2.23-1.07 milhões de anos atrás) uma nova vaga de colonizadores teria chegado ao Danúbio, vindos da Mesopotâmia e dos mares Negro e Cáspio. Os mesmos autores sugerem que este segundo evento de colonização promoveu contactos secundários entre as espécies consideradas como relíquias do Messiniano (endémicas dos rios mediterrâneos) e os invasores vindos da Mesopotâmia, o que poderia explicar as evidentes discrepâncias entre os dados morfológicos e moleculares, também muitas vezes referidas por outros autores para esta linhagem.

Doadrio e Carmona (2003b, 2004) chegaram a resultados completamente diferentes. De acordo com estes autores a principal radiação da linhagem *Chondrostoma* seria muito mais antiga, tendo ocorrido provavelmente no Miocénico Médio (no Tortoniano, há cerca de 11 milhões de anos atrás). A diversificação da linhagem seria posterior, no Pliocénico, coincidindo com a formação do sistema de cursos de água actual. Estes resultados rejeitam então a hipótese "Lago Mare" defendida por Bianco (1990) e apoiada por Durand et al. (2003). Esta enorme discrepância de resultados foi explicada por Doadrio e Carmona (2003b, 2004) ao mostrarem que Durand et al. (2003) utilizaram incorrectamente o relógio molecular, considerando o 1% de divergência entre linhagens como se fosse a taxa de mutação dentro de cada linhagem. Adicionalmente, dados sobre um outro género de Leuscicinae (*Squalius*, Sanjur et al., 2003) revelaram também um padrão de diversificação mais antigo do que o Messiniano, mais uma vez contrariando a hipótese "Lago Mare", numa linhagem independente mas de espécies da mesma subfamília.

Estes dados têm sido também confirmados pela análise e datação de fósseis de ciprinídeos da Península Ibérica, todos pré-messinianos. Os fósseis mais antigos de exemplares de Leuciscini datam do Oligocénico Superior (de la Peña, 1995) e incluem exemplares de *Rutilus* em Espanha (datados do Tortoniano, de la Peña, 1995), de *Squalius* em Portugal (datados do Serravaliano, Miocénico Médio, Gaudant, 1977a) e de *Paleoleuciscus* em Espanha (um do Aquitaniano, há cerca de 20-23 milhões de anos atrás, Daams, 1976, e vários do Tortoniano, há cerca de 7-11 milhões de anos atrás, observação pessoal. M. Böhme). A descoberta de mais fósseis pré-messinianos poderia ajudar na análise dos padrões de dispersão dos peixes europeus mas o número reduzido de fósseis no Sudeste de Espanha e também na Itália (de la Peña, 1995) devido à sua submersão neste período, tem representado um entrave para a aquisição deste tipo de dados. É de realçar que estes fósseis de *Rutilus* podem pertencer na realidade a peixes da linhagem *Chondrostoma*, uma vez que historicamente tem havido uma grande instabilidade com muitas espécies ibéricas de *Rutilus* a serem reclassificadas como pertencendo ao género *Chondrostoma*, muitas vezes com base na evidência molecular (e.g. Zardoya e Doadrio, 1998).

No que respeita à fase "Lago Mare" do Mediterrâneo a sua importância foi sugerida para as espécies do subgénero *Luciobarbus*, para as quais se considera que esta fase no Messiniano seja a responsável pela actual distribuição das suas espécies (Tsigenopoulos et al., 2003). As espécies deste subgénero pertencem ao género *Barbus* e encontram-se distribuídas na Península Ibérica, Noroeste de África (de Marrocos à Tunísia), Médio Oriente e Grécia (e.g. Tsigenopoulos et al., 2003). Os mesmos autores consideram que a dispersão destas espécies através do Mediterrâneo poderia ter sido favorecida pelas suas características biológicas, em particular a sua tolerância à salinidade. No entanto, como se referiu acima, a dispersão deste grupo, em particular de África para a Península Ibérica, parece ter seguido uma via diferente.

1. 5 O género *Chondrostoma*

A presente tese aborda vários dos problemas paleobiogeográficos apresentados acima, utilizando a linhagem que tradicionalmente correspondia ao género *Chondrostoma*, fazendo-o numa multiplicidade de escalas, que vão num extremo ao conjunto do grupo, e no outro à área restrita da Península Ibérica. Apesar do estatuto genérico do grupo ser contestado no presente trabalho (ver capítulo 2), não restam dúvidas de que se trata de um grupo monofilético da subfamília Leuciscinae, estreitamente aparentado com os géneros *Delminichys* (Freyhof et al., 2006), *Telestes* e *Rutilus* (Zardoya e Doadrio, 1998, 1999) entre outros. Por comodidade para o leitor, na presente introdução, ao fazer a apresentação da biologia do grupo e das espécies que o integram, mantém-se a nomenclatura antiga, embora se exponha no capítulo 2 as razões que levaram à redefinição deste género.

A história do género *Chondrostoma* tem sido extremamente conturbada, provavelmente devido à extrema diversidade morfológica que se observa nos peixes do grupo. Assim, como se referiu acima, muitas espécies ibéricas tinham sido classificadas inicialmente no género *Rutilus* e só a evidência molecular levou à sua reclassificação. Dada a importância dos peixes que têm vindo a ser classificados no género *Chondrostoma* para o presente trabalho revêem-se em seguida os aspectos mais relevantes da biologia do grupo.

Os peixes do género *Chondrostoma* distribuem-se pelo Sul e Centro da Europa, desde o Oceano Atlântico até ao Mar Cáspio e desde o Mediterrâneo até ao Báltico. Este género está ainda presente na Ásia Menor, Cáucaso e Mesopotâmia (e.g. Elvira, 1997; Durand et al., 2003).

O número de espécies incluídas neste género varia de acordo com os autores, devido ao uso de diferentes critérios de diagnose (i.e. morfológicos e osteológicos ou moleculares). Na sua revisão do género *Chondrostoma* publicada em 1997, Elvira reconheceu 26 espécies como pertencendo a este género. No mesmo ano, Bogutskaya descreveu *Chondrostoma beysehirense*, uma nova espécie para as águas turcas (Bogutskaya, 1997). Ambos os autores basearam os seus trabalhos em critérios apenas morfológicos e osteológicos, considerando a presença de uma boca ínfera e dotada de um estojo córneo como o caracter mais importante no reconhecimento das espécies deste género.

Por outro lado, os trabalhos de Zardoya e Doadrio (1998,1999) sobre os ciprinídeos da Europa e da Península Ibérica, realizados com base no gene mitocondrial do citocromo *b*, revelaram que espécies previamente incluídas no género *Rutilus* Rafinesque, 1820, formam um grupo monofilético com as espécies incluídas por Elvira (1997) no género *Chondrostoma*. É de realçar que outras espécies, não incluídas por Elvira no trabalho supracitado, tinham sido já incluídas no género *Chondrostoma* com base em caracteres morfológicos e citogenéticos, por Collares-Pereira (1980, 1983), casos de *C. lusitanicum* e *C. lemmingii* Steindachner, 1866. Só as investigações moleculares supracitadas permitiram abrir caminho a uma solução global, ao mostrar que na Península Ibérica não existiam espécies do género *Rutilus* e que todas as espécies anteriormente incluídas neste género se agrupavam no mesmo clado que as espécies do género *Chondrostoma*. Ao mesmo tempo, estes avanços moleculares levantavam um problema novo e difícil para a taxonomia destes peixes, já que no plano morfológico deixava de haver caracteres unificadores e distintivos, ficando a delimitação do género numa situação complexa e problemática.

Mais recentemente foram descritas, apenas para a Península Ibérica, três novas espécies: *C. almacai* Coelho et al., 2005, *C. oretanum* Doadrio e Carmona, 2003a e *C. occidentale* Robalo et al., 2005a (capítulo 2). Uma nova espécie para a Bacia do Douro, cujas populações foram até agora atribuídas a *C. lemmingii*, aguarda descrição (Doadrio e Elvira, não publicado). Todas estas espécies apresentam boca curva e ausência de estojo córneo.

Esta discrepância no número de espécies incluídas no género *Chondrostoma*, consoante o tipo de dados usados, levou à conclusão por parte de Durand et al. (2003) que os caracteres morfológicos e osteológicos se encontrariam desacoplados da informação presente nos genes. Doadrio e Carmona (2004) chegam mesmo a afirmar que os caracteres relacionados com a estrutura da boca carecem de utilidade em termos filogenéticos.

No total, se incluirmos todas as espécies consideradas como pertencendo ao género *Chondrostoma*, independentemente do tipo de dados em que se baseou a sua inclusão, obtemos 35 espécies. Embora não exista disponível na literatura, até agora, um trabalho que reúna todas estas espécies, os trabalhos sobre filogenia do género *Chondrostoma* publicados até à data desta tese (e.g. Durand et al., 2003; Doadrio e Carmona, 2004) identificaram as seguintes linhagens, embora as relações entre elas não tenham ficado esclarecidas: *toxostoma*, *lemmingii*, *polylepis*, *arcasii*, *nasus*, *soetta* e *genei*. Em todos estes trabalhos as relações filogenéticas dentro do género resultaram numa politomia. As politomias são multifurcações numa árvore filogenética (por oposição a bifurcações). Podem representar falta de sinal filogenético (*"soft polytomies"*) ou uma cladogénese extremamente rápida e/ou simultânea (*"hard polytomies"*) (e.g. Page e Holmes, 1998). Nalguns casos uma sequência muito rápida de eventos de cladogénese pode ser quase indistinguível de uma politomia, se não existem mutações suficientes para distinguir os clados formados (Slowinski, 2001; Poe e Chubb, 2004). Noutros casos, o uso combinado de marcadores moleculares independentes pode revelar relações filogenéticas escondidas (Page e Holmes, 1998; Poe e Chubb, 2004).

Durand et al. (2003) justificaram a politomia encontrada no género *Chondrostoma* com uma sequência de eventos rápidos de dispersão e colonização das espécies durante a fase "Lago Mare" do Messiniano. Segundo os mesmos autores esta politomia seria representativa de uma rápida cladogénese, que não teria deixado marcas detectáveis a nível genético. Aliás, esta suposta radiação muito rápida de *Chondrostoma* constituía um dos argumentos considerados mais fortes por Durand et al. (2003) para defender a hipótese do "Lago Mare".

Doadrio e Carmona (2003b) consideram que a politomia encontrada não reflecte obrigatoriamente uma cladogénese rápida e que poderia eventualmente ser resolvida com outros marcadores moleculares.

1.5.1 Espécies ibéricas do género *Chondrostoma*: biogeografia, relações filogenéticas, filogeografia e comportamento

A fauna da Península Ibérica é muito rica em ciprinídeos, apresentando uma grande proporção de endemismos, muitos com elevado estatuto de conservação (Espanha, Doadrio, 2001; Portugal, Cabral et al., 2005). Só em Portugal existiam em 2005, 9 espécies do género *Chondrostoma*, presentes em 4 das 7 linhagens identificadas por Doadrio e Carmona (2004).

Os exemplares das espécies *C. polylepis*, *C. willkommii* e *C. duriense* atingem cerca de 50cm de comprimento máximo, apresentam a boca ínfera, recta e provida de estojo córneo (Coelho, 1987; Elvira, 1997) e realizam migrações pré-reprodutoras (Granado-Lorenzio e García-Novo, 1981; Coelho, 1987; Rodriguez-Ruiz e Granado-Lorencio, 1992; Doadrio, 2001). Estão distribuídas em alopatria no território continental português da seguinte forma: *C. duriense* Coelho, 1985 no Norte do país, *C. polylepis* Steindachner, 1864 no Centro e *C. willkommii* Steindachner, 1866 no Sul. Destas, apenas *C. willkommii* apresenta um estatuto de conservação preocupante em Portugal uma vez que está classificada como Vulnerável no recente *Livro Vermelho dos Vertebrados de Portugal* (Cabral et al., 2005). Em todos os trabalhos publicados usando dados moleculares estas três espécies formam um grupo monofilético (Zardoya e Doadrio, 1998, 1999; Durand et al., 2003; Doadrio e Carmona, 2004).

Outra das linhagens que consta nos trabalhos supracitados é a constituída pelas espécies *C. oligolepis* (= *C. macrolepidotum*[1], Steindachner, 1866, ver capítulo 4) e *C. arcasii* (Steindachner, 1866), espécies irmãs anteriormente incluídas no género *Rutilus* (Zardoya e Doadrio, 1998; Doadrio, 2001). Estes pequenos *Chondrostoma* cujo comprimento máximo ronda os 16cm (Collares-Pereira, 1983) são endémicos do Norte e Centro da Península Ibérica. De acordo com o *Livro Vermelho dos Vertebrados de Portugal* (Cabral et al., 2005) apenas *C. arcasii* está dado como Em Perigo, apesar de no mesmo livro se referir que a sua identificação é muitas vezes difícil por confusão com *C. oligolepis* (ver também Casado, 1995 e Collares-Pereira 1979, 1983) e pela sua capacidade de gerar híbridos provavelmente férteis com *C. duriense* e *C. polylepis* (Collares-Pereira, 1983; Collares-Pereira e Coelho, 1983; Gante et al., 2004). Collares-Pereira (1983) considera que *C. arcasii* se encontra praticamente

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[1] Apesar de em muita da literatura publicada surgir o nome de *Chondrostoma macrolepidotus* este nome não se encontrava bem formado, tendo resultado do hábito anterior de designar esta espécie como *Rutilus macrolepidotus*. A sua passagem para o género *Chondrostoma* deveria implicar a passagem para o epíteto específico *macrolepidotum*. Com o abandono do epíteto específico *macrolepidotus*, por este se encontrar indisponível (ver capítulo 4), este problema de terminologia nunca chegou a ser resolvido.

ausente de Portugal, com possíveis excepções nalguns afluentes da Bacia do Minho e Douro. Estudos moleculares recentes realizados por Doadrio e Carmona (2004) consideram mesmo que esta espécie se encontra ausente de Portugal. Os mesmos autores sugerem a presença de espécies por descrever nas Bacias do Douro e Minho. Doadrio (2001) descreve a área de distribuição de *C. arcasii* como abrangendo em Espanha a área compreendida entre as bacias do Rio Ulla (na Galiza, Noroeste da Península Ibérica) e do Rio Júcar (para Este, já na vertente mediterrânica). Também refere a sua presença nas grandes bacias do Douro, Tejo e Ebro (parte espanhola).

C. oligolepis, que não está, de acordo com o Livro Vermelho, sujeito a qualquer grau de ameaça está, no que respeita à sua distribuição, restrito à região mais a oeste da Península Ibérica, na região mais Atlântica de Portugal. De acordo com Collares-Pereira (1983), *C. oligolepis* encontra-se distribuído desde o Rio Lima (Noroeste de Portugal) até ao Rio Alcoa (mais a sul, entre as cidades de Leiria e Lisboa). Posteriormente, Diogo (1999) estendeu esta área de distribuição aos rios Real e Alcabrichel (ainda mais a sul). As populações do rio Alcabrichel, bem como as dos Rios Sizandro e Safarujo, na área entre Torres Vedras e Mafra revelaram-se posteriormente muito distintas de *C. oligolepis*, tendo sido incluídas numa nova espécie *C. occidentale* (Robalo et al., 2005b, capítulo2).

Outra linhagem de pequenos *Chondrostoma* endémicos da Península Ibérica é a constituída pelas espécies *C. lemmingii* (Steindachner, 1866) e *C. lusitanicum* Collares-Pereira, 1980 e ainda pelas recém descritas *C. oretanum* Doadrio e Carmona, 2003 e *C. almacai* Coelho, Mesquita e Collares-Pereira, 2005. De acordo com Cabral et al. (2005) *C. lemmingii* encontra-se Em Perigo e em Portugal tem a sua área de distribuição restrita a alguns afluentes orientais da bacia do Tejo (Collares-Pereira, 1983; Marques, 2002) e à maioria dos principais afluentes do Guadiana (e.g. Collares-Pereira et al., 2002). Encontra-se também presente nas pequenas bacias de Quarteira, Gilão e Almargem (no Leste Algravio, Mesquita e Coelho, 2002). Em Espanha, encontra-se presente nas bacias do Tejo, Guadiana, Guadalquivir e Odiel, assim como nos afluentes mais a sudoeste da Bacia do Douro (Doadrio, 2001), embora recentemente se tenha constatado que estas populações do Douro correspondem na realidade a uma espécie muito distinta de *C. lemmiingii* (Doadrio e Carmona, dados não publicados). Populações de alguns afluentes da Bacia do Guadalquivir foram elevadas ao estatuto de espécie (*C. oretanum*, Doadrio e Carmona, 2003) com base em critérios moleculares, morfológicos e osteológicos. Na sua descrição foi proposto o estatuto de Criticamente Em Perigo (CR) para esta espécie.

C. lusitanicum é uma espécie endémica de Portugal que apresenta o mesmo estatuto e que ocorre nas pequenas bacias a norte do Rio Tejo (Samarra, Cheleiros e Colares), na bacia do Tejo (nos afluentes do seu curso inferior) na bacia do Sado e nas pequenas bacias litorais entre Sado e Mira (Cabral et al., 2005). De acordo com os dados apresentados por Fluviatilis (2003), as populações do Tejo e Sado encontram-se extremamente fragmentadas, tendo apenas sido detectados exemplares em 11 de 81 pontos de amostragem (ver também Robalo et al., no prelo a, capítulo 8). De acordo com o Livro Vermelho dos Vertebrados estes dados implicam uma redução muito significativa da sua área de distribuição, provavelmente devido à degradação dos seus habitats e da qualidade da água, à implementação de infra-estruturas hidráulicas, regularização dos caudais, captação de água e ainda à interacção com espécies não indígenas. As populações do Mira e do Arade (em tempos consideradas como pertencendo a esta espécie) foram recentemente (Coelho et al., 2005) descritas como uma nova espécie, *C. almacai*, também ela com uma distribuição muito restrita (apenas ocorre nestas bacias da região sul de Portugal), apresentando também o estatuto de Criticamente Em Perigo (CR).

A linhagem reconhecida em Doadrio e Carmona (2004) como *toxostoma* inclui as espécies *C. toxostoma* Vallot, 1837, *C. arrigonis* Steindachner, 1866, *C. miegii* Steindachner, 1866 e *C. turiense* Elvira, 1987, quase todas (à excepção da primeira) com as suas áreas de distribuição nos rios mediterrânicos do Este de Espanha. *C. arrigonis*, *C. miegii* e *C. turiense* são ciprinídeos de tamanho médio (até 30 cm de comprimento) que apresentam o lábio inferior provido de uma lâmina córnea arqueada bem evidente (Doadrio, 2001). *C. arrigonis* encontra-se classificada como Em Perigo e é endémica da bacia do Rio Júcar. *C. miegii* não se encontra ameaçada em termos de conservação e ocorre nos rios das vertentes cantábrica oriental e mediterrânica, existindo ainda uma população no Rio Tejo. *C. turiense* é considerada Em Perigo, sendo endémica das bacias dos rios Túria e Mijares. Estas espécies foram classificadas como subespécies de *C. toxostoma* por Lozano-Rey (1935) e depois consideradas como espécies válidas por Elvira (1997). A distribuição de *C. toxostoma* abrange agora apenas as áreas do sul e centro de França (Doadrio, 2001).

Importa, no contexto das *Chondrostoma* distribuídas na Península Ibérica, e principalmente no que respeita às que se encontram distribuídas no território Português, deixar aqui uma palavra sobre conservação dos habitats dulçaquícolas. Das 8 espécies de *Chondrostoma* que constam no *Livro Vermelho dos Vertebrados de Portugal*, 6 apresentam um estatuto de conservação elevado (Criticamente Em Perigo, CR ou Em Perigo, EN). Na sua grande maioria este estatuto é justificado por um decréscimo e/ou fragmentação acentuados das populações, devido a vários tipos de intervenção humana directa ou indirecta, sobre os cursos dos rios ou sobre a qualidade das águas. A maioria dos cursos de água portugueses (principalmente os do centro e sul do país) sofre grandes oscilações dos seus caudais ao longo das várias estações do ano, o que faz com que os peixes tenham que sobreviver muitas vezes em pequenas "poças" ou mesmo junto às nascentes dos rios. Este facto, junto com o aumento das temperaturas e o uso indevido das águas torna a sobrevivência das populações muito difícil e favorece a sua fragmentação.

A construção de barragens que interrompe os cursos de água, embora forneça um ambiente lacustre favorável à reprodução de várias espécies, também impede as migrações pré-reprodutoras de outras, criando artificialmente um ambiente propício à hibridação das espécies deste género (e mesmo à hibridação entre géneros diferentes) devido à sua reconhecida plasticidade na formação de híbridos.

Se juntarmos a este panorama a constante introdução de espécies exóticas, por vezes deliberadamente por parte de pescadores desportivos, ou a sua chegada pelos cursos de água que vêm de Espanha, não é muito difícil chegar à conclusão de que é necessário um grande e enérgico esforço para conservar a ictiofauna dulçaquícola endémica da Península Ibérica no geral e de Portugal em particular. A aplicação da legislação já existente, a nível nacional e comunitário, seria um primeiro passo muito positivo neste sentido, com o objectivo fundamental de proteger toda esta diversidade.

Em Espanha a situação, no que respeita a aspectos de conservação, não se revela muito diferente. No *Atlas y Libro Rojo de los Peces Continentales de España* (Doadrio, 2001) das 8 espécies de *Chondrostoma* reconhecidas até essa data no território espanhol, apenas duas espécies (*C. miegii* e *C. polylepis*) não apresentavam qualquer estatuto de conservação. *C. arrigonis* e *C. turiense* são consideradas Em Perigo e todas as restantes são incluídas na categoria Vulnerável. Também as ameaças à conservação das espécies são as acima referidas para as populações portuguesas.

No que respeita ao conhecimento básico sobre as espécies mais abordadas nesta tese, ainda existe muito trabalho para fazer. Embora os estudos de genética molecular tenham nos últimos anos revelado muito sobre a singularidade de determinadas populações, ainda há muito a investigar no que respeita às distribuições das espécies existentes, monitorização das suas populações, descrição de novas espécies, descrição dos comportamentos reprodutores e da biologia da reprodução no geral, etc. Em termos muito gerais é esse o objectivo desta tese, fornecer o máximo de informação possível sobre os peixes do género *Chondrostoma*, com principal ênfase nas pequenas *Chondrostoma* presentes na Península Ibérica.

Antes de enumerar esses objectivos vale a pena sublinhar algo que decorre de forma muito clara mesmo da breve apresentação deste grupo de ciprinídeos que se expôs nos parágrafos precedentes. Das 7 linhagens reconhecidas no género *Chondrostoma* (Doadrio e Carmona, 2004), 4 são estritamente ibéricas ou quase, correspondendo no seu conjunto a uma importante percentagem das espécies. Esta simples observação sugere que uma parte muito substancial da radiação do grupo teve lugar na península e que uma compreensão global da sua filogenia envolve uma investigação detalhada das linhagens ibéricas, dos tempos prováveis de colonização da península e da biogeografia das diferentes linhagens nos territórios português e espanhol. A presente tese tenta cumprir este desiderato, proceder a uma abordagem a múltiplos níveis, da filogenia global do grupo à filogenia de espécies ibéricas particulares, tendo como pano de fundo a hipótese de que uma compreensão adequada da colonização da Península Ibérica por estes peixes terá implicações da maior relevância para o conhecimento da paleobiogeografia da ictiofauna dulçaquícola primária da Europa, em particular da bacia Mediterrânica.

1. 6 Objectivos

1- Elaborar uma filogenia do género *Chondrostoma*, que esclareça as relações de parentesco entre os clados reconhecidos até agora por vários autores (e.g. Doadrio e Carmona, 2004);
2- Contribuir para um maior conhecimento das entidades taxonómicas do género *Chondrostoma* presentes na Península Ibérica (e sua distribuição geográfica), recorrendo a dados morfológicos e moleculares;
3- Compreender as relações entre as espécies do clade *arcasii* (*C. arcasii* - *C. oligolepis*);
4- Realizar a filogeografia da espécie *C. lusitanicum*;
5- Realizar a filogeografia da espécie *C. lemmingii*;
6- Descrever os comportamentos reprodutores e agonísticos de algumas espécies ibéricas deste género;
7- Contribuir com sugestões para a conservação das espécies.

1.7 Lista dos trabalhos que integram a tese

Esta tese é constituída por vários capítulos que correspondem a artigos científicos já publicados ou no prelo à data da sua entrega. Embora estes trabalhos tenham sido publicados em conjunto com outros investigadores, a autora participou activamente em todas as fases da sua concretização, desde a recolha dos dados à elaboração dos manuscritos.

Tendo como objectivo evitar a heterogeneidade das apresentações gráficas de cada revista optou-se por uma uniformização que, no geral e sempre que possível, segue as normas da revista Molecular Phylogenetics and Evolution para o texto e referências em toda a tese. Afora esta uniformização respeitou-se integramente o conteúdo dos artigos originais.

1 - Robalo, J.I., Almada, V.C., Faria, C., 2003. First description of agonistic behaviour in *Chondrostoma polylepis* (Pisces: Cyprinidae) with notes on the behaviour of other *Chondrostoma* species. Etología 11, 9-13.

2 - Carvalho, V., Robalo, J.I., Almada, V.C., 2003. A description of the reproductive behaviour of the endangered Iberian cyprinid *Chondrostoma lusitanicum* Collares-Pereira 1980 in captivity. Etología, 10: 23-25.

3 - Sousa-Santos, C., Robalo, J. I., Collares-Pereira, M.J., Almada, V. C., 2005. Heterozygous indels as useful tools in the reconstruction of DNA sequences and in the assessment of ploidy level and genomic constitution of hybrid organisms. DNA Seq. 16, 462-467.

4 - Robalo, J., Almada, V., Santos, C., Moreira, M.I., Doadrio, I., 2005. New species of the genus *Chondrostoma* Agassiz, 1832 (Actynopterigii, Cyprinidae) from western Portugal. Graellsia 61, 19-29.

5 - Robalo, J., Doadrio, I., Almada, V., Kottelat, M., 2005. *Chondrostoma oligolepis*, new replacement name for *Leuciscus macrolepidotus* Steindachner, 1866 (Teleostei: Cyprinidae). Ichthyol. Explor. Freshwaters 16, 47-48.

6 - Robalo, J., Santos, C., Almada, V., Doadrio, I., 2006. Paleobiogeography of two Iberian endemic cyprinid Fishes (*Chondrostoma arcasii - Chondrostoma macrolepidotus*) inferred from mitochondrial DNA sequence data. J. Hered. 97, 143-149.

7 - Robalo, J., Sousa-Santos, C., Levy, A., Almada, V.C., 2006. Molecular insights on the taxonomic position of the paternal ancestor of the *Squalius alburnoides* hybridogenetic complex. Mol. Phylogenet. Evol. 39, 276-281.

8 - Robalo, J., Almada, V. , Levy, A., Doadrio, I., 2007. Re-examination and phylogeny of the genus *Chondrostoma* based on mitochondrial and nuclear data and the definition of 5 new genera. Mol. Phylogenet. Evol. 42, 362-372.

9 - Robalo, J., Doadrio, I., Valente, A., Almada, V., no prelo. Identification of ESUs in the Critically Endangered Portuguese minnow *Chondrostoma lusitanicum* Collares-Pereira 1980, based on a phylogeographical analysis. Conserv. Genet. Online first. DOI: 10.1007/s10592-006-9275-x.

10 - Vinyoles, D., Robalo, J. I., de Sostoa, A., Almodóvar, A., Elvira, B., Nicola, G. G., Fernández-Delgado, C., Santos, C. S., Doadrio, I., Sardà-Palomera, F., Almada, V.C. , no prelo. Spread of the alien bleak *Alburnus alburnus* (L., 1758) in the Iberian Peninsula: the role of reservoirs. Graellsia.

11 - Robalo, J., Doadrio, I., Valente, A, Almada, V., no prelo. Insights on speciation patterns in the genus *Iberochondrostoma* (Cyprinidae): evidence from mitochondrial and nuclear data. Mol. Phylogenet. Evol.

Parte II

Questões filogenéticas e taxonómicas

Capítulo 2

New species of the genus *Chondrostoma* Agassiz, 1832 (Actynopterigii, Cyprinidae) from western Portugal

2.1 Abstract

A new cyprinid species endemic to western Portugal, *Chondrostoma occidentale* sp. n., is described based on fish collected in the small costal drainages of the Rivers Alcabrichel, Sizandro and Safarujo. The new species belongs to a monophyletic clade that also includes *C. oligolepis* and *C. arcasii*, with which it shares many molecular and morphological features. However, it can be distinguished from these two *Chondrostoma* species by a combination of the following characters: 40-43 ($\bar{x} = 40.9$) canaliculate scales on the lateral line; 6-7 ($\bar{x} = 6.9$) above the lateral line; 2-3 ($\bar{x} = 2.9$) scales below the lateral line. The axilary pelvic scale is smaller than or at most equal to the base of the pelvic fin. The coronoid process of the dentary is very thick. The minimum divergence distances in cytochrome *b* between *C. occidentale* and *C. oligolepis* and *C. arcasii* was “p” = 4.8-5.9%. This new species should be considered Critically Endangered (CR) according to the IUCN Red List Categories.

2.2. Introduction

The Iberian Peninsula harbours a very diverse cyprinid fauna with most species being endemic (Doadrio, 2001). A substantial proportion of this diversity corresponds to fish of the genus *Chondrostoma* (sensu Zardoya and Doadrio, 1999) with at least 11 described endemic species (Doadrio and Carmona, 2003) and some others awaiting formal description (e.g. Carmona et al., 2000; Mesquita et al., 2001). A monophyletic clade has been recognized for a long time in this genus (Zardoya and Doadrio, 1998; Doadrio, 2001) comprising two species formerly included in the genus *Rutilus* Rafinesque, 1820, *Chondrostoma arcasii* (Steindachner, 1866) and *Chondrostoma macrolepidotum* (Steindachner, 1866) (the name of this species was preoccupied by *Leuciscus macrolepidotus* Ayres 1854 and a new replacement name has been provided for *C. macrolepidotum* (Steindachner, 1866) by Robalo et al., 2005 as *Chondrostoma oligolepis* Robalo, Doadrio, Almada and Kottelat, 2005). These two species have proved difficult to distinguish due to some overlap in many morphometric and meristic characters and have been the subject of several comparative studies (Casado, 1995; Collares-Pereira, 1979, 1983; Zardoya and Doadrio, 1998). Collares-Pereira (1983) showed that *C. arcasii* is almost absent in Portugal, excepting perhaps the basin of the Miño River and some populations in the Távora River, a tributary of the left margin of the Duero basin. Thus, according to Collares-Pereira (1983) the distribution of *C. oligolepis*, ranges from the basin of the Limia River (in north-western Portugal) south to the Alcoa River (located between the cities of Leiria and Lisbon). This species is also present in the Nabão River (Tagus basin). However, subsequent field work showed that *C. oligolepis* is also present to the south of the Alcoa River, in the small independent coastal drainages of Real and Alcabrichel Rivers (Diogo, 1999).

Chondrostoma arcasii is mainly distributed in Spain, ranging from the basin of the River Ulla in the north-western area of the Iberian Peninsula (Galicia) eastwards to the basin of the River Júcar in the Mediterranean slope, and being present in the large basins of the Duero, Tagus and Ebro (Doadrio, 2001).

In a survey of the clade *C. arcasii* – *C. oligolepis* encompassing all its geographical distribution in Portugal and Spain, the authors of the present work (Robalo et al., in press) could confirm with molecular data (sequences of the mitochondrial cytochrome *b* gene) that *C. oligolepis* ranges from the River Limia south to the River Tornada. However, to the south of this area, in the small rivers Alcabrichel, Sizandro and Safarujo, the same authors found populations of the genus *Chondrostoma*, belonging to the *oligolepis* - *arcasii* clade, in which the cytochrome *b* sequences were very divergent from the previously known species. Doadrio and Carmona (2004) published evidence that corroborates this conclusion.

In this paper, we present evidence showing that the features of these populations warrant their inclusion in a new species and give its formal description.

2.3 Material and Methods

For morphometric purposes we studied the following material of *C. oligolepis*, belonging to the collection of UIE/ISPA: 18 individuals from Lima basin, 15 individuals from Mondego basin, 20 individuals from Tornada basin and 17 individuals from Alcoa basin. Syntipes from *C. oligolepis*, deposited in the collections of Naturhistorischen Museum Wien (NMW 49815: 1-4, although only three syntypes are mentioned by Steindachner in the description), were also analysed. As far as *C. arcasii* is concerned, 23 individuals from the River Adaja in the Duero basin (Spain) were studied from the collections of Museo Nacional de Ciencias Naturales (MNCN 156860-157023). The material of *Chondrostoma* nov sp included a total of 60 adults: 48 from the Sizandro basin and 12 from the Safarujo basin. Although the genetic comparisons showed that the population of Alcabrichel belongs to the same monophyletic clade that includes the populations of Sizandro and Safarujo (Doadrio and Carmona, 2004; Robalo et al., in press) we didn't find preserved specimens from Alcabrichel suitable for morphometric and meristic analysis.

From this material we selected 24 individuals as type series, deposited in Museo Nacional de Ciencias Naturales (MNCN), Madrid, Spain. Additional material is deposited in Instituto Superior de Psicología Aplicada (ISPA), Lisboa, and in Museu Nacional de Historia Natural, Lisboa, Portugal.

The diagnostic and description are based on the material of the type series. For a schematic representation of the locations of the drainages from which fish were analysed see Fig. 2.1.

The morphological comparisons involved only fish of the new species and *C. oligolepis* and *C. arcasii*, because previous genetic studies (Doadrio and Carmona, 2004; Robalo et al., in press) had shown that they belong to a very well supported monophyletic clade. In turn, Zardoya and Doadrio (1998, 1999) had shown, with genetic data, that this clade, although lacking the horny blade on the lower lip characteristic of the genus

Chondrostoma (as defined by Elvira, 1987, 1997), must be included in this genus, which emerges as a very robust monophyletic group.

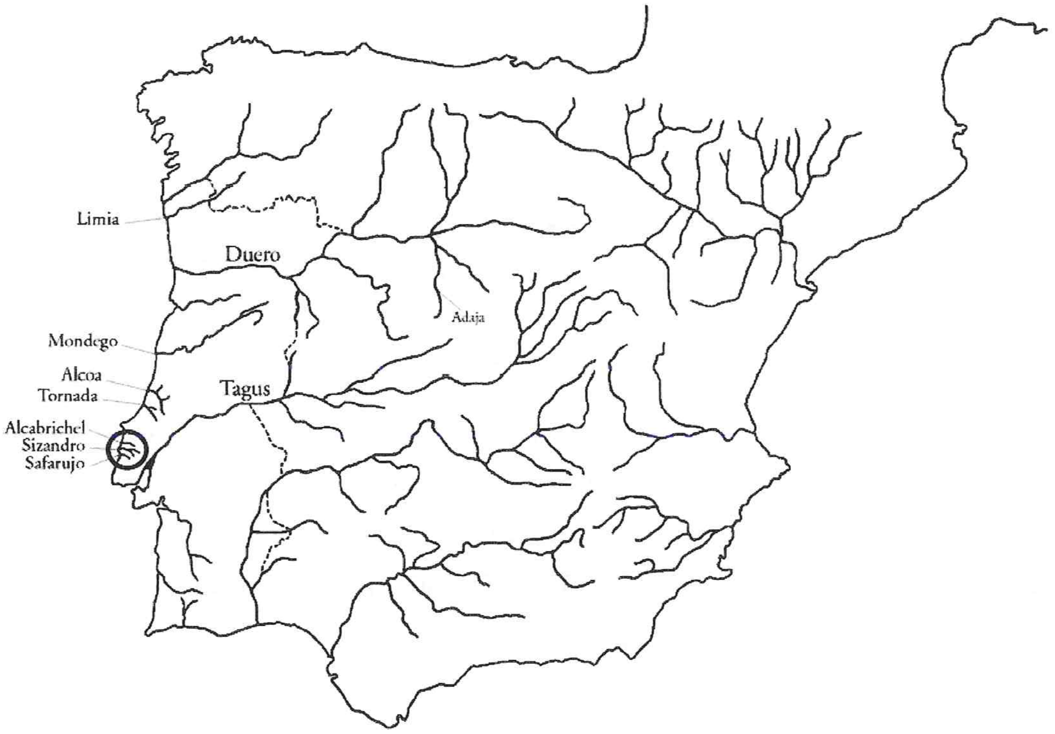

Fig. 2.1 Schematic map of the Iberian Peninsula indicating the locations of the drainages mentioned in the text.

This finding prompted the transfer of these species from the genus *Rutilus* to the genus *Chondrostoma*. Thus, we limited our comparisons to *C. oligolepis* and *C. arcasii* because they are the species of *Chondrostoma* that are genetically and morphologically closest to the new species and the ones from which its differentiation had to be demonstrated.

For genetic purposes we studied 6 individuals of *Chondrostoma* nov sp and 8 individuals of *C. oligolepis*. As the northern limit of the distribution of *C. lusitanicum* Collares-Pereira, 1980 is located a few kilometres to the south of the River Safarujo (Collares-Pereira, 1983; Costa Pereira, 1995) we decided to include three samples of *C. lusitanicum* from the rivers nearest to our study area, to avoid possible confusion between our fish and *C. lusitanicum*, another small bodied member of the genus *Chondrostoma*, that also lacks the horny blade in the lower lip.

For osteological purposes we studied the following material: *C. arcasii*: 18 individuals from Queiles River (Ebro Basin), 3 individuals Abion River (Duero Basin), 3 individuals Pedro River (Duero Basin), 1 individual Ontigola Lagoon (Tajo Basin), 1 individual Gritos River (Júcar Basin). *Chondrostoma oligolepis*: 2 individuals Alcoa River (Alcoa Basin), 2 individuals Mondego River (Mondego Basin). *Chondrostoma* nov. sp.: 1 individual Safarujo River (Safarujo Basin), 2 individuals Sizandro River (Sizandro Basin).

Data Collection

All fish of *Chondrostoma* nov sp were collected for the present study. Part of the material was collected by electrofishing. To enable us to use live fish, animals where anesthetized with a weak solution of MS222. They were placed on a white surface and lateral views of the specimens where taken with a SONY DSC-S75 digital camera. A small piece of fin was clipped off for genetic analysis and the basic meristic characters were determined. After being placed in clean water more than 99% of the fish fully recovered. To allow direct comparison between live and preserved specimens the fish from the MNCN where photographed with the same camera in lateral view.

Morphology

Meristics - The following meristic characters were compared: canaliculate scales of the lateral line (SLL), scales of the upper transverse row (SUTR), scales of the lower transverse row (SLTR), branched dorsal fin rays (DR) and branched anal fin rays (AR). In addition, the following characters were analysed in the type series used to describe the new species: pectoral fin rays (PR), ventral fin rays (VR), pharyngeal teeth (PT), gill rackers (GR), abdominal and caudal vertebrae.

Morphometry - Morphometric data were collected from the digital pictures using the software package IMAGE tool (UTHSCSA V. 3.00), using the standard length (SL) of the fish taken at the time of photographing to calibrate all measurements.

The pictures that yielded doubtful measurements, either because a preserved specimen was deformed or because the picture had poor quality were discarded.

Fourteen morphometric variables were analysed.

The following abbreviations were used for these morphometric characters: SL, standard length; HL, head length; HH, head height; PrOL, preorbital length; ED, eye diameter; PsOL, postorbital length; DOP, distance measured on a vertical line from the upper edge of the orbit to the upper limit of the head; PrDD, predorsal distance; PrVD, preventral distance; PrAD, preanal distance; MHCP, minimum height of the caudal peduncle; BDP, body depth at the level of the insertion of pectoral fins; BDA, body depth at the level of the insertion of anal fins and HA, angle centred at the tip of the snout formed by the straight lines that go from there to the highest and lowest points of the head. The relative size of the axilary pelvic scale was qualitatively evaluated whenever possible. In addition, the following measurements were also performed on the type material: PrPD, prepectoral distance; CPL, caudal peduncle length; APL, anal peduncle length; PVL, pectoral-ventral length; VAL, ventral-anal length; DFL, dorsal fin length; DFH, dorsal fin height; PFL, pectoral fin length; VFL, ventral fin length; AFL, anal fin length; AFH, anal fin height; CFL, caudal fin length; BD body depth.

Differences in body shape between populations were analysed using discriminant analysis. Since indices may not vary linearly with size, causing differences of size to yield false differences between populations, we decided to use as raw data residuals of log-log regressions of the variables, using the standard length (SL) of the fish as the independent variable (Oliveira and Almada, 1995). HA was not subjected to this transformation since it is an angular measure. Residuals of the regressions were used to perform a discriminant analysis, in which the new species was compared to *C. arcasii* and *C. oligolepis*. A discriminant analysis was also performed separately on the meristic data. As the discriminant analysis depended on residuals of relationships of the morphometric characters with standard length, some proportions important to the characterization of the shape of the fish were not captured and were compared separately. These comparisons were performed using Mann-Whitney U Test and adopting a significance level of $p<0.05$. To compensate for the performance of multiple comparisons, Bonferroni corrections were applied to all analyses. All these statistical procedures were performed using STATISTICA (V. 5.5, StatSoft, 1996).

Osteology

The osteological characters were studied from cleared and stained specimens (Wassersug, 1976).

Genetics

Total genomic DNA was extracted from a piece of fin clip or muscle by the standard proteinase K and phenol/chloroform extraction method (Sambrook et al., 1989). A total of 1140 bp of the cytochrome b gene was amplified using the primers presented by Machordom and Doadrio (2001). Details of DNA extraction, PCR and sequencing may be requested from the authors. The details of the procedures and the results of the molecular analyses will be published elsewhere (Robalo et al., in press).

After alignment with Clustal X (Thompson et al., 1997), distances between samples were computed with PAUP* 4.0 (Swofford, 1998). AMOVA was performed using Arlequin version 2.0 (Schneider et al., 2000) in order to compare the samples of the fishes from Alcabrichel, Sizandro and Safarujo Rivers with *C. oligolepis*, the only member of the clade that occurs in the vicinity of these drainages.

2.4 Results

The discriminant analysis of the meristic data yielded highly significant results (Wilks' Lambda = 0.11; $F_{(8, 294)} = 73.53$; $p<0.00001$). 85% of *Chondrostoma* nov sp where correctly classified, with only 5 misclassifications involving *C. oligolepis* and *Chondrostoma* nov sp, 4 misclassifications between this group and *C. arcasii* and none between *C. oligolepis* and *C. arcasii*.

Discriminant analysis of the morphometric data also yielded robust results (Wilks' Lambda = 0.17; $F_{(30.206)} = 9.82$; $p<0.00001$). 95% of *Chondrostoma* nov sp where correctly classified, with 2 misclassifications between *C. oligolepis* and *Chondrostoma* nov sp, 1 misclassification between *Chondrostoma* nov sp and *C. arcasii* and none between *C. oligolepis* and *C. arcasii*.

Concerning genetic comparisons, the within group average p distance was 0.27% for *C. lusitanicum* (n = 3), 0.45% for *C. oligolepis* (n = 8) and 0.28% for *Chondrostoma* nov sp (n = 6). *C. occidentale* had a between group distance of 5.83% with *C. oligolepis*, which is of the same order than that between many widely recognized pairs of cyprinid fish species (Zardoya and Doadrio, 1998, 1999). The distance between *Chondrostoma* nov sp and *C. arcasii* is of similar order than that between *C. occidentale* and *C. oligolepis*. This distance is even greater than that between *C. arcasii* and *C. oligolepis* (Robalo et al., submitted). The distance of *Chondrostoma* nov sp to *C. lusitanicum* is much greater, 9.78%.

The results of AMOVA were highly significant and showed that the variation between *C. oligolepis* and *Chondrostoma* nov sp was much higher than that within groups (95.19% and 4.81%, respectively, FST=0.95, p<0.00001). In addition, not a single haplotype was shared between the two groups.

***Chondrostoma occidentale*, new species (Fig. 2.2)**

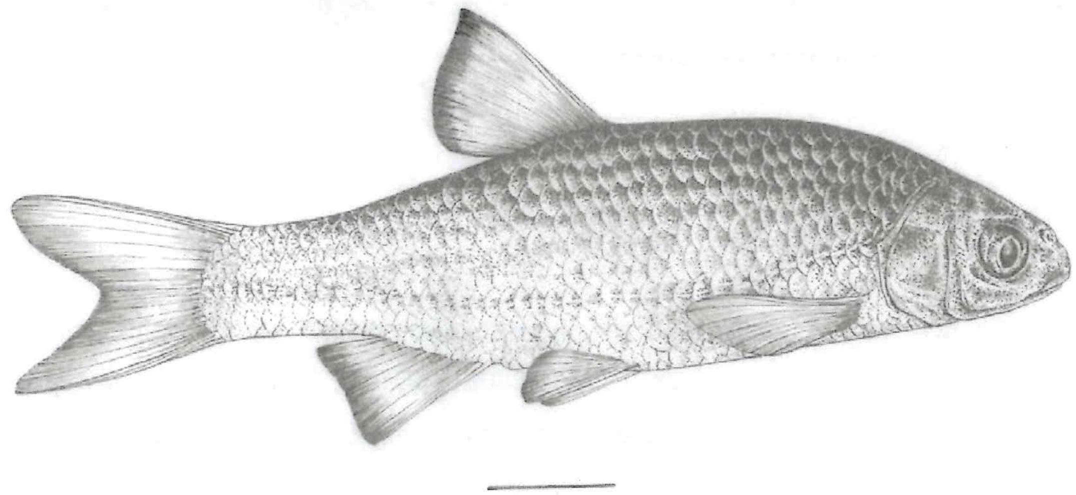

Fig. 2.2 *Chondrostoma occidentale* sp. nov. Holotype. MNCN244125. 72.5 mm SL. Safarujo River, Mafra, Safarujo basin, Portugal.

HOLOTYPE: MNCN244125. 72.5 mm SL. Safarujo River, Mafra, Safarujo basin, Portugal. Leg. V. Almada, J. Robalo, C. Santos. 9.X.2002.

PARATYPES: MNCN244123, 1 individual Safarujo River. Mafra, Safarujo basin, Portugal. Leg. V. Almada, J. Robalo, C. Santos. 9.X.2002. MNCN244127-128, 2 individuals Sizandro River, Torres Vedras, Sizandro Basin, Portugal. Leg. V. Almada, J. Robalo, C. Santos. 9.X.2002. MCN246658-677, 20 individuals, Sizandro River, Torres Vedras, Sizandro Basin, Portugal. Leg.V. Almada, I. Doadrio, P. Garzón, J. Robalo, C. Santos.

Diagnosis

Chondrostoma occidentale is a member of the *C. arcasii* - *C. oligolepis* species group. This group of arched mouth fish lacks the horny blade on the lower lip and has a lateral line scale which is much lower than other bladeless species of *Chondrostoma* (<45 on lateral line). *Chondrostoma occidentale* differs from *C. arcasii* and *C. oligolepis* by the following combination of characters: 40-43 ($\bar{x}$ = 40.9) canaliculate scales on the lateral line; 6-7 scales ($\bar{x}$ = 6.9) above the lateral line; 2-3 ($\bar{x}$ = 2.9) scales below the lateral line (see Table 2.1). Axillary pelvic scale shorter than or equal to the insertion of the pelvic fin. The coronoid process of the dentary is very thick (Fig. 2.3). The minimum divergence distances in cytochrome *b* between *C. occidentale* and the remaining members of the group was "p" = 4.8-5.9%. The body proportions that differed significantly in *C. occidentale*, either from *C. oligolepis* and/or *C. arcasii* are presented in Table 2.2.

Description

DR III $\bar{x}$ = 6.9 ± 0.3 (7-8), AR III $\bar{x}$= 6.9 ± 0.3 (6-7), PR I 12-13, VR I 7-8, SLL $\bar{x}$= 40.9 ± 0.9 (40-43), SUTR $\bar{x}$= 6.9 ± 0.37 (6-7), SLTR $\bar{x}$= 2.29 ± 0.30 (2-3), PT 5-5, GR $\bar{x}$= 13.6 ± 0.4 (12-14), 20-21 abdominal vertebrae, 17 caudal vertebrae. Morphometric and meristic characters of the type material are given in Table 1. A small sized species that rarely reaches 100 mm of standard length.

In Table 2.2 the meristic and morphometric characters of *C. occidentale* are contrasted with those of *C. oligolepis* and *C. arcasii* to help the characterization of the new species. In this table, information on *C. occidentale* includes that of the type series and all other specimens analysed.

When we state that a given count or ratio for *C. occidentale* is greater or smaller than in other species we are only considering data that are statistically significant. Although there is some overlap in the meristic characters between *C. occidentale* and the two other species compared in this study, all scale counts are significantly different, both between *C. occidentale* and *C. oligolepis*, and between *C. occidentale* and *C. arcasii*.

The body is elongated and moderately compressed. In terms of body shape *C. occidentale* is, in many respects, intermediate between *C. oligolepis* and *C. arcasii* (Table 2.2). It differs from *C. oligolepis*, the species that is geographically closer, in the following characters: *C. occidentale* has a more elongated body, a feature that is expressed by the ratio BDP/SL. The head is lower and more elongated. The profile of the head and snout of *C. occidentale* tends to differ from that of *C. oligolepis* as expressed by the angle centred on the tip of the snout and formed by the straight lines that go from there to the highest and lowest points on the head (HA). This angle in *C. occidentale* is usually smaller than in *C. oligolepis* and it is even smaller in *C. arcasii*. The eyes tend to be located higher on the head. They are also located more posteriorly than in *C. oligolepis* and in *C. arcasii*, as shown by the ratio ED/PrOL. As the ratio ED/SL did not differ significantly between *C. occidentale* and *C. oligolepis* (see Table 2.2) the relationships considered above are not artificially caused by differences in eye diameter.

Taken together, these proportions mean that *C. occidentale* has as more pointed and long snout than *C. oligolepis*. The height of the caudal peduncle is contained 8.61-11.41 ($\bar{x}$ = 9.78) times in the standard length. The caudal peduncle is proportionately and significantly narrower than in *C. oligolepis*. In *C. occidentale*, the insertion of the anal fin is located more posteriorly than in *C. oligolepis*.

Pigmentation pattern

The general pattern of coloration in *C. occidentale* is, broadly speaking, similar to those of *C. oligolepis* and *C. arcasii*. In the three species, the body is darker above the lateral line than bellow, a yellow iridescent band runs along the flanks and there is reddish pigmentation at the base of all fins, especially on the paired ones.

In *C. occidentale* there are, however, details of the colour pattern that distinguish it from *C. oligolepis* and *C. arcasii*. The upper part of the body is much lighter than in *C. oligolepis*, *C. arcasii* showing the darkest pattern. The pigmentation along the lateral line in

C. occidentale is less conspicuous than in the remaining species of the group and the pigmentation at the base of the fins is lighter and more orange than red. Below the lateral line, the body is brighter, presenting a silvery hue.

Table 2.1 Number of individuals, Means, Ranges and Standard deviations of the meristic and morphometric variables for *C. occidentale* paratypes and holotype. For abbreviations see the Material and Methods section.

***Chondrostoma occidentale* nov.sp.**

Variable	**Holotype**	**Paratypes (n=23)**		
		Mean	Range	SD
SLL	42	40.9	40-43	0.9
SUTR	7	6.9	6-7	0.3
SLTR	3	2.9	2-3	0.3
DR	7	6.9	6-7	0.3
AR	7	6.9	6-7	0.3
SL	72.5	66.2	44.8-93.4	14.0
HL	17.5	15.7	11.5-22.1	2.9
PrOL	5	4.7	3.4-7	0.9
ED	5.1	4.2	3.2-5.3	0.6
PsOL	8.5	7.3	5.3-10.5	1.4
ID	8.5	5.9	3.7-8.3	1.1
HH	13.7	11.3	7.3-15.7	2.2
PrDD	40.2	35.1	22.6-52.2	8.1
PrPD	17.7	16.7	11.5-23	3.3
PrVD	35.3	32.9	22-47.2	6.8
PrAD	48.4	45.3	30.1-67	10.1
CPL	27.7	26.1	16.9-39.7	6.0
APL	15.3	15.0	9.8-22.7	3.4
PVL	16.5	15.1	9.7-21.5	3.2
VAL	11.2	10.7	6.8-15.8	2.7
DFL	9.1	7.1	5.1-10.4	1.3
DFH	15	12.6	8.9-16.5	2.1
PFL	15.3	12.6	8.5-17.1	2.5
VFL	12.9	10.7	6.6-14.3	2.5
AFL	8.6	6.9	4.3-9.1	1.3
AFH	11.7	11.0	7.3-15.2	2.5
CFL	15	12.5	8.2-17.9	2.5
BD	20	16.2	10.1-25	3.7
MHCP	7.8	7.2	4.8-9.9	1.5

Table 2.2 Number of individuals, Means, Ranges and Standard deviations of the meristic variables and morphometric indices that yielded significant differences between *Chondrostoma occidentale* and *C. oligolepis* and between *C. occidentale* and *C. arcasii.* Signs of > and < in *C. occidentale* cells are oriented towards the species with which the corresponding measure has yielded a significant difference and represent the relation between these values. Only comparisons that yielded p<0.05 after Bonferroni corrections were included. The ratio ED/SL was also included although there were no significant differences in comparisons of this ratio. It was kept in the table because if not properly controlled for this measurement other ratios could have become artificially biased. For abbreviations see the Material and Methods section.

	C. oligolepis					*C. occidentale*					*C. arcasii*			
	N	**Mean**	**Range**	**Std.Dev.**		**N**	**Mean**	**Range**	**Std.Dev.**		**N**	**Mean**	**Range**	**Std.Dev.**
SLL	70	38.10	35-41	1.44	<	59	42.68	39-47	1.52	<	23	44.83	43-48	1.59
SUTR	70	6.94	6-8	0.42	<	60	7.19	6.5-8	0.37	<	22	7.50	7-8.5	0.49
SLTR	70	2.89	2-3.5	0.28	<	60	3.21	3-4	0.31	<	23	4.28	4-5	0.29
DR	69	7.17	6-8	0.42	>	60	7.02	7-8	0.13		23	7.00	7	0.00
AR	69	7.42	7-8	0.50	>	60	7.10	6-8	0.35	>	23	6.78	6-7	0.42
ED/PROL	52	1.25	0.63-1.73	0.21	>	51	1.13	0.85-1.39	0.12	<	12	1.25	1.08-1.45	0.13
HH/SL	52	0.19	0.17-0.21	0.01	>	51	0.18	0.15-0.22	0.01		12	0.18	0.17-0.19	0.00
PrOL/SL	52	0.06	0.04-0.07	0.01		51	0.06	0.04-0.07	0.01	>	12	0.05	0.04-0.06	0.01
ED/SL	52	0.07	0.04-0.08	0.01		51	0.07	0.05-0.08	0.01		12	0.06	0.05-0.07	0.01
PsOL/SL	52	0.11	0.08-0.13	0.01		51	0.11	0.04-0.16	0.01	<	12	0.12	0.11-0.13	0.01
DOP/SL	52	0.03	0.02-0.05	0.01	>	49	0.02	0.01-0.04	0.01		12	0.02	0-01-0.03	0.01
MHCP/SL	52	0,11	0.09-0.13	0.01	>	51	0.10	0.09-0.12	0.01		12	0.10	0.09-0.11	0.01
BDP/SL	39	0.22	0.20-0.25	0.01	>	29	0.21	0.19-0.23	0.01		12	0.21	0.20-0.23	0.01
BDA/SL	52	0.18	0.16-0.20	0.01		51	0.18	0.16-0.22	0.01	>	12	0.16	0.14-0.18	0.01
HH/HL	52	0.83	0.72-0.99	0.05	>	51	0.79	0.63-1.00	0.06		12	0.77	0.69-0.85	0.05
PrOL/HL	52	0.24	0.19-0.35	0.03		51	0.25	0.20-0.37	0.03	<	12	0.22	0.19-0.25	0.02
ED/HL	52	0.30	0.22-0.35	0.02	>	51	0.28	0.21-0.38	0.03		12	0.27	0.24-0.30	0.02
DOP/HL	52	0.14	0,07-0,19	0.03	>	49	0.10	0.05-0.16	0.03	>	12	0.07	0.03-0.11	0.02
HH/BDP	39	0.87	0,79-0.95	0.04	>	29	0.84	0.76-0.90	0.04		12	0.84	0.77-0.87	0.03
PrAD/SL	52	0.69	0,63-0,74	0.02	<	51	0.70	0.62-0.73	0.02		12	0.70	0.68-0.75	0.02
HÁ	53	59.09	52,24-63,58	2.99	>	55	55.82	47.53-60.46	3.31		12	56.20	52.95-60.11	1.97

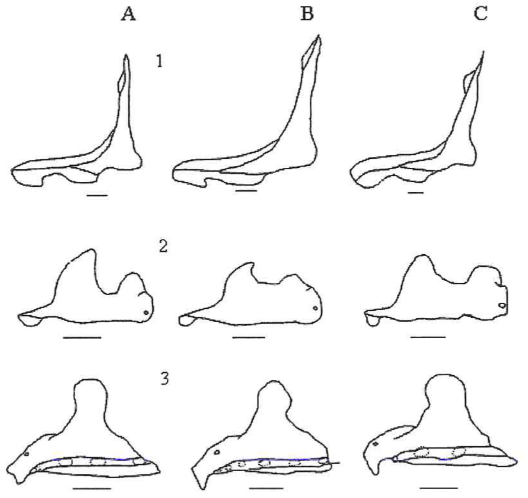

Fig. 2.3 Osteological characters of the cleithrum (1), maxilla (2) and dentary (3) of *Chondrostoma arcasii* (A), *C. oligolepis* (B) and *C. occidentale* sp. n. (C). Scale bar: 1 mm.

Osteology

Concerning osteology the only interspecific differences found are referred below. The superior process of the cleithrum is narrower than in *C. arcasii* and *C. oligolepis*. In the maxilla the small palatine process is similar to that of *C. oligolepis*. As regards the dentary, the coronoid process is considerably thicker than in the other two species. The number of pores of the dentary varies between 2 and 3, being similar to *C. arcasii* (Fig. 2.3).

Etymology

The area of Portugal where this species occurs is known as the Oeste, meaning West in English. The latin name occidentale emphasizes the fact that this species is endemic to this region.

Distribution

The species is endemic to Estremadura, central western Portugal and is restricted to the rivers Sizandro, Safarujo and Alcabrichel.

Common name

Ruivaco do Oeste.

Remarks

The rivers where *C. occidentale* is found are small coastal streams that have a greatly reduced water flow in the dry summer period. In this situation, the fish become concentrated in a few pools where they seek shelter in shady areas under the cover of vegetation.

Aquarium observations (Robalo, unpublished) showed that *C. occidentale* forms spawning aggregations shedding eggs near stones and vegetation. The eggs are adhesive. Breeding occurs in spring, especially in late April and May, when ripe fish were also collected in the field.

Chondrostoma occidentale is the only cyprinid fish occurring in these streams. The only fish collected with *C. occidentale* were *Cobitis paludica* and *Anguilla anguilla.*

Conservation

The populations of the rivers where the species occurs are in a critical situation. The drainages receive discharges of large amounts of sewage from pig farms, distilleries and the very intense agriculture of the surrounding area. Several massive fish kills have been documented in recent years. Intensive sampling by electrofishing failed to yield fish in these drainages, except for small isolated pockets located upstream of the major sources of pollution.

A taxon is considered Critically Endangered (CR) according to the IUCN Red List Categories (IUCN, 2001) when the best available evidence indicates that it meets any of the criteria A to E listed in Section V and it is therefore considered to be facing an extremely high risk of extinction in the wild. In our opinion, given the restricted number of known, fragmented and isolated populations, the low numbers of individuals present in each population and all the threats referred to above, this species should be cataloged in category CR following the criteria B1ab (i,ii,iii)+2ab (i,ii,iii).

2. 5 Discussion

The results presented in this paper show that the populations of the rivers Sizandro and Safarujo differ from their northern neighbour *C. oligolepis* in morphology, osteology and in their mitochondrial genome, although each morphological variable taken separately does not allow an absolutely certain discrimination of the fish due to the presence of a few borderline individuals. These populations are still genetically more distant from their southern neighbour, *C. lusitanicum*. Although attempts of calibration of a molecular clock for the cytochrome *b* of cypriniform fish have varied considerably, several authors have agreed in recent years with the estimate of 1.05% divergence per MYA (0.53 per lineage per MYA) (Doadrio and Carmona, 2004; Dowling et al., 2002; Durand et al., 2003). Thus, the distances observed in this study clearly indicate that the populations of the rivers Alcabrichel, Sizandro and Safarujo diverged from *C. oligolepis* 5.55 MYA ago.

The recognition of *C. occidentale* as a new species means that the distribution of *C. oligolepis* ranges from the River Limia, in the north, south to the River Tornada. Concerning the population of the River Alcabrichel, its mitochondrial DNA places it unambiguously with *C. occidentale*. Meristic and morphometric data for this population are urgently needed to clarify its taxonomic status.

2.6 Acknowledgements

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Capítulo 3

Heterozygous indels as useful tools in the reconstruction of DNA sequences and in the assessment of ploidy level and genomic constitution of hybrid organisms

3.1 Abstract

In this paper we describe a simple approach using double peaks in chromatograms generated as artefacts in the vicinity of heterozygous indels, to identify the specific sequences present in individual strands of a given DNA fragment. This method is useful to assign bases in individuals that are heterozygous at multiple sites. In addition, the relative sizes of the double peaks help to determine the ploidy level and the relative contribution of the parental genomes in hybrids. Our interpretation was confirmed with the analysis of artificial mixtures of DNA of two different species. Results were robust with varying PCR and sequencing conditions. The applicability of this method was demonstrated in hybrids of the *Squalius alburnoides* complex and in heterozygotes of *Chondrostoma oligolepis*. Far from being limited to these fish models and the gene where it was tested (beta actin), this sequence reconstruction methodology is expected to have a broader application.

3.2 Introduction

When an organism is heterozygous for several linked sites in a given DNA fragment that is being sequenced, one major problem is to assign the individual bases in each heterozygous position to each of the parental genomes. This problem is found both in intraspecific studies and especially in interspecific hybrids whose parental sequences often differ in many nucleotide positions.The presence of a heterozygous indel in a fragment of nuclear DNA generates a disturbance in the sequencing process characterized by a succession of false double peaks. Bhangale et al. (2005) used the double peaks generated to identify indels in a set of 330 human genes. Once identified, the length of the indel was inferred from the pattern of peaks by performing a pairwise alignment of bases corresponding to the two allelic sequences, obviating the need of having a previous knowledge of both of the sequences involved (Bhangale et al., 2005). In this paper, we explore further potentialities of this approach. First, we show with intra and interspecific data how the two parental haplotypes can be read from the chromatogram if we previously know its characteristic indels. In addition, we evidence how this method can help to determine the ploidy of each hybrid and to access the relative contributions of the parental genomes, constituting a useful alternative tool to quantitative PCR methods.

3.3 Methods

To test this method in interspecific hybrids, we used individuals of the Iberian minnow *Squalius alburnoides* complex. Its hybrid origin resulted from interspecific crosses between *S. pyrenaicus* females (PP) and males from an unknown species (AA), generating 2n = 50, 3n = 75 and 4n =100 hybrid forms (reviewed in Alves et al., 2001) and reconstituted diploid nonhybrids with the nuclear AA genome of the missing paternal ancestor (Alves et al., 2002, Robalo et al., in press), which are morphologically distinct from the diploid hybrid form of the complex. In order to be able to analyse the hybrid nuclear genomes, a total of 31 individuals of the parental species were analysed: 11 *S. pyrenaicus*, nine *S. carolitertii* and 11 diploid nonhybrid *S. alburnoides* (GenBank: AY943863–AY943896). Samples of *S. carolitertii* were used since in the river basins where *S. pyrenaicus* is absent, although the mtDNA found in *S. alburnoides* fish is also *S. pyrenaicus*-like, the complex seems to be maintained by crosses with males of *S. carolitertii* (CC) and by diploid hybrid males (CA) (Cunha et al., 2004; Pala and Coelho, 2005). Samples from 19 hybrids of *S. alburnoides* (eight diploids, 10 triploids and one tetraploid) were used. The ploidy of the hybrids was previously determined by flow cytometry using fresh fin clips, following an adaptation of the method proposed by Lamatsch et al. (2000) (Collares-Pereira, 1985).

To illustrate the applicability of the method in intraspecific studies we sampled 13 individuals of *Chondrostoma oligolepis* (formerly known as *Chondrostoma macrolepidotum*) another Iberian minnow with 2n = 50 (Collares-Pereira, 1985).

Total genomic DNA was extracted from fin clips preserved in ethanol by an SDS/proteinase-k based protocol (adapted from Sambrook et al., 1989). A total of 927 bp of the beta-actin gene was amplified using the primers For-5'-ATGGATGATGAAATTGCCGC-3' and Rev-5'-AGGATCTTCATGAGGTAGTC-3' (Robalo et al., in press). The amplification process was conducted as follows: 35 cycles of [94ºC(30 s),55ºC(40 s) and 72ºC(1 min 30 s)]. Amplification and sequencing of DNA from six diploids and six triploids was repeated using different PCR conditions: 35 cycles of [94ºC(30 s), 42ºC(40 s), 72ºC(1 min 30 s)].The amplified fragment is homologous to a region of the beta-actin gene of *Cyprinus carpio* (GenBank: M24113), between the positions 1622 and 2550, including introns B and C and three exons. Each sample was sequenced in both directions with the same primers used for PCR. Sequences were aligned with BioEdit v.5.0.6.

Ploidy assessment methodology

To test the hypothesis that unbalanced proportions of parental genomes can be detected in non-quantitative PCR products, we produced six groups of artificial hybrids simulating hybrid forms of the *S. alburnoides* complex. Each group included PA, PAA and PPA forms made with mixtures of re-suspended DNA from the same genome donors (previously sequenced and all differentiated by specific point mutations): 9µl of each DNA suspension from the A - and P - genome donors to produce PA hybrids; 6 µl of DNA suspension from the P-genome donor and 12 µl from the A-genome donor for PAA hybrids; and the reversed quantities for PPA hybrids. The contribution of each parental complement to the hybrid genome was quantified by the "measuring method": measuring both overlapping peaks in each position (using ImageTool 2.0 UTHSCSA with a screen resolution of 1024 X 768 pixels) and calculating the ratio "height of peak from P/(height of peak from P + height of peak from A)" (P/P + A ratio). The provenience of the higher overlapping peak was also registered to calculate the percentage of P-peaks that were greater than A-peaks ("P-count")—"count method".

Different DNA concentrations caused by variations in the extraction procedure could be responsible for excesses of one of the genomes. The value of the P/A ratio for each position measured in an artificial diploid was used as a "correction coefficient" for the peak heights measured in the chromatograms of the artificial triploids made with the same genome donors. These corrected P-peak values were then used to calculate the P/P + A ratio in each position. In natural hybrids the P/A correction was unnecessary.

3.4 Results

There was no clear distinction between *S. pyrenaicus* and *S. carolitertii* for the analysed gene segment (one to four mutations between pairs of haplotypes) thus they were here designated as "*S. pyrenaicus / S. carolitertii*" (PP genome). The chromatograms of the parental species showed single peaks, except for one to four single nucleotide polymorphisms for some fish.

All hybrids sequenced showed double peaks in segments that varied between 544 and 667 bp (58.7 and 72.0% of the amplified fragment, respectively), involving the bases we expected to find if we overlapped the parental genomes. We assumed that analysing about a hundred double peaks should provide a sufficient number of distinct points to allow an adequate statistical analysis. Thus, a segment of 176 bp was randomly selected in the double peaks region, containing 118 positions with overlapping peaks (the remaining were single peaks as a result of the addition of equal bases—see Fig. 3.1).

Reconstruction of the parental sequences in a hybrid

In the presence of one heterozygous indel in a fragment of nuclear DNA, the sequencer starts to read two bases in the same position, a situation that generates a pattern of overlapping peaks in the chromatogram. In these regions of double peaks, the bases are out of phase as many positions as the number of bases of the indel (Fig. 3.1a), a condition that will be maintained until a second indel of opposite direction counterbalances the first, which could be many dozens or hundreds of bases downstream from the initial indel. Thus, starting at the position where the first heterozygous indel occurs, it is possible to read the complements involved in the formation of a hybrid genome.

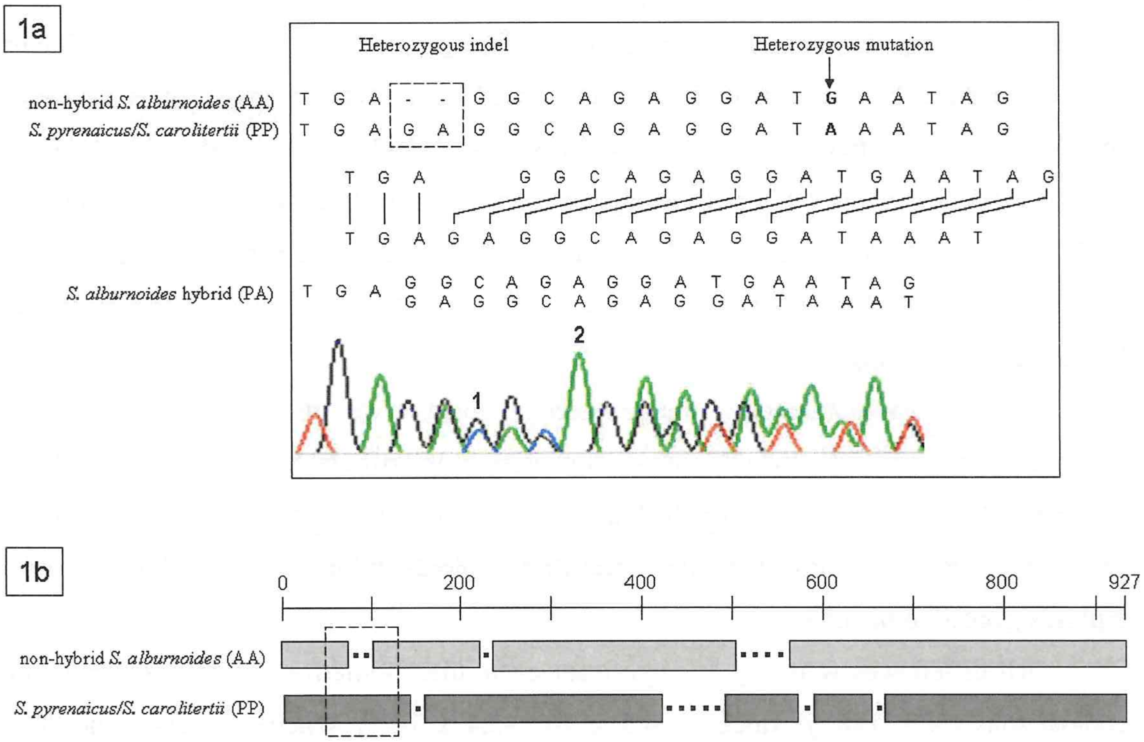


Fig. 3.1(1a) Demonstration of the disturbance generated by the first heterozygous indel in the sequencing process of *a S. alburnoides* individual with P and A complements. When the bases in both alleles are the same. The chromatogram shows a single higher peak reflecting the presence of double quantities of the same base, which means that the sequences can be read even in the homologous regions flanked by overlapping peaks. 1—double peak; 2—single peak resulting from the addition of equal bases. 3 (1b) Schematic representation of the characteristic indels of the parental species in the analysed fragment of 927 bp (deletions are represented by black dots). Indel size ranged from one to five bases (mean = 1.88 ± 1.68). The broken line box indicates the localization of the indel represented in Fig. 1a.

S. pyrenaicus / *S. carolitertii* and nuclear nonhybrid *S. alburnoides* differed by a total of seven indels. The reconstruction of the genomes of the hybrids was possible because each parental species had a characteristic number and location of indels (Fig. 3.1b). It was also favoured by the existence of several point mutations characteristic of each parental species (in homozygous condition) that marked out highly conserved regions and made possible to ascribe unambiguously each peak in a double peaks region to the correct parental genome.

Information on the ploidy and hybrid genome constitution

Since the height of a peak in a chromatogram reflects approximately the amount of a specific base in that position, one can expect that different genome constitutions exhibit chromatograms with different peak heights. Exception is made when there is a suppression of signal at a given position because during the sequencing process the reading of a specific base may be affected by the constitution of the preceding one (for example, it is frequent to observe weak G's after C's or A's—see Hills et al., 1997 for more information on peak patterns). However, this "effect of the adjacent base" affects all the samples in the same way, as demonstrated by a strong correlation of the heights of the peaks between samples (for six samples, Spearman-R ranged between 0.88 and 0.97, for 70 analysed nucleotide positions of a homozygous segment).

After the reconstruction of the genomes involved, it was possible to assess the ploidy level and to determine the hybrid genome constitution. In artificial hybrids we obtained P/P + A mean values of 0.29 ± 0.08 for PAA and 0.70 ± 0.07 for PPA hybrids. For all of the six groups the P/P + A values of the PAA and PPA triploids did not overlap with those of the diploids (forced to be 0.50).

All differences were highly significant even after Bonferroni correction for multiple comparisons (N = 118, *p* values = 4.5×10^{-20}–2.4×10^{-16}, Wilcoxon tests). The "count method" was also applied and the resulting P-count was significantly different: 0.71% ± 0.84 for PAA and 96.32% ± 5.76 for PPA hybrids ($p = 0.0036$, N1 = N2 = 6, Mann- Whitney test).

The data for each group of artificial hybrids are summarized in Table 3.1.

The application of the "count method" to natural hybrids showed that diploids had higher peaks attributable to P-genome (55.77% ± 6.88; range 47.01% – 65.25%), which were significantly different from the 8.13% ± 0.95 (range 6.84%–9.40%) calculated for the PAA triploids ($p = 0.0005$, N1 = 8, N2 = 9, Mann-Whitney test). Among the triploids we found a morphologically distinct individual that had extremely high values of P-count (95.69%), suggesting a PPA genome. The P-count for the single tetraploid (1.69%) was lower than the smallest value for PAA triploids (6.84%), suggesting a PAAA constitution.

Table 3.1 Results from the measuring and count methods applied to the six groups of artificial hybrids. Mean values and standard deviations for P/P + A ratios (both raw and uncorrected values) and P-counts calculated from the analysis of 118 sites are presented for each group. Total means and standard deviations of the P/P þA ratios and P-counts for the three types of artificial hybrids are also presented.

		Measuring method					**Count method**	
		raw P/P+A ratio			**corrected P/P+A ratio**		**P-count (%)**	
group	PA	PAA	PPA	cPA	cPAA	cPPA	PAA	PPA
1	0.45 ±0.13	0.33 ±0.11	0.63 ±0.13	0.50	0.37 ±0.06	0.69 ±0.06	1.74	99.12
2	0.30 ±0.18	0.15 ±0.09	0.48 ±0.21	0.50	0.29 ±0.07	0.70 ±0.11	0.85	97.46
3	0.31 ±0.12	0.18 ±0.08	0.58 ±0.17	0.50	0.32 ±0.04	0.77 ±0.08	0.00	99.15
4	0.47 ±0.12	0.34 ±0.10	0.65 ±0.10	0.50	0.36 ±0.03	0.68 ±0.02	0.00	100.00
5	0.27 ±0.20	0.05 ±0.04	0.58 ±0.27	0.50	0.16 ±0.10	0.79 ±0.13	1.69	97.46
6	0.35 ±0.17	0.16 ±0.11	0.42 ±0.21	0.50	0.26 ±0.06	0.59 ±0.10	0.00	84.75
total mean	0.36	0.20	0.56	0.50	0.29	0.70	0.71	96.32
total sd	0.08	0.11	0.09	0.00	0.08	0.07	0.84	5.76

Sections of chromatograms of the hybrids are shown in Fig. 3.2. The "measuring method" also returned significant differences: mean values of P/P + A for PA and PAA hybrids were, respectively, 0.53 ± 0.01 (range 0.51 – 0.55) and 0.34 ± 0.01 (range 0.33 – 0.36) (p = 0.0005, N1 = 8, N2 = 9, Mann-Whitney test). Values of P/P + A ratio for the morphologically distinct triploid (0.70) and for the tetraploid (0.23) also differed markedly from the remaining fish, corroborating the genomic constitutions suggested by the "count method".

The comparison between the measures taken for the same individuals in two different PCR and sequencing runs demonstrated a repeatable distinction between diploids and triploids. In the second PCR and sequencing of the same material, the mean P/P + A values were still significantly different between diploids and triploids: 0.58 ± 0.03 and 0.39 ± 0.01, respectively (p = 0.0039, N1 = N2 = 6, Mann-Whitney test). The results showed a high correlation between the P/P + A values obtained for the two chromatograms from the same individual for diploids and triploids (Spearman-R ranged between 0.66 and 0.77 and between 0.76 and 0.83, respectively). A comparison of the results obtained when lower numbers of double peaks are analysed showed that all the differences between ploidies were recovered and the values showed only slight deviations from those obtained with the entire data series (Fig. 3.3). Indeed, even groups of 20 overlapping peaks provided estimates that were sufficiently accurate to assign the proportion of each genome in different ploidy groups. This means that even disturbances that are much shorter than the ones used in this study may recover basically the same ploidy information.

Application to intraespecific heterozygotes

Concerning intraspecific heterozygotes, four of the *C. oligolepis* samples sequenced revealed one heterozygous indel of seven bases in the same fragment of the beta-actin gene, generating a double peaks region of 823 bp. The reconstructed parental haplotypes were recovered in the remaining individuals (GenBank: AY943897–AY943905): the strand with the deletion was present in homozygosity in seven individuals (CO1 genome), and the other strand was present in two individuals homozygous for the insertion (CO2 genome). Measures of the peaks in the chromatograms of the heterozygotes yielded a CO1/CO1 + CO2 ratio of 0.58 ± 0.03, a value that is comparable to that obtained for the PA diploids of *S. alburnoides* from the same PCR and sequencing run ($p = 1.000$, N1 = 6, N2 =4, Mann - Whitney test).

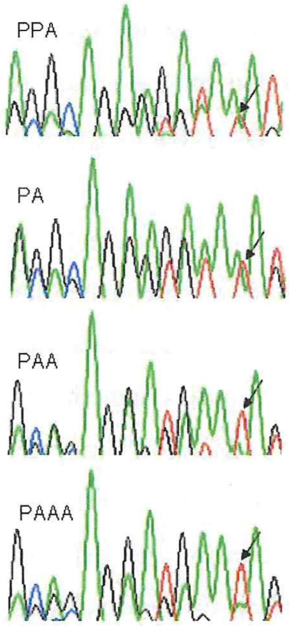
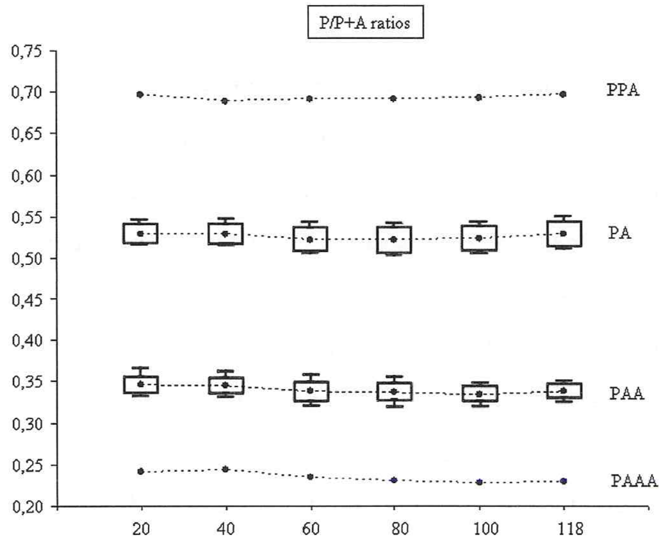


Fig. 3.2 Sections of the chromatograms of *S. alburnoides* natural hybrids where different dosages of A and P genomes are evident. The arrow indicates a base attributed to the non-hybrid *S. alburnoides* progenitor (A genome) that increases its relative height from PPA to PA to PAA to PAAA.



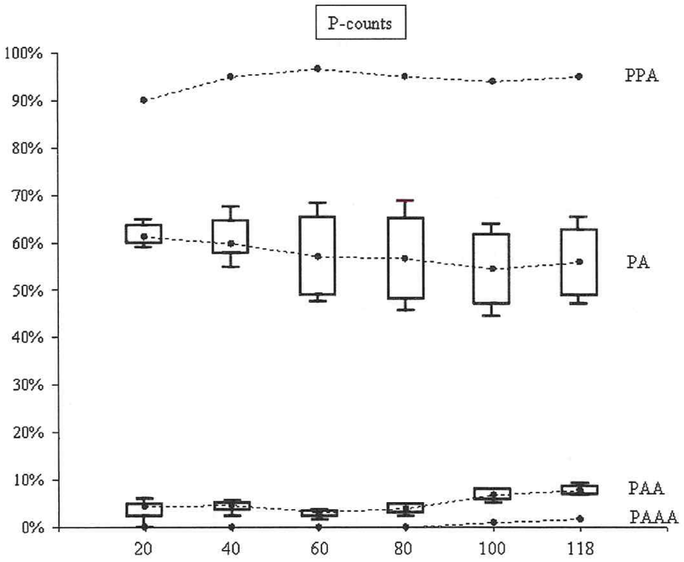


Fig. 3.3 Plot of means, standard deviations, minimums and maximums of the P/P + A ratios and P-counts in PA diploids (N = 8), PAA triploids (N = 9), PPA triploid (N = 1) and PAAA tetraploid (N = 1), as a function of the number of double peaks analysed (20, 40, 60, 80, 100 and 118).

3.5 Discussion

The results demonstrated that the pattern of double peaks generated by heterozygous indels proved to be useful: (i) to reconstruct the parental sequences involved in large DNA segments of individuals that are heterozygotes for several linked sites and of interspecific hybrids; (ii) to determine the ploidy of the hybrids and (iii) to identify the relative contributions of the parental sequences in nondiploid hybrids. The method was repeatable in different PCR and sequencing conditions and the interpretation of ploidy and genomic proportions was always consistent. To control for possible variations we recommend that: in all PCR and sequencing runs at least one diploid hybrid should be included to serve as a standard control to that run; and results should be confirmed with forward and reverse sequencing. One may ask which of the two methods described in this paper is the more accurate. The "count method" is much less time consuming and in the present study discriminated all the genomic constitutions that had been identified with the measuring method. However, we suggest that the "measuring method" should be preferred over the "count method" since it presents the great advantage of controlling for varying PCR artefacts and PCR conditions and to obviate the effects of neighbouring bases on the height of each peak. In addition, the "measuring method" is more reliable and powerful than the "count method" to discriminate different hybrid forms in which on of the parental complements is predominant—for instance, when we want to discriminate between a PAA and a PAAA individual. When compared with allozyme electrophoresis, our method avoids killing the specimens and problems of regulation of gene expression. Although crude when compared with quantitative PCR procedures, it is sufficiently precise and inexpensive to be considered a useful tool in the study of interspecific hybrids and in population genetics. DNA segments that harbour several and closely located indels provide ideal material for the application of this method as they provide several reference points that allow recovery of the specific sequences starting from both directions and minimize the risk of error caused by recombination. The process of sequence reconstruction is also favoured by the existence of several characteristic point mutations fixed for each parental species. These mutations mark out highly conserved regions and make it possible to ascribe unambiguously each peak in a double peaks region to the correct parental genome. Although advantageous, these conditions are not essential and their absence would not necessarily make the method impracticable. In fact, as referred by Bhangale et al. (2005), since the length of the indel is inferred from the pattern of double peaks, the process does not require the presence of homozygotes for both of the alleles in the surveyed sample, a situation that widens its applications.

As flow cytometry methodology can only determine the ploidy level of the samples and not their exact genome composition, mainly when parental species have similar DNA contents, our approach also constitutes a valuable complementary tool for the analysis of hybrids with either balanced or non balanced parental genomes.

3.6 Acknowledgements

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Capítulo 4

Chondrostoma oligolepis, new replacement name for *Leuciscus macrolepidotus* Steindachner, 1866 (Teleostei: Cyprinidae)

Leuciscus macrolepidotus Steindachner, 1866b is a primary junior homonym of *Leuciscus macrolepidotus* Ayres, 1854 and therefore permanently invalid (International Code of Zoological Nomenclature, art. 57.2) and must be replaced. *Leuciscus macrolepidotus* Ayres is a valid species of the genus *Pogonochthys* (see Nelson et al., 2004). We propose *Chondrostoma oligolepis* as a new replacement name for *L. macrolepidotus* Steindachner, 1866b. The new specific epithet is derived from the Greek and means with few scales. It is treated as a noun in apposition.

Chondrostoma oligolepis, known for a long time as *Rutilus macrolepidotus* or *C. macrolepidotus*, was originally described as *Leuciscus macrolepidotus* by Steindachner, (1866b). This description presents a number of problems. It is included at the end of an account of *L. arcasii* Steindachner, 1866a [now *Chondrostoma arcasii*]. At the end of this account, Steindachner (1866b: 10) commented that he had also two specimens from "the creek near Alcobazar" and one specimen from "Cintra" which generally agreed with his *L. arcasii* but had fewer and larger scales along the lateral line (33-36, vs. 40-46) and which he considered as a distinct species, *L. macrolepidotus*. These three specimens are syntypes and were deposited in the collections of Naturhistorisches Museum Wien (NMW). The three syntypes are stored together (NMW 49815) and there is no way to tell which one originates from which locality. The situation is further complicated by the presence of a fourth specimen in NMW 49815, while Steindachner explicitly mentioned only 3 syntypes. Thus, one of the four specimens is not a syntype but there is no way to identify it. Steindachner (1866c: pl. 1

Fig. 4) published a figure of *L. macrolepidotus* in the next part of his report; the caption mentions the locality as "from the creeks near Alcobazar and Cintra (Portugal)". This suggests that the specimens were already mixed at the time the drawing was completed. Steindachner did not indicate the size of the syntypes. The specimens in NMW 49815 are 72, 77, 84, and 91 mm SL. Among these 4 specimens, the only one which is unambiguously identifiable as a syntype is the one figured by Steindachner. Steindachner's plate usually shows specimens in their natural size. The specimen represented on the figure is 91 mm SL and is the 91 mm SL specimen. This specimen is designated as lectotype; it is now catalogued as NMW 49815-2. "Alcobazar" is probably Alcobaça, a locality on River Alcoa. The indication of "Cintra" is problematic. Intensive search in the small rivers around Sintra only yielded *C. lusitanicum* Collares-Pereira, 1980 and we do not know of any record of *C. oligolepis* in the area. Examination of the four specimens in NMW 49815 showed that they unambiguously correspond to our present concept of *C. oligolepis*, with meristic characters that diverge markedly from those of the remaining *Chondrostoma* species present in Portugal. As there is no way to find out the exact locality of the syntypes, strictly speaking the type locality encompasses the two localities listed by Steindachner (Code art. 76.1). As the species is not known from Sintra but is present in Alcobaça, this last locality should be used as type locality for practical purposes.

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Capítulo 5

Molecular insights on the taxonomic position of the paternal ancestor of the *Squalius alburnoides* hybridogenetic complex

5.1 Introduction

Many hybrid fish lineages result from intrageneric crosses and are often asexual, being considered as "evolutionary dead-ends" (Vrijenhoek, 1998). This seems not to be true in the *Squalius alburnoides* (Iberian minnow) Steindachner, 1866 complex. This complex, that seems to have originated in a cross between distant species, has a remarkable variability in ploidy level and genomic composition, includes fertile fish of both sexes and some of its forms retain meiosis and recombination (Alves et al., 2001).

The *S. alburnoides* complex probably originated from crosses between S. pyrenaicus females and males from an unknown species. The complex includes 2n = 50, 3n = 75, and 4n = 100 hybrid forms (reviewed in Alves et al., 2001) with varying proportions of the two parental genomes (denoted by P and A, corresponding to the *S. pyrenaicus* and paternal ancestor genomes, respectively). Reconstituted diploid non-hybrids (AA genome) are also produced and are morphologically distinct from the diploid hybrid form of the complex (PA). These non-hybrid fish, normally males (females seem to be extremely rare), exhibit the nuclear genome of the paternal ancestor (AA) and *S. pyrenaicus*-like mtDNA. In the absence of an identified paternal species this suggests that they are reconstituted from the hybrids (Alves et al., 2002). The oogenesis of triploid females with PAA genomes frequently involves discarding the *Squalius* (P genome), followed by normal meiosis and recombination, generating A gametes. When these gametes fuse with the sperm of AA males, which also undergo normal meioses, new AA male progeny is generated. In the absence of *S. pyrenaicus*, such as in the northern basins of Portugal, this complex seems to be maintained by crosses with males of *S. carolitertii* (CC) and by diploid hybrid males (CA), although the mtDNA found in *S. alburnoides* fish is *S. pyrenaicus*-like (Cunha et al., 2004; Pala and Coelho, 2005).

The Leuciscini presently found in the Iberian Peninsula include mainly species of the genera *Squalius*, *Chondrostoma*, and *Anaecypris*. Studies based on allozymes showed that *S. alburnoides* did not originate from intrageneric crosses between *Squalius* species and also ruled out members of the genera *Chondrostoma* and *Anaecypris* as paternal ancestors (Alves et al., 1997; Carmona et al., 1997). Recent studies still unpublished (Gilles, Dowling, Alves, Coelho, and Collares-Pereira) using introns from two nuclear genes seem to suggest that perhaps A. hispanica-like individuals may represent the paternal ancestor of this complex.

The aim of the present work was to investigate which of the genera considered is phylogenetically closest to the paternal species that originated this complex. This approach is based on the amplification of a segment of the beta-actin nuclear gene from a number of genera closely related to *S. pyrenaicus* and from the non-hybrid males of *S. alburnoides*. The topology obtained with beta-actin was compared to relatively complete phylogenies of the European cyprinids based on cyt *b* (e.g., Briolay et al., 1998; Gilles et al., 1998; Zardoya and Doadrio, 1999) and with our own reconstruction using a set of species comparable to that used with beta-actin.

5.2 Methods

Total genomic DNA was extracted from fin clips preserved in ethanol by an SDS/proteinase-k-based protocol (adapted from Sambrook et al., 1989).

For the beta-actin gene a total of 1062 bp was amplified using the primers For–5'-ATGGATGATGAAATTGCCGC-3' and Rev–5'-AGGATCTTCATGAGGTAGTC-3' (J. Robalo, unpublished). The amplification process was conducted as follows: 35 cycles of 94 °C (30 s), 55 °C (40 s), and 72 °C (1 min 30 s). Further details may be requested from the corresponding author. The amplified fragment is homologous to a region of the beta-actin gene of *Cyprinus carpio* (GenBank Accession No. M24113), including introns B and C and three exons.

For the cyt *b* gene a total of 1044 bp was amplified using the primers LCB1 5'-AATGACTTGAAGAACCACCGT-3' (Brito et al., 1997) and HA–5'-CAACGATCTCCGGTTTACAAGAC-3' (Schmidt and Gold, 1993). PCR conditions followed those in Cunha et al. (2004).

For both genes, each sample was sequenced in both directions with the same primers used for PCR. Sequencing reactions were performed by Macrogen Inc. in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer.

All accession numbers from the present and previous studies are listed in Table 5.1.

Sequences were aligned with BioEdit v.5.0.6.

The aligned sequences were analysed using distance (minimum evolution, ME), maximum likelihood (ML), maximum parsimony (MP), and Bayesian methods. For ME and ML analyses, we performed a hierarchical likelihood ratio test (LRT), using the program ModelTest 3.6 (Posada and Crandall, 1998) to Wnd the model of evolution that best Wtted our data. These phylogenetic analyses were performed using PAUP 4.0 (Swofford, 1998) and MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bootstrap analysis were used to assess the relative robustness of branches of the MP (1000 replicates), the ME (1000 replicates), and the ML (100 replicates) trees. For the Bayesian analysis, cyt b data were partitioned by codon base position, and a GTR+I+Γ model was used for third base positions and a HKY model for first and second base positions. For beta-actin, gaps were coded as separate characters (Simmons and Ochoterena, 2000), using Gap-Coder (Young and Healy, 2003). Data were partitioned and separate models used for each region, thus: GTR+I+Γ model used for third base positions of exon and intron region, HKY model for first and second base positions of exons, and single rate for gap characters. For both genes, four separate analyses were performed (one with four million generations and three with one million generations) with four chains per analysis. The first 50 000 generations were discarded as "burn-in," the remaining generations were sampled every 100 generations, and majority-rule consensus trees were calculated from samples at stationarity.

To compare topologies recovered with both genes, the same species were used in both analyses, except for the genera *Phoxinus* and *Misgurnus* where, for lack of corresponding sequences for both fragments, we used different congeneric species.

Table 5.1 Species considered in this study and their GenBank accession numbers (cyt *b* and beta-actin gene)

Species Name	**GenBank accession number Cyt*b* gene**	**GenBank accession number Beta-actin gene**
Phoxinus lagowskii steindachneri	AB162650	
Misgurnus anguillicaudatus	AF051868	
Misgurnus mizolepis		AF270649
Leuciscus idus	AY026397	DQ061947*
Scardinius erythrophthalmus	AY509848	DQ061949*
Phoxinus oxycephalus		AF200957
Anaecypris hispanica	AJ427814	DQ061936*
Chondrostoma genei	AF533766	DQ061938*
Chondrostoma lemmingii	DQ089654*	DQ061940*
Chondrostoma lusitanicum	AY254584	DQ061941*
Chondrostoma oligolepis	DQ061932*	DQ061942*
Chondrostoma polylepis	AF045982	DQ061945*
Chondrostoma prespensis	AF090747	DQ061944*
Chondrostoma soetta	AY568623	DQ061939*
Chondrostoma turiensis	AY568619	DQ061946*
Telestes souffia	AY509862	DQ061950*
Rutilus rutilus	DQ061933*	DQ061948*
Squalius aradensis	AF421825	DQ061937*
Squalius carolitertii	AF045994	AY943882*
Squalius pyrenaicus	AF421826	AY943882*
Squalius torgalensis	DQ061934*	DQ061937*
Squalius alburnoides		AY943863*
Cyprinus carpio	NC001606	M24113
Gobio gobio	AY426589	DQ061935*

We decided to include a phylogeny for the cyt *b* gene because we could not get beta-actin sequences for all the species used in previous cyt *b* studies. Thus, there was a risk that our reduced data set for beta-actin was not directly comparable to cyt *b* studies with more taxa and different outgroups.

Species from the genus *Misgurnus* (Cobitidae) were used as outgroups.

The sequence of *S. alburnoides* chosen to integrate this paper is the most common haplotype found through out the species area of distribution, both in hybrids and in reconstituted non-hybrid males, and was recovered from 51 fishes out of 103 (unpublished data). All other haplotypes differ from this most common one by few mutational steps that rarely exceed one and are unlikely to affect the results presented. This sequence was found in homozygous condition in many reconstituted non-hybrid males which allowed us to reconstruct it from the chromatograms without ambiguities. As we also obtained homozygous sequences for the maternal ancestor of *S. alburnoides*, *S. pyrenaicus*, we were in a position to identify the overlapping patterns of the two species when they were present in hybrids. In this respect, the beta-actin gene proved a very useful marker because it possesses a combination of three very convenient features: (a) well-preserved exons that provide good landmarks to align the sequences and identify homologous regions; (b) introns that are sufficiently variable to accumulate information useful in phylogenetic reconstructions; and (c) the presence of frequent indels. These indels, when in heterozygous condition, make possible the reconstruction of the two sequences that overlap in the chromatogram of a hybrid fish. Indeed, was recently published a method that permits the recognition and reconstruction of different nuclear DNA sequences in the same chromatogram through the detection of indels (Bhangale et al., 2005) that with some alterations (Sousa Santos et al., in press) makes possible to distinguish copies of the gene of maternal origin (similar to those found in *S. pyrenaicus*) and reconstruct the remaining ones, of paternal origin. For both genes, the saturation of transitions and transversions was checked by plotting the absolute number of changes of each codon position against uncorrected sequence divergence values (p). There was no evidence of saturation in the ingroup (graph not shown).

5.3 Results

Beta-actin gene

Among all the sequences studied, 283 sites were variable and 77 were parsimony informative.

The general time-reversible model with among-site rate heterogeneity HKY+G (HKY, Hasegawa et al., 1985) was selected by ModelTest as the best fit to the data. Base frequencies were A = 0.22, C = 0.24, G = 0.20, and T = 0.34. Among-site rate variation was approximated with the gamma distribution shape parameter .= 0.27. The proportion of invariable sites (I) was 0.7.

MP analysis resulted in a consensus tree of 392 steps (Consistency Index, CI = 0.87; Homoplasy Index, HI = 0.12; Retention Index, RI = 0.70). The results of the four phylogenetic inference methods are summarized in Fig. 5.1.

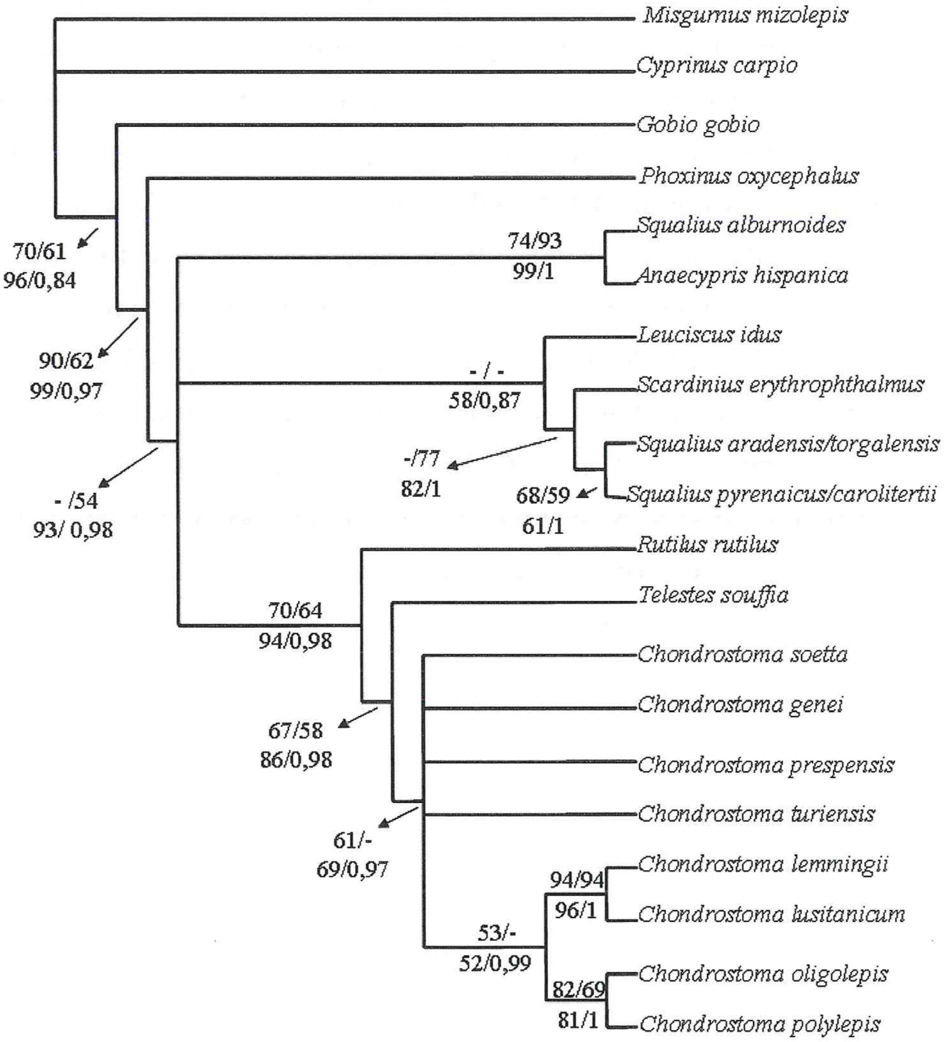

Fig. 5.1 This figure is based on the MP tree. Phylogenetic relationships among the species based on cyt *b* sequences. For each branch, numbers above represent the bootstrap values obtained for ME and ML; numbers below indicate those for MP and the posterior probabilities for Bayesian inference.

Cyt *b* gene

Among all the sequences studied, 445 sites were variable and 363 were parsimony informative.

The general time-reversible model with among-site rate heterogeneity GTR+I+G (Lanave et al., 1984; Yang, 1994) was selected by ModelTest as the best fit to the data. The rate matrix parameters estimated were: R (a) = 1.10, R (b) = 38.6, R (c) = 0.63, R (d) = 1.81, and R (e) = 1.0. Base frequencies were A = 0.29, C = 0.31, G = 0.11, and T = 0.28. Among-site rate variation was approximated with the gamma distribution shape parameter . = 1.02. The proportion of invariable sites (I) was 0.52. MP analysis resulted in a consensus tree of 1596 steps (CI = 0.43; Homoplasy Index, HI = 0.57; Retention Index, RI = 0.41). The results of the four phylogenetic inference methods are summarized in Fig.5. 2.

Phylogenetic reconstructions with all inference methods show some important congruent features for both genes (Figs. 5.1 and 5.2) and for the most part agreed with the findings of Briolay et al. (1998), Gilles et al. (1998), and Zardoya and Doadrio (1998, 1999). The division between the subfamilies Cyprininae and Leuciscinae was recovered with Cyprininae in a basal position. *Gobio gobio* is more closely related to Leuciscinae than to Cyprininae. Phoxinins and leuciscins emerge in a single clade, although in the present work the position of the *Phoxinu*s species varied according with the reconstruction method.
The beta-actin gene also confirms the polyphyly of the old genus *Leuciscus*, *Telestes* (= *Leuciscus*) *souffia* being undoubtedly related with the genus *Chondrostoma*. *Leuciscus idus* remains in an unresolved situation, at least with the taxa included in this analysis. The Iberian *Squalius* remain divided in the two groups: *S. aradensis–S. torgalensis* and *S. pyrenaicus – S. carolitertii*.

In the monophyletic genus *Chondrostoma* the beta-actin gene did not resolve the polytomy found in the previous cyt *b* studies of Doadrio and Carmona (2004), Durand et al. (2003), and Zardoya and Doadrio (1999).

Trees derived from the two genes disagree in the placement of *Scardinius erythrophtalmus.*

In the beta-actin tree, *S. alburnoides* is, according with all methods, strongly associated with *Anaecypris hispanica* (in the cyt *b* tree it is not present because its mitochondrial DNA is *Squalius*-like).

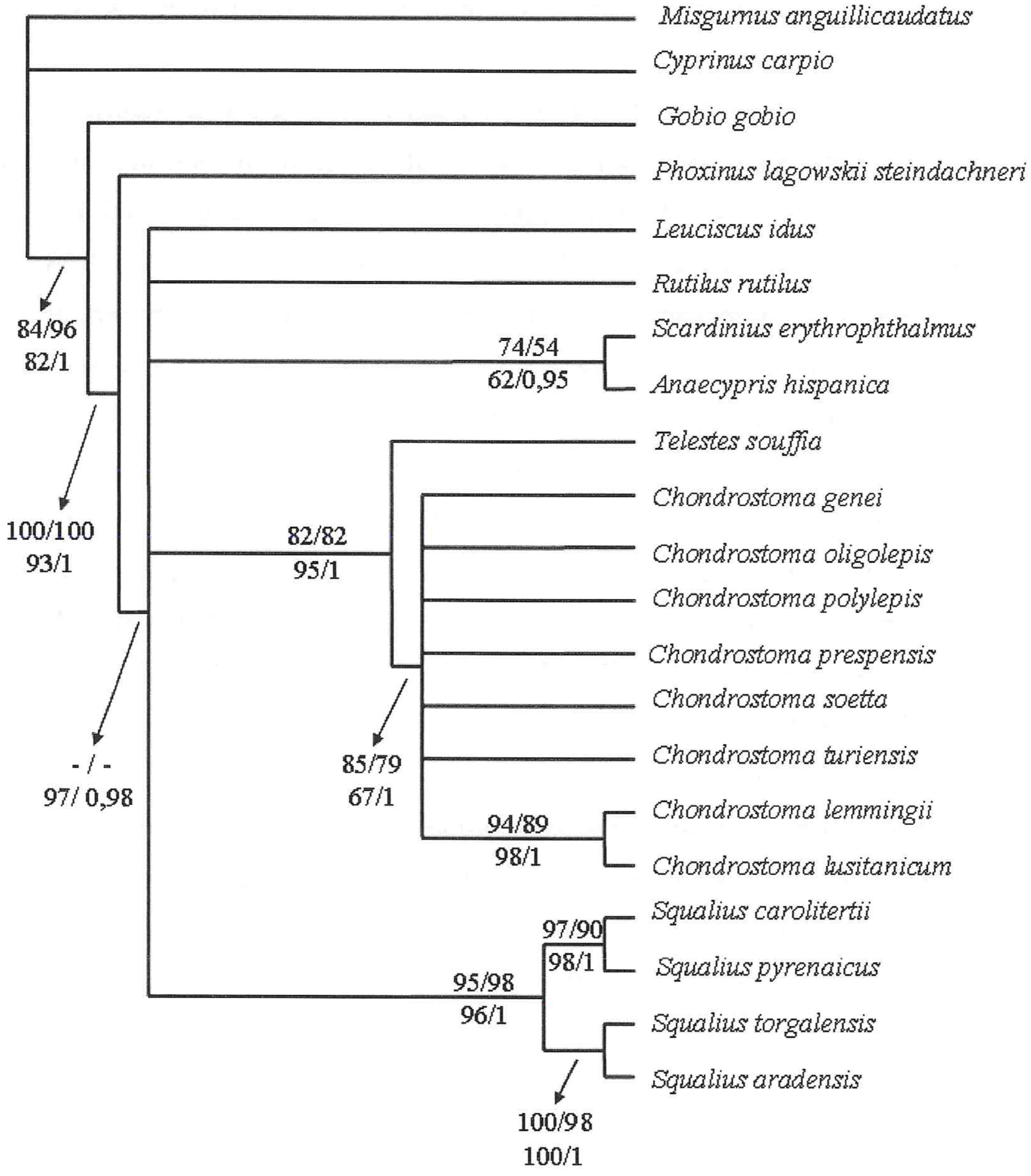

Fig. 5.2. This figure is based on the MP tree. Phylogenetic relationships among the species based on beta-actin sequences. For each branch, numbers above represent the bootstrap values obtained for ME and ML; numbers below indicate those for MP and the posterior probabilities for Bayesian inference.

5.4 Discussion

The results of the present study clearly point to two main conclusions: (1) the paternal ancestor of *S. alburnoides* did not belong to the genera Squalius or *Chondrostoma* and (2) of the species included in this study, *A. hispanica* is the closest to the paternal ancestor (as already suggested by Gilles, Dowling, Alves, Coelho, and Collares-Pereira, unpublished).

During the history of *S. alburnoides* some recombination may have taken place between the *Squalius* and *alburnoides* genomes and thus the beta-actin sequences of the reconstituted non-hybrid males may not be identical to the sequence of the paternal ancestor. The patristic distance between *S. alburnoides* and *Squalius pyrenaicus / S. carolitertii* beta-actin sequences is 1.48% while that between *A. hispanica* and *S. pyrenaicus / S. carolitertii* is 1.80%. The p distance between *S. alburnoides* and *A. hispanica* is 1.48%. Although the hypothesis of some recombination cannot be excluded, the fact that all inference methods recovered the *S. alburnoides* beta-actin sequence with that of *A. hispanica*, with very high bootstrap support, indicates that the signal still present in the beta-actin gene is sufficiently strong to trace its phylogenetic relationships.

Our suggestion is that, in the past, one species of the same clade of *A. hispanica* may have hybridized with *S. pyrenaicus* and originated *S. alburnoides*. The morphology of the reconstituted AA males supports this conclusion: they are similar to *A. hispanica* in size, general body shape, and coloration, but they also differ from it in several important characters (e.g., structure of the lateral line, lateral scale counts, and number of gill rakers) (Collares-Pereira, 1983). The recent discovery of frequent natural intergeneric hybrids between Squalius cephalus and a species of *Chalcalburbus* (Ünver and Erk'Akan, 2005), a member of the same clade as *A. hispanica* (Zardoya and Doadrio, 1999), supports our hypothesis of an intergeneric hybrid origin.

In spite of its hybrid origin *S. alburnoides* seems to be, from an evolutionary perspective, a very successful fish. It is often much more abundant than other sympatric *Squalius*. Alves et al. (2001) argue that the continuous shifting between forms, with P and A nuclear genomes being cyclically lost, gained or replaced by new genomes, allows the introduction of new genetic material. The evolutionary potential of this species, in terms of recombination and maintenance of genetic variability, may be even enhanced by the presence of tetraploid fishes of both sexes in some natural populations. Indeed, PPAA tetraploids seem to undergo normal meiosis. Crosses between them mean that sexual reproduction is restored, while their crosses with other forms of the complex are a way to introduce recombination in the whole system (Alves et al., 2001).

The beta-actin gene evolves at a much slower rate than the cyt *b* gene. For example, the difference between *S. pyrenaicus* and *S. carolitertii* was 6.13% for the cyt *b* gene, while the majority of fish from both species share the same haplotype for the beta-actin gene. The distance between species of *Squalius* and *Chondrostoma* averaged 14.05% for the cyt b and 1.77% for beta-actin gene. Because of its slow rate of evolution, the beta-actin may prove potentially very useful in studies of cyprinid phylogeny, particularly in resolving intrageneric and intertribe relationships.

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Capítulo 6

Re-examination and phylogeny of the genus *Chondrostoma* based on mitochondrial and nuclear data and the definition of 5 new genera

6.1 Abstract

Previous molecular phylogenetic studies of the genus *Chondrostoma* (Cyprinidae: Leuciscinae) were unable to resolve the relationship among its major species groups. In this paper we present a phylogeny for this genus, based on five mitochondrial genes and the nuclear gene beta-actin, comprising a total of 4068 bp. Bayesian inference using all gene fragments yielded a fully resolved phylogeny, compatible with topologies obtained from individual fragments using maximum parsimony and minimum evolution. Mapping of morphological characters critical to the rasping feeding mode of most *Chondrostoma* species indicates that they evolved several times, and questions the use of these characters in the traditional definition of the genus. Our findings led us to the definition of the following new genera: *Achondrostoma*, *Iberochondrostoma*, *Pseudochondrostoma*, *Protochondrostoma* and *Parachondrostoma*. Our data contradict the hypothesis of a rapid radiation during Lago Mare phase, suggested by previous studies.

6.2. Introduction

The genus *Chondrostoma* belongs to the family Cyprinidae, subfamily Leuciscinae (Zardoya and Doadrio, 1999; Durand et al., 2003; Nelson, 2006). This subfamily includes a large number of species distributed in mainland Eurasia (excluding Arabia, India, and Southeast Asia), Japan, and North America (Howes, 1991). Fish from the genus *Chondrostoma* are distributed throughout south and central Europe, from the Atlantic to the Caspian Sea, from the Mediterranean to the Baltic Sea. The genus is also present in Asia Minor, the Caucasus, and Mesopotamia (e.g., Elvira, 1997; Durand et al., 2003). Distribution maps are provided by Elvira (1987), Durand et al. (2003) and Doadrio and Carmona (2004).

The number of species included in the genus varies according to the nature of data used to diagnose species, i.e., morphological and osteological features or molecular characters. Using mainly osteological characters, Elvira (1997), in his review of the genus, recognized 26 species. In that same year Bogutskaya (1997) described a new species from Turkey (*C. beysehirense*), raising to 27 the number of species described in pure morphological grounds. Durand et al. (2003) characterized the *Chondrostoma* genus "by a mouth clearly subterminal, with transverse or arched slit, without barbell, and with the upper jaw forming a muzzle well-arched, with very hard oral lips and a sharp boarder".

Molecular studies of cyprinids of Europe and Iberian Peninsula, based on the mitochondrial cyt *b* gene (Zardoya and Doadrio, 1998, 1999), showed that the species included by Elvira (1997) in the genus *Chondrostoma* form a monophyletic clade that also includes species previously ascribed to the genus *Rutilus* Rafinesque, 1820. Some of these species had already been included in the genus *Chondrostoma* in earlier investigations (e.g. *C. lusitanicum* Collares-Pereira, 1980; *C. lemmingii* Steindachner, 1866, Collares-Pereira, 1983). The variation of criteria among authors and the discrepancies between molecular and morphological data generated a troublesome situation concerning the delimitation of the genus *Chondrostoma*, which remains as yet unresolved.

The recent findings of new *Chondrostoma* species in the Iberian Peninsula illustrate well the current state of affairs. Four new species of *Chondrostoma* have been described, which lack the horny layer on the lower lip, considered by some authors to be an important diagnostic feature of the genus (Elvira, 1987, 1997): *C. almacai* (Coelho et al., 2005), *C. occidentale* (Robalo et al., 2005), *C. oretanum* (Doadrio and Carmona, 2003a) and *C. sp.* (a species from the Duero basin which had been ascribed to *C. lemmingii* in earlier studies, Doadrio and Elvira, unpublished). Thus, data obtained from morphology and from cyt *b* sequences are decoupled in this genus (Durand et al., 2003) and some authors consider that the mouth-related characters lack phylogenetic utility (Doadrio and Carmona, 2004). Overall, 35 species are included in the genus, if we consider those that had been placed there on morphological and or molecular grounds. All molecular studies using the mitochondrial cyt *b* gene led to the identification of a number of very well-supported lineages of *Chondrostoma* (e.g. Doadrio and Carmona, 2004), although the relationships among these groups were unresolved. The recovery of polytomies rather than bifurcating relationships may reflect lack of phylogenetic signal or the presence of rapid cladogenetic events. A rapid succession of several independent cladogenetic events may be empirically indistinguishable from a lineage-level polytomy, if internodes are so short that no mutations occur to distinguish daughter clades (Slowinski, 2001; Poe and Chubb, 2004). If internodes are large enough, using several independent gene fragments, even though each fragment many contain a faint phylogenetic signal, one may be able to detect a central tendency among gene histories, revealing previously hidden phylogenetic relationships (Page and Holmes, 1998; Poe and Chubb, 2004).

There is a wide consensus pointing to an Asian origin of the cyprinid colonization of Europe during the Oligocene, when the Turgai Sea (which separated the two continents) dried out (Briggs, 1995). Concerning the colonization of the South European peninsulas, two hypotheses have been proposed: (1) dispersion around the Mediterranean Sea during the Lago Mare phase of the Messinian Salinity Crisis (originally proposed by Bianco, 1990) and (2) an older and more gradual colonization of the Mediterranean waters through river captures from central Europe (originally proposed by Banarescu, 1992).

The Lago Mare hypothesis assumes that a major leuciscin radiation, which shaped the major lineages of the Mediterranean leuciscins, occurred during the Messinian salinity crisis (about 5.5 MYA) when the Mediterranean suffered a drastic drop in salinity allowing a very quick dispersal of leuciscins around the entire basin (Bianco, 1990).

The second hypothesis assumes that leuciscins invaded central Europe from Asia and colonized the southern peninsulas in the Oligocene, before the alpine orogeny isolated central from south Europe (formation of the Alps, Pyrenees, among other mountain chains) (Banarescu, 1992).

Recently, Doadrio and Carmona (2003b) called attention to the fact that the two hypotheses are not entirely incompatible. The southern dispersal route, suggested by Bianco (1990), has widespread acceptance and has now been incorporated into Banarescu's north dispersal theory (Banarescu and Coad, 1991). The current synthesis to explain the biogeography of European cyprinid fishes is based on two assumptions: (1) European cyprinids originated in Asia and (2) subsequently dispersed via two routes. Central European taxa dispersed from the north in the early Miocene, through water captures (rivers or lakes), while endemic freshwater fauna from the Mediterranean area dispersed via the southern route across the Mediterranean Sea, when it was oligohaline in the late Miocene.

Several authors have attempted to test these hypotheses using molecular data from different genera and different molecular clock calibrations for cyt *b* (Zardoya and Doadrio, 1999; Durand et al., 2003; Doadrio and Carmona, 2003b, 2004). For the genus *Chondrostoma* and considering a molecular clock of 1% divergence per MY, Durand et al. (2003) found evidence that suggests a relatively recent colonization event of the western Palaearctic area in the Messinian (5.5 MYA), thus favouring the Lago Mare hypothesis (Bianco, 1990). The inability to solve the polytomous relationships among the main clades of *Chondrostoma* was taken by Durand et al. (2003) as evidence for its very rapid radiation. Doadrio and Carmona (2003b, 2004) using a similar value for the molecular clock achieved different results. According to these authors, the main *Chondrostoma* lineages originated in the Middle Miocene (in the Tortonian, approximately 11 MYA) and diversified only during the Pliocene (during the formation of the current river system), thus rejecting the Lago Mare dispersal hypothesis for this genus. The discrepancy between studies may be due to an inadequate interpretation of the molecular clock on behalf of Durand et al. (2003), namely using the 1% rate of sequence divergence between lineages as the rate of accumulation of mutation within each lineage (Doadrio and Carmona, 2003b, 2004).

Using the mitochondrial genes 12S, 16S, cyt *b*, d-loop (control region), and tRNA-Phe and the nuclear gene beta-actin, and including a number of additional species we address the heretofore polytomous relationship among major *Chondrostoma* lineages and provide a new, dichotomous phylogenetic hypothesis. A total of 22 of the 35 species included in the genus were analysed. In addition, we mapped the morphological traits traditionally used to define the genus, to infer their evolutionary history and to test whether they are sufficient to diagnose *Chondrostoma* as a monophyletic entity. Because these characters relate to mouth structures, strongly implied in the feeding mode of many *Chondrostoma* species, they are likely liable to parallel or convergent evolution. Indeed, if the ancestors of *Chondrostoma* fed by scrapping algae from rocks any modifications of the mouth that would improve its rasping efficiency could possibly be favoured by selection. Thus, we also mapped dorsal and anal fins rays and lateral scale counts, traits that are probably independent of feeding mode.

6.3. Methods

Taxon sampling

The taxa analysed in this study, their collection sites and their corresponding GenBank sequence Accession Nos. are listed in Table 6.1. We also included *Anaecypris hispanica*, *Rutilus rutilus* and *Telestes souffia*. Except in the few cases when not enough biological material was available, the DNA used for PCR and sequencing of all fragments came from the same individual. A. hispanica was used as outgroup in all analyses. This species was chosen because it is a leuciscin that, in previous molecular studies, using both mitochondrial and nuclear DNA, proved to be basal to the remaining species analysed (Zardoya and Doadrio, 1999; Robalo et al., 2006a). This option was adopted to leave the species more closely related with *Chondrostoma* in the ingroup, to test if *Chondrostoma* was shown by the analysis to be form a monophyletic clade. Voucher specimens are preserved in the fish collections of Museo Nacional de Ciencias Naturales (MNCN) and the Unidade de Investigação em Eco-Etologia, Instituto Superior de Psicologia Aplicada (UIEE/ISPA).

DNA analysis

Total genomic DNA was extracted from fin clips preserved in ethanol by an SDS/proteinase-k based protocol (adapted from Sambrook et al., 1989). For the beta-actin gene a total of 939 bp was amplified using the primers BactFor 5'-ATGGATGATGAAATTGCCGC-3' and BactRev 5'-AGGATCTTCATGAGGTAGTC-3' (Robalo et al., 2006a). PCR conditions followed those in Robalo et al. (2006a). The amplification process was conducted as follows: 35 cycles of [94 °C (30 s), 55 °C (40 s) and 72 °C (1 min 30 s)]. The amplified fragment is homologous to a region of the beta-actin gene of *Cyprinus carpio* (GenBank: M24113), including introns B and C and three exons.

For the cyt *b* gene a total of 1029 bp was amplified using the primers LCB1–5'-AATGACTTGAAGAACCACCGT-3' (Brito et al., 1997) and HA–5'-CAAC GATCTCCGGTTTACAAGAC-3' (Schmidt and Gold, 1993). PCR conditions followed those in Cunha et al. (2004). The amplification process was conducted as follows: 25 cycles of [94 °C (1 min), 50 °C (1 min) and 72 °C (2 min)].

A mitochondrial fragment was amplified using the primers DL1 5'-ACCCCTGGCTCCCAAAGC-3' (Liu et al., 2002) and 12Sstartrev 5'-GCTGGCACGAGTTTTACCGGC-3' (Robalo, unpublished), which recovers the d-loop—control region (915 bp), the tRNA-Phe gene (69 bp) and the beginning of the 12S gene (167 bp). PCR conditions followed those in Liu et al. (2002). The amplification process was conducted as follows: 2 min at 94 °C, 40 cycles of [94 °C (30 s), 50 °C (30 s) and 72 °C (1.5 min)]. An additional fragment of the 12S gene (395 bp) was amplified, using the primers 12S For 5'-AAC TGG GAT TAG ATA CCC CAC-3' and 12SRev 5'-GGG AGA GTG ACG GGC GGT GTG-3' for a total of 562 bp of this gene. For the 16S gene a total of 554 bp was amplified using the primers 16SFor 5'-AAG CCT CGC CTG TTT ACC AA-3' and 16SRev 5'-CTG AAC TCA GAT CAC GTA GG-3'. For 12S and 16S rDNA, primers and PCR conditions follow those in Almada et al. (2005). The amplification process was conducted as follows: 4 min at 94 °C, 30 cycles of [94 °C (1 min), 55 °C (1 min) and 72 °C (1 min)], 10 min at 72 °C.

For all genes, each sample was sequenced in both directions using the PCR primers. Sequencing reactions were performed by Macrogen Inc., (Seoul, Republic of Korea) in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer.

Data analysis

Sequences aligned with BioEdit v.5.0.6. were analysed using distance (minimum evolution, ME) and maximum parsimony (MP) methods, using PAUP*4.0 (Swofford, 1998). ME (neighbour-joining, NJ) trees were generated using maximum likelihood distances and random tie breaks. Molecular evolution models were selected using AIC criterion, as implemented in Modeltest (Posada and Crandall, 1998).

Table 6.1 Taxa analysed in this study, their sites of origin and their GenBank Accession numbers

	River/Basin/Country	**12s**	**16s**	**D-loop-tRNA-Phe-12S**	**Cyt *b***	**Beta-actina**
Chondrostoma						
almacai	Arade and Mira/ Arade and Mira/ Portugal	DQ447669	DQ447693	DQ455031	AF045986*	DQ447717
arcasii	Adaja/ Duero/ Spain	DQ455023	DQ447689	DQ455042	DQ447730	DQ447711
arrigonis	Cabriel/ Jucar/ Spain	DQ447684	DQ447699	DQ455025	DQ447734	DQ447714
duriense	Sabor/ Duero/ Portugal	DQ447673	DQ447705	DQ455033	AF045983*	DQ447715
genei	Arno/ Arno/ Italy	DQ447677	DQ447706	DQ455038	AF533766*	DQ061938
lemmingii	Almonte/ Tagus/ Spain	DQ447668	DQ447707	DQ455027	DQ447733	DQ447716
lusitanicum	Colares and Samarra/ Colares and Samarra/ Portugal	DQ447670	DQ447694	DQ455043	AY254584*	DQ447718
knerii	Trebizat/ Neretva/ Bosnia-Herzegovina	DQ447680	DQ447702	DQ455030	DQ447739	DQ447724
miegii	Cadagua/ Nervion/ Spain	DQ447665	DQ447692	DQ455026	DQ447732	DQ455049
nasus	Mures/ Rhone/ France	DQ447667	DQ447691	DQ455047	DQ447729	DQ447726
occidentale	Alcabrichel/ Alcabrichel/ Portugal	DQ447672	DQ447695	DQ455044	AY254585*	DQ447720
oligolepis	Tornada/ Tornada/ Portugal	DQ447671	DQ447696	DQ455032	AY254679*	DQ447713
oretanum	Robledillo/ Guadalquivir/ Spain	DQ447678	DQ447700	DQ455041	DQ447737	DQ447722
oxyrhynchum	Rubas/ Rubas/ Russia	DQ447676	DQ447708	DQ455035	AF095606*	DQ447721
phoxinus	Suiça/ Cetina/ Bosnia-Herzegovina	DQ447679	DQ447701	DQ455029	DQ447738	DQ447723
polylepis	Azambuja/ Tagus	DQ447674	DQ447703	DQ455034	AF045982*	DQ06194*
prespense	Prespa Lake/ Prespa/ Greece	DQ447682	DQ447697	DQ455046	DQ447735	DQ061944*
sp	Yeltes/ Duero/ Spain	DQ447666	DQ447690	DQ455048	DQ447741	DQ447712
soetta	Po/ Po/ Italy	DQ447681	DQ447709	DQ455045	DQ447740	DQ061939*
turiense	Mijares/ Mijares/ Spain	DQ447683	DQ447698	DQ455040	DQ447731	DQ061946*
vardarense	Aoos/ Aoos-Vjose/ Greece	DQ447675	DQ447704	DQ455039	AF090749*	DQ447719
wilkommii	Arenoso/ Guadalquivir/ Spain	DQ447685	DQ447710	DQ455028	DQ447736	DQ447725
Anaecypris hispanica	Caia/ Guadiana/ Portugal	DQ447662	DQ447686	DQ455024	AJ427814*	DQ061936
Telestes souffia	Saone/ Rhone/ France	DQ447663	DQ447688	DQ455037	DQ447728	DQ061950
Rutilus rutilus	Açores/ Portugal	DQ447664	DQ447687	DQ455036	DQ447727	DQ061948

MP topologies were sought using a 10 replicate heuristic search with random stepwise additions followed by tree-bisection–reconnection (TBR) branch swapping. The incongruence length difference test (Farris et al., 1995, as implemented in PAUP* 4.0) was used to access the homogeneity of the 12S, 16S and tRNA-Phe genes, and the exons and introns of beta-actin. As no statistically significant differences were found, MP and ME analyses were performed separately on the following subsets: 12S–16S-tRNA-Phe, cyt *b*, d-loop and beta-actin.

For each gene, the saturation of transitions and transversions was checked by plotting the absolute number of changes of each codon position against uncorrected sequence divergence values (p). There was no evidence of saturation in the ingroup (graphs not shown). Thus, we gave equal weights to transitions and transversions when performing the MP trees. Giving transversions 10 times the weight of transitions did not affect the results.

Bootstrap analyses (1000 replicates) were used to assess the relative robustness of branches of the ME and the MP trees (Felsenstein, 1985). Bayesian analysis was performed using MCMC as implemented in Mr. Bayes 3.1 (Ronquist and Huelsenbeck, 2003), with four independent runs of five Metropolis-coupled chains of 2 000 000 generations each, to estimate the posterior probability distribution. The full sequence matrix was partitioned per gene fragment, and the nuclear beta-actin gene was partitioned into an exon and intron partition, making a total of seven data partitions. Independent model parameters (GTR + Γ+ I) were estimated for each partition. The heating parameter was set to 0.15, topologies were sampled every 100 generations, and a majority-rule consensus tree was estimated after discarding the first 105 generations. To access congruence between the more fully resolved Bayesian phylogeny (based on all gene fragments) and the ME and MP trees based on partial data sets, we compared the latter trees with trees obtained by the same method, but imposing the Bayesian topology as a constraint. Independently, we adopted the same procedure to compare constrained and unconstrained ME and MP trees of the partial datasets using relevant features of the Bayesian tree as constraints: the monophyly of the Iberian species, topological relationships within the Iberian clade, monophyly of the Italo-Balkanic group (*C. kneri*, *C. phoxinus*, *C. soetta*), monophyly of the nasus group, monophyly of all non-Iberian species with exclusion of the basal *C. genei* and dichotomy between *C. genei* and a clade comprising all remaining *Chondrostoma* species. Constrained and unconstrained trees were compared statistically using Kishino and Hasegawa (1989) and Shimodaira and Hasegawa (1999) tests, as implemented in PAUP.

Character mapping

Information concerning several meristic characters was gathered from the literature (Collares-Pereira, 1983; Elvira, 1987; Doadrio, 2001) and from available specimens: presence/absence of horny layer on lower lip, position and arching of mouth, number of scales on the lateral row, and number of dorsal and anal fin rays. Character history was mapped on the best resolved phylogenetic tree, i.e., the Bayesian topology. Ancestral states were reconstructed under a maximum-parsimony method, considering ordered character states with equally weighted transitions, using Mesquite v. 4.5.2 (Maddison and Maddison, 2005).

6.4 Results

A total of 4068 bp was amplified. Of these, 3129 bp correspond to mitochondrial DNA and 939 bp to the nuclear gene of beta-actin. Details of MP and ME analyses are summarized in Table 6.2. No bootstrap analysis was performed on MP reconstruction for beta-actin, due to the high number of most parsimonious trees obtained (7910 trees). In Fig. 6.1, we present the results of the Bayesian analysis using the entire data set. This phylogeny presents a complete resolution of relationships within the genus, and is entirely compatible with all other trees obtained using MP and ME inference on each DNA fragment (see supplementary material for MP e ME trees, and statistical comparison of these trees with the Bayesian topology). These partial trees do not contradict the Bayesian phylogeny; they are simply unable to resolve certain relationships and many clades are not recovered with good bootstrap support.

Since there were no incompatibilities between the Bayesian tree of the entire data set and each of the remaining trees, we will concentrate in the analysis of that tree. We recovered seven major lineages, also recognized by Doadrio and Carmona (2004): four Iberian clades (the *C. toxostoma*, *C. lemmingii*, *C. polylepis*, and *C. arcasii* groups), named after a member species; and three Euro-asian groups (the *C. nasus* and *C. soetta* groups, and the monospecific *C. genei* lineage). *Chondrostoma genei* is basal to all *Chondrostoma* species. The first bifurcation separates the Iberian species from the remaining. In the Iberian clade, the basal position is occupied by the Spanish species *C. miegii*, *C. arrigonis* and *C. turiense* (*toxostoma* lineage). Although we did not have samples of *C. toxostoma* all previous studies stressed its close proximity to the remaining members of this group (e.g. Durand et al., 2003; Doadrio and Carmona, 2004), so we assume that it very likely belongs in this clade. The remaining species of the Iberian group form a clade that is sister to the *toxostoma* group.

Table 6.2 Results of the ME and MP analysis

	12S-16S-tRNAPhe	Cyt *b*	D-loop	Beta-actin
MP analysis				
Number of parsimony informative characters	67	255	118	23
Number of most parsimonious trees obtained	34	3	5	7910
Consistency index	0.60	0.48	0.57	0.87
Tree length	230	907	469	74
Retention index	0.64	0.54	0.58	0.87
Homoplasy index	0.40	0.53	0.43	0.14
Rescaled consistence index	0.38	0.26	0.33	0.75
ME analysis				
Model selected by Modeltest (AIC criteria)	TrN+I+G	GTR+G+I	TIM+I+G	K81uf+I
Nucleotide proportions	A=0.29	A=0.28	A=0.32	A=0.21
	C=0.26	C=0.31	C=0.21	C=0.25
	G=0.23	G=0.14	G=0.14	G=0.22
	T=0.22	T=0.28	T=0.32	T=0.32
Assumed proportion of invariable sites	0.79	0.57	0.53	0.87
Alpha	0.64	1.14	0.57	Equal rates

This clade splits into two branches: one comprising the *lemmingii* lineage (*C. lusitanicum*, *C. almacai*, *C. oretanum* and *C. lemmingii*), the other comprises two sister clades. *C. arcasii*, *C. oligolepis*, *C. occidentale* and the undescribed *C. sp*. form the *arcasii* lineage. Its sister is the *polylepis* lineage which includes the large bodied and straight mouth Iberian species *C. duriense*, *C. polylepis* and *C. willkommii*. The non-Iberian clade, that groups the remaining species included in the analysis, splits into two sister groups. In one of them the Italian *C. soetta* groups with the species *C. knerii* and *C. phoxinus* from Bosnia (*soetta* lineage). The sister clade contains the central European *C. nasus* and the related species *C. oxyrhynchum*, *C. prespense* and *C. vardarense* (*nasus* lineage). Since in previous studies this *nasus* lineage has been always consistently recovered based on the cyt *b* gene, we assume that it is likely that this group includes *C. nasus* and all the remaining species of the Balkans, Anatolia and Mesopotamia.

The tree discussed above, was already anticipated although with weaker support by the study of Doadrio and Carmona (2004) and fully resolves the phylogeny of the genus. Partial trees are less resolved although some clades were recovered when using all gene fragments and methods. The same holds for the monophyly of the genus *Chondrostoma*, and its relationship with *Telestes* and the more distantly related *Rutilus*.

The character distribution of the horny layer on the lower lip and ventral position of the mouth are coincidental (Fig. 6.2a). For all characters examined, except fin rays, the Iberian *C. lemmingii* and *C. arcasii* groups exhibit character states similar to those inferred for the common ancestor of the genus. Yet this appears to be due, at least in some cases, not to the retention of but a reversal to the ancestral state of the genus, i.e., these lineages exhibit a non-ventral mouth with no horny layer (as outgroups and inferred ancestor). The ancestor of the Iberian *Chondrostoma* is inferred to exhibit a ventral mouth with a horny layer on the lower lip, as most species of the genus. It is noteworthy that while the *arcasii* group is most closely related to the *polylepis* group, it often exhibits character states quite different from its sister group, yet similar to other Iberian *Chondrostoma*, e.g., absence of horny lip, arched mouth, low number of anal and dorsal fin rays. The *polylepis* group tends to resemble the non-Iberian groups. The morphological characters that were studied, and which are frequently used for species description in *Chondrostoma*, appear to be quite labile within the genus. A thick horny layer on the lower lip evolved at least twice from a thinner one, and perhaps once from an ancestor with no horny lip. A straight mouth also evolved several times from an arched condition, apparently often accompanying the thickening of the lower lip. Finally, the high lateral scale counts, typical of large bodied *Chondrostoma*, also arose recurrently from lower counts, common in small bodied *Chondrostoma*.

6.5 Discussion

The high level of resolution obtained with the full data set using Bayesian analysis, together with its compatibility with the trees derived from the different data subsets using MP and ME, clearly rules out the hypothesis that we are faced with a simultaneous formation of multiple lineages from the same ancestor in the phylogeny of *Chondrostoma*.

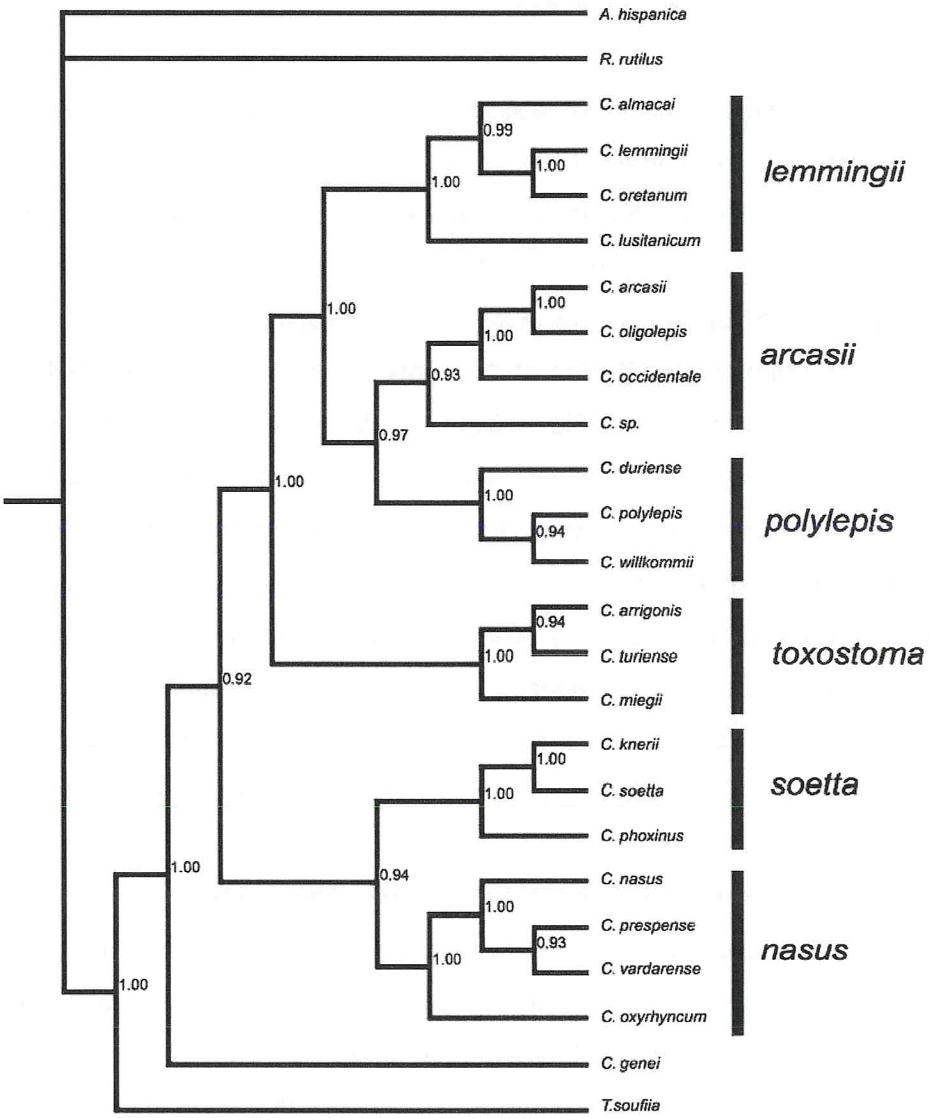

Fig. 6.1 Bayesian tree with posterior probabilities representing the phylogeny of the genus *Chondrostoma*. Lineages referred in the text are represented by black bars on the right.

This conclusion had been previously argued by Doadrio and Carmona (2004) who, using the cyt *b* gene alone, had obtained a broadly similar, albeit less well-supported, Bayesian tree of the genus. The fact that much more mitochondrial data and a nuclear gene confirm and strengthen their finding is, in our view, good evidence that the dichotomous resolution is not an artefact.

Obviously, the rejection of a hard polytomy is not in itself proof that a rapid succession of branching events did not take place. The hypothesis of Durand et al. (2003)

assumes that the basic cladogenic events in *Chondrostoma* took place in a very short period in the Messinian period, when most of the Mediterranean coastal area was a series of freshwater bodies. Such a hypothesis is compatible with a rapid succession of dichotomous events; temporally so close that with less data they were not resolved. However, when we compare the timing of the major cladogenic events, obtained with the correct application of the same molecular clock as Doadrio and Carmona (2004), we find that the basal radiation in the *Chondrostoma* occurred much earlier than the Messinian, about 12 MYA. Enforcing this molecular clock upon the Bayesian phylogeny indicates that the Iberian *Chondrostoma* originated more than 11 MYA, shortly after the origin of the entire clade.

Doadrio and Carmona (2004) also showed that the different *Chondrostoma* lineages diversified at considerably different times (e.g., 7 MYA for the arcasii lineage to 3 MYA for the *C. toxostoma* lineage), thus rejecting the idea of an almost simultaneous radiation in the Messinian.

In the Iberian Peninsula, fossil cyprinids date to the Oligocene (De la Peña, 1995) and fossils apparently belonging to the genus *Chondrostoma* are found in Miocene deposits before the Messinian (Doadrio, unpublished). The timing of the splits between the different Iberian lineages, are also much older than the Messinian, a fact also previously noted by Doadrio and Carmona (2004). All together, the available evidence points to a radiation of *Chondrostoma* prior to the Messinian.

The Bayesian phylogenetic hypothesis also has interesting biogeographic implications. We will address briefly the situation of the different groups.

Iberian *Chondrostoma*: The *arcasii* lineage speciated in north and central Iberia, as a consequence of historical events involving the extensive endorheic lagoon system present in the area during the Cenozoic (Robalo et al., 2006b). Its sister clade, the *lemmingii* lineage, occurs from the Tagus to the south and southwest. Sister to these clades, the *toxostoma* lineage occurs mainly in the Mediterranean rivers of eastern Spain with the related *C. toxostoma* in south and central France. The Iberian Peninsula seems to have been the area with the highest level of diversification of major lineages of *Chondrostoma*. In addition, the almost non-overlapping distributions of the *arcasii*, *lemmingii* and *toxostoma* groups suggest that they evolved in allopatry, although secondary contacts are presently found in some rivers (Doadrio, 2001).

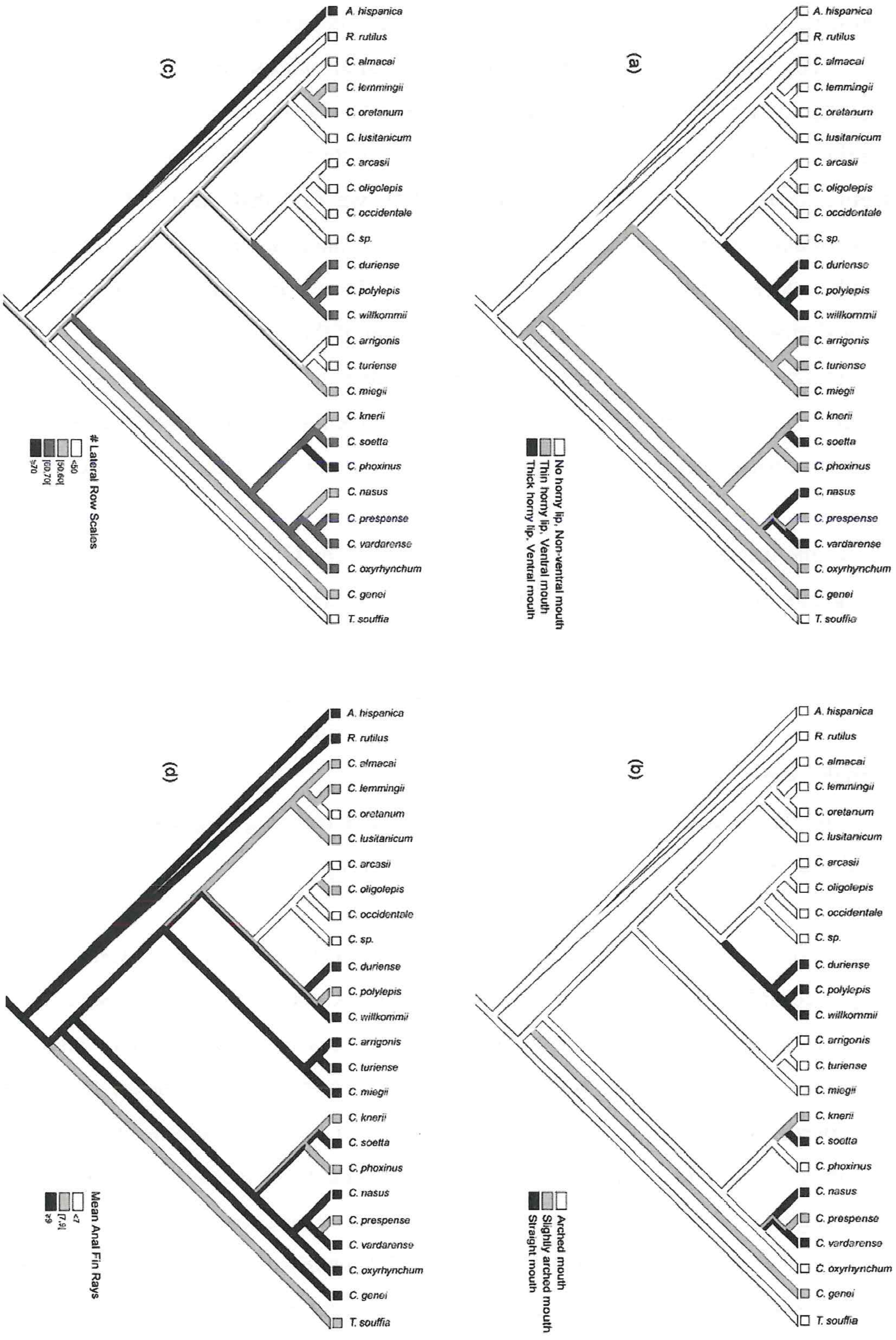

Fig. 6.2 Character mapping of mouth features (a and b), lateral row scales (c) and anal fin rays (d).

Italobalkanic lineages: *C. genei* and the *soetta* clade occur in an area around the central part of north Mediterranean. The genetic distance between these nearly sympatric clades suggests that rather than being the product of a dispersal during the Lago Mare phase of the Messinian, these groups may be the remnants of a much more widespread fauna occurring in Central Europe. Interestingly, basal species of other lineages of cyprinids and cobitids are found in this region, from north Italy to western Balkans, including Croatia, Bosnia and Albania (Ludwig et al., 2001; Perdices and Doadrio, 2001; Sanjur et al., 2003). Some cyprinid lineages have high levels of diversity in this area and a new genus was recently described (Freyhof et al., 2006). All these findings support the view that the freshwater ichthyofauna of this region is considerably old, may have been relatively spared by glaciations, and may have had a wider distribution in Central Europe, before the Plio–Pleistocene cooling.

Nasus lineage. This clade presents a distribution ranging from the Middle East to Western Europe, reaching the North Sea. How may one explain such a broad east–west distribution? In western Europe, the group is represented only by *C. nasus*. The greatest radiation of the clade occurred in the eastern part of its range. Like many other cyprinid lineages the nasus lineage must have dispersed from the Danube and its surroundings, and then probably invaded Western Europe (through the Rhine) during the Pleistocene (Banarescu, 1989). The distributions of other lineages like *Rutilus*, *Squalius*, *Alburnus*, among others, define similar pictures (Banarescu, 1989; Zardoya and Doadrio, 1999; Sanjur et al., 2003): they are highly diverse in southeastern Europe, particularly in Greece, yet one species ranges more broadly into west and even northern Europe (e.g., *R. rutilus*, *Squalius cephalus* and *Alburnus alburnus*). It is possible that the *nasus* group evolved in the Paratethys Sea. Interestingly, a member of the *nasus* clade presents the ancestral condition of arched mouth and occupies a basal position in the group, *C. oxyrhynchum*, occurs in waters draining to the Caspian Sea (which is a derivative of the Paratethys Sea, together with the Black Sea, Briggs, 1995). This Paratethyan origin would also explain the distribution of *C. nasus*, a fish typical of the Danube (a river draining into the Black sea) and of the Rhine (that may have received it from the Danube). The group may have invaded Greece and Turkey through Pliocenic river captures, which are well-documented for other fish taxa (e.g., *Squalius*, Sanjur et al., 2003).

Such a scenario raises an interesting question. Often, when discussing the models of colonization of Europe by cyprinids, we think of the fauna of Central Europe as it is now. It may well be, however, that the fish species we see presently in Central Europe are basically a recent product of migrations from the east and that much of the original fauna was extirpated by the successive glaciations of the Pleistocene. If other taxa support this hypothesis, opposing "perimediterranean" versus "central European" dispersals may be a false dilemma.

The ideas discussed above bear directly on the evolution of the genus *Chondrostoma*. The evolution of mouth structures and lateral scale counts suggested by Fig. 6.2 implies recurrent evolution of a ventral rasping mouth reinforced by a horny layer that forms a cutting edge on the lower lip and its change from a curved to a straight line, which is the more derived condition. In the Iberian clade, it is impressive to find that the more specialized forms of the polylepis group hybridize with species of the arcasii group (which its characterized by lower scale counts, near terminal mouth and absence of horny layer), originating apparently fertile hybrids (Gante et al., 2004). Members of the *C. polylepis* and *C. nasus* groups share traits that lack an obvious relationship with feeding, unlike the structure of the mouth, including high lateral scale count, large size, and migration of breeding fish to headwaters to spawn.

How can one explain the repeated evolution of so many traits? One alternative to convergent evolution is that these traits introgressed into the ancestral lineage of the polylepis group by hybridization. Assuming our earlier proposal of extinction of much of the pre-glacial fauna of central Europe, there may well have been, former contacts among ancestors of the Iberian *arcasii – polylepis* groups with fish closer to *C. nasus*, originating hybrid lineages in Iberia. Alternatively, the polylepis group, distributed on the western part of the Iberian Peninsula, may have resulted from an ancient introgression by members of the *toxostoma* group, which occupies the eastern part of the Peninsula. The species of this group also have large bodies, perform reproductive migrations to headwaters and possess a rasping mouth with a horny layer - albeit an arched one. In this latter scenario, the straight mouth of the polylepis group would be a derived condition.

Judging from the present behaviour of the fish involved, this scenario seems less likely, as there is no record of hybridization between members of the *polylepis* and *toxostoma* lineages when they occur in sympatry, whereas the toxostoma and arcasii lineages do hybridize (Doadrio, unpublished). Finally, parallel evolution of large body and high scale counts in the polylepis and nasus groups cannot be ruled out with the data presently available, even if reasons for such recurrent change are unclear.

Only studies with multiple unlinked nuclear markers may help to decide between the hypotheses of past hybridization and parallel evolution. However, past hybridization should not be invoked to explain the failure of previous studies to resolve the phylogeny of the group, as all of them dealt with mitochondrial DNA that, in principle, has not recombined.

The present study supports the view that the genus *Chondrostoma* needs a major taxonomic revision. It shows that the morphological characters traditionally used to define the genus are labile and probably evolved several times in distinct lineages. The discrepancies evident in the literature concerning the delimitation of the genus, the conflicts between molecular and morphological data, and the fact that different clades originated at different times are, in our view, arguments against the traditional definition of *Chondrostoma*. We believe that the best way to resolve this complicated situation is to restrict the scope of the genus, erecting a number of new genera that form well defined monophyletic groups, and for which molecular and morphological criteria are in agreement. It may be argued that this study is not a revision of the genus, as 13 of the 35 species of *Chondrostoma* were not included in the analysis. This risk is however minimized if we consider that of the 13 species absent in the present study, six had already been studied from a molecular perspective, using the cyt *b* gene. As mentioned above, *C. toxostoma* forms a very well defined clade with *C. miegii*, *C. arrigonis* and *C. turiense* (Doadrio and Carmona, 2004). Five other species (*C. angorense*, *C. cyri*, *C. holmwoodii*, *C. meandrense* and *C. regium*) are integrated without ambiguities in the clade that contains *C. nasus* (Durand et al., 2003; Doadrio and Carmona, 2004). Thus, there are seven species for which molecular information is lacking. These are, however, a cohesive group in morphological terms (Bogutskaya, 1997; Elvira, 1997) that share the traits possessed by all members of the *C. nasus* group for which molecular data exists. In addition, all seven species are distributed in the eastern part of the range of *Chondrostoma* (Turkey and nearby areas). Thus, it is very unlikely that further molecular studies of this seven species will affect the genera defined below, which are mainly Iberian in distribution, with an italo-balkanic taxon. When all these arguments are considered, it becomes clear that the species group for which we reserve the name *Chondrostoma* in our proposal is the only one that may eventually be affected by future molecular analysis. We believe that taking the risk of advancing the recognition of new genera, which are supported by molecular and morphological data, is preferable to the current situation. Indeed, at present, ichthyologists are faced with two incongruent alternatives: (1) defining the genus on morphological grounds, as adopted by Elvira (1987, 1997) makes the genus paraphyletic; (2) the definition of the genus on pure molecular grounds suffers from the same lack of information for the seven species, as yet unstudied genetically and generates a morphologically very heterogeneous taxa.

Our proposal of classification is as follows:

Genus *Chondrostoma* Agassiz, 1832

Type species. *Cyprinus nasus* L., 1758

Etymology referring to the characteristic horny layer on the lower lip.

Included species. *Chondrostoma nasus* (L., 1758); *Chondrostoma soetta* Bonaparte, 1840; *Chondrostoma knerii* Heckel, 1843; *Chondrostoma phoxinus* Heckel, 1843; *Chondrostoma regium* (Heckel, 1843); *Chondrostoma variabile* Jakowlew, 1870; *Chondrostoma oxyrhynchum* Kessler, 1877; *Chondrostoma cyri* Kessler, 1877; *Chondrostoma holmwoodii* (Boulenger, 1896); *Chondrostoma colchicum* Derjugin, 1899; *Chondrostoma kubanicum* Berg, 1914; *Chondrostoma kinzelbachi* Krupp, 1985; *Chondrostoma angorense*, Elvira, 1986; *Chondrostoma meandrense* Elvira, 1986; *Chondrostoma scodrensis* Elvira, 1986; *Chondrostoma beysehirense* Bogutskaya, 1997; *Chondrostoma vardarense* Karaman, 1928; *Chondrostoma orientale* Blanco and Banarescu, 1982; *Chondrostoma prespense* Karaman, 1924.

Diagnosis. Straight or arched mouth with horny layer on the lower lip; without ascendent process on premaxilla; dentary with a coronoid process anteriorly orientated and with a short anterior process; ethmoides wider than long; 52–78 canaliculated scales on the lateral line; 7–12 scales above the lateral line; 5–6 scales below the lateral line; 8 branched rays in the ventral fin; 7–10 branched rays in the dorsal fin; 8–11 branched rays in the anal fin; 7–5/5–6 pharyngeal teeth; 19–36 gill rackers on the first branchial arch. Upper branch from fifth ceratobranchial enlarged.

Distribution. From the Rhine, Danube and Po basins to the east reaching southwest Iran.

Genus *Achondrostoma* N. Gen

Type species. *Leuciscus* (*Leucos*) *arcasii* Steindachner, 1866

Etymology referring to the absence of horny plate on the mouth.

Included species. *Achondrostoma arcasii* (Steindachner, 1866), *Achondrostoma oligolepis* (Robalo et al., 2005), *Achondrostoma occidentale* (Robalo et al., 2005).

Diagnosis. Arched mouth without horny layer on the lower lip; process from premaxilla well developed and upward oriented; dentary with a coronoid process posteriorly oriented and with a long anterior process; lengh of the ethmoides greater than the width; 33–46 canaliculated scales on the lateral line; 6–8 scales above the lateral line; 2– 4 scales below the lateral line; 7–8 branched rays in the ventral fin; 7 branched rays in the dorsal fin; 7 branched rays in the anal fin; 5–5 pharyngeal teeth; 10–15 gill rackers on the first branchial arch. Upper branch of fifth ceratobranchial not enlarged.

Distribution. Endemic from North and central Iberian Peninsula, ranging from the Atlantic eastwards to the Mediterranean.

Genus *Iberochondrostoma* N. Gen

Type species. *Leuciscus lemmingii* Steindachner, 1866

Etymology referring to Iberian Peninsula where this genus is distributed.

Included species. *Iberochondrostoma lemmingii* (Steindachner, 1866); *Iberochondrostoma lusitanicum* (Collares-Pereira, 1980); *Iberochondrostoma oretanum* (Doadrio and Carmona, 2003); *Iberochondrostoma almacai* (Coelho et al., 2005).

Diagnosis. Arched mouth without horny layer on the lower lip; well developed and upward orientated process from premaxilla; dentary with a coronoid process posterioly orientated and with a long anterior process; length of the ethmoides greater than the width; 46–60 canaliculated scales on the lateral line; 11–12 scales above the lateral line; 5–6 scales below the lateral line; 7–8 branched rays in the ventral fin; 6–7 branched rays in the dorsal fin; 6–7 branched rays in the anal fin; 6–5/5 pharyngeal teeth; 24–27 gill rakers on the first branchial arch. Upper branch of fifth ceratobranchial enlarged.

Distribution. Endemic from South and Central Iberian Peninsula on Atlantic slope.

Genus *Pseudochondrostoma* N. Gen

Type species. *Chondrostoma polylepis* Steindachner, 1865

Etymology refers to its morphological similarity with the genus *Chondrostoma* as consequence of homoplasy in multiple traits.

Included species. *Pseudochondrostoma polylepis* (Steindachner, 1865); *Pseudochondrostoma willkommii* (Steindachner, 1866); *Pseudochondrostoma duriense* (Coelho, 1985).

Diagnosis. Straight mouth with horny layer on the lower lip; well developed and upturned process from premaxilla; dentary with a coronoid process anteriorly orientated and with a short anterior process; ethmoides wider than long; 59–78 canaliculated scales on the lateral line; 10–12 scales above the lateral line; 4–6 scales below the lateral line; 8 branched rays in the ventral fin; 8–9 branched rays in the dorsal fin; 8–10 branched rays in the anal fin; 7–5/5–6 pharyngeal teeth; 19–35 gill rakers on the first branchial arch. Upper branch of fifth ceratobranchial enlarged.

Distribution. Endemic from the Atlantic slope of the Iberian Peninsula.

Genus *Protochondrostoma* N. Gen

Type species. *Leuciscus genei* Bonaparte, 1939

Etymology from the basal position of this genus in the group of genera that formerly comprised *Chondrostoma*.

Included species. *Protochondrostoma genei* (Bonaparte, 1939).

Diagnosis. Horny layer on the lower lip; arched mouth; dentary with the coronoid process anteriorly orientated and short anterior process; premaxilla with anterior process well developed and anteriorly orientated; ethmoides more wide that long; 50–62 canaliculated scales on the lateral line; 8–9 scales above the lateral line; 4–6 scales below the lateral line; 8 branched rays in the ventral fin; 8 branched rays in the dorsal fin; 9–10 branched rays in the anal fin; 5–5 pharyngeal teeth; 14–18 gill rackers on the first branchial arch. Distribution: Po and Adige Basins in Italy and Slovenia.

Genus *Parachondrostoma* N. Gen

Type species. *Chondrostoma miegii* Steindachner, 1866

Etymology refers to the combination of traits that are similar to those of *Chondrostoma* with others that emphasize tha distinctiveness of the two genera.

Included species. *Parachondrostoma toxostoma* (Vallot, 1837); *Parachondrostoma miegii* (Steindachner, 1866); *Parachondrostoma arrigonis* (Steindachner, 1866); *Parachondrostoma turiense* (Elvira, 1986).

Diagnosis. Horny layer on the lower lip; arched mouth; dentary with the coronoid process anteriorly orientated and short anterior process; premaxilla with anterior process well developed and upward oriented; ethmoides wider than long; 44–62 canaliculated scales on the lateral line; 7–9 scales above the lateral line; 4–6 scales below the lateral line; 8 branched rays in the ventral fin; 8 branched rays in the dorsal fin; 8–11 branched rays in the anal fin; 7–5/6–5 pharyngeal teeth; 16–35 gill rakers on the first branchial arch.

Distribution. Central and North Mediterranean rivers from the Iberian Peninsula and Loire, Garonne, Adour, Herault, Rhône Aude and Var in France.

Key to Genera

1

With horny layer on the lower lip	2
Without horny layer on the lower lip	5

2

Without ascendent process in premaxilla	*Chondrostoma*
With ascendent process in premaxilla	3

3

Straight mouth	*Pseudochondrostoma*
Arched mouth	4

4

Ascendent process of premaxilla oriented anteriorly	*Protochondrostoma*
Ascendent process of premaxilla orientated upward	*Parachondrostoma*

5

33–46 canaliculated scales on the lateral line	*Achondrostoma*
46–60 canaliculated scales on the lateral line	*Iberochondrostoma*

6.6 Acknowledgments

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Parte III

Filogeografia de alguns peixes ibéricos

Capítulo 7

Paleobiogeography of two Iberian endemic cyprinid fishes (*Chondrostoma arcasii-Chondrostoma macrolepidotus*) inferred from mitochondrial DNA sequence data

7.1 Abstract

We tested different hypotheses related to the origin and evolution of the endemic Iberian fishes *Chondrostoma arcasii* and *Chondrostoma macrolepidotus* from northern and central regions of the Iberian Peninsula. We evaluated the monophyly of the populations within each species and sought to determine if diversification of the populations coincided in time with the formation of the Iberian drainages dating back to the upper Pliocene (2.5–1.8 million years ago). A molecular phylogenetic analysis of the mitochondrial cytochrome *b* gene showed that the different populations of the northern Iberian Peninsula are clustered into five phylogroups and do not fit into the dichotomy *C. arcasii* - *C. macrolepidotus*. We propose that species differentiation occurred prior to the upper Pliocene formation of the present hydrographic basins and that endorheic basins, a system of inland lakes found in Spain during the Mio-Pliocene, played an important role in this diversification and differentiation process.

7.2 Introduction

In the Iberian Peninsula, as in other southern European peninsulas, the primary freshwater fish fauna is dominated by cyprinids and is characterized by a high level of endemism, a low number of genera, and a high number of species per genus (Doadrio, 2001). The prevalence of endemic species and the low number of genera have been explained by the persistence of important barriers that strongly reduce the possibilities of colonization of Iberia by freshwater fishes. Surrounded by the Atlantic and the Mediterranean and connected to the bulk of Europe by a zone of very high mountains, the Pyrenees, Iberia was colonized by only a few lineages that succeeded in surmounting these barriers (e.g., Doadrio, 2001).

This high level of diversity and endemism in the Iberian cyprinids resulted from cladogenetic processes that occurred within the peninsula after its colonization by these few lineages (Doadrio, 2001). How to explain this high level of diversity of endemic cyprinids? The present-day hydrographic network of the peninsula is geologically recent. During the upper Miocene and lower Pliocene many of the modern rivers did not exist as such, and several endorheic (closed) basins drained the bulk of freshwater in Iberia (Andeweg et al., 1999). One way to explain the diversity of the fish fauna is to hypothesize that it originated at least as early as the Mio-Pliocene due to the isolation of the numerous inland lakes that then existed and the heterogeneity of the habitats provided by the lacustrine environments. Alternatively, the diversification of the fauna may be more recent, resulting from the separation of different watersheds as the modern river network was formed. These two hypotheses, although not being mutually exclusive, lead to distinct predictions. If speciation was associated with the formation of the modern rivers, we predict that (1) the separation of most sister species must be of Pliocene origin and their separation times must correlate well with the formation of the different river systems and (2) different rivers must harbor different sister species widely distributed along their basins.

If most of the diversification occurred in the isolated inland lakes of Mio-Pliocene, we predict that (1) the timing of the cladogenetic events must predate the formation of the modern exorheic drainage pattern and (2) sister species may co-occur as the result of the connections between different ancient lakes, which took place after speciation.

In this paper, we assess the relative importance of these two hypotheses using a phylogenetic and biogeographic analysis of the sister species *Chondrostoma arcasii* (Steindachner, 1866b) and *Chondrostoma macrolepidotus* (Steindachner, 1866a), two cyprinid fishes endemic to the north central Iberian Peninsula. *C. macrolepidotus* is restricted to the western part of the Iberian Peninsula on the Atlantic slope of Portugal, while *C. arcasii* inhabits north and central Spain on both the Mediterranean and Atlantic slopes (Collares-Pereira, 1983; Doadrio, 2001). The two species are difficult to distinguish morphologically (Casado, 1995; Collares-Pereira, 1979, 1983), and Zardoya and Doadrio (1998) provided molecular evidence contradicting their monophyly, although showing that taken together they form a monophyletic clade. Thus, this paper also aims to contribute to a better understanding of their relationship. We used samples that cover, for the first time, the entire distribution area of the two species. Our analysis was based on the sequences of the mitochondrial cytochrome *b* gene.

7.2 Materials and Methods

One hundred and two specimens were collected throughout the entire range of the *C. arcasii-C. macrolepidotus* species group. Voucher specimens were deposited in the collections of the Museo Nacional de Ciencias Naturales, Madrid, Spain. Total genomic DNA was extracted from a piece of fin clip or muscle by the standard proteinase K and phenol/chloroform extraction method (Sambrook et al,. 1989). From each individual DNA sample, two overlapping fragments of the cytochrome *b* gene (total of 1,036 bp) were amplified via polymerase chain reaction (PCR). The primers used for cytochrome b were those mentioned in Machordom and Doadrio (2001). The amplification process was conducted as follows: 94ºC (2 min), 35 cycles at 94ºC (45 s), 48ºC(1 min), 72ºC (5 min). PCR mixtures were prepared in 25-µl volumes with a final concentration of 0.5 mM each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgC12, and 1 unit of Taq DNA polymerase (Perkin-Elmer, Boston, MA). After checking PCR products on 1.5% agarose gels, they were cloned using the pGEM-T vector (Promega, Madison, WI) into Escherichia coli JM109 and were sequenced using the FS-Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA). DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers. All samples were sequenced on an Applied Biosystems 3700 DNA sequencer following the manufacturer's instructions. Chromatograms_and alignments were visually checked and verified, and there were no gaps in the resulting DNA sequences. Sequences have been deposited in GenBank. Accession numbers and capture localities are presented in Table 7.1.

Data Analysis

The aligned sequences were analyzed using distance [minimum evolution (ME)], maximum likelihood (ML), and maximum parsimony (MP) methods. Bayesian phylogenetic inference was also performed, using MRBAYES 3.0 (Huelsenbeck and Ronquist 2001) by simulating a Markov chain for 1,000,000 cycles. MP analysis was performed using heuristic searches with 10 random stepwise additions and tree bisection reconnection branch swapping. Results were based on a 9:1 Ti/Tv weight, following the empirically determined Ti/Tv ratio for *C. arcasii* - *C. macrolepidotus*. To find the best model of evolution that fit our data for ML and ME analyses, we performed a hierarchical likelihood ratio test (LRT), using the program MODELTEST 3.04 (Posada and Crandall 1998). All phylogenetic analyses, except Bayesian inference, were performed using PAUP* 4.0 (Swofford, 1998). Bootstrap analyses were used to assess the relative robustness of branches of the ML (1,000 replicates), ME (1,000 replicates), and MP (500 replicates) trees.

Sequences of *Chondrostoma lusitanicum* (from River Samarra, Portugal), *Telestes soufia*, and *Rutilus rutilus* (both from River Saone, France) (GenBank accession numbers AY254584, Y10439, and Y10440, respectively) were used as outgroups.

The saturation of transitions and transversions was checked by plotting the absolute number of changes of each codon position against uncorrected sequence divergence values (p). There was no evidence of saturation in the ingroup (graph not shown).

To examine whether the different *C. arcasii* - *C. macrolepidotus* populations evolved at the same rate, we conducted an LRT with and without the molecular clock constraint, using PUZZLE (Strimmer and von Haeseler, 1996). We also conducted a relative-rate test (Sarich and Wilson, 1973; Wu and Li, 1985) among the main clades, with *C. lusitanicum* as outgroup, using the program RRTREE 1.1 (Robinson-Rechavi and Huchon, 2000). The PUZZLE program found no taxa with statistically longer branches, and the results of the relative-rate tests were not significant. Thus, the data were compatible with the use of a molecular clock.

Dowling et al. (2002) based on multiple fossil data and sequence comparisons estimated a divergence rate of about 0.53% per lineage per million years for the cytochrome *b* gene of cyprinids. This figure of about 1% of pairwise divergence per million years was adopted in other phylogenetic studies of cyprinids (Durand et al., 2003; Doadrio and Carmona 2004). We also used this calibration to estimate both divergence dates and the principal events that caused speciation in *C. arcasii* and *C. macrolepidotus* populations.

To calculate divergence times, we performed an ML analyses based on the HKY85 model (Hasegawa et al., 1985) with empirical Ts/Tv ratios and base frequencies using PUZZLE version 4.0.1 (Strimmer and von Haeseler 1996) with the clocklike option to obtain a clock-constrained tree (in which all root-to-tip distances have equal value).

7.4 Results

The 102 specimens yielded 57 distinct haplotypes. Among all the sequences studied, 299 sites were variable and 197 were parsimony informative. According to codon position, the most informative was the third (168 parsimony informative characters), followed by the first (21 characters).

The general time reversible model with among-site rate heterogeneity GTRI+G+I (Lanave et al. 1984; Yang 1994) was selected by MODELTEST as the best fit to the data. The rate matrix parameters estimated were R(a) = 7.27, R(b) = 1.59, R(c) = 3.57, R(d) = 7.47, and R(e) = 4.98. Base frequencies were A = 0.274, C = 0.274, G = 0.155, and T = 0.292. Among-site rate variation was approximated with the gamma distribution shape parameter alpha = 3.02. The proportion of invariable sites was I = 0.61.

MP analysis resulted in a single most parsimonious tree of 576 steps. The results of the four phylogenetic inference methods are summarized in Fig. 7.1, together with the geographic representation of the groups obtained. The topologies recovered by the four methods shared most features.

The ML tree was taken as the base for Fig.7.1, where bootstrap values for the ML, MP, and ME analyses and Bayesian inference with posterior probabilities are shown. Phylogenetic reconstructions for all inference methods showed two monophyletic lineages supported by high bootstrap and posterior probability values, separated by an average HKY85 distance of d = 6.5%. Contrary to what could be expected from the current taxonomy of the group, none of these lineages correspond to *C. arcasii* or *C. macrolepidotus*. Indeed, C. macrolepidotus and most populations of *C. arcasii* are included in the first of the two major lineages evidenced by the analysis (Groups I – III in Fig. 7.1).

Table 7.1 Specimens of the *C. arcasii* - *C. macrolepidotus* complex included in this study Their water body, drainage, locality/country, number of specimens, number of haplotypes per locality, and GenBank accession number. P, Portugal; S, Spain. The specimens from Lagoa do Canário (Azores) came from a population of *C. macrolepidotus* that originated in the Mondego River and was introduced to São Miguel Island in the 19th century. We decided to include them because they represent material present in Mondego prior to the advent of sport fishing, which may have encouraged some fish transfers between basins.

Water body	Drainage	Locality/Country	Number of specimens	Number of haplotypes	Accession Numbers
Alcabrichel	Alcabrichel	Lourinhã/P	4	4	AY254585-88
Lagoa do Canário	Mondego **	São Miguel, Azores/P	5	1	AY254589-90; AY254652-53
Ceira	Mondego	Góis/P	3	3	AY254648-50
Alva	Mondego	Sobral/P	1	1	AF045980
Alcoa	Alcoa	Alcobaça/P	4	2	AY254591-94
Cávado	Cávado	Barcelos/P	5	4	AY254595-99
Paiva	Duero	Castro D'Aire/P	1	1	AY254600
Távora	Duero	Vila da Ponte/P	5	1	AY254601-05
Araviana	Duero	Olvega/S	1	1	AY254606
Arlanza	Duero	Castillo de la Reina/S	1	1	AY254607
Aranzuelo	Duero	Arauzo de Torres/S	1	1	AY254608
Pisuerga	Duero	Salinas de Pisuerga/S	1	1	AY254609
Cubillo	Duero	Zael/S	1	1	AY254610
Adaja	Duero	Niharra/S	1	1	AY254611
Duraton	Duero	Saldaña de Ayllon/S	1	1	AY254612
Piron	Duero	Peñarrubias de Piron/S	1	1	AY254613
Sabor	Duero	Torre de Moncorvo/P	5	4	AY254614-18
Maçãs	Duero	-/P	1	1	AY254619
Bernesga	Duero	Beberino/S	2	2	AF045979; AY254620
Jalón	Ebro	Medinaceli/S	1	1	AY254621
Araquil	Ebro	Ciordi/S	1	1	AY254622
Júcar	Júcar	Gritos/S	1	1	AY254623
Estorãos	Lima	Ponte de Lima/P	4	1	AY254624-27
Lis	Lis	Porto de Mós/P	4	2	AY254628-31
Mijares	Mijares	Olba/S	1	1	AY254632
Valbona	Mijares	Valbona/S	2	2	AY254633-34
Coura	Minho	Vilar de Mouros/P	12	4	AY254635-46
Sil	Minho	Orallo/S	1	1	AY254647
Palancia	Palancia	Bejis/S	2	1	AY254654-55
Real	Real	Óbidos/P	4	2	AY254656-59
Safarujo	Safarujo	Mafra/P	6	1	AY254660-65
São Pedro	São Pedro	S. Pedro de Moel/P	3	2	AY254666-68
Sizandro	Sizandro	Torres Vedras/P	5	2	AY254669-73
Dulce	Tagus	Pelegrina/S	1	1	AY254674
Trabaque	Tagus	Villaconejos/S	1	1	AY254675
Gébalo	Tagus	Alcaudete de la Jara/S	2	2	AY254676-77
Lozoya	Tagus	Pinilla del Valle/S	2	2	AF045981; AY254678
Tornada	Tornada	Tornada/P	1	1	AY254679
Ulla	Ulla	Ponte Ulla/S	2	2	AY254680-81
Zela	Vouga	Vouzela/P	3	3	AY254682-84

C. macrolepidotus shows a clear geographic structure: Group I includes all the northern populations south to Mondego, while the remaining populations of the same species south of Mondego form Group III. Bayesian analysis suggests that these two groups form a monophyletic clade, sister to Group II that includes the majority of populations of *C. arcasii*. Interestingly, fishes from some Mediterranean rivers are grouped with those of Duero, while a few others are grouped with those of the Tagus. The Lozoya River is often not grouped with other tributaries of the Tagus basin because it tends to contain faunistic elements that are more representative of the Duero River, such as the species *Cobitis calderoni* (Doadrio, 2001).

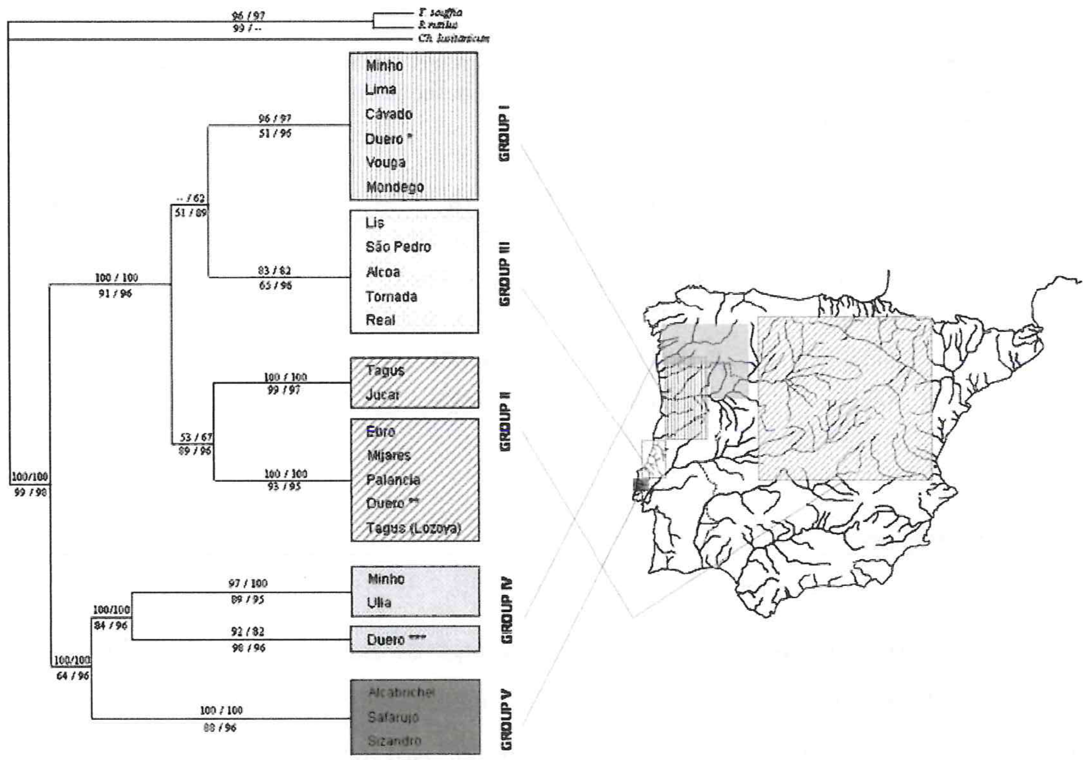

Fig. 7.1 Figure based on the ML tree. Phylogenetic relationships among the populations analyzed based on cytochrome *b* sequences (left) and schematic map of Iberia with the distributions of the phylogroups recovered by the phylogenetic analysis (right). Numbers above branches represent the bootstrap values obtained for ME and ML; numbers below branches indicate those for MP and the posterior probabilities for Bayesian inference. Statistics for the parsimony analysis are tree length 5 576 steps, consistency index (CI) 5 0.632, retention index 5 0.936, homoplasy index (HI) = 0.368, rescaled consistency index = 0.592, and F-ratio = 0.137 (without informative characters: CI = 0.565 and HI = 0.435). Most topological features were recovered by the four inference methods. When a particular branch was not recovered by a certain method, one hyphen replaces the corresponding bootstrap value. Each branch tip represents all the individuals sharing the same haplotype. In the boxes, the names of the rivers where the respective group of haplotypes was found are listed. Only one fish per haplotype was included in the figure, although the analysis was based on all the specimens. Duero*, lower section of the basin (in Portugal, rivers Paiva and Távora); Duero**, upper part of the basin (Spain, all tributaries upstream Bernesga); Duero***, tributaries around the border between Spain and Portugal (rivers Bernesga, Maçãs and Sabor).

Surprisingly, the second major lineage includes populations that are geographically very distant, some located in northwestern Spain and Portugal (Group IV, traditionally ascribed to *C. arcasii*), while others (Group V, analyzed here for the first time) are in the extreme southwest of the entire distribution area. Both are separated by many hundreds of kilometers and show an average HKY85 distance of d = 4.18%. The HKY85 distances among the five major phylogroups recovered in the analyses are presented in Table 7.2, together with the nucleotide diversity values for each phylogroup. It is important to note that except in the Minho drainage all the phylogroups described here were not found in sympatry, as can be seen in Fig. 7.1.

Table 7.2 HKY85 distances among the five major phylogroups recovered in the analyses_Average values are shown in the lower half, while the corresponding ranges are shown in the upper half. The nucleotide diversity values for each phylogroups are presented in the diagonal.

Groups	**I**	**II**	**III**	**IV**	**V**
I	0.0028 +/- 0.0017	1.81%- 3.48%	1.14%-1.81%	6.33%-6.97%	6.23%-7.40%
II	2.32%	0.0120 +/- 0.006	1.61%-3.48%	6.42%-8.14%	6.64%-8.36%
III	1.33%	2.52%	0.0033 +/- 0.002	6.01%-6.75%	6.12%-7.19%
IV	6.67%	7.31%	6.38%	0.0033 +/- 0.002	3.88%-4.99%
V	6.64%	7.27%	6.50%	4.39%	0.0024 +/- 0.0015

7.5 Discussion

Our results clarify the unexpected findings of Zardoya and Doadrio (1998), who questioned the monophyly of *C. arcasii* and *C. macrolepidotus*. Indeed, it becomes apparent that *C. arcasii* is a polyphyletic group, including the two major lineages identified (Groups II and IV). At the same time, our study also provides evidence that in the southwest of the distribution area of the species group there are populations that do not fit either in *C. arcasii* or in *C. macrolepidotus* (Group V). Group V populations emerge farther south of the distribution range of *C. macrolepidotus*, in the small coastal rivers of Alcabrichel, Sizandro, and Safarujo. This peculiar distribution (with populations in the extreme northwest and southwest limits of the group range) strongly suggests that the lineage must have had a much wider distribution in the past and that the populations represent relic pockets that were separated vicariantly.

In other freshwater fishes, phylogroups detected with molecular methods have subsequently been ascribed to different species (Hendry et al. 2000). It is probable that fishes belonging to Groups IV and V also correspond to distinct but yet undescribed species. A formal taxonomic revision of the entire group is urgently needed.

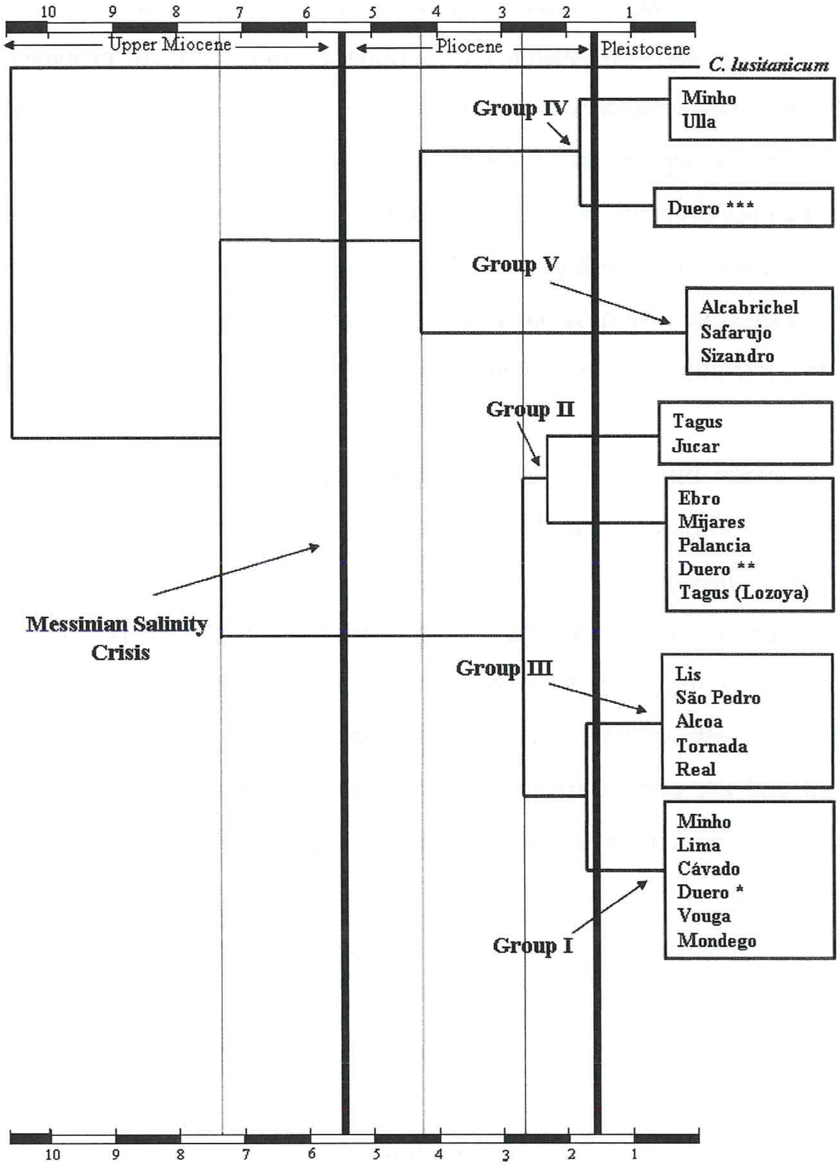

Fig. 7.2 Clock-constrained ML tree showing the major cladogenetic events in the history of Iberian cyprinids. The scale bars show the time scale resulting from a calibration of the molecular clock (1% per MYA).For the explanation of the boxes at the tip of the branches, see Fig. 1. Duero*, lower section of the basin (in Portugal rivers Paiva and Távora); Duero**, upper part of the basin (Spain, all tributaries upstream Bernesga); Duero***, tributaries around the border between Spain and Portugal (rivers Bernesga, Macxa˜s, and Sabor).

The divergence time that separates the two main lineages identified in this study of more than 7 million years, clearly suggests that this cladogenetic event took place in the Miocene, while the separation of phylogroups IV and V took place more than 4 million years ago (MYA), in the lower Pliocene (Fig. 7.2). These events predate the formation of the present hydrographic network (Andeweg et al., 1999). In addition, as predicted by our second hypothesis, members of the two main lineages co-occur in the same basin, namely, in the

Duero basin. Thus, our results indicate that a substantial proportion of the diversification of the group occurred before the present river drainages were formed, probably in the inland lakes that drained Iberian waters during the Mio-Pliocene. Our results also identified more recent events like the separation of the phylogroups II and I–III that occurred less than 3 MYA. Thus, the high diversity presently found in these Iberian cyprinids is likely the result of old cladogenetic events that took place in Miocene times and more recent events of Pliocene and possibly Pleistocene origin. If our conclusions are correct, the two scenarios presented in this paper both contributed to the present level of diversity.

We are aware that all the interpretations presented above depend critically on the validity of our calibration of the molecular clock. Different authors have calibrated molecular clocks for the cytochrome *b* gene of cyprinids using fossil or geological data. Zardoya and Doadrio (1999) calibrated a molecular clock of 0.76% divergence per lineage per million years, using the opening of the Gibraltar strait (5.5 MYA, Krijsman et al., 1999) and the formation of the Korinthos strait in Greece. Subsequently, on the basis of the opening of the Gibraltar strait and the genetic divergence between populations of the genus Barbus from Africa and Iberian Peninsula, Machordom and Doadrio (2001) estimated a molecular clock of 0.66% per lineage per million years. Dowling et al. (2002) and Smith et al. (2002) provided a calibration of approximately 0.5% divergence per lineage per million years for the cyt *b* gene, based on a series of comparisons for which divergence rates and fossil ages are available. A comparison involving North African and Iberian species of the genus Cobitis (which belongs to a family closely related to the cyprinids) yielded a divergence rate of 0.42% per lineage per million years (Perdices and Doadrio, 2001). Thus, although older estimates of the divergence rates between lineages of cyprinids for the cyt *b* gene were higher (e.g., 0.76% per lineage per million years; Zardoya and Doadrio, 1999), the more recent estimates converge on a divergence rate of around 1% sequence divergence per pairwise comparison per million years or 0.5% divergence within lineage per million years, the figure that we adopted in this study. However, even if we adopted the more conservative calibration of 0.76% per lineage per million years of Zardoya and Doadrio (1999), the separation of the two main lineages would still be of late Miocene-lower Pliocene origin, a timing that would not invalidate our conclusions.

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7.7 References

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Capítulo 8

Identification of ESUs in the Critically Endangered Portuguese minnow *Chondrostoma lusitanicum* Collares-Pereira 1980, based on a phylogeographical analysis

8.1 Abstract

In this paper, we analysed the phylogeography of the Critically Endangered fish *Chondrostoma lusitanicum* (Cyprinidae) endemic to Portugal. A fragment of the mitochondrial cyt *b* gene and one from the nuclear beta-actin gene were sequenced. The results support recognition of 3 ESU's distributed as follows: 1) Sado River, 2) the Tagus River basin and 3 independent nearby coastal rivers (collectively named ''West'') and 3) Lagoa de Albufeira, a small coastal lagoon between Tagus and Sado basins.

8.2 Introduction

Chondrostoma lusitanicum is a small cyprinid endemic to Portugal and considered Critically Endangered (Cabral et al., 2005), whose range is restricted to the basins of lower Tagus (e.g. Rivers Trancão, Maior, Jamor, Lage and Ossos, see Fig. 8.1), Sado, and a number of small independent drainages that are in the neighbourhood of those rivers. It also occurs in Lagoa de Albufeira, a very small coastal lagoon between Tagus and Sado (Fig. 8.1). Its habitat is composed mostly by small Mediterranean type streams which are prone to very severe droughts, while the proximity of most streams to big cities like Lisbon and Setúbal means that these waters are subjected to very strong pollution caused by urban, industrial and agricultural sources. In this context, it is urgent to delineate a conservation strategy for the species and the prospect of *ex-situ* reproduction for populations that live in streams where the habitat became unsuitable must be considered.

To achieve this goal a proper identification of the units to be conserved is an essential prerequisite. The first attempt to address this issue with DNA data was made by Mesquita et al. (2001). However, only mitochondrial data were included, sample size was very limited (only 22 individuals of *C. lusitanicum* were analysed) and geographical coverage was very incomplete (e.g. only two fish were analysed in the entire Tagus basin and only one of the independent small drainages where the species occurs was included).

In this paper we present data on the phylogeography of the species, covering most of its range, in order to identify the ESUs (Evolutionary Significant Units, sensu Waples, 1991) that make up the species. According to Waples (1991) an ESU can be defined as a population or population group with historical isolation and adaptive distinction from other populations within the species, and therefore representing a significant portion of the evolutionary legacy of the species.

8.3 Methods

Individuals of *C. lusitanicum* were sampled from ten sites (Fig. 8.1, Table 8.1). Sixty-five individuals were sequenced for the cyt *b* and 42 for the beta-actin gene (Table 8.1).

Total genomic DNA was extracted from ethanol preserved fin clips by an SDS/proteinase-k-based protocol (adapted from Sambrook et al. 1989). 726 bp and 871 bp were amplified for the cyt *b* and beta-actin genes respectively. Primers and PCR conditions used are those reported in Robalo et al. (2006). Sequencing reactions were performed by Macrogen Inc.

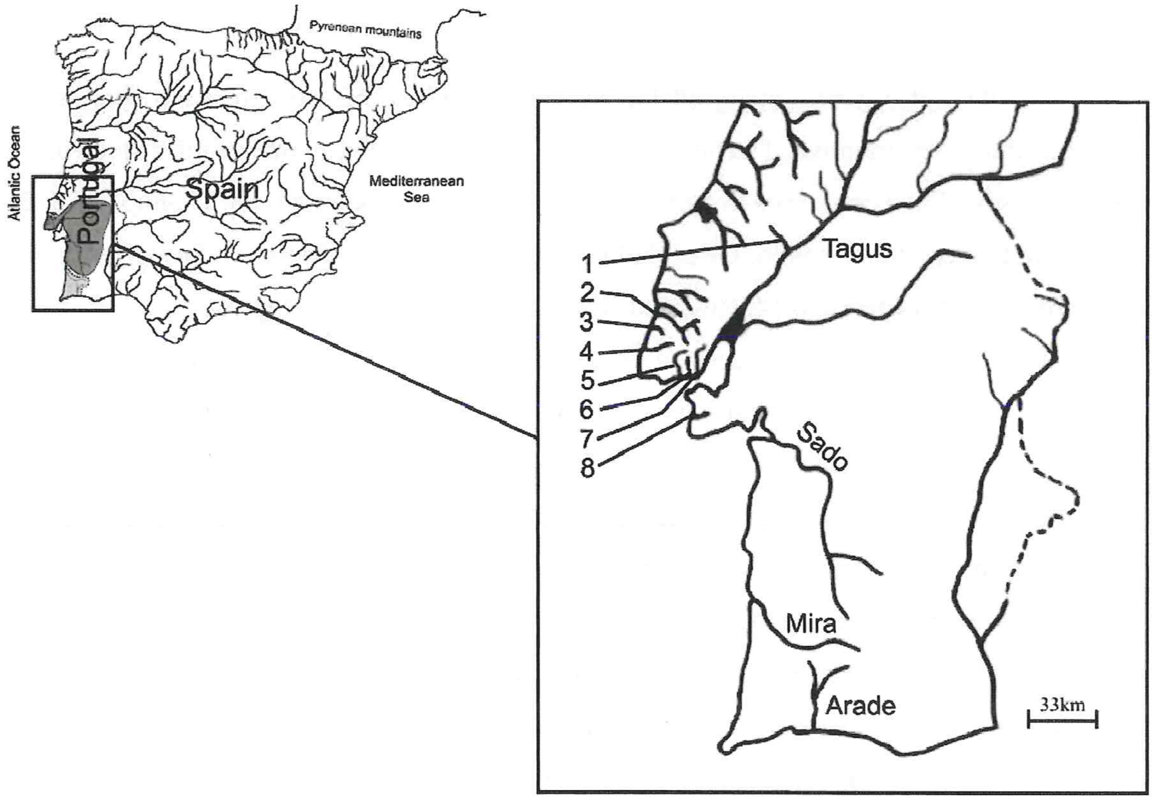

Fig. 8.1 Map of the Iberian Peninsula with the distribution of *C. almacai* (darker area) and *C. lusitanicum* (lighter shading) and detail of the main drainages sampled. Labels of collecting sites in the map: (1) Maior, (2) Lizandro, (3) Samarra, (4) Colares, (5) Lage, (6) Ossos, (7) Jamor, (8) Lagoa de Albufeira

All sequences were deposited in GenBank. Sequences were aligned with Clustal X (Thompson et al. 1997) and analysed using distance (neighbour-joining, NJ) and maximum parsimony (MP) using PAUP 4.0 (Swofford, 1998). The uncorrected p distance was used. *Chondrostoma almacai* Coelho, Mesquita, Collares-Pereira, 2005 was used as outgroup.

ARLEQUIN version 3.01 (Schneider et al., 2000) was used to estimate genetic diversity, mean number of pairwise differences for each pair of populations and to perform analysis of molecular variance (AMOVA; Excoffier et al., 1992). Mean numbers of pairwise differences were corrected by subtracting the average within population differences for the populations of each pair, as implemented in ARLEQUIN. Subsequently these values were transformed in percent sequence divergence after dividing the number of pairwise differences by the length of the DNA fragment analysed.

8.4 Results

For the cyt *b* gene, among all the sequences studied, 65 sites were variable and 55 were parsimony informative. Twenty-two haplotypes were found in the samples studied. MP analysis resulted in a consensus tree of 75 steps (Consistency index = 0.88; Homoplasy Index = 0.12; Retention Index = 0.96; CI excluding uninformative characters = 0.86; HI excluding uninformative characters = 0.14: Rescaled consistency index = 0.85) (Fig. 8.2).

The phylogenetic trees derived from the cyt *b* gene and using the two inference methods converged in the same basic topology. They support the monophyly of *C. lusitanicum*. The phylogenetic tree consistently differentiates two groups, one including fish from the Sado basin and another including fish from Lagoa de Albufeira and all populations that we grouped as ''West''. For cyt *b*, the mean divergence between Sado and ''West'' and between Sado and Lagoa de Albufeira, after correcting for within population variation, is 2.01% and 1.9% respectively. AMOVA performed considering a single group with three populations: ''West'', Lagoa de Albufeira and Sado, showed that among population variation explained 87, 19% of the variance and was highly significant ($P < 0.001$). All comparisons involving pairs of populations were significant at the same p level (FST values, ''West''– Lagoa de Albufeira = 0.41; ''West''– Sado = 0.92; Lagoa de Albufeira–Sado = 0.89). Concerning the genetic diversity, most indices support the conclusion that Sado and Lagoa de Albufeira are the most diverse populations, contrasting with the low diversity of the ''West'' (see Table 8.1). Overall, Sado seams to be the most diverse population. Lagoa de Albufeira comes next, which is surprising because a water body of a few hectares harbours more diversity than all samples from ''West'', which cover many tens of kilometres of eight different rivers (Table 8.1).

For the beta-actin gene, only two haplotypes were found: one for each group, ''West'' (including all the individuals from Lagoa de Albufeira) and Sado. The samples from Sado differ from those of ''West'' and Lagoa de Albufeira by two fixed mutations, one substitution and one indel of two bases. *C. almacai* (including individuals both from Arade and Mira basins) presented their own haplotype.

Table 8.1 Collecting sites, number of individuals and number of haplotypes for cyt *b* and beta-actin genes. Number of polymorphic sites, gene diversity, nucleotide diversity and mean number of pairwise differences per group of populations studied, for cyt b gene. n^1 – number of samples per drainage; n^2 – number of samples per group of populations considered in this study. Names marked with * represent tributaries of the lower Tagus. For location of sample sites see Fig. 8.1. GenBank Accession Nos. DQ845488-DQ845562 and DQ898223-36 for cyt *b* gene and DQ845564-DQ845600 and DQ898237-48 for beta-actin gene)

					Cyt *b*				**Beta-actin**	
	n^1	n^2	Number of haplotypes	Number of polymorphic sites	Gene diversity	Nucleotide diversity	Mean number of pairwise differences	n^1	n^2	Number of haplotypes
C. lusitanicum										
“West”		40	7	8	0.3279 +/- 0.0962	0.0016 +/- 0.0012	1.1309 +/- 0.7512		33	1
Trancão *	3							2		
Maior *	5							5		“West” and Lagoa de Albufeira share the same haplotype
Jamor *	4							3		
Lage*	5							4		
Ossos*	3							2		
Colares	7							4		
Samarra	9							3		
Lizandro	4							3		
Lagoa de Albufeira	12	12	4	6	0.6364 +/- 0.1277	0.0027 +/-0.0019	1.9697 +/- 1.1964	7	7	1
Sado	14	14	7	7	0.8242 +/- 0.0781	0.0019 +/- 0.0014	1.3956 +/- 0.9099	9	9	1
C. almacai		23	4	3	0.4862 +/- 0.1053	0.0008 +/- 0.0007	0.5771 +/- 0.4837		7	1
Mira	18							5		
Arade	5							2		

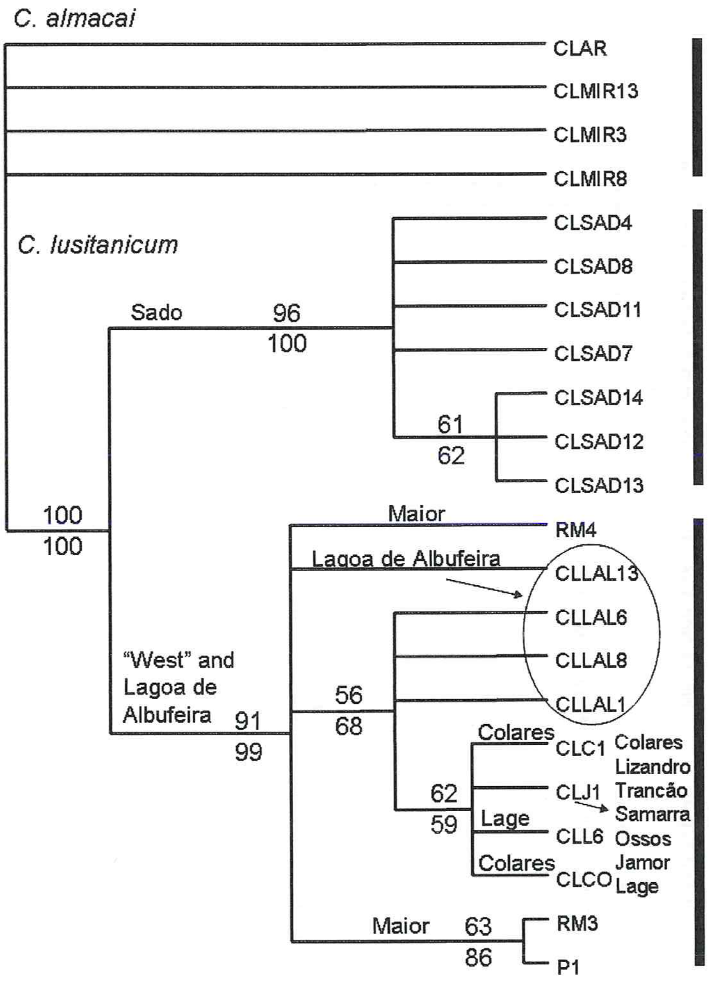

Fig. 8.2 Tree of cyt *b* based on MP analysis. Each haplotype was included only once in the tree, regardless of the number of fish that presented it. Numbers above branches represent bootstrap values for MP (1,000 replicates) and numbers below branches represent bootstrap values for NJ (100 replicates). C. almacai haplotypes were used as outgroups. Distribution of haplotypes per drainages is also presented in the terminal branches. Black vertical bars represent the three beta-actin haplotypes. Labels, numbers of fish and distribution for each haplotype: CLSAD14: 5 fish, Sado; CLSAD12: 1 fish, Sado; CLSAD13: 1 fish, Sado; CLSAD4: 4 fish, Sado; CLSAD8: 1 fish, Sado; CLSAD11: 1 fish, Sado; CLSAD7: 1 fish, Sado; CLLAL13: 3 fish, Lagoa de Albufeira; CLLAL6: 1 fish, Lagoa de Albufeira; CLLAL8: 1 fish, Lagoa de Albufeira; CLLAL1: 7 fish, Lagoa de Albufeira; CLC1: 1 fish, Colares; CLJ1: 32 fish, Colares, Lizandro, Trancão, Samarra, Ossos, Jamor and Lage; CLL6: 1 fish, Lage; CLCO: 1 fish, Colares; RM3: 2 fish, Maior; P1: 2 fish, Maior; RM4: 1 fish, Maior; CLAR: 1 fish, Arade; CLMIR13: 16 fish, Arade and Mira; CLMIR3: 5 fish; Mira; CLMIR8: 1 fish, Mira.

8.5 Discussion

Two divergent groups were distinctly identified within the very small distribution area of *C. lusitanicum*: one from Sado and another from ''West'' and Lagoa de Albufeira.

As stated above, for the beta-actin gene, the samples from Sado differ from those of ''West'' and Lagoa de Albufeira by two fixed mutations. This difference is not trivial. For instance, in *Squalius pyrenaicus* and *Squalius carolitertii*, two sister species that according with cyt *b* data diverged about 4–6 MYA (Sanjur et al., 2003) the same sequence is still present in the majority of fish of both species sampled so far (Robalo et al., 2006). For the cyt *b* gene, the mean divergence between Sado and ''West'' and between Sado and Lagoa de Albufeira, after correcting for within population variation, was 2.01% and 1.9% respectively. Assuming a clock calibration of 1.05% divergence between lineages per million years (Dowling et al. 2002) we find that the population from Sado diverged from the group ''West''/ Lagoa de Albufeira about 2 million years ago in the Plio-Pleistocene boundary. Taken together, the available evidence suggests that we may be in the presence of two distinct species, stressing the need of further genetic and morphological investigations. Regardless of the decision on this issue we believe that this paper provides strong evidence that the populations from the Sado must be recognized as an ESU that deserves specific conservation measures. From the groups studied this group showed the highest level of genetic diversity, despite the fact that the fish came from a single location.

The populations of ''West'' and Lagoa de Albufeira share the same beta-actin haplotype and the corrected p distance between the two groups for cyt *b* (0.13%) suggests a divergence time of about 130 000 years. This group includes two distinct ESUs, which are supported by the FST values for cyt *b*. Indeed, not a single haplotype was shared among them. In spite of its minute size the ESU of Lagoa de Albufeira presents a comparatively high level of genetic diversity which, in our view, justifies its preservation.

Within the ''West'' group, the River Maior shared no haplotypes with the remaining rivers. If the few fish from the River Maior are excluded, the remaining fish from the ''West'' form a very homogenous group with extremely low genetic diversity dominated by a single haplotype and occupying the tributaries of the estuarine part of the Tagus drainage and a few independent coastal streams located near the Tagus. Indeed, a single haplotype for cyt *b* (CLJ1) was found in 32 of 39 fish coming from different drainages. The present paper is in apparent contradiction with Mesquita et al. (2001), which suggested the separation of fish from the Tagus from those from Samarra, one of the independent drainages also sampled in our study. It is important to note, however, that Mesquita et al. (2001) only sampled two fish from Tagus and do not state in which part of the basin the fish were collected. In the present study, the Tagus fish that cluster with other drainages of the ''West'' are those that run to the saline estuary (Trancão, Ossos, Ribeira da Lage e Jamor). Thus, we suggest that while, based on the available data, at least three ESUSs are granted: Sado, Lagoa de Albufeira and ''West'', further sampling in the Tagus basin may lead to subdivision of this last ESU. The low diversity of this ESU is unlikely to be caused only by repeated bottlenecks as suggested by Mesquita et al. (2001), but also, at least in part, by the likely young age of the populations of the area, as suggested by the sharing of a common haplotype by the great majority of fish of seven different rivers.

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Capítulo 9

Insights on speciation patterns in the genus *Iberochondrostoma* (Cyprinidae): evidence from mitochondrial and nuclear data

9.1 Abstract

In this paper the patterns of cladogenesis in the cyprinid fish genus *Iberochondrostoma* were analysed using a mitochondrial (cytochrome *b*) and a nuclear (beta-actin) gene fragment. The two genes yielded discordant results. While the cytochrome *b* gene yielded a fully dichotomous tree, where all species of the genus are monophyletic, the much slower beta-actin gene yielded star-like relationships. However, when information from both genes was considered together, the data suggested the persistence of a very large central unit from which at least two peripheral clades arose at different times. This pattern which is akin to peripatric speciation was shown to be compatible with the paleogeographical information available. It is suggested that combining the techniques of phylogeny and phylogeography and the use of multiple markers varying in their rate of evolution may enrich our understanding of speciation and evolution of clades beyond species level.

9.2. Introduction

Although much progress has been achieved, a considerable amount of work is still needed to fully bridge the gap between studies of the genetic relationships among populations and those involving taxa beyond the species level.

In phylogeographic studies emphasis has been placed on the relationships among populations, commonly represented by haplotype networks or star-like tree topologies. These patterns are largely caused by incomplete lineage sorting and absence of reciprocal monophyly (e.g. Avise, 2000). On the contrary, phylogenetic analysis, especially when it is based on the rapidly evolving mitochondrial genes tends to result in trees presenting dichotomous structures with monophyletic groups. Implicit in many phylogenetic analyses is the assumption that in speciation the ancestral species tend to originate two sister species not very different in size. In this perspective, polytomies are viewed as being usually soft, being due to insufficient sampling, either in the amount of DNA or in the number of taxa. Hard polytomies in turn, would result from a very rapid succession of speciation events which left no record in a given genetic marker (e.g. Page and Holmes, 1998; Poe and Chubb, 2004). Another scenario is however conceivable. Consider a species with a broad geographical distribution and with a large effective population size. Such a "large" species can in different occasions, give rise to new peripheral species with small geographical areas and effective sizes without substantial changes in its genetic structure. Indeed, the larger the effective population size the slower the lineage sorting will be and the split of a small subset like the formation of a new "small" peripheral species may be of little consequence for the genetic pool of the large population which gave rise to it. In such situations "large" species may originate different descendent species at rather separated times. This speciation pattern can be detected using two or more genetic markers with different rates of evolution and may represent one of the possible explanations for a phenomenon frequently reported in phylogenetic studies: the fact that in many cases, different genes yield different tree topologies (e.g. Russo et al., 1996; Mattern, 2004). The combination of phylogenetic and phylogeographic techniques using large DNA datasets and comprising genes with different rates of evolution may help to recognize the modes of speciation that took place in a clade, namely the hypothesis of peripatric speciation (Mayr, 1982). In this speciation pattern we expect to find a species with a central position presenting a high level of genetic diversity and ancestral polymorphisms and a few species in its periphery each one with low genetic diversity, small effective population size and geographical area, but revealing much more complete lineage sorting. The same pattern is unlikely to occur if a species gives rise to two species of similar size.

In this paper we try to test the hypothesis of peripatric speciation using the genus *Iberochondrostoma* as a model. This genus was recently described (Robalo et al. , 2007a) and constitutes an excellent model because it is well characterized from the morphological and molecular point-of-view and has a compact distribution area that occupies the West, centre and South of the Iberian Peninsula, being present almost without exception in all Atlantic basins between Tagus and Guadalquivir. Three of the 4 species included in this genus are considered as Critically Endangered (Doadrio, 2001; Doadrio and Carmona, 2003a; Cabral et al., 2005; see Fig. 9.1) thus the study of their genetic structures assumes a great importance for their conservation (Mesquita et al., 2001; Robalo et al., 2007b).

Iberochondrostoma includes the following species at the present moment: *I. lemmingii* (Steindachner, 1866); *I. lusitanicum* (Collares-Pereira, 1980); *I. oretanum* (Doadrio and Carmona, 2003a) and *I. almacai* (Coelho, Mesquita and Collares-Pereira, 2005) (for the species distribution see Fig. 9.1). At least an additional one is awaiting formal description (the populations of *I. lusitanicum* from the Lower Tagus, adjacent drainages, in this paper collectively named West, and Lagoa de Albufeira, Robalo et al., 2007b). The old reports of *I. lemmingii* in the Douro drainage have been shown to correspond to a different fish of a distinct genus (*Achondrostoma*, Robalo et al., 2007a) which is being formally described as a new species.

The hypothesis that the evolution of this genus may have been characterized by the persistence of a large central species which gave rise to several peripheral small daughter species at different times is suggested by the present geographic distribution of the members of the genus (Fig. 9.1). In addition, it is known that, in the Miocene, many Iberian waters of the area where *Iberochondrostoma* species live drained to a number of inland lakes, the modern river system being very recent (Plio-Pleistocene) (Friend and Dabrio, 1996; Andeweg, 2002). Thus, the disappearance of the ancient lakes and the association of their remnants to different river systems is compatible with the pattern of speciation outlined above.

This paper aims to describe the phylogeography of the entire genus *Iberochondrostoma*, using two molecular markers, with different rates of evolution. The mitochondrial cytochrome *b* (cyt *b* gene) was selected as a rapidly evolving gene, very commonly used in phylogenetic (e.g. Zardoya and Doadrio, 1998, 1999) and phylogeographic studies (Salzburger et al., 2003; Robalo et al., 2006a, 2007b). On the contrary, the nuclear beta-actin gene was selected due to its slow evolution rate (Robalo et al., 2006b, 2007a).

The hypothesis of speciation by peripheral isolation leads to the following predictions: 1) the presence of a central species of large size with several small species surrounding it; 2) more complete lineage sorting in the peripheral species than in the central one, promoted by their smaller sizes; 3) a much higher level of diversity and the persistence of ancestral polymorphisms in the central species.

9.3 Methods

Taxon sampling

The taxa analysed in this study, their collection sites and their corresponding GenBank sequence Accession Nos. are listed in Table 9.1 (For collection sites location see Fig. 9.1). Except in the few cases when not enough biological material was available, the DNA used for PCR and sequencing of both fragments came from the same individual. Voucher specimens are preserved in the fish collections of Museo Nacional de Ciencias Naturales (MNCN) and the Unidade de Investigação em Eco-Etologia, Instituto Superior de Psicologia Aplicada (UIEE/ISPA).

DNA analysis

Total genomic DNA was extracted from fin clips preserved in ethanol by an SDS/proteinase-k based protocol (adapted from Sambrook et al., 1989).

For the cyt *b* gene a total of 738 bp was amplified using the primers LCB1–5'-AATGACTTGAAGAACCACCGT-3' (Brito et al., 1997) and HA–5'-CAAC GATCTCCGGTTTACAAGAC-3' (Schmidt and Gold, 1993). PCR conditions followed those in Cunha et al. (2004). The amplification process was conducted as follows: 25 cycles of [94 °C (1min), 50 °C (1min) and 72 °C (2min)].

For the beta-actin gene a total of 891 bp was amplified using the primers BactFor -5'-ATGGATGATGAAATTGCCGC-3' and BactRev – 5' -AGGATCTTCATGAGGTAGTC-3' (Robalo et al., 2006b). PCR conditions followed those in Robalo et al. (2006b). The amplification process was conducted as follows: 35 cycles of [94 °C (30 s), 55 °C (40 s) and 72 °C (1 min 30 s)]. The amplified fragment is homologous to a region of the beta-actin gene of *Cyprinus carpio* (GenBank: M24113), including introns B and C and three exons.

Sequencing reactions were performed by Macrogen Inc. in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer.

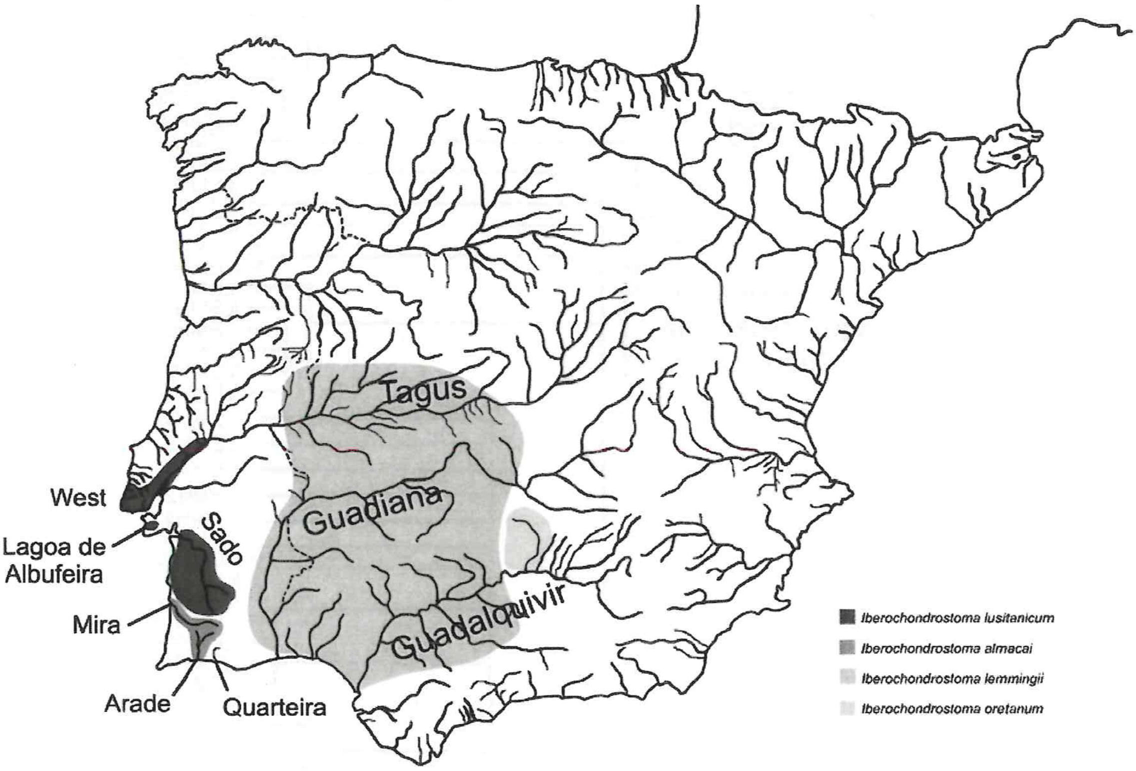

Fig. 9.1 – Map of the Iberian Peninsula with the distribution of the *Iberochondrostoma* species.

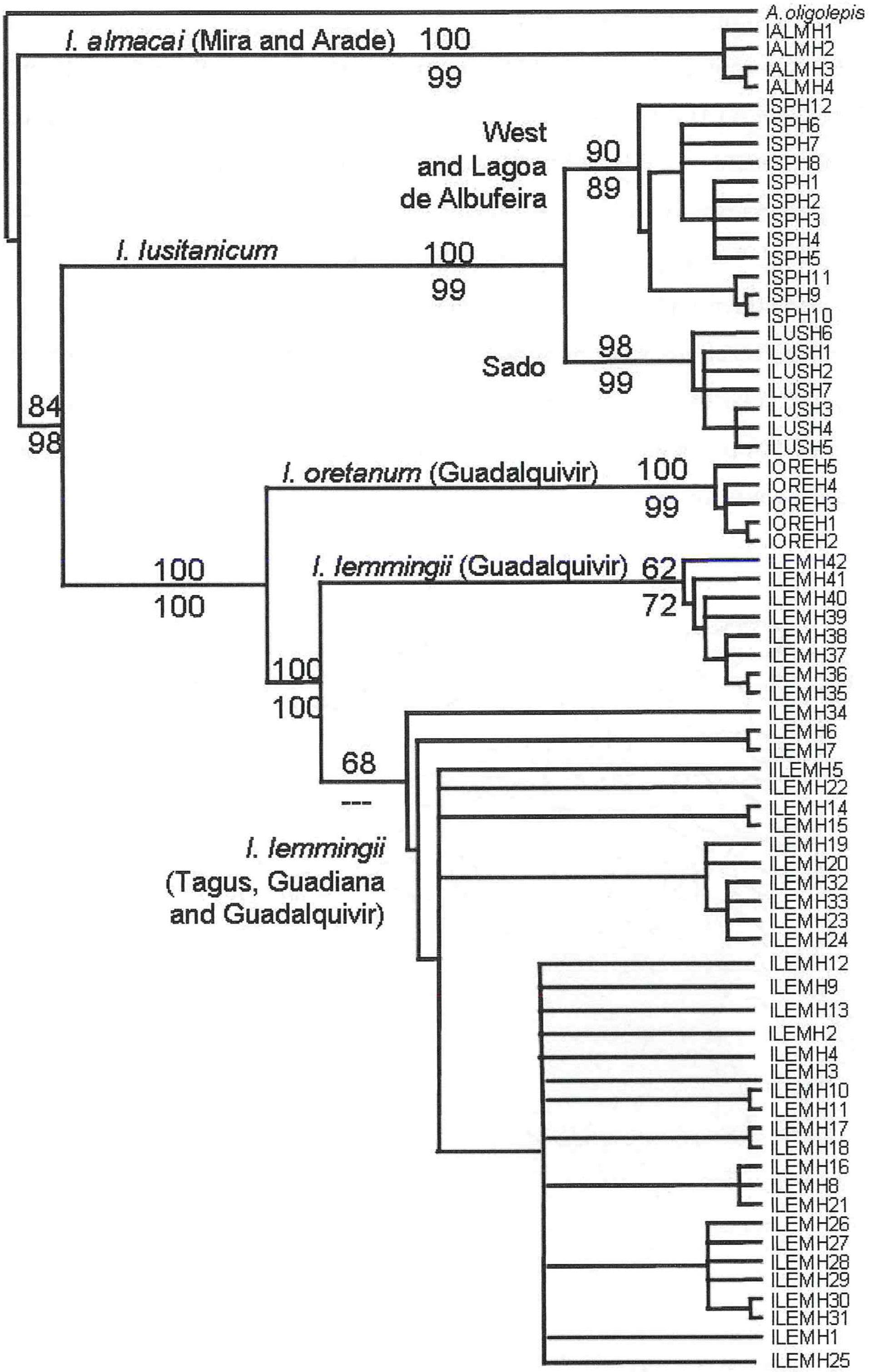

Fig. 9.2 Tree of cyt *b* based on MP analysis. Each haplotype was included only once in the tree, regardless of the number of fish that presented it. Numbers above branches represent bootstrap values for MP (1000 replicates) and numbers below branches represent bootstrap values for NJ (100 replicates). Haplotypes are the same considered in TCS (for haplotype labels see the legend of Fig. 9.3)

Table 9.1 Samples analysed in this study, their collection sites and their corresponding GenBank Accession Numbers for cytochrome *b* and beta-actin genes. Accession Numbers marked with * were retrieved from GenBank.

Species	Basin	River/ Locality/ Country	Cyt *b* Number of samples/ GenBank Accession Numbers	Beta-actin Number of samples/GenBank Accession Numbers
I. lemmingii	Quarteira	Quarteira / Paderne / Portugal	3/ EF520187; EF520188; EF520190	5/ EF520237; EF520239; EF520240; EF520242; EF520223
	Guadiana	- / - / Portugal	3/ X99423*; EF520189; EF520191	
		- / - / Spain	1/ AF045987	
		Jabalón / Bazan / Spain	3/ EF524514; EF520147; EF520148	3/ EF520224, EF520226, EF520225
		Esteras / Saceruela / Spain	7/ EF520136; EF520142; EF520144; EF520145; EF520149; EF520181; EF520192	5/ EF520230; EF520251; EF520252; EF520253; EF520257
		Gargantiel / Almadenejos / Spain	3/ EF520162; EF520166; EF520179	1/ DQ061940*
		Estomiza / Gamonoso / Spain	1/ DQ089654*	1/ EF520241
		Siruela / Tamurejo / Spain	4/ EF520160; EF520175; EF520182; EF520198	1/ EF520244
		Queijigares / Fontanosas / Spain	7/ EF520127; EF520129; EF520134; EF520161; EF520196; EF520201; EF520214	3/ EF520256; EF520250; EF520258
		Alcarrache / Higuera de Vargas / Spain	1/ AY568605*	
		Guadalmez / La Bienvenida / Spain	1/ EF520159	
		Valdeazogues / Almadenejos / Spain	1/ EF520163	
		Navalatienda / Valdemanco de Esteras / Spain	1/ EF520165	
		Guadamez / Valle / Spain	2/ EF520168; EF520177	
		Alcudia / El Alamillo / Spain	1/ EF520184	

(Table 9.1 Cont.)

Species	Basin	River/ Locality/ Country	Cyt *b* Number of samples/ GenBank Accession Numbers	Beta-actin Number of samples/GenBank Accession Numbers
I. lemmingii	Guadiana	Maillo / Navas de Estena / Spain	3/ EF520193; EF520194; AF045988	
		Sillo/ Cumbres de San Bartolomé / Spain	3/ EF520195; EF520202; EF520217	
		Estenilla / Gamonoso / Spain	2/ EF520203; EF520219	
		Gévora / Alburquerque / Spain	1/ EF520213	
		Higuerón/ Cañaveral de León / Spain	1/ EF520218	
	Tagus	Magasca / Trujillo / Spain	13/ EF520128; EF520130; EF520178; EF520172; EF520131; EF520135; EF520169; EF520170; EF520171; EF520183; EF520173; EF520180; EF520200	9/ EF520227; EF520246; EF520245; EF520229; EF520233; EF520234; EF520238; EF520249; EF520254
		Almonte/ Jaraicejo/ Spain	2/ EF520156; DQ447733*	2/ DQ447716*; EF520247
		Pesquero/ Valverde del Fresno / Spain	6/ EF520153; EF520154; EF520155, EF520157; EF520185; EF520186	2/ EF520228; EF520248
		Tietar / Talayuela / Spain	2/ AY568604*; EF520158	
		Alburrel / Valencia de Alcantara / Spain	1/ AY568603*	
		Huso / Aldeanueva de San Bartolomé / Spain	1/ EF520199	
		Albuera / Almendral / Spain	1/ EF520206	
		Aurela/Santiago de Alcantara/Spain	2/ EF520215; EF520216	
		Vid/Torrejón el Rubio/Spain	1/ EF520221	
	Guadalquivir	Arenoso / Montoro / Spain		1/ EF520222
		Matapuercas / Adamuz/ Spain	4/ EF520133; EF520138; EF520146; EF520174	2/ EF520231; EF520255

(Table 9.1 Cont.)

Species	**Basin**	**River/ Locality/ Country**	**Cyt *b*** **Number of samples/ GenBank Accession Numbers**	**Beta-actin** **Number of samples/GenBank Accession Numbers**
I. lemmingii	Guadalquivir	Tablillas / Brazatortas / Spain	8/ EF520137; EF520139; EF520140; EF520141; EF520143; EF520164; EF520176; EF520197	3/ EF520232; EF520235; EF520236
		Ovejuna / - / Spain	3/ AY568608*; EF520204; EF520205	
		Belmez / Albardado / Spain	6/ AY568607*; EF520208; EF520209; EF520210; EF520211; EF520212	
		- / - / Spain	1/ EF520167	
		Montemayor/Cañaveral de León/Spain	1/ EF520220	
		Molinos/Llerena/Spain	1/ EF520207	
I. oretanum	Guadalquivir	Robledillo/ Solana del Pino / Spain	5/ DQ447737*; EF520150; EF520151; EF520152; AF045989	1/ DQ447722*
		- / - / Spain	1/ EF520132	1/ EF520243
I. lusitanicum	Sado	Sado / Canal Caveira / Portugal	14/ DQ845549* - DQ845562*	9/ DQ845592* - DQ845600*
	West	Trancão / S. Julião do Tojal / Portugal	3/ DQ845523*; DQ845532*; DQ845542*	2/ DQ845579*; DQ845580*
		Maior / - / Portugal	5/ DQ898232*- DQ898236*	5/ DQ898237*- DQ898241*
		Jamor / Oeiras / Portugal	4/ DQ898223*- DQ898226*	3/ DQ898246*- DQ898248*
		Lage / - / Portugal	5/ DQ898227*- DQ898231*	4/ DQ898242*- DQ898245*
		Ossos / Rinchoa/ Portugal	3/ DQ845524*; DQ845544*	2/ DQ845588*; DQ845590*;
		Colares / Colares/ Portugal	7/ DQ845514*; DQ845525*; DQ845527*; DQ845530*; DQ845535*; DQ845537*; DQ845538*; DQ845546*	4/ DQ845581*- DQ845583*; DQ845589*

(Table 9.1 Cont.)

Species	**Basin**	**River/ Locality/ Country**	**Cyt *b*** **Number of samples/ GenBank Accession Numbers**	**Beta-actin** **Number of samples/GenBank Accession Numbers**
I. lusitanicum	West	Samarra / Praia da Samarra / Portugal	9/ DQ845522*; DQ845526*; DQ845528*; DQ845529*; DQ845531*; DQ845536*; DQ845539*; DQ845541*; DQ845543*	3/ DQ845578*; DQ845584*; DQ845591*
		Lizandro/ Cheleiros/ Portugal	4/ DQ845533*; DQ845534*; DQ845540*; DQ845545*	3/ DQ845585*- DQ845587*
	Lagoa de Albufeira	Lagoa de Albufeira / Herdade da Apostiça/ Portugal	12/ DQ845511*- DQ845513*; DQ845515*- DQ845521*; DQ845547*- DQ845548*	7/ DQ845564*- DQ845570*
I. almacai	Mira	Mira / Santa Luzia / Portugal	18/ DQ845489*- DQ845494*; DQ845496*- DQ845498*; DQ845500*; DQ845502*- DQ845504*; DQ845506*- DQ845510*	5/ DQ845571*- DQ845573*; DQ845576*- DQ845577*
	Arade	Odelouca /Alferce / Portugal	5/ DQ845488*; DQ845495*; DQ845499*; DQ845501*; DQ845505*	2/ DQ845574*; DQ845575*

Data analysis

Sequences were aligned with Clustal X (Thompson et al., 1997). Phylogenetic analyses were performed with PAUP 4.0 (Swofford, 1998) using maximum parsimony (MP) and distance (neighbour-joining, NJ). For the cyt *b* gene the molecular evolution model was selected using AIC criterion, as implemented in Modeltest v. 3.6. (Posada and Crandall, 1998). For the beta-actin gene the uncorrected p distance was used instead of selecting a distance with Modeltest because it makes no sense to use a complex model to characterize relationships among very closely related species when using a gene with a very slow mutation rate. For instance, *Squalius pyrenaicus* and *Squalius carolitertii*, two sister species that according with cyt *b* data diverged about 4-6 MYA (Sanjur et al., 2003) did not suffered lineage sorting for beta-actin sharing haplotypes (Robalo et al., 2006b).

Bootstrap analysis was used to assess the relative robustness of branches of the MP (1000 replicates) and the NJ (100 replicates) (Felsenstein, 1985).

We excluded shared haplotypes for a given gene for the phylogenetic analysis. *Achondrostoma oligolepis* was used as outgroup in all analyses (GenBank Accession Numbers: AY254679 for cyt *b* gene and DQ447713 for beta-actin gene).

The following methods were used only in the cyt *b* dataset due to the low mutation rate of the beta-actin gene.

Relationships among haplotypes were analyzed with a parsimony network estimated by the software TCS version 1.18 (Clement et al., 2000).

ARLEQUIN software package version 3.01 (Schneider et al., 2000) was used to estimate the genetic diversity in the species studied, to access population differentiation and to perform neutrality tests. Analysis of molecular variance (AMOVA; Excoffier et al., 1992) and pairwise *F*ST were performed whenever sample sizes were adequate. Fu's *F*s (Fu, 1997) and Tajima's D (Tajima, 1989) tests were performed to test for possible bottlenecks and population expansion. Mismatch analysis (Rogers and Harpending, 1992; Rogers, 1995) was not attempted because only for the Guadiana did the model fit the data so we felt unjustified to explore them in this direction. Mean numbers of pairwise differences were corrected by subtracting the average within population differences for the populations of each pair, as implemented in ARLEQUIN. Subsequently these values were transformed in percent sequence divergence after dividing the number of pairwise differences by the length of the DNA fragment analysed.

The analysis of the Single Nucleotide Polymorphisms (SNPs) of the beta-actin was made by mapping, in the aligned sequences, all mutations present (fixed or in heterozigozity) in all haplotypes. The synapomorphic states were inferred by comparing the sequences with those of two outgroups: *Achondrostoma oligolepis* (GenBank Accession Number: DQ447713; representing the sister clade of *Iberochondrostoma*, Robalo et al., 2007a) and *Protochondrostoma genei* (GenBank Accession Number: DQ061938, representing the most ancestral genus of the ones formerly comprised in *Chondrostoma*, Robalo et al., 2007).

9.4. Results

For the cyt *b* gene 167 sites were variable and 10 were parsimony informative. Seventy haplotypes were found in the 197 samples studied. MP analysis resulted in 100 trees retained, with a consensus tree of 249 steps (Consistency index = 0.66; Homoplasy Index = 0.34; Retention Index = 0.92; CI excluding uninformative characters = 0.55; HI excluding uninformative characters = 0.45: Rescaled consistency index = 0.61) (Fig. 9.2). The MP and NJ trees for cyt *b* had the same basic topology. The model selected by Modeltest corresponded to GTR+G+I with the following assumed nucleotide frequencies: A=0.2655; C=0.2673; G=0.1609; T=0.3063. The assumed proportion of invariable sites was 0.5154 and the distribution of rates at variable sites was equal to the gamma (continuous) distribution with shape parameter (alpha) = 1.5260

For the beta-actin gene 16 sites were variable and 5 were parsimony informative. Twenty one haplotypes were found in the 86 samples studied. MP analysis resulted in 100 trees retained, with a consensus tree of 23 steps (Consistency index = 0.70; Homoplasy Index = 0.30; Retention Index = 0.46; CI excluding uninformative characters = 0.42; HI excluding uninformative characters = 0.58: Rescaled consistency index = 0.32) (Tree not shown).

Only the cyt *b* tree resulted in a clear dichotomous topology at least at species level (Fig. 2). In this tree the monophyly of *Iberochondrostoma* was confirmed with *I. almacai* in a basal position. Its sister clade splits in two well supported monophyletic groups, one comprising *I. lusitanicum* from Sado and its geographical neighbour *I. lusitanicum* from the West and Lagoa de Albufeira, while the other comprises *I. lemmingii* and *I. oretanum*. In the populations of *I. lemmingii* we find, however, some geographical differentiation. All haplotypes (except one) from the Rivers Belmez and Ovejuna (Spain, Guadalquivir Basin) are included in a well supported separated clade, suggesting the presence of different haplotypes in the same basin. The remaining haplotypes of *I. lemmingii* (including those from other Guadalquivir drainages) do not show any relevant geographical association.

For the cyt *b* dataset AMOVA was performed considering a single group with seven populations: *I. lemmingii* (Tagus), *I. lemmingii* (Guadiana), *I. lemmingii* (Guadalquivir), *I. lusitanicum* (West), *I. lusitanicum* (Lagoa de Albufeira), *I. lusitanicum* (Sado) and *I. almacai*. Populations of *I. lemmingii* from the Quarteira drainage and from the *I. oretanum* species were not included in this analysis due to their low number of samples. The results showed that among population variation explained 91.87% of the variance and was highly significant ($P < 0.001$; FST=0. 92). AMOVA was also performed considering three groups, corresponding to the species *I. lemmingii*, *I. lusitanicum* and *I. almacai*. The results showed that among groups variation explained 85.54% of the variance, among populations within groups 8.45% and within populations 6.02% ($P < 0.001$; FSC=0.58; FST=0.94; FCT =0.86).

All comparisons involving pairs of populations were significant at the same p level (Table 9.2). Concerning the molecular diversity indices the highest ones are generally found in the southeast in the populations of *I. lemmingii* from Guadiana and Guadalquivir and in *I. oretanum*. The smallest ones are found in the populations of *I. lusitanicum* from Tagus and in *I. almacai* (Table 9.3).

The values of Tajima D and Fu *Fs* only suggest population growth in the populations of *I. lemmingii* from Guadiana and *I. lusitanicum* from Sado (Table 9.3).

The results of TCS applied to the cyt *b* haplotypes (using a 95% confidence interval, Fig. 9.3) show that each species has its own network. In *I. lemmingii* the main haplotype, inferred as the ancestral one, is well represented in all basins where the species occurs. The Guadalquivir seems to represent an ancient centre of diversification, with haplotypes similar to the ancestral one but also others very different in the populations of Belmez and Ovejuna. The vast majority of haplotypes from Guadiana are very close to the presumed ancestral one. *I. lemmingii* haplotypes from Tagus are also in general close to the ancestral, differing in a maximum of 6 mutations.

Table 9.2 FSTs for pairs of populations.

	I. lemmingii Tagus basin	*I. lemmingii* Guadiana basin	*I. lemmingii* Guadalquivir basin	*I. lusitanicum* West	*I. lusitanicum* Lagoa de Albufeira	*I. lusitanicum* Sado Basin	*I. almacai*
I. lemmingii Tagus basin	0.00000						
I. lemmingii Guadiana basin	0.14917	0.00000					
I. lemmingii Guadalquivir basin	0.22064	0.18993	0.00000				
I. lusitanicum West	0.94839	0.94796	0.91399	0.00000			
I lusitanicum Lagoa de Albufeira	0.93104	0.93583	0.87120	0.38752	0.00000		
I. lusitanicum Sado basin	0.93844	0.94118	0.88308	0.91670	0.88812	0.00000	
I. almacai	0.96075	0.95998	0.92357	0.97703	0.97537	0.98077	0.00000

Table 9.3 Number of samples sequenced for cty *b* gene, number of haplotypes found in each population, molecular diversity indices, Tagima's D and Fu's *Fs* values and their significance levels.

	N	Number of haplotypes	Number of polymorphic sites	Gene diversity	Mean number of pairwise differences	Nucleotide diversity	Tajima's D	Tajima's D p	Fu *Fs*	Fu *Fs* p
I. lemmingii Tagus basin	29	11	17	0.7167 +/- 0.0839	3.192118 +/- 1.698584	0.004403 +/- 0.002608	-0.90454	0.19400	-2.12922	0.16300
I. lemmingii Guadiana basin	46	21	38	0.8647 +/- 0.0438	2.825121 +/- 1.517649	0.003897 +/- 0.002324	-2.31028	0.00000	-12.94177	0.00000
I. lemmingii Quarteira	3	1	0	0.0000 +/- 0.0000	0.000000 +/- 0.000000	0.000000 +/- 0.000000	0.00000	1.00000	n.a.	n.a
I. lemmingii Guadalquivir basin	24	14	31	0.8841 +/- 0.0564	7.148551 +/- 3.473630	0.009860 +/- 0.005341	-0.52481	0.32900	-1.97370	0.21800
I. oretanum	6	5	7	0.9333 +/- 0.1217	2.800000 +/- 1.714643	0.003862 +/- 0.002731	-0.50439	0.36000	-1.41795	0.09500
I. lusitanicum West	40	8	12	0.4013 +/- 0.0979	1.305128 +/- 0.832867	0.001795 +/- 0.001273	-0.99364	0.16000	-2.31000	0.09000
I .lusitanicum Lagoa de Albufeira	12	4	7	0.6364 +/- 0.1277	2.136364 +/- 1.275780	0.002947 +/- 0.001981	0.40482	0.43600	1.08500	0.72400
I. lusitanicum Sado basin	14	7	7	0.8242 +/- 0.0781	1.395604 +/- 0.909935	0.001925 +/- 0.001409	0.09026	0.08800	-3.26000	0.00500
I. almacai	23	4	3	0.4862 +/- 0.1053	0.577075 +/- 0.483719	0.000796 +/- 0.000744	-0.74000	0.21200	-1.11000	0.12000

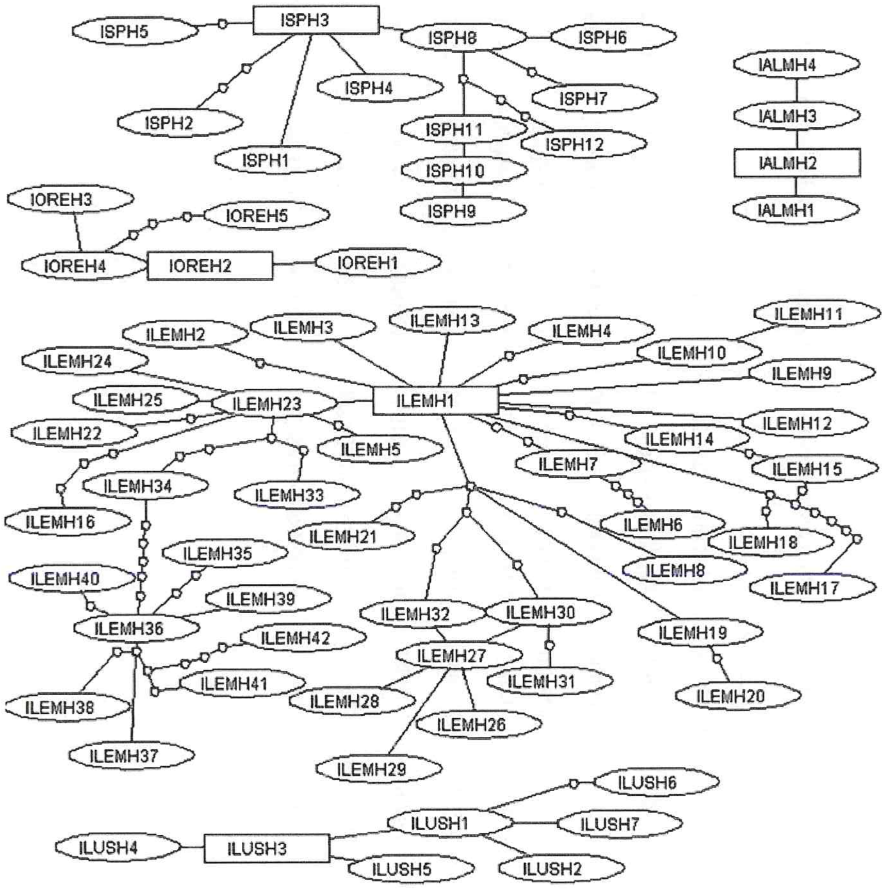

Fig. 9.3 TCS network. Labels: ILEM = *I. lemmingii*; ILUS = *I. lusitanicum* (Sado); ISP = *I. lusitanicum* (West and Lagoa de Albufeira) ; IORE = *I. oretanum*; IALM = *I. almacai*. ILEMH1 = 42 fish, Tagus, Guadiana, Quarteira, Guadalquivir; ILEMH2 = 1 fish, Guadiana; ILEMH3 = 2 fish, Guadiana; ILEMH4) = 1 fish, Guadiana; ILEM5) = 2 fish, Guadiana; ILEMH6 = 1 fish, Guadiana; ILEMH7 = 1 fish, Guadiana; ILEMH8 = 1 fish, Tagus; ILEMH9 = 1 fish, Guadiana; ILEMH10 = 2 fish, Guadiana; ILEMH11 = 2 fish, Guadalquivir;; ILEMH12 = 2 fish, Guadiana; ILEMH13 = 1 fish, Guadiana; ILEMH14 = 1 fish, Guadiana; ILEMH15 = 1 fish, Tagus; ILEMH16 = 1 fish, Guadiana; ILEMH17 = 1 fish, Guadiana; ILEMH18 = 1 fish, Guadiana; ILEMH19 = 2 fish, Guadiana; ILEMH20 = 1 fish, Guadalquivir; ILEMH21 = 1 fish, Guadalquivir; ILEMH22 = 1 fish, Guadiana; ILEMH23 = 9 fish, Guadiana, Guadalquivir; ILEMH24 = 1 fish, Guadiana; ILEMH25 = 1 fish, Guadiana; ILEMH26 = 2 fish, Guadiana, Tagus; ILEMH27 = 1 fish, Tagus; ILEMH28 = 1 fish, Tagus; ILEMH29 = 1 fish, Tagus; ILEMH30 = 5 fish, Tagus; ILEMH31 = 1 fish, Tagus; ILEMH32 = 1 fish, Guadiana; ILEMH33 = 1 fish, Tagus; ILEMH34 = 1 fish, Guadalquivir; ILEMH35 = 1 fish, Guadalquivir; ILEMH36 = 1 fish, Guadalquivir; ILEMH37 = 1 fish, Guadalquivir; ILEMH38 = 1 fish, Guadalquivir; ILEMH39 = 1 fish, Guadalquivir; ILEMH40 = 1 fish, Guadalquivir; ILEMH41 = 1 fish, Guadalquivir; ILEMH42 = 1 fish, Guadalquivir; IOREH1 = 2 fish, Guadalquivir; IOREH2 = 1 fish, Guadalquivir; IOREH3 = 1 fish, Guadalquivir; IOREH4 = 1 fish, Guadalquivir; IOREH5 = 1 fish, Guadalquivir; ISPH1 =1 fish, Tagus and adjacent drainages; ISPH2 =1 fish, Tagus and adjacent drainages; ISPH3 =31 fish, Tagus and adjacent drainages, Lagoa de Albufeira; ISPH4 =1 fish, Tagus and adjacent drainages; ISPH5 =1 fish, Tagus and adjacent drainages; ISPH6 =1 fish, Lagoa de Albufeira; ISPH7 =1 fish, Lagoa de Albufeira; ISPH8 =7 fish, Lagoa de Albufeira; ISPH9 = 2 fish, Tagus; ISPH10 =2 fish, Tagus; ISPH11 = 1 fish, Tagus; ISPH12 = 3 fish, Lagoa de Albufeira; ILUSH1 = 4 fish, Sado; ILUSH2 = 1 fish, Sado; ILUSH3 = 5 fish, Sado; ILUSH4 = 1 fish, Sado; ILUSH5 = 1 fish, Sado; ILUSH6 = 1 fish, Sado; ILUSH7 = 1 fish, Sado; IALMH1 = 1 fish, Arade; IALMH2 = 16 fish, Arade, Mira; IALMH3 = 5 fish, Mira; IALMH4 = 1 fish, Mira.

The analysis of the beta-actin SNPs (Table 9.4) showed that heterozygosity is only present in haplotypes of the *I. lemmingii* populations (i.e. all mutations present are fixed in *I. lusitanicum*, *I. almacai* and *I. oretanum*). Of the 21 mutation sites mapped, 7 corresponded to fixed mutations, 2 to insertions and 1 to a deletion. Twelve sites were polymorphic and showed some degree of heterozigosity. From these, 7 were present in haplotypes representing only one specimen with no geographical meaning and 5 represented ancestral polymorphisms (i.e. are present in individuals of the different populations of *I. lemmingii*). Concerning the meaning of the fixed mutations, we can find a derived mutation that gathers all *Iberochondrostoma*, another that groups *I. almacai* and haplotypes from the River Jabalón in the upper Guadiana basin, and still another characteristic of *I. lusitanicum*. As referred by Robalo et al. (2007b), the haplotypes of *I. lusitanicum* from West and Lagoa de Albufeira and *I. lusitanicum* from Sado differ in two fixed mutations, each being synapomorphic of each of the clades. *I. oretanum* also has a derived and fixed characteristic mutation, but the same mutation appears in a few *I. lemmingii* from the same basin (Guadalquivir), although only in heterozygosity, a situation that may have resulted from a possible hybridization event or may indicate that the mutation occurred before the separation between *I. lemmingii* and *I. oretanum*. Although a fixed mutation was found that groups *I. lusitanicum*, *I. almacai* and the haplotypes from the River Jabalón in the Guadiana basin it is important to point out that the fixed allele shared by these fish corresponded to the primitive state. For the same locus all the remaining *I. lemmingii* and *I. oretanum* shared the same derived allele.

9.5 Discussion

The results obtained with the cyt *b* gene confirm the monophyly of *Iberochondrostoma* as well as the monophyly of each species included in this genus.

Concerning the phylogeography of *I. lemmingii* the first conclusion is that, based on the present results, there is poor geographical differentiation (with many haplotypes shared among drainages) between Tagus, Guadiana and Guadalquivir, with the exception of the fishes from the Rivers Belmez and Ovejuna from the Guadalquivir Basin (Figures 9.2 and 9.3). Their position in relation to the remaining *I. lemmingii* will need further evaluation because they were not present in the beta-actin dataset due to lack of samples. In *I. lemmingii*, the Guadalquivir tributaries Belmez and Ovejuna are the only ones to present distinctive haplotypes. All other basins and rivers share several haplotypes and present low corrected pairwise differences among populations, providing at the same time evidence for ancestral polymorphisms and indicating that the events that severed the last connections among these three basins (Tagus, Guadiana and Guadalquivir) must have been very recent. Assuming a molecular clock of 1% between lineages per MY (e.g. Dowling et al., 2002; Doadrio and Carmona, 2004; Robalo et al., 2007a) the populations of *I. lemmingii* of Tagus and Guadiana were connected until about 80 000 years ago.

The basin of the Guadalquivir seems to be a major diversification centre at least at some of its drainages. Apart from the already mentioned populations of the Rivers Belmez and Ovejuna, *I. oretanum* also had its origin in the Guadalquivir basin.

Taken together, the results derived from cyt *b* gene show a very broadly distributed species, *I. lemmingii*, present in the upper part of the Tagus, Guadiana and Guadalquivir, from which a small albeit genetically diverse *I. oretanum*, splited about 2 MYA.

The populations of *I. lemmingii* are clearly more diverse genetically than those of the remaining species of the genus, except perhaps *I. oretanum*. The number of samples of *I. oretanum* is however too low to draw firm conclusions on this issue. The more peripheral *I. almacai* and *I. lusitanicum* from West and Lagoa de Albufeira show the lowest diversity indices.

I. lemmingii retained several ancestral polymorphisms with several haplotypes shared among major basins.

I. almacai and *I. lusitanicum* correspond to much older splits in *Iberochondrostoma*. The timing of separation of *I. almacai* from the remaining fish is about 6/7 MYA and the split of *I. lusitanicum* from *I. lemmingii* took place about 4.5 /5.5 MYA. These old phylogroups are all peripheral in geographical location (occurring in the southwest and west of Iberia respectively), all occupy areas that are much smaller than that of *I. lemmingii* and present low levels of genetic diversity when compared with those of *I. lemmingii*. This overall pattern is consistent with a scenario of peripatric speciation (Mayr, 1982) with a broadly distributed species located well inside the Iberian Peninsula, originating small peripheral species at different times. Indeed, all 3 predictions presented in the introduction were matched by the results presented above.

Table 9.4 SNPs found and analysed in the beta-actin gene. Legend: Ilembact = haplotypes for *I. lemmingii*; Iorebact = haplotypes for *I. oretanum*; Ispbact = haplotypes for *I. lusitanicum* (West and Lagoa de Albufeira); Ilusbact = haplotypes for *I. lusitanicum* (Sado); Ialmbact = haplotypes for *I. almacai*. IlembactH1 = 3 individuals; Tagus, Guadalquivir; IorebactH1 = 1 fish, Guadalquivir; IlembactH2 = 2 fish, Tagus, Guadalquivir; IlembactH3= 1 fish, Guadalquivir; IlembactH4 = 1 fish, Tagus; IlembactH5 = 1 fish, Tagus; IlembactH6 = 3 fish, Tagus, Guadiana; Ispbact H1 = 40 fish, Tagus and adjacent basins, Lagoa de Albufeira; IlusbactH1 = 9 fish, Sado; IlembactH7 = 1 fish, Guadiana; IlembactH8 = 1 fish, Guadiana; IlembactH9 = 1 fish, Guadiana; IalmbactH1 = 7 fish, Mira, Arade; IlembactH10 = 1 fish, Tagus; IlembactH11 = 1 fish, Guadalquivir; IlembactH12 = 10 fish, Quarteira, Guadiana, Guadalquivir; IlembactH13 = 1 fish, Guadalquivir; IlembactH14 = 1 fish, Guadiana; IlembactH15 = 1 fish, Tagus; IlembactH16 = 8 fish, Tagus, Guadiana; IlembactH17 = 1 fish, Tagus.

Haplotypes \ bp	73	101	108	114	122	138	204	296	385	424	447	455	482	518	520	528	604	608	611	677	720-21
IlembactH1	A		A	C	T	C	G	A	C	G	T	C		T	T	C	C	A	C	G	CT
IorebactH1	A		A	C	T	C	G	A	C	G	T	C		A	T	C	C	A	C	G	CT
IlembactH2	A		A	C	T	C	G	A	C	G	Y	C		T	T	C	C	A	C	G	CT
IlembactH3	A		A	C	T	C	G	A	C	G	Y	C		W	T	C	C	A	C	G	CT
IlembactH4	A		A	C	T	C	R	R	C	G	C	C		T	T	Y	C	A	Y	G	CT
IlembactH5	A		A	C	T	C	G	A	C	G	C	C		T	K	C	C	A	C	G	CT
IlembactH6	A		A	C	T	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IspbactH1	G		A	C	G	C	G	A	C	G	C	C		T	T	C	A	A	C	G	CT
IlusbactH1	G		A	C	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	
IlembactH7	A	T	A	C	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH8	A	T	A	S	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH9	A	T	A	C	G	C	G	A	C	A	C	C		T	T	C	C	A	C	G	CT
IalmbactH1	A	T	A	C	G	C	G	A	C	G	T	T	T	T	T	C	C	G	C	G	CT
IlembactH10	A		T	C	T	G	G	A	C	G	C	C		T	T	C	C	A	C	K	CT
IlembactH11	A		T	C	T	G	G	A	C	G	C	C		T	T	C	C	A	C	T	CT
IlembactH12	A		T	C	T	G	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH13	A		W	C	T	S	G	A	C	G	Y	C		W	T	C	C	A	C	G	CT
IlembactH14	A		W	C	T	S	G	A	Y	G	Y	C		K	T	C	C	A	C	G	CT
IlembactH15	A		W	C	T	S	G	A	C	G	Y	C		T	T	C	C	A	C	G	CT
IlembactH16	A		W	C	T	S	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH17	A		W	C	T	S	G	A	C	G	C	C		T	T	C	C	A	C	K	CT
A. oligolepis	A		A	T	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
P. genei	A		A	T	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT

This picture is also supported by the analysis of the SNPs in the beta-actin gene. As stated in the results there is not a single synapomorphic allele specific to *I. lemmingii*. On the contrary, all the small phylogroups or species (*I. lusitanicum*, *I. almacai* and *I. oretanum*) have fixed derived alleles, some of which occur in heterozygosity in *I. lemmingii.* This pattern is the one we would expect if we had a very large population or set of populations with frequent connections in which lineage sorting would proceed slowly and a number of "daughter" peripheral populations in which lineage sorting and allele substitution would proceed much faster (Kimura, 1983). We suggest that the SNP that reflects the oldest split is the one that unites *I. almacai* and *I. lusitanicum*. Indeed it is fixed in all these species and is found in *I. lemmingii*, although with very low frequency, in the River Jábalon (upper Guadiana). An ancestral polymorphism in the ancient populations that gave rise to what is now *I. lemmingii* would have become fixed in the colonists that dispersed to the west and southwest of the Iberian Peninsula. This interpretation is consistent with what we know on the geology of Iberia. The present-day river system is very recent, being of Plio-Pleistocene age and took its modern shape and exorheic condition after the peninsula tilted to the west, causing many of its water sheds to drain into the Atlantic. As stated above, in the Miocene, most freshwaters of Iberia drained to several inland lakes. Rivers like Douro, Tagus and Guadiana did not exist in their present configuration and each of them comprised several drainage systems feeding the inland several lakes (e.g. Andeweg, 2002). For instance, the upper Tagus was connected to the upper Guadiana up to the Pleistocene (Moya - Palomares, 2002) but was separated from the lower Tagus in several occasions during the Mio–Pliocene (Cunha et al., 1993). The lower Tagus, in turn, had a southward extension that approached the south of Portugal in the area that presently corresponds to the Sado basin (Teresa Azevedo, personal communication). We suggest that in the upper Miocene (judging from the cyt *b* data) fish from the upper Tagus, upper Guadiana and belonging to the stock that gave rise to *I. lemmingii* manage to reach the lower Tagus. From the lower Tagus / Sado, this ancestral stock would have spread to the southwest of Portugal where it originated *I. almacai*. It is interesting to note that fossils of *Squalius*, another cyprinid genus, are known from the Miocene of the lower Tagus (Póvoa de Santarém, Gaudant, 1977) and in *Squalius* there are also species with very restricted ranges in the southwest of Portugal (*S. torgalensis* and *S. aradensis*; Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998) implying a connection of the lower Tagus/Sado with the bulk of the Peninsula and a corridor that allowed the dispersal of fish to southwest Portugal. The separation of the *Squalius* species of southwest Portugal points to a similar timing as the separation of *I. almacai* (around 6 MYA, Sanjur et al., 2003; Doadrio and Carmona, 2003b). The differentiation of the Tagus and Sado during the Pliocene promoted the speciation of *I. lusitanicum* from West / Lagoa de Albufeira and *I. lusitanicum* respectively. There is ample geological evidence that the lower Tagus and Sado were connected at some periods and both rivers suffered very pronounced marine transgressions (Pimentel, 1997; Andeweg, 2002) that would have pushed their populations of *Iberochondrostoma* to the respective headwaters, accelerating lineage sorting and genetic differentiation. In the Pliocene, the connection between upper and lower Tagus was severed (Cunha et al., 1993; Andeweg, 2002) avoiding that new waves of *I. lemmingii* reinvaded the lower reaches of the Tagus. Even nowadays, with the Tagus being a continuous river, *I. lemmingii* is restricted to the Spanish part of the Tagus and a few tributaries near the Portuguese boarder, while *I lusitanicum* from West and Lagoa de Albufeira is restricted to the tributaries closest to the estuary.

Overall, the paleobiogeographical information available is compatible with the scenario of a large species giving rise to two smaller ones at different periods and further fragmentation of one of these smaller entities.

Two notes of caution are needed:

1) This sequence of events assumes that the few SNPs involved did not suffer recurrent mutations that would cause homoplasy. The low number of polymorphic sites is in our view, consistent with this possibility;
2) The low genetic diversity of the peripheral species may have also resulted from bottlenecks that occurred after their differentiation, namely strong marine transgressions in the western rivers, causing drastic reductions in population size.

The use of other nuclear markers will help to test the validity of the model presented in this paper.

In conclusion we note that what seemed to be a well resolved dichotomous tree inferred from cyt *b* did not correspond to the picture obtained with a slowly evolving nuclear gene. The slow rate of evolution of this fragment and its fourfold higher effective population size (Zhang and Hewitt, 2003) apparently allowed the preservation of old signatures, of past events that the rapid lineage sorting, that probably took place in the cyt *b* gene, did not preserve.

We suggest that phylogeographic reconstruction, in particular using SNPs applied to slowly evolving nuclear genes, in combination with phylogenetic analysis, may help us to get a better understanding of the evolutionary history of clades beyond the species level, an essential step to study speciation.

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Parte IV

Observações sobre comportamentos reprodutores e agonísticos

Capítulo 10

First description of agonistic behaviour in *Chondrostoma polylepis* (Pisces: Cyprinidae) with notes on the behaviour of other *Chondrostoma* species

10.1 Abstract

In this paper we describe for the first time the occurrence of agonistic behaviour in *Chondrostoma polylepis* (Pisces: Cyprinidae) in captivity. In this species agonistic behaviour was present in adults of both sexes and juveniles, during spring and summer, well after the end of the breeding season. Qualitative observations revealed the presence of a similar pattern of agonistic behaviour in *Chondrostoma duriensis*. Despite intense observation effort, no signs of agonistic behaviour were detected in three other *Chondrostoma* species of the same geographical area, *C. macrolepidotus*, *C. lemmingii* and *C. lusitanicum*. It is hypothesized that agonistic behaviour in *C. polylepis* and *C. duriensis* may be functionally linked to their feeding ecology.

10.2 Introduction

Although the family Cyprinidae is "the largest family of freshwater fishes and, with the possible exception of Gobiidae, the largest family of vertebrates" (Nelson, 1994), reports on the occurrence of agonistic behaviour are relatively infrequent in this taxon. Most references report only male aggression in the context of reproduction (for an example of agonistic behaviour in a temperate cyprinid occurring out of the reproductive context see Rincón and Grossman, 2001). Among north American minnows, although egg broadcasting without territoriality is found in the majority of the species and was considered ancestral (Jonhston, 1999), several instances of male breeding territoriality, nest guarding and even nest-building have been reported (e.g. *Campostoma anomalum pullum*, Miller, 1962; species of the genera *Luxilus*, *Semotilus*, *Nocomis*, Jonhston, 1999). Among European cyprinids, agonistic behaviour by breeding males seems even more rare (e.g. *Leucaspius delineatus*, *Rhodeus sericeus amarus* Wheeler, 1969; *Abramis brama*, Poncin et al., 1996; *Rutilus rutilus*, Wedekin, 1996). In temperate cyprinids, it seems safe to state that agonistic behaviour and territoriality in non reproductive contexts are rare phenomena, a situation that, as Barlow (1993) points out seems to hold in many other freshwater fish groups. In European cyprinid species, we could not find a single reference in the literature on agonistic behaviour occurring outside the breeding season, except for Wheeler (1969) statement about *Leuciscus cephalus*, that "large chubs are usually solitary possessing and defending from others of the same species, a "territory" in the river".

In Portuguese freshwaters there are at least six, possibly seven, cyprinids of the genus *Chondrostoma* Agassiz, 1835 (SNPRCN, 1992; Zardoya and Doadrio, 1998), all endemic to the Iberian Peninsula.

In this paper we describe the patterns of agonistic behaviour observed in captive groups of *Chondrostoma polylepis* Steindachner, 1865 and present qualitative behavioural observations on four other *Chondrostoma* species of the same geographical area, *Chondrostoma duriensis* Coelho, 1985, *Chondrostoma macrolepidotus* Steindachner, 1866, *Chondrostoma lemmingii* Steindachner, 1866 and *Chondrostoma lusitanicum* Collares-Pereira, 1980, and discuss hypotheses about their function.

10.3 Methods

A group of 30 juveniles of *C. polylepis*, bred in captivity from a stock originated on a population of the Tagus basin, were kept in a public aquarium (Aquário Vasco da Gama). Fishes were born in 1999 and were observed during 2000 and 2001. The 600l tank was illuminated 8h per day and was equipped with biological filters. The bottom of the tank was covered with a layer of sand and several large flat stones, and enriched with some aquatic plants. Fishes were fed with Artemia sp. and red chironomid larvae.

Behavioural descriptions were made using ad libitum and focal observations (sensu Martin and Bateson, 1993). A total of 40h of *ad libitum* observations were made: 20h during the winter period and 20h that were video recorded (with a Sony Hi8 CCD-V600 E camera), during the spring and summer period. In addition, also using video recording, a total of 189 min. of focal observation was made. During the focal observations, the location of the focal fish in the tank and all the agonistic encounters in which it participated were recorded. Each focal observation lasted 3 min. and this procedure was repeated for 10 fishes in each observation period (30 min.). If a fish went out of sight, or could be confused with other, its focal observation was discarded. As the fishes were not tagged these observations must not be considered independent, as some fishes could be observed more than once in a session, although differences of size and the presence of small scars on the body and fins considerably reduced this risk.

A fish was classified as the loser of an encounter if at the end of the interaction it withdrew or fled from the opponent, or was threatened or attacked without retaliation. When both fishes withdrew without an apparent asymmetry, the outcome was classified as inconclusive. Groups of *C. polylepis*, *C. duriensis*, *C. macrolepidotus*, *C. lemmingii* and *C. lusitanicum* were observed to check for the presence of agonistic behaviour. In Table 10.1 we summarize, for each species, the information about the number of individuals observed, number of groups observed, total *ad libitum* observation hours and total capacity of the tanks in which observations were made. The fishes were kept in outdoor tanks with natural photoperiods and temperatures.

For each species we tried to cover the entire spectrum of juvenile sizes and some of them were collected when about 2 cm long and were reared in the tanks to full maturity. The observations were conducted throughout the year and were distributed throughout the day. As the tanks were located outdoors and were only observed during daylight hours the observation schedules changed according with the annual variations of the daylight cycle.

Table 10.1 Summary of the information on the fishes used in this study. For each species, the number of individuals and groups, total observation time (ad libitum) and capacity of the observation tanks. (*information referring to the group studied in Aquário Vasco da Gama).

Species	*C. popylepis*	*C. duriensis*	*C. macrolepidotus*	*C. lemmingii*	*C. lusitanicum*
Number of individuals	20+30* juveniles 25 adults	30 juveniles 33 adults	50 juveniles 150 adults	6 juveniles 12 adults	23 juveniles 90 adults
Groups	3+1*	4	15	3	4
Observation time	60+30*h	80 h	>300h	40 h	>200 h
Tanks	80L, 450 L and 600L*	15 to 450 L	15 to 450 L	80 L	80 L and 450 L

The fish were observed in a variety of contexts, including the spawning of adults of *C. lusitanicum* and *C. macrolepidotus*. As the agonistic and the reproductive behaviour of these species was previously unknown we decided to perform *ad libitum* observations and tried to describe and record all types of behaviour that we could differentiate and the relative positions of the fishes when they were observed interacting.

10.4 Results

Agonistic Ethogram (in parenthesis we present the frequencies of occurrence of each behaviour observed, in a total of 178 behavioural acts recorded during 40 agonistic interactions analysed from video records):

Charge – One fish moves rapidly toward another by movements of the caudal peduncle, with all fins closed (30%).

Butting – One fish buts with his snout in another fish, generally in the posterior ventral region or in the caudal peduncle. Sometimes it can give successive buts. Butting often occurred during charges and the attacked fish was frequently hit at the caudal peduncle or the posterior end of the dorsal fin (25%).

Fleeing – One fish moves rapidly away from another (32%).

Chase – One fish rapidly pursues another that is fleeing (8%).

Serpentine – Two fishes swimming rapidly in circles, or semi-circles, both direct their head to the other fish tail, while orienting its own tail away from the other (Fig. 10.1). The fish look like if they are trying to bite one another at the caudal peduncle. When one of the fishes bites the opponent this movement ceases. Sometimes there are more than two fishes interacting (2%).

Threaten – One fish initiates charging but stop soon after (2%).

Mouth to mouth – two fishes move rapidly toward the opponent, apparently directing an attack to the opponent's mouth (0.6%).

Like death – A fish lays on its side in the water column, when other is swimming around butting it (0.6%).

Normally, the fishes that participate in an interaction became darker, with the lateral line more conspicuous. The sequences of agonistic behaviour shown by these fish usually followed the pattern: charge → butting →fleeing →chase (ilustrated in Fig. 10.2).

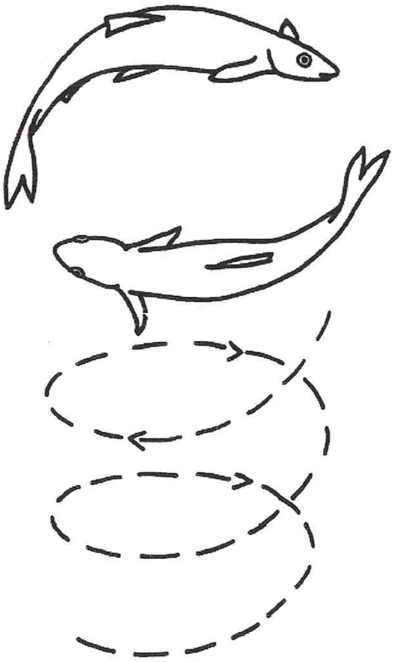

Fig. 10.1 Serpentine.

Normally the agonistic interactions had a short duration (mean=1.23 s; S.D.=0.57; range: 1.00-3.00 s; n=40). They were performed in the water column with no apparent association with stones or other objects present in the tank. After an interaction the fishes tended to return to the place where they were previously. The average number of agonistic interactions was 1.62 per fish per min. (S.D.=1.33; range: 0-9; n=63 fishes). In 80% of the agonistic interactions analysed using the videotape recording (n=40), the fish that initiated the interaction was the winner.

As feeding could affect the behaviour of the fishes, many observations were made well before and after feeding had taken place.

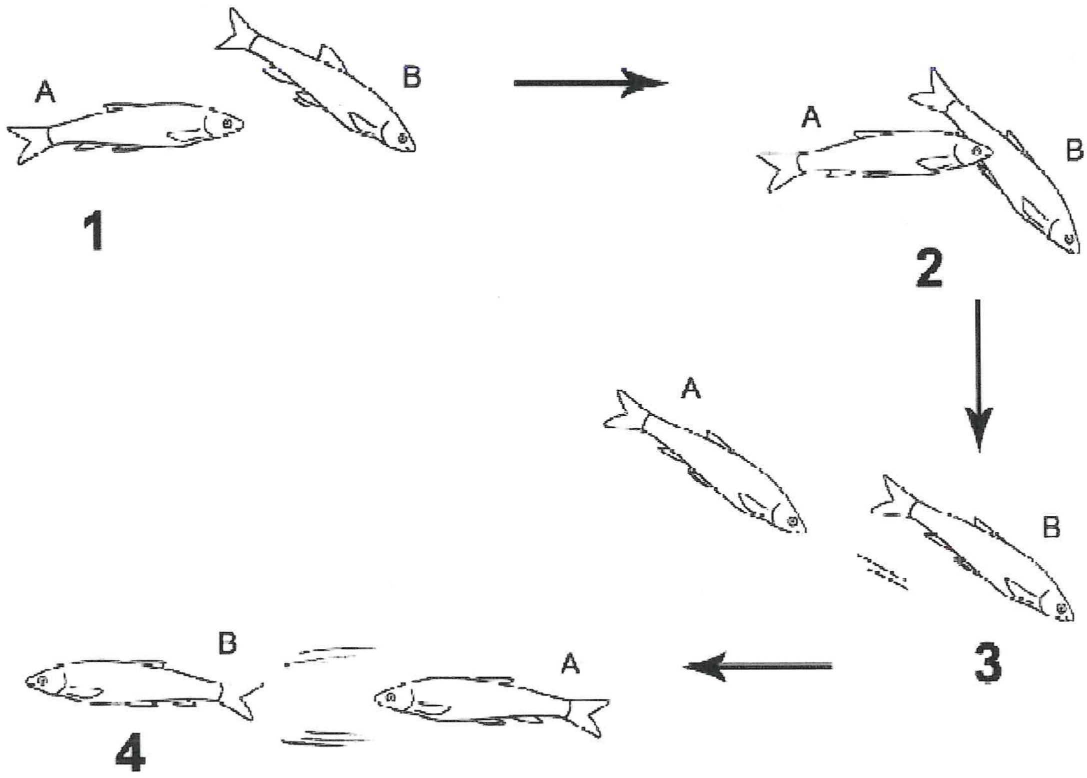

Fig. 10.2 Illustration of the most common sequence of agonistic behaviour. 1- Charge; 2- Butting; 3- Fleeing; 4- Chase.

As we didn't know to what extent group size and tank size could affect the behaviour of the fish, each species was observed in tanks of different size and in groups that ranged from a minimum of six individuals to a maximum of 40 individuals. As the aim of the study was descriptive no attempt was made to standardize the number of group size and number of tank sizes per group sizes.

When fishes were placed in the 600l tank, in February 2000, they formed one school, without showing any aggressive behaviour. One week later, they began to be more aggressive and spaced, appearing to hold small territories, continuously swimming in circles in a restricted area (patrolling). During all the summer period they showed this aggressive behaviour, but in September/October they began to school again. After winter, in April they began to show aggressive behaviours again. First, only one or two fishes stayed out of the school expelling other fishes away from their territories by charging and butting, sometimes driving all the school to the surface. The aggressive behaviour tended to spread to more fishes as time passed: one week after four fishes were out of the school; 20 days after, six fishes were out; 50 days after, there was no school at all. The first two fishes that initiated this process seemed to be the largest.

Although no quantitative data were collected, our observations showed that the agonistic behaviour of *C. duriensis* corresponds to the descriptions provided for *C. polylepis.* Juveniles of this species having only 2 cm already displayed overt aggression. In both species, both males and females performed agonistic behaviour. This behaviour was not linked to reproduction, since it occured outside the breeding season and in fishes with no signs of being ripe. In groups of large fish, even in a 450l tank, only a few fish were able to keep territories and the losers of fights showed very serious wounds, especially near the base of the dorsal fin and had to be removed to avoid more serious injury.

In *C. macrolepidotus*, *C. lemmingi* and *C. lusitanicum,* we could not identify any kind of behaviour that could be plausibly classified as agonistic. During the reproduction of *C. lusitanicum* (Carvalho et al., 2002) and *C. macrolepidotus* (J. Robalo, unpublished data), no territoriality was observed in males and no inter-male aggression could be found. We assume that, with the variety of group and tank sizes, the large numbers of individuals studied and the large amount of observation time dedicated to each species, it is safe to conclude that agonistic behaviour is absent or at least very uncommon in *C. lemmingii*, *C. lusitanicum* and *C. macrolepidotus*.

10.5 Discussion

The results of the present study provide strong evidence that species of the genus *Chondrostoma* vary sharply in the expression of agonistic behaviour, which is frequent and intense in *C. polylepis* and *C. duriensis* and virtually absent in *C. macrolepidotus*, *C. lemmingii* and *C. lusitanicum*. In addition, in the species in which it is present, aggression is not limited to males or to reproductive contexts, being already present in very small juveniles. Although the evidence does not prove that the agonistic behaviour observed corresponds to territorial defence, the observations presented above suggest that territoriality may occur in *C. polylepis* and *C. duriensis*.

These two species are very closely related (Zardoya and Doadrio, 1998) and together with a third Iberian species, *Chondrostoma willcommii*, belong to an ecologically specialized group of nase, that like the central European *Chondrostoma nasus* have a ventral straight mouth with the lower lip reinforced by an horny blade (e.g. Coelho, 1987; Doadrio, 2001). These fishes feed largely on algae, detritus and other materials that they scrape from the surface of rocks and other substrata (Wheeler, 1969; Lobón-Cerviá and Elvira, 1981; Bellido et al., 1989). They often occur in deep waters with moderate currents, exploiting the algal growth on large stones and boulders. This feeding habitat does not correspond to the spawning ground of these species, as they are known to leave their feeding habitats to spawn (Granado-Lorenzio and García-Novo, 1986; Coelho, 1987; Rodriguez-Ruiz and Granado-Lorencio, 1992; Doadrio, 2001). We hypothesised that the agonistic behaviour of these species may be linked to the defence of areas rich in algal growth on objects like large rocks that may provide some shelter against the currents. We suggest that in the feeding habitat, defending a territory may be a very profitable strategy that may allow the fishes to occupy places sufficiently sheltered from the main current on which algal production is enough to support the fishes.

This hypothesis would be especially plausible for the summer conditions, when many fishes are forced to congregate in pools, where algal growth may quickly become a scarce resource. It is interesting to note that, in our observations, the highest levels of aggression were observed in the summer and aggression virtually ceased in the winter. If future field work confirms our interpretation, the behaviour of *C. polylepis* would be analogous to the defence of algal gardens by herbivorous cichlids (Barlow, 1993) and to the territoriality of salmonids (Keenleyside and Yamamoto, 1962; Grant and Kramer, 1990) with the difference that while most salmonids tend to rely on drifting food these nase species may find adequate food on the surface of rocks and boulders that provide them shelter. An example of aggression on a drift feeding cyprinid is provided by Rincón and Grossman (2001), for *Clinostomus funduloides*.

In preliminary field observations of *C. duriensis* (J.Robalo, unpublished data) fish were observed defending algae covered stones against other fish.

The remaining species of *Chondrostoma* observed in this study that lack agonistic behaviour, also lack the morphological and behavioural specializations of head and mouth for feeding on algae and have an omnivorous diet (e.g. Doadrio, 2001). They are frequently found in waters with abundant vegetation and week currents in which the feeding mode of *C. duriensis* and *C. polylepis* is unlikely to be functional and where territorial defence may be uneconomical.

Although the data are not sufficient to draw definitive conclusions on the validity of this hypothesis, we think it is worth testing, with field experiments and observations in the future.

10.6 Acknowledgements

We thank the Centro Aquícola da Azambuja, Professors Alexandre Valente, Manuela Coelho and Maria João Collares-Pereira for offering some of the fishes used in this study and the Aquário Vasco da Gama, especially Fátima Gil for maintaining the fishes and for her valuable suggestions. We also thank Carla Santos, Vera Carvalho, Manuel Robalo, João Paulo Fonseca and Paulo Morais for help during the field work and in maintaining aquaria. We also thank Dr. Ignacio Doadrio (MNCN – Madrid) for the translation of the abstract and Teresa Bento who kindly made the figures. Preliminary sketches were provided by Ana Margarida Silva. Part of this study was supported by Fundação para a Ciência e Tecnologia (FCT) as a part of the Plurianual Program (UI&D 331/94) and C. Faria was also supported by a grant from FCT (PRAXIS XXI/BD/5666/95). Direccão Geral das Florestas provided the necessary authorizations for field work.

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Capítulo 11

A description of the reproductive behaviour of the endangered Iberian cyprinid *Chondrostoma lusitanicum* Collares-Pereira 1980 in captivity

11.1 Abstract

In this paper we describe, for the first time, the reproductive behaviour of the rare Iberian cyprinid fish *Chondrostoma lusitanicum*, endemic to Portugal. The species is an egg broadcaster releasing adhesive eggs. Spawning aggregations may involve large numbers of fish but within a group, short sequences of courtship and spawning typically involve one female and one or a few males. Males follow females touching them frequently and at spawning press the female body against the available objects. Males perform circles that may help to retain a female in a given area. A special behaviour is described for females with the likely function of attracting males and initiating courtship.

11.2 Introduction

The family Cyprinidae is the largest family of freshwater fishes (Nelson, 1994). Most known taxa in the Iberian Peninsula are endemic (Alves and Coelho, 1994) and classified as endangered (10 out of 15) in the Portuguese Vertebrate Red Data Book (SNPRCN 1992), mainly due to anthropogenic factors leading to habitat destruction.

Chondrostoma lusitanicum Collares-Pereira 1980 is a small cyprinid endemic to Portuguese waters, present in shallow streams with medium flow currents and some vegetation on the banks (e.g. Alves and Coelho, 1994). It is listed as rare in the Portuguese Vertebrate Red Data Book (SNPRCN 1992) and it has a restricted distribution area, covering some small coastal streams, slightly north of the Tagus mouth, some scattered tributaries of Tagus and Sado drainages and the southern drainages of Mira and Arade (Collares-Pereira, 1983; Alves and Coelho, 1994; Pereira, 1995). As a rare species, it is important to understand all aspects of biology for proper conservation. However, the reproductive biology of this species is unknown and its breeding behaviour and that of other closely related species of Iberian *Chondrostoma* is as yet undescribed.

The aim of this paper is to describe the reproductive behaviour of *C. lusitanicum* in captivity, considering the fact that knowledge about the reproduction modes of the species may be important in conservation strategies (Johnston, 1992).

11.3 Methods

Twenty fishes, approximately half of each sex, were collected by hand net from a small independent coastal occidental stream (Ribeira da Samarra), near Lisbon, in the summer of 1998 and were raised in an outdoor aquarium (150×50×70 cm) under natural conditions of light and temperature. The aquarium was provided with large stones and water plants and the fishes were fed with fish commercial flakes and chironomid larvae. Although not monitored daily, the temperature ranged from about 10 to 22/24 ºC. All fishes were adult (70-100 mm total length) and sexually mature at the time of this study. These values are common in adults collected in the field. The species reaches a maximum of about 145 mm (according with the extensive sample of Collares-Pereira (1983)). About 100 hours of *ad libitum* observations (*sensu* Martin and Bateson, 1993) were made. When it seemed likely that a reproductive event could take place, judging from the swollen abdomen of the females and a general increase of the activity of the fishes, both in movements and number of interactions, videotape recording was performed with a Sony Hi8 CCD-V600 E camera. A total of 120 min of recordings was used to allow subsequent description of courtship and spawning. In the analysis of the film we concentrated in six reproductive sequences, for which it was possible to follow the participants until spawning, although many other interactions took place but could not be followed with sufficient detail. The duration of the interactions was, whenever possible, measured with a digital stopwatch.

11.4 Results

A major reproductive event took place on 25 April 2000 in the afternoon (around 5 p.m.) at a temperature of 18°C and apparently involved most, or all, the fishes present in the aquarium (males and females). It was preceded by a general increase in the movements of the fishes and of the number of interactions among them. In the context of this high level of activity, pairs or small groups of fishes engaged in brief sequences of courtship and spawning. All sequences were initiated when a male approached a female, laterally or from below. The male began following the female, initially touching her urogenital region with his snout and gradually moving to the flanks and ending in the head region. At this phase, in four out of six interactions, one or more males joined the pair (usually two, three in one case). Several males could follow the female until the end of the reproductive sequence or abandon her, being or not replaced by others. These sequences occurred with the fishes a few cm above the substratum. Alternatively, the fishes could use a vertical surface like the wall of the aquarium (two out of six reproductive sequences). In that case, they moved up and down in circles, keeping their proximity to the wall and swimming with the body tilted in such a way that the abdomen was turned to the wall of the aquarium. When followed by the male(s) females generally tried to escape moving rapidly, being normally pursued through the whole aquarium. The sequences ended either in spawning (four sequences) or by male withdrawal (two sequences). Spawning occurred when a male(s) pressed the female against substratum, large stones or plant clutches, with the individuals keeping their bodies laterally compressed against each other. In such instance, a female was observed spawning simultaneously with three males. However, at this stage, other males may join the group. A maximum number of eight individuals could be counted with certainty in one such group, at least two of them being females. In these groups, the number and the rapid movements of the fish made sex identification and counting of many of the participants uncertain.

In those large groups there was a rapid turnover of fishes that entered and left the aggregations, which dispersed and re-formed several times. Although the exact number of fishes could not be determined from the video recordings, it can be stated safely that at least two such spawning aggregations occurred simultaneously in different parts of the aquarium, involving the majority of fishes. We are sure that many of the females spawned during the observations, at least four or five, although we could not determine their exact number. During spawning, males and females quivered simultaneously and although eggs and sperm release was not visible, fertilized eggs were collected from the sites under the spawning fishes just after these breeding events. These eggs were slightly adhesive, sticking to gravel, stones and aquatic vegetation. The duration of reproductive sequences varied between 18 and 60 sec (n=6, average=27.3 sec). After such a spawning sequence, the fishes normally dispersed, until the onset of the next sequence. In some cases, they stayed in the place where spawning had occurred, revolving the substratum apparently searching for eggs to eat.

Although the reproductive sequences followed the general pattern described above, a few details are worth mentioning. As long as we could see, no agonistic interactions were present between males, both outside and within reproductive sequences, even when several males were following one female.

Although females usually tried to escape males during reproductive interactions, they also seem to display a particular swimming pattern in the water column that apparently was conspicuous enough to attract male attention. Indeed, all instances in which females were observed performing this behaviour were followed by males approach. This pattern occurred at the onset of four of the six reproductive interactions recorded, and was also seen several times during observations, in open areas with good visibility. It was mainly characterized by oblique head up little, but fast, back and forth swimming movements with constant beating of pectoral and caudal fins.

In what concerns male behaviour there was a component that, although not always present in reproductive sequences (in the six sequences it was recorded only two times), was performed normally in interactions between one female and one single male. In this pattern the male displayed a few circles around the female limiting her movements. Between bouts of circles the male touched her flanks and abdomen with his snout.

11.5 Discussion

The increase of the general activity of fishes in the onset of the breeding episode observed was also described as an indicator for several cyprinid species (e.g. Svärdson (1952), *Rutilus rutilus*; Breder and Rosen (1966) *Cyprinus carpio*). The reproduction mode observed is broadcasting (e.g. release and abandonment of eggs and sperm over an unprepared substrate; Johnston, 1999), common to many other cyprinids, referred as primitive for the north American species of minnows and for fish in general by Johnston and Page (1992).
One of the salient features of our observations is the absence of agonistic behaviour in male-male interactions, also related to absence of territoriality. Although this phenomenon is not documented for the Portuguese cyprinid fishes of the genus *Chondrostoma*, there are several descriptions of territorially and aggressive behaviour associated with reproduction in many other species of this family (Miller (1962), *Campostoma anomalum pullum*; Gale (1986), *Cyprinella lutrensis*; Poncin et al. (1996), *Abramis brama*; Wedekin (1996), *Rutilus rutilus*; Johnston (1999), North American genera *Rhinichthys*, *Cyprinella*, *Luxilus*, *Campostoma*, *Semotilus* and *Nocomis*).

Another interesting feature in the present observations was the existence of a unique female behaviour, that when displayed in the water column could apparently capture male attention and elicit the start of a reproductive sequence. The dynamics of the movements involved and its conspicuousness suggest a possible function as a signal of readiness to mate and an eventual release of pheromones (as suggested by Miller (1962) for *Campostoma anomalum pullum*), but that remains to be tested. As far as we know, such soliciting behaviour by females was not described before for cyprinid fishes. We believe that the role of females in the initiation of courtship deserves special attention, because traditionally much more attention has been given to the action of males in the courtship of fishes, probably in part because it is easier to detect.

The bouts of circles displayed by males around females also deserve further investigation. They occurred normally in interactions involving pairs (one male one female) and we suggest that they play a role in limiting female movements, therefore preventing female escape, a normal feature in the sequences observed.

The behaviour patterns and the spawning sequences described above seem to be specially suited for the type of environment in which this species occurs. The complex swimming movements, with the body tilted to one side, near vertical surfaces, are likely only possible in quiet or slow moving waters. The same applies to the stereotyped head up swimming movements of females that we suggest are courtship signals for the males and to the circles performed by the male around the female. Finally, the eggs would be quickly swept away in conditions of strong currents.

The notes presented are descriptive and show only the general pattern of reproductive behaviour of this species. More accurate information is needed. One of the problems that require a solution is the almost virtual absence of sexual dimorphism (not common in cyprinids, e.g. Miller (1962), *Campostoma anomalum pullum*; Breder and Rosen (1966), *Gobio gobio* and *Phoxinus phoxinus*; Poncin et al. (1996), *Abramis brama*; Wedekin (1996), *Rutilus rutilus*), with the exception of the swollen abdomen of the females in spawning condition. This feature is not always visible and it is almost impossible to use as a diagnosing character in groups of several rapidly moving individuals, when they spawn in large numbers.

Despite the still preliminary character of this note it is important to consider that reproduction in *C. lusitanicum* occurred among individuals kept in captivity for about one and a half years. This may be a good indicator of the suitability of this species, if necessary, for captive breeding with conservation purposes.

11.6 Acknowledgements.

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Parte V

A introdução de ciprinídeos exóticos e as ameaças para a fauna dulçaquícola ibérica

Capítulo 12

Spread of the alien bleak *Alburnus alburnus* (L., 1758) in the Iberian Peninsula: the role of reservoirs

12.1 Abstract

Since its first record in a tributary of the Ebro basin in 1992, the bleak *Alburnus alburnus* has spread to almost the whole Iberian Peninsula. In this paper (based on electrofishing surveys in 598 localities), we provided information on the expansion of the bleak. We detail current distribution in the Ebro basin and in the rivers of the Eastern Pyrenees and we report its presence in other three major Iberian drainages. Currently, the bleak is sympatric with 15 Iberian endemic species (58% of the total Iberian endemic fish). The possible threats caused by the presence of the bleak to the rich Iberian ichthyofauna are discussed. The factors that promoted the extremely rapid expansion of this exotic cyprinid are examined, focusing on the role of reservoirs, which seem to favour its dispersal.

12.2 Introduction

One of the major threats to the freshwater fish fauna stems from the accidental or deliberate introduction of exotic species (Cambray, 2003). This threat is particularly important in the Iberian Peninsula, a major hotspot of European freshwater fish biodiversity, with the 74% of primary freshwater fish being endemic (Doadrio, 2001), and many of them considered as Critically Endangered in Portugal and/or Spain (Doadrio, 2001; Cabral et al., 2005). This valuable Iberian fish fauna is mainly threatened by habitat degradation and uncontrolled introductions of exotic fish. During the past decades, the Iberian Peninsula has been subjected to numerous fish introductions ainly for angling purposes, aquaculture, ornament, biological control or accidentally (Garcia-Berthou and Moreno-Amich, 2000; Elvira and Almodovar, 2001). In Spain, 25 exotic species have become established, representing almost 30% of the Spanish freshwater fish fauna (Elvira and Almodovar, 2001), while in Portugal 13 exotic species have been reported (Almaca, 1995; Gante and Santos, 2002), with increasing new records in both countries (Caiola and Sostoa, 2002; Benejam et al., 2005). Such a scenario claims to obtain detailed information on the distribution of the exotic fish, to evaluate the likely threat to the native fish fauna and to assess the mechanisms that are promoting this high level of introduction and expansion of exotics. The recent introduction and spread of the bleak might illustrate the speed and extent of the dispersal of exotics in Iberian waters.

The bleak *Alburnus alburnus* (Linnaeus, 1758) is a small cyprinid fish with a wide distribution in Europe, from the east slope of the Pyrenees to the Ural Mountains (Perez-Bote et al., 2004). In 1972, the bleak was accidentally introduced with other fish species from Britain to Cyprus, where it was established and breeding (Lever, 1990). In the Iberian Peninsula the bleak was repeatedly introduced as a forage species by anglers (CHE, 1997; Perez-Bote et al., 2004; Velasco et al., 2005). Its introduction was associated with the introduction of major predators such as the northern pike *Esox lucius* (Linnaeus, 1758), the largemouth bass *Micropterus salmoides* (Lacepede, 1802) (Elvira and Almodovar, 2001), the zander *Sander lucioperca* (Linnaeus, 1758), and the wels catfish *Silurus glanis* (Linnaeus, 1758) (Carol et al., 2003). In June 1992, the bleak was collected for the first time in the River Noguera Ribagorzana, a tributary of the Ebro basin, where it became established (Elvira, 1995). Four years later, it was also collected in five rivers of this basin (Cinca, Segre, Jalon, Guadalope and Matarranya) (CHE, 1997). In 1997 it was recorded for the first time in river basins from the Eastern Pyrenees slope (Muga River, northeastern Spain) (Cardona et al., 2002) (Fig. 12.1).

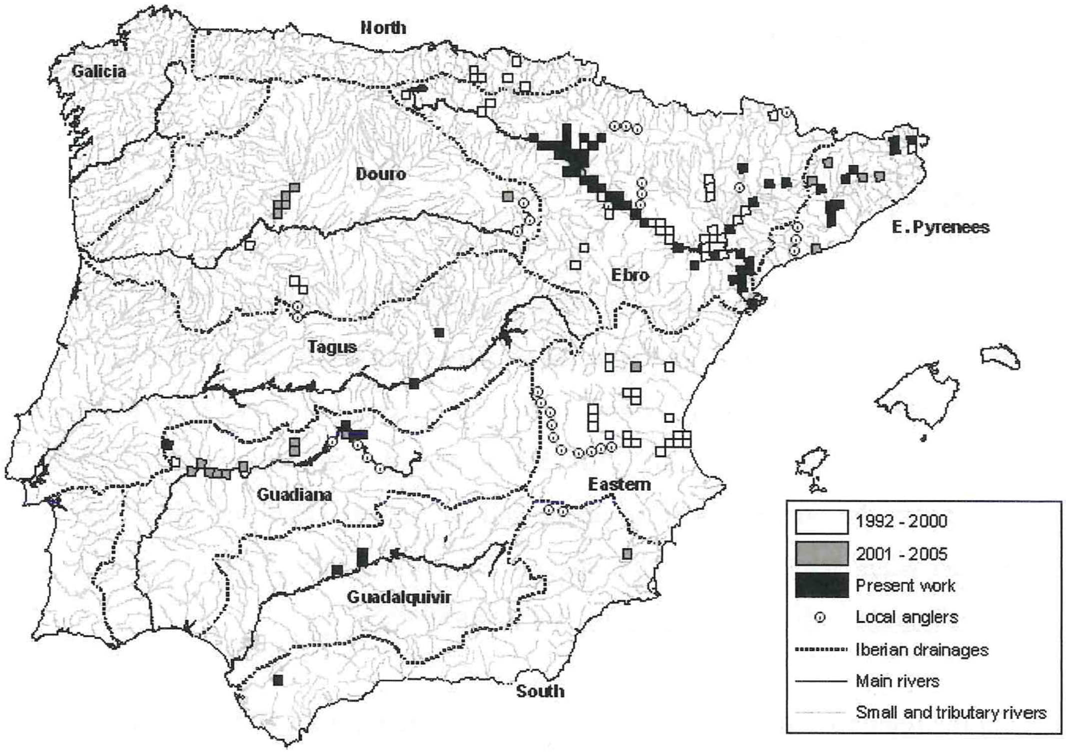

Fig. 12.1 Current distribution of the bleak in the Iberian basins according to published results (see the methods section for more details), data from the present work (species distribution from 2001 to 2006) and additional information from local anglers.

Doadrio (2001) documented this expansion in the Ebro basin and in Mediterranean Rivers from eastern Spain (Jucar, Turia and Mijares). Around 1999 the bleak appeared in two other major Iberian basins: the Douro in the north (Tormes River) (Velasco et al., 2005) and the Guadiana in the south (Campo Maior reservoir, Portugal), extending its range to the Spanish border (Perez-Bote et al., 2004). According to Carol et al. (2003), in 2003 the bleak was also introduced in several reservoirs located in three basins of the Eastern Pyrenees (Foix, Llobregat and Ter; Fig. 12.1). More recently, the bleak was recorded in three reservoirs of the Douro basin (Velasco et al., 2005), in the Segura basin (southeastern Spain; Andreu-Soler et al., 2004) and in two additional reservoirs in the Guadiana basin in Spain (Perez-Bote et al., 2004). The aim of this work is to describe and map the current distribution of the bleak in the Iberian Peninsula with several new records. We also analyse the likely threats of the presence of the bleak in Iberian waters to the rich endemic fish fauna. The factors that promoted the extremely rapid expansion of this exotic cyprinid are examined, focusing on the role of reservoirs, which could favour the dispersal of the species. Considering that reservoirs modify the flow regime, we expect a different occurrence pattern: downstream dams the bleak should show a random distribution whereas upstream the bleak should be not randomly distributed, with a high occurrence close to the dam.

12.3 Methods

A total of 598 localities were surveyed by electrofishing during 2001 to 2006: Eastern Pyrenees (N=222), Ebro (N= 273), Tagus (N=4), Guadiana (N=9) and Guadalquivir drainages (N=90). Two types of data are provided. In the Ebro basin and the Eastern Pyrenees basins regular surveys were conducted, that provided a detailed monitoring of bleak expansion. In other drainages, the surveys were irregular and in several cases the bleak was collected unexpectedly when searching for other species. Electrofishing was mainly conducted with a portable equipment with a current ranging from 300-800 V.

In the regularly surveyed area, river sections ranging from 50 to 200 m were sampled, representing the different mesohabitats and usually exceeding an area of 100 m2. All fish collected were identified and returned alive to the river.

All the published information was also surveyed, as well as information provided by anglers, whenever it was considered accurate. However, the information from anglers was not used in the statistical analysis and was treated separately, due to the risk of misidentifications. The information was recorded in digital maps (UTM 10 x 10 km) with the software ArcView.

Three temporal phases of the bleak expansion were mapped:

a) 1992-2000 (CHE, 1997; Doadrio, 2001; Cardona et al., 2002; Perez-Bote et al., 2004 and Velasco et al., 2005);

b) 2001-2005 (Carol et al., 2003; Andreu-Soler et al., 2004; Perez-Bote et al., 2004 and Velasco et al., 2005);

c) 2001-2006 (data from the present work).

To test the hypothesis that the reservoirs promote the dispersal of the bleak the distance (≤ 50 km) between locations where the bleak was found and the nearest dam was recorded. The position of the dam (upstream or downstream of the bleak site) was also recorded. In three cases where bleak sites were located between two dams at distances smaller than 50 km, the distance to each dam was recorded separately.

Five tests of distribution fitting were performed to determine the distribution types. Overall tests showed coincidence, so that only Kolmogorov-Smirnov test values are shown in results.

12.4 Results

Data are presented separately from the Eastern Pyrenees and Ebro basins, where regular surveys were conducted, and the less systematic information concerning the spread of the bleak in other Iberian basins.

The spread of the bleak in the eastern Pyrenees and Ebro

Inspection of Fig. 12.1 shows that during the study period the distribution of the bleak in the surveyed area markedly changed. The more significant changes can be summarized as follow: while in many rivers the bleak initially occurred near dams, it became widespread throught the entire river, invaded new tributaries and in many cases reached the headwaters. For instance, in the Llobregat and Ter rivers the presence of the bleak increased from 8% in 2000 up to 42% by the end of 2005. In the River Ebro the bleak was present in 624 km of the 910 km of its main course and in 35% of its tributaries (Fig. 12.1, Table 12.1). In the middle watercourse of the River Ebro, many young of the year (mean fork length ± SD = 33.2 ± 7.9 mm, N = 215) were found in August 2005. The size of the individuals in this sample ranged from 18 to 121 mm (furcal lenght).

Table 12.1 Number of localities sampled in each river and first year of capture of the bleak in the Eastern Pyrenees (EP), Ebro (E), Tagus (T), Guadiana (GA) and Guadalquivir (GR) basins. Localities = Numbre of localities (UTM 10X10 km)

Main river (basin)	River	Localities	Year
Fluvià (EP)		1	2002
Llobregat (EP)		3	2002
Llobregat (EP)	Cardener	3	2002
Llobregat (EP)	Riera Gavarresa	1	2002
Muga (EP)		1	2002
Muga (EP)	Llobregat	1	2002
Muga (EP)	Orlina	1	2002
Ter (EP)		1	2004
Ebro (E)		27	2001-2006
Ebro (E)	Mayor	1	2004
Ebro (E)	Ega	1	2003
Ebro (E)	Cidacos	1	2001
Ebro (E)	Aragón	6	2003
Ebro (E)	Aragón (Arga)	4	2003
Ebro (E)	Aragón (Zidacos)	2	2004
Ebro (E)	Alhama	1	2004
Ebro (E)	Guadalope	1	2006
Ebro (E)	Segre	3	2003
Ebro (E)	Segre (N. Ribagorzana)	1	2003
Ebro (E)	Segre (Llobregós)	1	2003
Ebro (E)	Matarranya	1	2003
Ebro (E)	Matarranya (Algars)	1	2003
Ebro (E)	Canaleta	1	2003
Guadiana (GA)	Caia	1	2005
Guadiana (GA)	Estena	1	2003
Guadiana (GA)	Estena (Estenilla)	1	2003
Tagus (T)	Tagus	1	2005
Tagus (T)	Manzanares	1	2006
Guadalquivir (GR)	Guadalmellato (Varas)	1	2006
Guadalquivir (GR)	Guadiato (Cabrilla)	1	2006

New records in other Iberian drainages

During our surveys (2001-2006), the bleak was found for the first time in the following drainages (Table 12.1, Fig. 12.1):

- River Fluvia (Les Caselles, 42°10.8'N 2°47.83'E). The isolated presence in this locality suggests a local introduction.
- River Tagus (Toledo, 39°51.67'N 4°01.57'W) and River Manzanares (a Tagus tributary in Madrid 40°25.1'N 3°42.35'W).
- Two tributaries of the median course of the Guadalquivir basin and a reservoir of the same basin (San Rafael de Navallana reservoir, in the main course of the Guadalquivir).
- Guadalcacin reservoir, in River Guadalete (South drainages).

- River Caia (a small tributary of the Guadiana basin). It is important to note that the ten bleaks captured in this location ranged from 30 to 150 mm (standard length), with four fish being young of the year. Since it is unlikely that small juveniles were introduced by anglers this size variation strongly suggests that the bleak is currently breeding in this river. The species has also been caught in the River Guadiana, close to the mouth of the Caia (Fluviatilis, 2003).

Additional information obtained from anglers

According to the information obtained from anglers during 2006, the bleak distribution is actually larger than the one referred in this work in all the basins surveyed. This information is showed in Fig. 12.1 and comprises the following water bodies:

- River Gaia (Eastern Pyrenees).
- Aragon and Gallego rivers and Santa Ana reservoir (Ebro basin). Aditionally, there is information that suggests the introduction of the bleak in a small mountain lake at an altitude of 1500 m (Estanque de Romedo) with the purpose of feeding the introduced charr *Salvelinus alpinus* (Linnaeus, 1758).
- Headwaters of the River Tormes (Douro basin).
- Headwaters of the River Jucar (Southeastern drainages).
- Guadiana basin: headwaters and Garcia Sola reservoir.
- South drainages: River Mundo and Talave reservoir.

Influence of dams and reservoirs in the spread of the bleak

Considering river sections of $\leq$ 50 km above and below dams we found that downstream dams bleak occurrence fits to an uniform distribution [Kolmogorov-Smirnov test = 0.12, $P>0.05$; Fig. 12.2 (a)] while upstream bleak occurrence fits to an exponencial distribution [Kolmogorov-Smirnov test = 0.15, $P>0.05$; Fig. 12.2 (b)]. The dispersal of bleak upstream dams (at distances $\geq$ 10 km) was not conditioned by the lack of river sections long enough (mean length of river sections upstream of the dams ± SD = 56.8 ± 38.6 km).

The cumulative presence of dams also showed a significant effect on bleak dispersal, so that when rivers were not regulated upstream dams bleak occurred close to them [median (interquartile range) = 5.5 (1.8-8) km], whereas in rivers with additional dams upstream a greater expansion was observed [median (interquartile range = 23.2 (6.2-28.8) km; Mann-Whitney *U*-test: U = 20, $n1$=10, $n2$=11, P<0.05].

Sympatric species

A list of autochthonous fish collected with the bleak is presented in Table 12.2, as well as the total number of species recorded for each drainage.

Seventy-four per cent (N=26) of the auchthoctonous species of the Iberian Peninsula are endemic.

In our surveys, the bleak is sympatric with at least 15 endemic species (58%). From those, 12 have some threatened status. For example, *Anaecypris hispanica* (Steindachner, 1866), which is Critically Endangered, as a distribution area that completely overlaps with that of the bleak (Table 12.2). The majority of the remaining species, mostly cyprinids, are classified at least as Vulnerable in Spain and/or in Portugal. If one considers the number of endangered endemic species present in all water bodies that are now starting to be colonized by the bleak, the value rises to 56% for Portugal (two species Critically Endangered, six In Risk of Extinction and two Vulnerable) and to 78% for Spain (four species in Risk of Extinction and 14 Vulnerable) (Table 12.2).

These figures comprise several species from the genera *Parachondrostoma* and *Squalius* (Table 12.2), namely *P. arrigonis* (Steindachner, 1866) and *P. turiense* (Elvira, 1987), both considered in Risk of Extinction and with an overlap of 100% with the bleak distribution (Table 2). From all endemic species, six have distributions overlapping 100% with that of the bleak (Table 12.2). Moreover, the bleak co-occurs at least with 12 species of other exotic fish, potentially increasing this pressure on the native species.

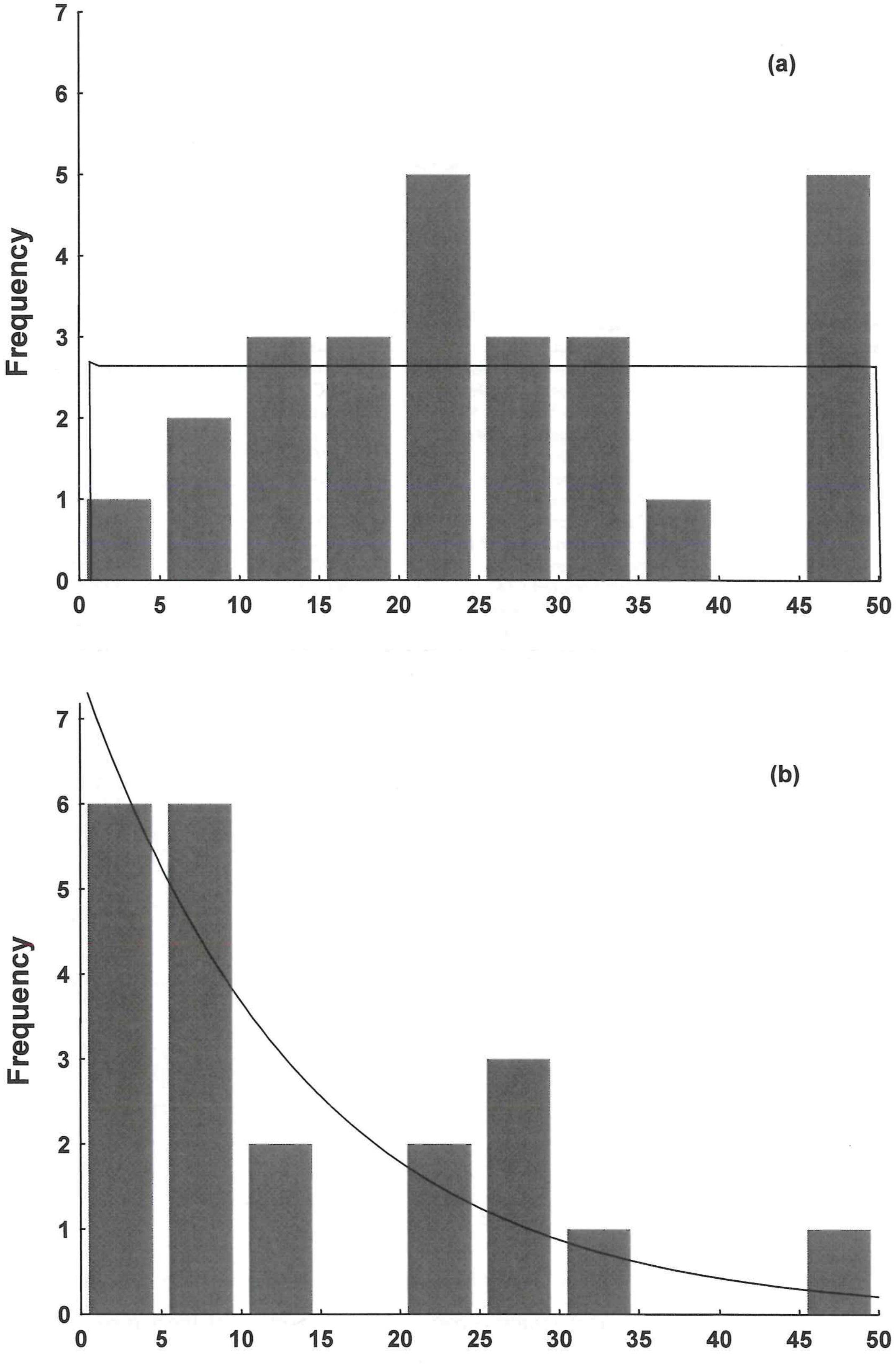

Fig. 12.2 Frequency distribution of sites containing bleak near dams (at distance ≤ 50 km) considering intervals of 5 km. The bleak distribution downstream (a) and upstream dams (b) is presented.

Table 12.2 List of autochthonous (non-migratory) fish species of the Iberian Peninsula potentially sympatric with the bleak, their current conservation status and number and percentage of basins where the bleak was recorded [(1) Endemic to Portugal, (2) Endemic to Spain, (3) Endemic to the Iberian Peninsula, (4) Endemic to Spain and south France]. * Sympatric species. Conservation status according to the Spanish (Doadrio, 2001) and Portuguese Red Data Books (Cabral et al., 2005); categories and criteria follow IUCN (2001). CR = Critically Endangered, EN = Endangered, VU = Vulnerable, LC = Least Concern, NT = Non-Threatened, NE = Not Evaluated. For the former *Chondrostoma* species, we followed the new classification proposed by Robalo et al. (2007)

Family	**Species**	**Current Status** Spain/Portugal	**N° of drainages**
Petromyzontidae	*Lampetra planeri*	CR/CR	2 (33%)
Salmonidae	*Salmo trutta* *	VU/LC	11 (39%)
Cyprinidae	*Achondrostoma arcasii* * (●)	VU/EN	10 (63%)
	Achondrostoma oligolepis (○)	-/LC	2 (17%)
	Anaecypris hispanica * (●)	EN/CR	1 (100%)
	Barbus bocagei (●)	NT/LC	2 (14%)
	Barbus comizo * (●)	VU/EN	2 (100%)
	Barbus graellsii * (θ)	NT/-	6 (75%)
	Barbus guiraonis (θ)	VU/-	3 (33%)
	Barbus haasi * (θ)	VU/-	5 (45%)
	Barbus meridionalis * (▣)	VU/-	3 (50%)
	Barbus microcephalus * (●)	VU/NT	2 (100%)
	Barbus sclateri * (●)	NT/EN	3 (25%)
	Barbus steindachneri (○)	-/NT	2 (100%)
	Gobio lozanoi * (▣)	VU/-	12 (75%)
	Iberochondrostoma lemmingii * (●)	VU/EN	4 (80%)
	Iberochondrostoma lusitanicum (○)	-/CR	1 (20%)
	Parachondrostoma arrigonis (θ)	EN/-	1 (100%)
	Parachondrostoma miegii * (θ)	NT/-	6 (30%)
	Parachondrostoma turiense (θ)	EN/-	2 (100%)
	Phoxinus phoxinus *	NT/-	6 (21%)
	Pseudochondrostoma duriense (●)	VU/LC	1 (11%)
	Pseudochondrostoma polylepis (●)	NT/LC	3 (43%)
	Pseudochondrostoma willkommii * (●)	VU/VU	3 (50%)
	Squalius alburnoides * (●)	VU/VU	4 (50%)
	Squalius carolitertii (●)	VU/LC	2 (18%)
	Squalius cephalus *	VU/-	6 (50%)
	Squalius palaciosi (θ)	EN/-	1 (25%)
	Squalius pyrenaicus * (●)	VU/EN	7 (26%)
	Tinca tinca	NT/NE	9 (75%)
Cobitidae	*Cobitis calderoni* * (●)	VU/EN	3 (43%)
	Cobitis paludica * (●)	VU/LC	9 (29%)
Balitoridae	*Barbatula barbatula* *	VU/-	4 (57%)
Gasterosteidae	*Gasterosteus gymnurus* *	EN/EN	5 (24%)
Blenniidae	*Salaria fluviatilis* *	EN/EN	4 (67%)

12.5 Discussion

The results of the present work clearly show that the bleak is currently present in all of the major Iberian basins and it is distributed across a large proportion of Iberian rivers. Up to now, only the north and northwest of the Iberian Peninsula seem to be free of the species, although little is known about the current status of the fish fauna in these areas. What are the causes behind this extremely rapid expansion of the bleak? New introductions seem to be the result of deliberate actions by anglers that use the bleak either as prey for piscivorous species or as live bait which is frequently released to the water after angling sessions. It is worth to keep in mind that the piscivorous fish are also exotics, which means that anglers are presumably introducing the predators and their prey, thus replacing the natural fish assemblages by increasingly artificial ones. Concerning the rapid spread within rivers where the bleak was already present for some years, it remains unclear to what extent the expansion results from persistent introductions in new localities or from the successful breeding and dispersal. However, a breeding population in the Iberian Peninsula is described by Carbonero et al. (2006) in the River Tormes. The finding of juveniles in the Ebro and Guadiana rivers in the present work strongly suggests the successful establishment of breeding populations. Thus, the mean size of the small specimens recorded in the Ebro and Guadiana rivers were similar to those described by Carbonero et al. (2006) for young of the year in the River Tormes. In Cyprus, its high fecundity allowed the bleak to outcompete other species (Welcomme, 1988). According with J. Carbonero (com. pers., 2006) the same situation exists in the Iberian rivers.

Other factors that may contribute to the adaptability of the bleak include its ability to exploit a widespread spectrum of prey (Vollestad, 1985; Chappaz et al., 1987; Biro and Musko, 1995; Vasek and Kubecka, 2004; Mehner et al., 2005) and its temperature tolerance (from mountain lakes to the River Ebro with summer temperatures around 30oC).

In the Iberian rivers of Mediterranean type water regulation, dam construction and excessive water extraction have contributed to a progressive substitution of rheophilic habitats by more lentic ones, and alterations have occurred simultaneously with the bleak expansion (Copp, 1990; Elvira et al., 1998). In this study a relationship between the expansion of the bleak and the construction of dams has been found. Thus, our findings suggest that the species dispersal is more significant in regulated rivers. In non-regulated rivers upstream of dams, the majority of the bleak were located in close proximity to dams (or just in the reservoirs), suggesting that they play an important role in bleak expansion. In its original distribution area the bleak occupies habitats with slow water (Brabrand, 1983). The attenuation of the natural flow fluctuations in water bodies caused by dams has already been associated with the presence of other introduced species (Bernardo et al., 2003; Clavero et al., 2004).

The negative effects of invasive fish species on native fish, communities and ecosystems is widely recognized (Cambray, 2003; Mills et al., 2004). The impact of an invader like the bleak needs to be assessed at different levels: genetic pool (hybridization), individuals (changes in life history and behaviour), populations (changes in abundance and distribution of native fish species) and communities (competition, species richness, diversity and trophic structure). Given the high level of endemics and the large number of critically endangered cyprinids, all factors that can potentially threaten the Iberian freshwater fish fauna need to be carefully monitored. In the case of the bleak two major issues are of special concern: its potential to outcompete native fish due to its very high reproductive output, and specially the potential danger stemming from the hybridization of the bleak with native fish. The bleak has proved to hybridise very easily with other cyprinids (Blachuta and Witkowski, 1984; Crivelli and Dupont, 1987), namely with species of *Squalius* (Wheeler, 1978; Witkowski and Blachuta, 1980; Kammerad and Wuestemann, 1989). The bleak is also a very close relative of the critically endangered *Anaecypris hispanica* (Robalo et al., 2006) and with the paternal line of the hybridogenetic *Squalius alburnoides*, which itself resulted from hybridization between *Squalius* and a close relative of the bleak and *Anaecypris* (Robalo et al., 2006). Thus, it is very likely that the co-occurrence of the bleak with several of the Iberian endemics will result in a serious introgression of allochthonous genes into the native populations.

The rapid expansion of the bleak and its potential risks for the native fish strongly argue for the need of systematic monitoring of the communities where the bleak has been introduced and the consideration of control plans if necessary. Many countries (including Portugal and Spain) have special laws on the introduction of alien species but these are not always obeyed. In the case of the bleak in Portugal and Spain, nothing can be achieved without the cooperation of the anglers and their associations. Thus, strong efforts aimed to compile adequate information are urgently needed.

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Parte VI

Discussão

Capítulo 13

Discussão

Pretende-se neste capítulo apresentar não só um resumo dos principais resultados, mas enquadrá-los nas hipóteses discutidas até agora sobre a colonização da Europa e das suas penínsulas meridionais pelos peixes dulçaquícolas primários, sem esquecer a colonização e especiação na Península Ibérica e as implicações taxonómicas dos artigos incluídos neste trabalho. Assim, vários temas serão abordados, numa perspectiva que parte do geral para o particular, das grandes implicações biogeográficas à escala europeia, para as pequenas implicações filogeográficas e/ou taxonómicas restritas a Portugal continental.

Aproveitar-se-à igualmente para fazer algumas considerações que se consideram relevantes para a conservação da ictiofauna dulçaquícola, tendo em especial atenção o território português.

13.1 Implicações biogeográficas relativas à interpretação dos diferentes modelos de colonização da Europa e das penínsulas meridionais europeias

Os resultados apresentados em Robalo et al. 2007 (capítulo 6) mostram que a principal radiação das grandes linhagens previamente incluídas no género *Chondrostoma* não ocorreu no Messiniano (como tinha sido sugerido por Durand et al., 2003) mas sim há cerca de 11 milhões de anos atrás no Tortoniano (confirmando a proposta anterior de Doadrio e Carmona, 2004), o que contradiz amplamente a hipótese de colonização "Lago Mare" proposta por Bianco (1990) (ver introdução, página 13). Uma conclusão concordante já tinha sido avançada para outros géneros pertencentes à subfamília Leuciscinae (e.g. *Squalius*, Sanjur et al., 2003). De acordo com os resultados desta tese e com alguns dos autores supracitados a colonização das penínsulas do sul da Europa pelos Leuciscinae parece ter decorrido mais de acordo com a hipótese proposta por Banarescu (1973, 1992, ver introdução, página 13), como resultado de colonizações antigas (desde o fim do Oligocénico até ao fim do Pliocénico, 35-1.7 milhões de anos atrás) e graduais através de capturas de rios (i.e. quando a erosão ou a alteração tectónica do território entre dois cursos de água, leva um deles a abandonar o seu leito, passando a correr no leito do outro) da Europa Central. Na actualidade, e como já havia sido referido na introdução, a fauna de Leuciscinae presente na Europa Central encontra-se representada por um baixo número de espécies (embora incluídas num número considerável de géneros) com amplas áreas de distribuição (e.g. Zardoya e Doadrio, 1999; Durand et al., 2003). Por outro lado, durante as glaciações no Pleistocénico as espécies da Europa Central parecem ter sofrido extinções em massa, de forma que as espécies de água doce hoje presentes nesse território: 1) sobreviveram localmente (e.g. Volckaert et al., 2002); 2) sobreviveram em refúgios no sul da Europa a norte das cadeias montanhosas (e.g. Nesbo et al., 1999) ou 3) ocupavam as penínsulas mediterrânicas durante as glaciações e posteriormente dispersaram (e.g. via Danúbio) (e.g. Slechtova et al., 2004).

A colonização recente da Europa Central é particularmente evidente quando se observa que as espécies mais basais dentro das árvores filogenéticas de cada género não se encontram actualmente nesta região. Por exemplo nos géneros *Protochondrostoma* e *Chondrostoma*, as espécies italobalcânicas *P. genei*, *C. soetta*, *C. phoxinus* e *C. knerii* (que ocorrem todas numa área em torno da parte central do Norte do Mediterrâneo) parecem ser representantes de uma fauna antiga que teria tido uma muito maior distribuição no passado pré-glaciar. Esta ideia apresentada por Robalo et al. (2007) é de certa forma corroborada pela presença de espécies basais de outras linhagens de ciprinídeos e de cobitídeos nesta zona (desde o Norte de Itália ao Oeste dos Balcãs, incluindo a Croácia, Bósnia e Albânia (Ludwig et al., 2001; Perdices e Doadrio, 2001; Sanjur et al., 2003). Algumas linhagens de ciprinídeos apresentam mesmo níveis elevados de diversidade nesta área e um novo género (*Delminychtys*) foi descrito muito recentemente (Freyhof et al., 2006). Todas estas evidências parecem apontar para a ideia de que a fauna desta região é consideravelmente antiga, tendo sido relativamente poupada às extinções glaciares, podendo inclusivé ter tido uma muito mais ampla distribuição na Europa Central antes do arrefecimento que ocorreu no Plio-Pleistocénico. Seguindo esta mesma linha de raciocínio, segundo a qual a Europa Central deve ter tido uma fauna diferente da actual, é de supor que extinções locais ou totais podem ter contribuído, a par de processos de recolonização a partir de zonas mais favoráveis, para a formação do que é hoje a ictiofauna dulçaquícola centro-europeia. Ao fazer esta afirmação há que ter o cuidado de sublinhar que as glaciações podem não ter sido o único factor de extinção da fauna de peixes de água doce na Europa. Gaudant (1977b) e M. Böhme (na sua compilação de dados de fósseis disponível online em http://www.wahre-staerke.com/) mostraram que a fauna Pliocénica da Europa Central tinha uma composição semelhante à actual, embora os detalhes relativamente à distribuição de cada espécie diferissem. É possível assim que as grandes alterações climáticas que tiveram lugar entre o Miocénico e o Pliocénico tenham tido um papel na constituição da ictiofauna moderna muito mais importante do que se costuma pensar habitualmente.

Se se considerarem as filogenias de ciprinídeos europeus até agora publicadas (e.g. Zardoya e Doadrio, 1998, 1999; Cunha et al., 2002) constata-se que o padrão emergente sugere que nas áreas meridionais a ictiofauna divergiu muito cedo, dando lugar a linhagens mais diversificadas e a alguns endemismos como *Delminychtys, Phoxinellus*, *Telestes* e outros descritos nesta dissertação.

No que respeita às espécies pertencentes ao género *Chondrostoma* (correspondente à linhagem *nasus* em Doadrio e Carmona, 2004), *C. nasus* apresenta uma ampla distribuição desde o Médio Oriente até ao Oeste da Europa atingindo o Mar do Norte. Segundo Robalo et al. (2007) esta ampla distribuição Este - Oeste pode ser explicada por uma radiação que teve origem na parte oriental da sua área de distribuição actual, desde o Danúbio e seus arredores, tendo atingido o oeste através do Reno durante o Pleistocénico. De facto, imediatamente após o último período glaciar, é muito fácil conceber contactos directos entre o Danúbio e o Reno.

Alternativamente, através dos lagos que o recuo dos glaciares deve ter deixado na grande planície europeia, é provável que tenham surgido várias vias de penetração ligando a Europa Oriental ao Báltico, que passou por fases oligohalinas (Briggs, 1995), o que pode ter ajudado a dispersar os ciprinídeos para os cursos de água que nele desaguam e para a região do Mar do Norte. É curioso verificar como as espécies de grande distribuição presentes em vários géneros (*Squalius cephalus*, *Rutilus rutilus*, *Gobio gobio*, *Alburnus alburnus*, *Barbus barbus* e *Scardinius erythrophthalmus*) conseguiram chegar às ilhas britânicas, seja quando o Tamisa era um afluente do Reno ou durante o degelo no Mar do Norte, através da via já referida que podia unir as planícies russas e o ocidente através do Báltico. Se esta hipótese estiver correcta é de prever que a divergência dos ciprinídeos nativos das ilhas britânicas relativamente aos da Europa Oriental deve ser muito recente, tendo ocorrido após a última glaciação.

Outras linhagens de peixes apresentam o mesmo tipo de distribuição, com uma elevada diversidade de espécies no Sudeste da Europa (particularmente na Grécia) (e.g., *Squalius*, *Alburnus*, entre outros, Banarescu, 1989; Zardoya e Doadrio, 1999; Sanjur et al., 2003) e uma espécie com uma grande área de distribuição para oeste e mesmo para o Norte da Europa (e.g., *Rutilus rutilus*, *Squalius cephalus* e *Alburnus alburnus*). É possível que o grupo *nasus* tenha evoluído no Paratethys. *C. oxyrhynchum,* que ocorre em águas que desaguam no Mar Cáspio (que em conjunto com o Mar Negro é um derivado do Paratethys; Briggs, 1995), é uma espécie basal no grupo e tem a condição ancestral do clade (boca arqueada). Esta origem poderia explicar a distribuição de *C. nasus*, um peixe típico do Danúbio (que desagua no Mar Negro) e do Reno (que deve tê-la recebido do Danúbio). É de realçar que tanto o Reno como o Danúbio têm as cabeceiras muito próximas, nos Alpes suíços. Também segundo Robalo et al. (2007) o grupo *nasus* pode ter invadido a Grécia e a Turquia através de capturas de rios no Pliocénico, que estão inclusive documentadas para outros géneros de peixes (e.g., *Squalius*, Sanjur et al., 2003).

Resumindo, a fauna actual da Europa Central pode ser um produto recente de migrações de este para zonas onde a fauna original pode ter sofrido extinções sucessivas provocadas pelas glaciações, sendo as populações perimediterrânicas representativas de faunas mais antigas (Robalo et al., 2007). Embora seja este provavelmente o padrão predominante pode ter havido também, como já foi referido, persistência de linhagens pré-glaciares fora das penínsulas, embora numa escala presumivelmente menor.

Importa salientar que os dados sobre as relações filogenéticas entre os géneros previamente incluídos no género *Chondrostoma* foram obtidos recorrendo a vários genes mitocondriais (incluindo o tradicionalmente utilizado citocromo *b*, que permitiu as comparações com os trabalhos previamente publicados por outros autores) e ao gene nuclear da beta-actina, de evolução mais lenta, o que permitiu reconstruir relações filogenéticas mais antigas (Robalo et al., 2007).

13.2 A colonização da Península Ibérica

De acordo com o já referido na introdução, a Península Ibérica, no que se refere aos Leuciscinae, parece ter sido colonizada por um número muito restrito de linhagens oriundas da Europa Central, que aí chegaram provavelmente em fases muito precoces da orogenia pirenaica, antes da Península Ibérica ter ficado isolada do resto da Europa no que respeita aos cursos de água. Devem ter entrado na península apenas três linhagens de Leuciscinae: uma que deu origem às espécies do género *Squalius*, outra às espécies hoje pertencentes aos géneros *Achondrostoma*, *Pseudochondrostotoma*, *Iberochondrostoma* e *Parachondrostoma* e ainda outra que teria dado origem aos peixes do actual género *Anaecypris*. De facto, quando os Leuciscinae ibéricos são colocados num contexto filogenético mais amplo (Zardoya e Doadrio, 1999; Cunha et al., 2002), constata-se que pertencem todos a estas grandes linhagens, à excepção de *Phoxinus phoxinus* que tem uma distribuição localizada no Norte da península (na Bacia do Ebro e talvez em alguns rios da cordilheira cantábrica). Fósseis encontrados na Península Ibérica corroboram esta hipótese (ver introdução, página 20).

Essas linhagens sofreram depois uma intensa diversificação local (compatível com o elevado número de espécies presentes actualmente na região) que, no entanto, parece ter tido uma contribuição muito diminuta para a formação da ictiofauna do resto da Europa. No gene nuclear da beta-actina, com uma taxa de evolução mais lenta do que os genes mitocondriais, encontram-se representadas estas linhagens (Robalo et al., 2006b) embora ainda sejam necessárias mais espécies para completar a amostragem.

Como também já havia sido referido na introdução (página 21), os Cyprininae presentes na Península Ibérica (*Barbus* do subgénero *Luciobarbus*) podem ter tido uma origem biogeográfica diferente (Doadrio, 1990; Tsigenopoulos et al., 2003).

A diversificação dos Leuciscinae na Península Ibérica parece ter ocorrido de forma ligeiramente diferente para as várias linhagens embora com alguns padrões convergentes.

Um ancestral aparentado com o do ancestral dos peixes hoje classificados como *Anaecypris hispanica* teria sido a espécie paterna putativa para o complexo hibridogenético *Squalius alburnoides*, sendo *S. pyrenaicus* a espécie materna putativa (Robalo et al., 2006b).

O ancestral dos géneros *Achondrostoma, Pseudochondrostotoma, Iberochondrostoma* e *Parachondrostoma* parece ter sido o mesmo e as distribuições dos géneros *Achondrostoma, Iberochondrostoma* e *Parachondrostoma* praticamente não se sobrepõem, o que faz pensar que devem ter evoluído em alopatria, embora existam actualmente contactos secundários nalguns rios (Doadrio, 2001).

Quanto à origem do género *Pseudochondrostoma* (endémico da Península Ibérica), a presença de caracteres partilhados entre este género e o género *Chondrostoma* (e.g. boca ínfera, recta e dotada de estojo córneo, tamanho máximo semelhante, migrações pré-reprodutoras) e a frequente hibridação entre as espécies de *Pseudochondrostoma* e *Achondrostoma* (Gante et al., 2004), sugere outras hipóteses (Robalo et al., 2007): 1) estes traços podem ser resultado de uma introgressão na linhagem ancestral de *Pseudochondrostoma* por hibridação; podem ter existido contactos entre peixes ancestrais do grupo *Pseudochondrostoma* - *Achondrostoma* (grupos irmãos nas árvore filogenéticas apresentadas por Zardoya e Doadrio, 1998, 1999; Durand et al., 2003; Doadrio e Carmona, 2003b; Doadrio e Carmona, 2004 e Robalo et al., 2007) e peixes do tipo *nasus* (género *Chondrostoma*) gerando uma linhagem híbrida na Península Ibérica; 2) alternativamente, o género *Pseudochondrostoma* pode ter resultado de uma introgressão ancestral com os membros do género *Parachondrostoma*, cujas espécies também apresentam corpos grandes e fazem migrações pré-reprodutoras.

Quanto à forma da boca, as espécies de *Parachondrostoma* possuem também uma boca com estojo córneo mas arqueada. De acordo com o último cenário a boca recta nos *Pseudochondrostoma* seria um caracter derivado, associado à alimentação (Robalo et al., 2007).

As espécies do género *Pseudochondrostoma* apresentam um elevado comportamento agonístico fora da época de reprodução, descrito em Robalo et al. (2003) (capítulo 10). Esta característica não se encontra documentada em nenhuma espécie dos géneros *Achondrostoma, Parachondrostoma* ou *Chondrostoma*, sendo um comportamento derivado (e.g. sinapomórfico deste género) provavelmente associado à defesa de fontes de alimentação (e.g.

pedras com coberto de algas, que raspam com a boca recta com estojo córneo) durante a Primavera e o Verão.

Na árvore filogenética apresentada em Robalo et al. (2007) o género irmão do clado *Achondrostoma / Pseudochondrostoma* é o género *Iberochondrostoma* que engloba as seguintes espécies: *I. lemmingii*, *I. oretanum*, *I. lusitanicum* e *I. almacai* (para mais informações sobre as espécies ver introdução, página 25). A história paleobiogeográfica e filogeográfica foi estudada em particular nesta tese para os géneros *Achondrostoma* (capítulo 7) e *Iberochondrostoma* (capítulos 8 e 9), com ênfase nas rotas de colonização e dispersão dentro da Península Ibérica. Os acontecimentos geológicos mais importantes que condicionaram estes eventos foram o endorreísmo do Cenozóico (e.g. Andeweg et al., 1999) e o padrão de diferentes conexões entre os principais rios da península, com ênfase nas ligações estabelecidas pelo Rio Tejo ao longo do tempo.

O género *Achondrostoma* parece ter tido origem no Norte e Centro da Península Ibérica como consequência do sistema de lagos endorreicos presente nesta área geográfica durante o Cenozóico (Robalo et al., 2006a). A ocorrência das espécies deste género apresenta na Península Ibérica uma distribuição no mínimo curiosa (ver introdução, página 24). Em Portugal *A. oligolepis* encontra-se dividido em dois grandes grupos: rios do Norte e rios do Oeste (excepto os rios Alcabrichel, Sizandro e Safarujo, onde estão as populações de *A. occidentale*). Considera-se importante salientar que num afluente do Tejo (Rio Nabão) estão presentes haplótipos correspondentes aos dois grandes grupos referidos. Em Espanha, *A. arcasii* ocupa as bacias do Tejo, Douro, Ebro e rios Mediterrânicos. Em termos de relações filogenéticas os peixes das bacias do Ebro e bacias Mediterrânicas são mais aparentados com os peixes da Bacia do Douro, enquanto os peixes da bacia do Rio Júcar se encontram mais aparentados com os da Bacia do Rio Tejo. É de notar que quando nos referimos a Tejo e Douro em Espanha, se tem em mente os trechos mais altos destes rios. De facto, o Douro junto à fronteira portuguesa não tem verdadeiros *A. arcasii* (ver abaixo) e a distribuição desta espécie no Tejo não se estende até Portugal.

Nas bacias do Douro e do Minho (populações nos trechos português e espanhol) encontram-se peixes pertencentes a diferentes grupos. Alguns indivíduos de ambos os rios encontram-se filogeneticamente muito aparentados com *A. occidentale*. A presença num mesmo clado de exemplares de populações de áreas geográficas tão afastadas (extremos noroeste e sudoeste) sugere que esta linhagem teria tido no passado uma distribuição mais ampla sendo as populações actuais apenas relíquias que teriam evoluído por vicariância nas periferias noroeste e sudoeste da primitiva área de distribuição. Segundo Robalo et al. (2006a, capítulo 7) a maioria das separações entre as espécies pertencentes a este género ocorreram antes da formação da actual rede hidrográfica (Andeweg et al., 1999), provavelmente nos lagos endorreicos presentes na península no Miocénico e Pliocénico. A formação da rede hidrográfica actual no Pleistocénico também terá contribuído mais recentemente para novas vagas de diferenciação entre populações. De facto, entre os rios Minho e Douro, a norte, e os Rios Alcabrichel, Sizandro e Safarujo, a sul, o território português é povoado por *A. oligolepis* que se encontra filogeneticamente muito mais próximo de *A. arcasii* do que de *A. occidentale* (2006a, capítulo 7). Este padrão de distribuição seria facilmente explicável se se considerarem os seguintes eventos: 1) a ruptura dos grandes lagos que drenavam grande parte das águas ibéricas (durante o Miocénico) e 2) o estabelecimento de um sistema fluvial que drena para o Atlântico (a partir do Plio-Pleistocénico). Estes acontecimentos podem ter permitido a entrada no território português de um membro da linhagem *arcasii* ancestral de *A. oligolepis*; que poderia ter provocado uma introgressão massiva nas populações ocidentais, o que faria de *A. oligolepis* um peixe de origem híbrida. A ser verdadeiro este cenário, *A. occidentale* e as populações aparentadas do Minho e Galiza seriam os "sobreviventes" que persistiram em zonas periféricas poupadas à introgressão pelos migrantes vindos de Leste. É interessante notar que no Rio Coura, na bacia do Minho, existe uma população onde co-existem e aparentemente se cruzam peixes com ADN mitocondrial de *A. oligolepis* e peixes com ADN mitocondrial aparentado com *A. occidentale* e com as *Achondrostoma* dos rios Galegos. É bem possível que no Rio Coura se esteja a assistir, em pequena, escala àquilo que aconteceu no passado numa escala muito mais vasta, nos rios do Norte e Centro de Portugal. Vale a pena aliás aproveitar este ponto para esclarecer um tópico taxonómico que tem gerado múltiplos mal-entendidos: os peixes do género *Achondrostoma* dos rios Galegos, do Minho e do Douro na região do Sabor são frequentemente referidas na literatura portuguesa como sendo *A. arcasii* ou potenciais híbridos de *A. oligolepis* com *P. duriense* (e.g. Collares-Pereira, 1983; Gante et al., 2004). Estes peixes, de perfil mais alongado e com maior número de escamas do que *A. oligolepis*, pouco têm que ver com as populações de *A. arcasii* genuínas do alto Tejo e Douro espanhol. Além disso, como já se referiu, do ponto de vista do seu ADN mitocondrial estão muito mais próximas de *A. occidentale*, que é também um peixe mais alongado e com um número elevado de escamas. Se se assumir que o nome *A. arcasii* será reservado para as populações do Douro espanhol, Ebro e Rios Mediterrânicos, etc, pode afirmar-se que não existe *A. arcasii* em Portugal, mas sim uma ou duas espécies ainda por descrever, no Minho e

Galiza e no Douro fronteiriço, nomeadamente no Rio Sabor. De acordo com Robalo et al. (2006a, capítulo 7) os tempos de divergência entre as populações de *A. occidentale* e as populações com ela aparentadas no Minho e Galiza são da ordem dos 4 milhões de anos e seriam de cerca de 7 milhões de anos entre este clado e o clado que inclui todas as restantes populações de *A. arcasii* e de *A. oligolepis* de Portugal e Espanha.

Já no que respeita ao género *Iberochondrostoma* os padrões de conexões entre os principais rios da península parecem explicar um padrão de especiação de tipo peripátrico (Mayr, 1982).

As populações de *I. lusitanicum* apresentam uma diferenciação geográfica entre o Tejo (e pequenas ribeiras costeiras independentes na sua vizinhança, incluindo a Lagoa de Albufeira) e o Sado, sugerindo a existência de uma nova espécie no Tejo (e ribeiras adjacentes e Lagoa de Albufeira) (Robalo et al., no prelo a, capítulo 8). De acordo com Robalo et al. (no prelo a) e usando o mesmo relógio molecular utilizado em todos os trabalhos que constam desta tese (cerca de 1% de divergência entre linhagens por cada milhão de anos, originalmente proposto por Dowling et al., 2002), estas espécies teriam divergido há cerca de 2 milhões de anos atrás, na fronteira entre o Pliocénico e o Pleistocénico.

No que respeita à filogeografia da espécie *I. lemmingii*, a primeira conclusão é que existe pouca diferenciação geográfica (com muitos haplótipos partilhados) entre as populações do Tejo, Guadiana e Guadalquivir, estimando-se que as populações do Tejo e do Guadiana tenham estado em contacto até há 80 000 anos atrás (Robalo et al., no prelo b, capítulo 9). A bacia do Guadalquivir parece ser o principal centro de diversificação pelo menos no que respeita a alguns dos seus afluentes. *I. oretanum* teve a sua origem no Guadalquivir (há cerca de 2 milhões de anos), bacia que inclui ainda hoje a totalidade da sua área de distribuição (Robalo et al., no prelo b, capítulo 9). De acordo com o relógio molecular utilizado, a separação de *I. almacai* das restantes espécies do género *Iberochondrostoma* teria ocorrido há cerca de 6 / 7 milhões de anos atrás e a separação de *I. lemmingii* de *I. lusitanicum* há cerca de 4.5 / 5.5 milhões de anos. Estas separações mais antigas formaram grupos mais periféricos em termos de localização geográfica (mais concretamente no Sudoeste e Oeste da Península Ibérica), todos em áreas mais restritas do que as ocupadas por *I. lemmingii* e apresentando também níveis de diversidade genética bastante inferiores. Estes dados levaram os autores (Robalo et al., no prelo b, capítulo 9) a sugerir para este género um cenário de especiação peripátrica (*sensu* Mayr, 1982) com uma grande espécie com uma ampla área de distribuição dando origem, em diferentes alturas, a pequenas espécies periféricas.

Esta interpretação coincide com o padrão de acontecimentos geológicos da península. Antes do sistema actual de rios (de idade Plio-Pleistocénica) os rios drenavam para grandes lagos (Friend e Dabrio, 1996; Andeweg, 2002). Os grandes rios da península como o Douro, o Tejo e o Guadiana não tinham a configuração que têm hoje. Por exemplo, no fim do Miocénico, o Alto Tejo estava separado do Baixo Tejo. Por sua vez, o Guadiana também não existia como sistema fluvial, estando representado por uma série de lagos. Um destes, no que é hoje o Guadiana Superior estava ligado ao alto Tejo (Moya-Palomares, 2002). O Baixo Tejo, por sua vez, tinha uma extensão na direcção do sul de Portugal na região da bacia do Sado (Teresa Azevedo, comunicação pessoal). Robalo et al. (no prelo b) sugerem que mais ou menos nesta época os peixes do Tejo e Guadiana superiores (pertencentes às populações que dariam origem a *I. lemmingii*) teriam atingido o Tejo inferior, provavelmente através de capturas de rios. A partir do baixo Tejo este ancestral teria descido até ao Sudoeste de Portugal, originando *I. almacai*. Esta teoria encontra-se suportada pelo facto de existirem fósseis de *Squalius* no Miocénico do baixo Tejo (Póvoa de Santarém, Gaudant, 1977a) e de neste outro género de ciprinídeos também existirem no sudoeste do país espécies com uma área de distribuição muito restrita e com aproximadamente o mesmo tempo de divergência da espécie ancestral do que o apresentado por *I. almacai* (*S. torgalensis* e *S. aradensis*; Coelho, Bogutskaya, Rodrigues e Collares-Pereira, 1998). Esta concordância existente em espécies de géneros diferentes implica a existência de uma ligação, posteriormente interrompida, entre as duas regiões do Tejo e de um corredor de dispersão para o Sudoeste de Portugal.

Os dois filogrupos de *I. lusitanicum* teriam sido derivados do stock que chegou ao Tejo inferior. Existem muitas evidências geológicas das ligações entre o Tejo e o Sado, assim como das intensas transgressões marinhas que ocorreram em ambas as bacias (Pimentel, 1997; Andeweg, 2002). Essas transgressões teriam levado as populações de *Iberochondrostoma* a permanecer nas cabeceiras dos rios, acelerando a diferenciação genética das populações.

No Pliocénico a ligação entre as duas partes do Tejo quebrou-se de novo, o que explica o facto de, em território português, só existir *I. lemmingii* nalguns afluentes próximos da fronteira (Robalo et al., no prelo b) e provavelmente ainda o facto de, nos *Achondrostoma*, só existir *A. arcasii* no Tejo espanhol e *A. oligolepis* no Tejo português.

I. oretanum teria derivado de *I. lemmingii* por uma rota diferente, que implicaria uma diferenciação na própria bacia do Guadalquivir (Robalo et al., no prelo b); no mesmo trabalho constata-se que os poucos rios onde se nota alguma diferenciação genética nas populações de *I. lemmingii*, são afluente do Guadalquivir.

Em síntese, a história de *Iberochondrostoma* parece ser caracterizada por uma grande espécie central (*I. lemmingii*) ocupando o alto Tejo, Guadiana e Guadalquivir, de onde, em diferentes períodos, por dispersão e fragmentação de populações periféricas, se originaram as restantes espécies.

13.3 Implicações taxonómicas do presente trabalho

O primeiro grande resultado desta tese, em termos taxonómicos, é a resolução da politomia do género *Chondrostoma*, confirmando com mais marcadores moleculares e com mais espécies um resultado previamente sugerido, embora numa árvore menos bem suportada, por Doadrio e Carmona (2004). Este trabalho (Robalo et al., 2007) propõe ainda uma grande revisão do género *Chondrostoma* com a definição de 5 novos géneros que correspondem no fundo ao reconhecimento das linhagens previamente descritas por Doadrio e Carmona (2003b, 2004) e Durand et al. (2003) (ver introdução, página 23) como géneros independentes. Esta necessidade surgiu, por um lado, como resposta à incapacidade de gerir a dualidade dos caracteres utilizados para reconhecer uma espécie como pertencente a este género (morfológicos e osteológicos *vs* moleculares). Por outro lado, Robalo et al. (2007) também demonstraram, através do mapeamento de caracteres, que os caracteres previamente utilizados para definir este género evoluíram provavelmente várias vezes e de forma independente em linhagens distintas, o que não os torna adequados para uso taxonómico. Os géneros definidos em Robalo et al. (2007) representam então todos eles linhagens monofiléticas coerentes do ponto de vista morfológico e molecular. Assim, o género *Chondrostoma* fica apenas restrito às espécies da linhagem representada por *Chondrostoma nasus*. Os novos géneros denominados *Pseudochondrostoma*, *Parachondrostoma*, *Iberochondrostoma*, *Achondrostoma* e *Protochondrostoma* correspondem respectivamente às linhagens *polylepis*, *toxostoma*, *lemmingii*, *arcasii* e *genei* (reconhecidas em Doadrio e

Carmona, 2004). Para uma lista das espécies incluídas em cada um dos géneros e as árvores filogenéticas ver Robalo et al. (2007) (capítulo 6, páginas 83 e 88).

As restantes implicações taxonómicas ocorreram maioritariamente no género *Achondrostoma* (Robalo et al., 2006a). A este género pertencem as seguintes espécies: *Achondrostoma arcasii*, apenas presente em território espanhol (como se defendeu acima, com base nas considerações filogenéticas e biogeográficas); *A. oligolepis* e *A. occidentale*. *A. oligolepis*, antes conhecido como *Chondrostoma macrolepidotus* foi renomeado em Robalo et al. (2005b) (trabalho que consta desta tese, capítulo 4) por o nome *macrolepidotus* se encontrar previamente ocupado.

Algumas populações de afluentes dos rios Douro e Minho cujos indivíduos eram descritos como pertencendo a *A. arcasii* aguardam o seu reconhecimento como novas espécies, estando já as suas descrições em fase de preparação (I. Doadrio, comunicação pessoal).

A. occidentale foi descrita em 2005 (Robalo et al., 2005a) num trabalho que também consta desta tese (capítulo 2). Esta espécie proposta como Criticamente em Perigo, tem uma distribuição muito restrita no Oeste de Portugal, mais concretamente nos pequenos cursos de água costeiros Alcabrichel, Sizandro e Safarujo. Esta nova espécie partilha muitas características com as restantes espécies do género *Achondrostoma*, distinguindo-se destas pela combinação das seguintes características: 40 - 43 ($\bar{x} = 40.9$) escamas canaliculadas na linha lateral, 6 - 7 ($\bar{x} = 6.9$) escamas acima da linha lateral; 2 - 3 ($\bar{x} = 2.9$) escamas abaixo da linha lateral; escama axilar mais pequena ou igual à base da barbatana pélvica. Do ponto de vista osteológico, o processo coronóide do osso dentário é muito espesso. No que respeita a dados moleculares, a divergência mínima encontrada para o gene do citocromo *b* entre esta nova espécie e qualquer das outras do seu género está entre 4.8 e 5.9% de divergência.

Dentro do género *Iberochondrostoma*, as populações de *I. lusitanicum* apresentaram uma diferenciação geográfica entre o Tejo (e pequenas ribeiras costeiras independentes na sua vizinhança, incluindo a Lagoa de Albufeira) e o Sado (Robalo et al., no prelo a,b). Esta diferenciação é de tal forma bem suportada que ambos os trabalhos referidos sugerem a necessidade urgente de se descrever uma nova espécie para o Tejo (e ribeiras adjacentes e Lagoa de Albufeira), uma vez que o holótipo de *I. lusitanicum* é da bacia do Sado (Collares-Pereira, 1980). Robalo et al. (no prelo b) revelaram ainda a existência de populações de *I.*

lemmingii com uma diferenciação considerável, em pelo menos 2 afluentes da bacia do Guadalquivir (Belmez e Ovejuna) que merecem, do ponto de vista dos autores, uma avaliação mais detalhada tendo em vista uma melhor caracterização destas populações. O facto do Guadalquivir ser considerado uma região de diversificação e de especiação (Robalo et al., no prelo b) reveste esta avaliação de uma maior importância.

Nos mapas presentes nas figuras 13.1 a 13.4 encontram-se representadas as distribuições das espécies dos géneros *Achondrostoma*, *Iberochondrostoma*, *Pseudochondrostoma* e *Parachondrostoma*, respectivamente, consideradas nesta tese. Estas áreas de distribuição encontram-se já actualizadas com os dados dos trabalhos publicados e que também constam desta tese. Optou-se por detalhar as distribuições centradas na Península Ibérica, tanto porque foi a área em que as alterações taxonómicas resultantes deste trabalho foram mais extensas e aprofundadas, como pelo facto da península ser a área em que se desenvolveram trabalhos filogeográficos detalhados.

13.4 Contribuições para a conservação das espécies

Apesar do grande impulso dado pela genética molecular aos trabalhos de filogenia, filogeografia e paleobiogeografia de peixes dos géneros citados nesta tese ainda existe muito trabalho de base por realizar, nomeadamente no que diz respeito à determinação da distribuição das espécies, ao seu comportamento (reprodutor e não só) e à sua relação com as, cada vez mais, espécies introduzidas.

A descrição de uma nova espécie no género *Iberochondrostoma* vai dividir a área de distribuição, já bastante reduzida, de *I. lusitanicum*, reforçando com certeza o seu já elevado estatuto de conservação. São necessárias medidas de conservação quase imediatas para salvaguardar as populações destas espécies e para preservar o seu património genético. Assim Robalo et al. (no prelo a) sugerem o reconhecimento de três ESUs: 1) Rio Sado, 2) Rio Tejo e ribeiras costeiras adjacentes e 3) Lagoa de Albufeira.

Num futuro próximo dever-se-ia considerar a reprodução *ex-situ* destas espécies (e de todas as outras que apresentem um estatuto de conservação elevado) como uma prioridade. No capítulo 11 (Carvalho et al., 2003) descreve-se o comportamento reprodutor de *I. lusitanicum* em cativeiro (para comparação com *A. oligolepis* e *A. occidentale*, ver também Pereira, 2007).

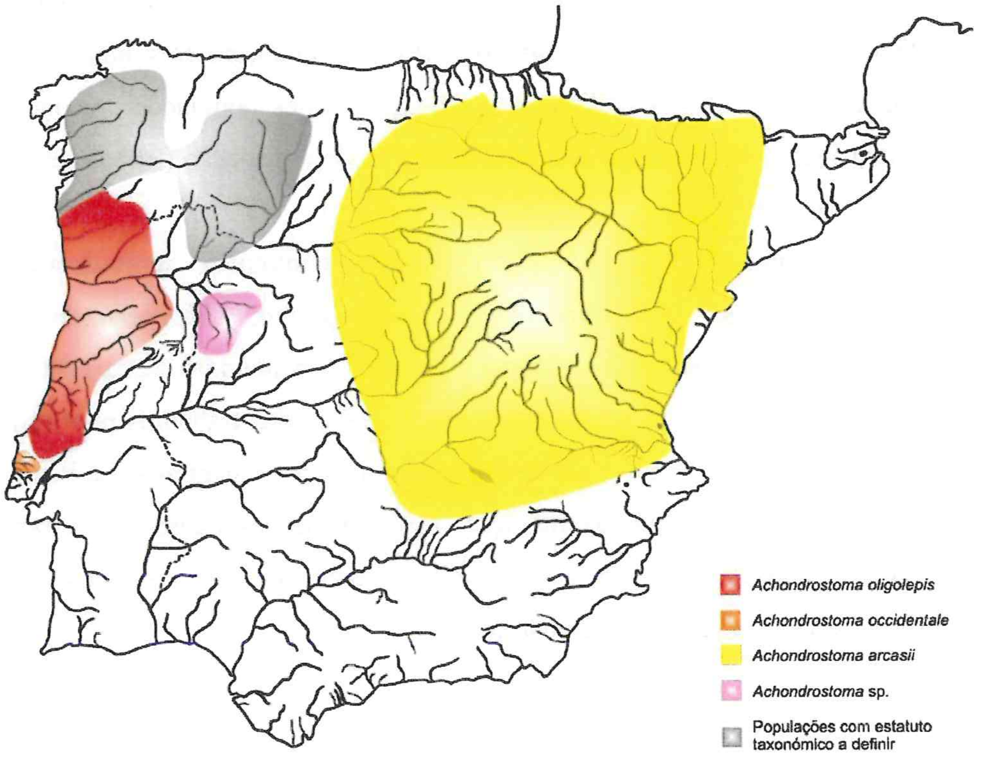

Fig. 13.1 Mapa de distribuição das espécies do género *Achondrostoma* na Península Ibérica

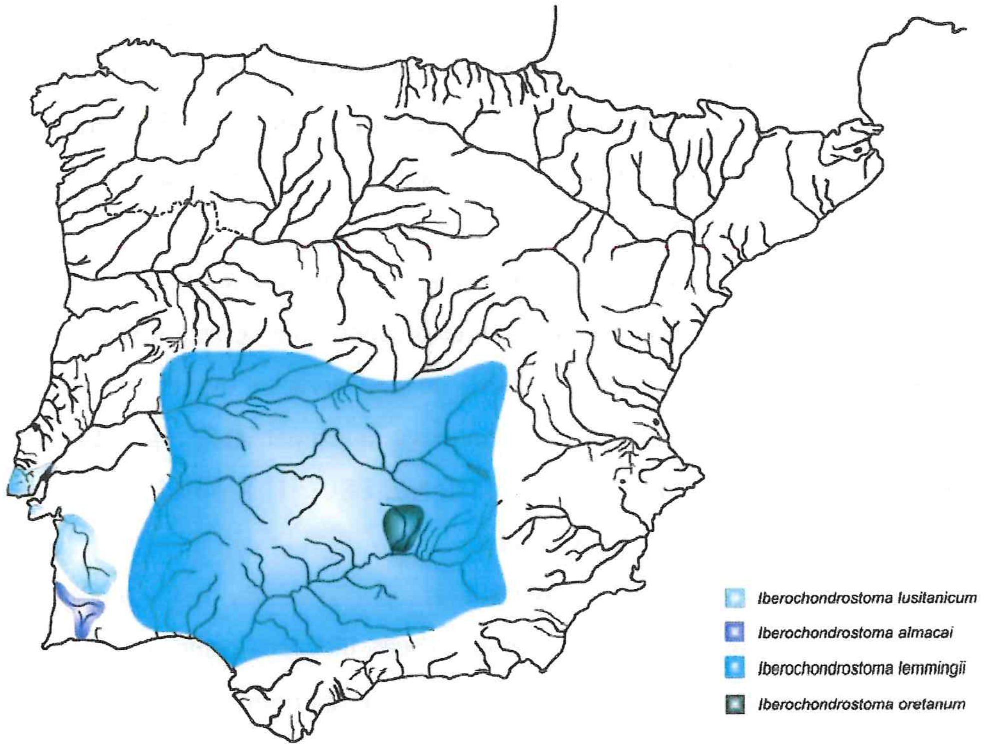

Fig. 13.2 Mapa de distribuição das espécies do género *Iberochondrostoma* na Península Ibérica

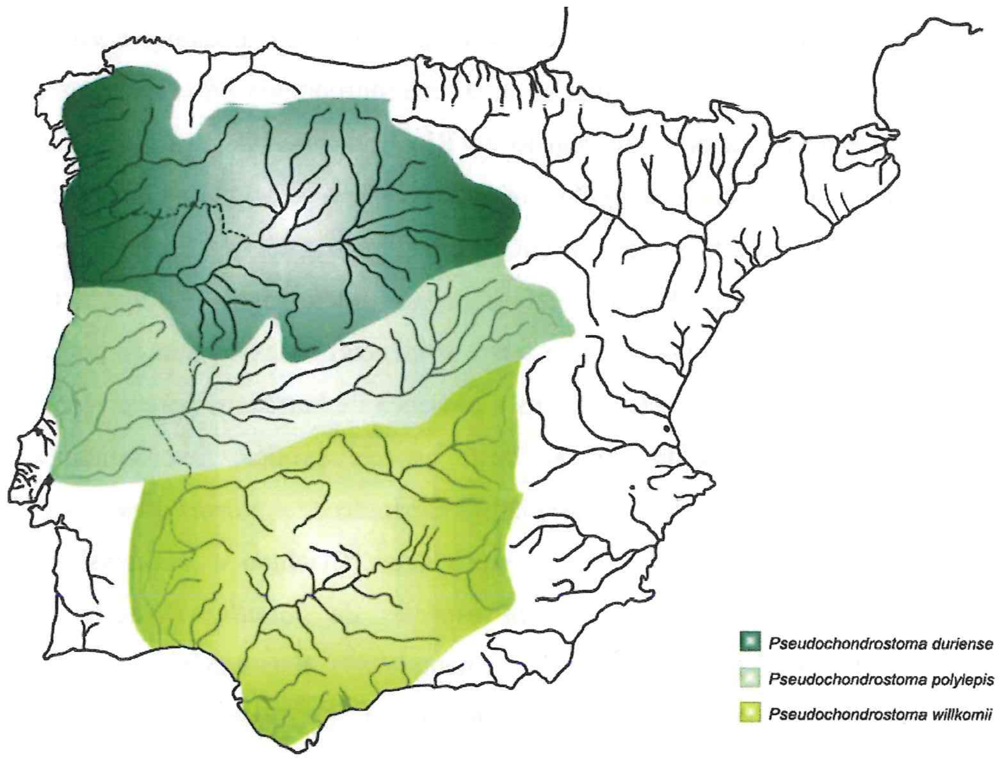

Fig. 13.3 Mapa de distribuição das espécies do género *Pseudochondrostoma* na Península Ibérica

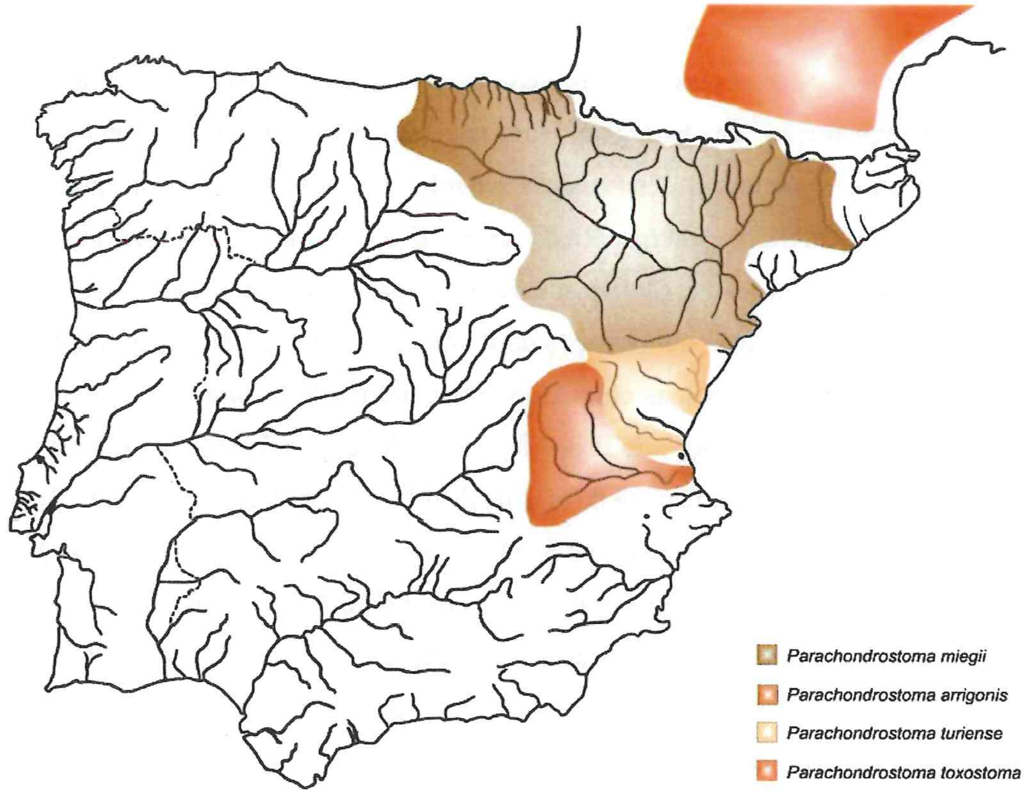

Fig. 13.4 Mapa de distribuição das espécies do género *Parachondrostoma* na Península Ibérica

No que respeita à introdução de espécies exóticas, o trabalho realizado por Vinyoles et al. (no prelo) (capítulo 12) apresenta a situação da espécie introduzida *Alburnus alburnus* na Península Ibérica. Desde o seu primeiro registo no Rio Ebro em 1992, esta espécie já se dispersou para toda a Espanha tendo atingido, em anos recentes, os primeiros cursos de água em Portugal. *A. alburnus* encontra-se neste momento em simpatria com 15 espécies de peixes endémicas, o que corresponde a 58% do total de peixes endémicos da Península Ibérica. Tanto em Espanha como em Portugal existem fortes evidências da existência de populações reprodutivamente activas. Segundo estes autores a presença de barragens parece representar um factor muito importante na distribuição desta espécie exótica, favorecendo a sua dispersão. Uma das maiores preocupações associada à expansão de *Alburnus alburnus* é a sua elevada capacidade de hibridação com outros ciprinídeos (Blachuta e Witkowski, 1984; Crivelli e Dupont 1987), nomeadamente com espécies de *Squalius* (Wheeler, 1978; Witkowski e Blachuta 1980; Kammerad e Wuestemann 1989). Esta espécie é também, de acordo com o revelado por um artigo desta tese (Robalo et. al. 2006b), um parente muito próximo da espécie *Anaeypris hispanica* e da linhagem paterna do complexo hibridogenético de *Squalius alburnoides* (Robalo et al. 2006b). Teme-se por isso que a presença destas espécies e de *Alburnus* nas mesmas águas resulte numa introgressão genética com consequências imprevisíveis para a sobrevivência das espécies endémicas. A implementação de um controle efectivo das espécies exóticas é um imperativo em termos de conservação.

Aliás, a construção de barragens suscita uma preocupação que impõe uma investigação urgente e sistemática. Têm vindo a ser referidos, tanto na literatura portuguesa como na espanhola, híbridos intergenéricos entre espécies de *Achondrostoma* e de *Pseudochondrostoma*. Gante et al. (2004) sugere aliás que os híbridos são possivelmente férteis tendo-se chegado a invocar a hibridação de *P. duriense* e *A. oligolepis* para explicar os peixes morfologicamente atípicos mas com ADN mitocondrial de *A. oligolepis* encontrados no Rio Távora. A área de distribuição de *P. polylepis* e *P. duriense* sobrepõe-se com a área de distribuição de *A. oligolepis*, grande parte da área de distribuição de *A. arcasii* e com a área de distribuição da(s) espécie(s) por descrever no Minho e Douro. Se a hibridação entre os peixes deste dois géneros for um fenómeno natural, que ocorre há milhares de anos com uma taxa semelhante à actual, o problema não inspirará preocupações de maior. No entanto, tem surgido a hipótese de ter sido a construção de barragens a promover um incremento importante deste tipo de hibridação.

Os peixes do género *Achondrostoma* não parecem efectuar migrações pré-reprodutoras enquanto as espécies de *Pseudocondrostoma* migram para águas de corrente rápida e fundo pedregoso para efectuar a desova. Se os reprodutores de *Pseudochondrostoma* se virem impedidos pelas barragens de subir os rios podem vir a encontrar-se sexualmente maduros nas mesmas águas onde desovam as espécies de *Achondrostoma*, o que possivelmente não aconteceria sem as barragens uma vez que os peixes do género *Achondrostoma* não parecem preferir as águas de corrente muito forte e fundo pobre em vegetação. Se forem as barragens a principal causa que promove a hibridação poderemos estar perante uma ameaça para peixes de ambos os géneros que poderão vir a encontrar-se dentro de algum tempo descaracterizados e com os seus mecanismos reprodutivos alterados de formas imprevisíveis.

Um fenómeno deste tipo foi documentado entre *Parachondrostoma toxostoma* e *C. nasus* (Costedoat et al., 2005). Sugere-se que a combinação de uma abordagem genética e uma abordagem ecológica poderá esclarecer no futuro este problema. Felizmente ainda existem na península alguns cursos de água sem barragens onde os dois géneros co-existem. Será assim possível proceder a uma comparação dos níveis de hibridação entre rios não regularizados e rios onde foram construídas barragens e comparar os níveis de hibridação a montante e a jusante dos obstáculos.

13.5 Contribuições metodológicas

Muitos dos trabalhos que constam desta tese recorreram a análises moleculares do gene da beta-actina. Este gene foi utilizado na resolução da politomia do género *Chondrostoma* e na definição dos novos géneros (Robalo et al., 2007), na filogeografia e definição dos ESUs da espécie *I. lusitanicum* (Robalo et al., no prelo a, capítulo 8) e como gene chave da apresentação de um método simples que, recorrendo a picos duplos num cromatograma (gerados como artefactos na vizinhança de indels em heterozigotia), permite identificar as cadeias individuais (Sousa-Santos et al., 2005, capítulo 3).

Adicionalmente, este método foi calibrado para ser usado para a determinação de ploidias e contribuições relativas dos genomas parentais em híbridos do complexo *Squalius alburnoides*. Também a posição filogenética deste complexo e o seu potencial ancestral paterno foi determinado através do uso deste gene nuclear que, sendo um gene de evolução lenta, pode ser bastante útil na clarificação das relações de parentesco entre géneros ou níveis taxonómicos superiores (Robalo et. al., 2006b). Adicionalmente, o seu uso na determinação das cadeias individuais foi testado em *A. oligolepis* (Sousa-Santos et al., 2005), representando uma nova abordagem filogeográfica que continuará muito para além do âmbito desta tese.

Finalmente, na análise dos padrões de especiação do género *Iberochondrostoma* este gene foi utilizado para o estudo de SNP's (*Single Nucleotide Polymorphisms*) (Robalo et al., no prelo b, capítulo 9)

As potencialidades deste gene e dos tipos de análise que permite são também um dos principais resultados desta tese. Um marcador mais lento e conservador pode permitir abordagens mais abrangentes e profundas da filogenia de grupos mais antigos, além de preservar marcas do passado que por questões de saturação dos marcadores ou de hibridação não se encontram patentes na análise de genes mitocondriais.

Não será talvez imodéstia afirmar também que o mapeamento de caracteres morfológicos em árvores filogenéticas foi nesta tese aplicado pela primeira vez aos ciprinídeos europeus de forma rigorosa. Esta abordagem, já esboçada em trabalhos de Zardoya e Doadrio (1998) permitiu esclarecer muitas das incongruências entre as classificações morfológicas e moleculares referentes ao antigo género *Chondrostoma*, ao mostrar que alguns traços que pareciam possuir alto valor diagnóstico como a presença de estojo córneo numa boca ínfera e o seu desenho rectilíneo são afinal caracteres evolutivamente mais lábeis do que se supunha, que parecem ter sido ganhos e perdidos mais do que uma vez na evolução deste grupo. Aliás, as investigações moleculares nos mais variados grupos de organismos têm certamente ajudado muito a repensar a forma como os biólogos utilizam o método comparativo, sempre que se utilizou a informação filogenética para mapear caracteres morfológicos. Tradicionalmente os biólogos costumavam ver as semelhanças morfológicas como expressões de homologia (i.e. reflexos de uma origem comum) ou de analogia (i.e. como consequência de evolução convergente) (Harvey e Pagel, 1991). Neste quadro conceptual a analogia era habitualmente vista como convergência a partir de pontos de partida bastante diferentes. O que as investigações modernas têm vindo a ilustrar de forma crescente é o facto de que organismos estreitamente aparentados, perante pressões selectivas semelhantes, tendem a responder com adaptações semelhantes. De facto, é tanto mais provável que duas linhagens sofram uma transformação semelhante perante uma dada pressão ambiental quanto maior fosse a sua semelhança e parentesco à partida. Estas situações de homoplasia criam, no entanto, grandes dificuldades quando se trata de averiguar se estamos perante homologias genuínas ou perante casos de evolução paralela (Harvey e Pagel, 1991). Só um mapeamento rigoroso da evolução dos caracteres numa filogenia confiável poderá ajudar a esclarecer estas situações.

13.6 Perspectivas para futuras investigações

Certamente que um trabalho científico terá valido a pena se ao conclui-lo se tiverem gerado mais boas perguntas do que as respostas que se obtiveram. O presente trabalho suscitou um grande número de questões que vale a pena resolver no futuro e que seria penoso expor aqui em detalhe. Vale a pena, porém, e de forma muito sintética, sublinhar alguns tópicos do que pode ser um programa de investigação a médio prazo neste domínio:

1 - ao nível europeu e integrando os trabalhos de filogenia molecular com os de paleontologia continuar a repensar o que seria a paleoictiofauna centro-europeia e os seus refúgios glaciares;
2 - a nível ibérico, testar a hipótese da grande introgressão de *Achondrostoma* vindos de leste aquando da formação da rede fluvial exorreicas;
3 - proceder à descrição das espécies de *Iberochondrostoma* e *Achondrostoma* que ficaram pendentes neste trabalho;
4 - averiguar se os fluxos migratórios em *Parachondrostoma* se deram na direcção de Espanha para França ou vice-versa:
5 - aprofundar a avaliação do possível impacto das barragens nos processos de hibridação entre *Achondrostoma* e *Pseudochondrostoma* e caracterizar biologicamente os híbridos;
6 - completar a caracterização genética das populações das bacias e cursos de água que ficaram por prospectar.

Como já foi referido na introdução desta tese, a conservação do elevado número de endemismos da ictiofauna dulçaquícola deve ser uma das nossas maiores prioridades. Mas é impossível preservar as espécies e a diversidade genética associada às suas diferentes populações sem preservar os cursos de água em que vivem e os habitats que rodeiam esses mesmos cursos de água. Todo o conhecimento obtido em termos de padrões de especiação, todo o conhecimento sobre a singularidade das espécies ibéricas e mesmo sobre as particularidades genéticas de algumas das suas populações de nada serve se não for aplicado à conservação deste património.

Alguns dos trabalhos incluídos nesta tese têm amostragens feitas em ribeiras que, apesar de se encontrarem deploravelmente degradadas, ainda têm peixes. Uma quantidade considerável delas tem poucos quilómetros de comprimento. E isso significa duas coisas: 1) que as suas já frágeis e fragmentadas populações de peixes têm pouco por onde fugir e 2) que a sua recuperação representa um gasto perfeitamente irrelevante do ponto de vista económico, mesmo para um poder local sem muitos fundos. Existe já muita legislação que protege habitats e espécies. Mas o seu incumprimento e a ausência de punição adequada a esse incumprimento estão a fazer desaparecer os nossos rios, em empreitadas com esse intuito ou a céu aberto. E com os rios vão os peixes. Para muitas espécies e populações ainda existe a hipótese da reprodução *ex-situ,* mas mesmo esta só tem sentido se existirem rios para repovoar. Resta-nos acreditar que todo este trabalho no passado e presente não terá sido em vão e sonhar, como se costuma dizer "com um futuro melhor".

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