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ORIGINAL PAPER

Phylogeography of the sea star *Marthasterias glacialis* (Asteroidea, Echinodermata): deep genetic divergence between mitochondrial lineages in the north-western mediterranean

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Abstract We explore the phylogeography of the broadcast spawner *Marthasterias glacialis* along south Europe and Azores. Sequences of the cytochrome *c* oxidase gene from 225 specimens, belonging to 10 localities, were analysed. We found 73 haplotypes grouped within two lineages (divergence 2.9%). One lineage was Atlanto–Mediterranean, whereas another one was exclusively Mediterranean. Estimation of lineages split goes back to 830,000–580,000 (±120,000) years ago. This suggests that sea-level oscillations during the Pleistocene glaciations promoted gene flow interruption, lineage divergence between basins and cryptic speciation. Secondary contact between populations allowed a recolonization of the Mediterranean by the Atlantic lineage. When animals of the Atlanto–Mediterranean lineage were considered separately, F_{st} index and AMOVA did not show significant differences between populations along either the Iberian Peninsula or basins. Isolation by distance between populations was not detected, and only populations of Plymouth and Azores showed significant differences to all the others. The remoteness of Azores islands might explain the structure of this population. Haphazard arrival of larvae and local extinctions rather than contemporary restricted gene flow might be responsible for the distinctive population structure of Plymouth.

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Introduction

It is currently well known that barriers to gene flow exist in the ocean realm generating different levels of population differentiation in marine species (Hellberg 1996; Lessios et al. 2001; Hedgecock et al. 2007). Gene flow between populations, promoted by migration and dispersion of both larvae and adults, does not only preclude local adaptation (Barton and Hewitt 1985) but also introduces new polymorphisms in the populations on which selection can potentially act. Therefore, dispersal distances may not only influence geographical range and genetic structure of the species, but also play an important role in population differentiation and speciation processes with profound consequences for the species' phylogeography (Solé-Cava and Thorpe 1991). Differences in species dispersal are primarily determined by the ontogeny of the organisms and are partially correlated with the time that larvae spend in the plankton. However, it has been demonstrated that even in species with apparently high dispersal capability, relatively strong population structure may occur (Launey et al. 2002; Zane et al. 2000). Potential long-distance movements may also be hindered by the existence of other physical and historical factors acting on population's connectivity. Observed patterns of genetic diversity distribution reflect the historical and contemporary interplay among ecological, demographic, behaviour, genetic, oceanographic, tectonic and climatic processes (Palumbi 1995; Benzie 1999).

Marine environments have been influenced by recurrent events of sea level fluctuations and climate changes. Cold glacial periods followed by warm interglacial periods during the Quaternary caused interruptions and reconnections between water masses with important implications for the species distribution, evolutionary history and speciation processes (Wares and Cunningham 2001). For instance, the Pleistocene ice ages (after 2.4 my) subdivided and promoted population differentiation of a number of Atlantic and Pacific coastal species by periodically closing their migratory routes (Cunningham and Collins 1998; Lessios et al. 2001, 2003).

Hydrological factors may also be one of the strongest forces shaping gene flow in the marine realm. Water current regimes at several scales, from the largest general circulation to the smallest local eddies, are capable to reduce dramatically the connection between nearby areas acting like physical barriers or, on the contrary, enhancing flow between distant areas. Thereby, marine circulation regimes may profoundly influence the distributional patterns of the oceans (Benzie 1999; Lessios et al. 2001; Le Gac et al. 2004; Addison and Hart 2004).

Marine areas separated by straits are an interesting focus of study since these specific zones, dominated by sudden changes on depth and strong current regimes, have been repeatedly subjected to disconnections and reconnections during the glaciation events (Wares and Cunningham 2001; Maggs et al. 2008). A number of studies on the phylogeography of marine invertebrates have been carried out at both sides of the Bass Strait that separates eastern from southern Australia (Waters and Roy 2003), the Bass Strait between South America and the Antarctic Peninsula (Hunter and Halanych 2008) and the Gibraltar Strait (Zulliger et al. 2009), which is the only connection between the Atlantic Ocean and the Mediterranean Sea and measures less than 11 miles wide. In the case of the Atlanto–Mediterranean transition, the Almeria–Oran Front (AOF) located inside the Mediterranean has been defined as the main boundary between Atlantic and Mediterranean surface waters, but the transition at the biological level still remains controversial (Borsa et al. 1997; Cimmaruta et al. 2005; Patarnello et al. 2007) and marine species display different patterns of genetic structure at both sides of this particular area (e.g. Quesada et al. 1995; Launey et al. 2002; Rios et al. 2002; Bargelloni et al. 2003). Population genetic analyses have been applied to a scarce number of echinoderm species along the Atlantic–Mediterranean area, but the available data demonstrated different levels of gene flow between basins depending not only on the species' life history but also on the genetic markers used. For instance, the high dispersal sea urchin *Paracentrotus lividus* showed a sharp break between populations at both sides of the AOF, as well as a higher genetic diversity in Mediterranean populations (Calderon et al. 2008). However, for the broadcast spawner *Astropecten aranciacus*, populations seemed to be isolated by distance but genetic divergences were not related with a gene flow interruption between basins (Zulliger et al. 2009).

Because we are far from understanding how the AOF distinctively affects gene flow of marine species, more phylogeographic data of Atlanto–Mediterranean species, with different life histories and dispersive capabilities, are needed in order to get a more comprehensive picture of the importance of this particular transition in shaping the distribution of genetic diversity at both basins.

Marthasterias glacialis (order Forcipulata, Asteriidae) is widely distributed in Europe, from Finnmark (the northernmost county of Norway) to the Mediterranean Sea (Harmelin et al. 1980; Nichols and Barker 1984; Savy 1987), being a common and emblematic species at both sides of the Gibraltar Strait, predator of bivalves and other echinoderms (Barker and Nichols 1983; Frid 1992; Guidetti 2004). Despite its abundance in the North Atlantic sublittoral habitats, it is not, in general, abundant along the Mediterranean littoral, rarely reaching densities up to 2 ind/50 m^2 (Savy 1987). According to Mortensen (1927), the British populations of the species have one single breeding season whereas, Mediterranean populations breed twice per year. *M. glacialis* is a broadcast spawner sea star, with long dispersal capability due to a planktotrophic larva, which remains in the water column for more than 3 months, passing through several larval stages. When adhesive structures appear, the larva attaches to the substrate and the metamorphosis commences (Barker and Nichols 1983).

The cytochrome *c* oxidase I (COI) gene has been widely used in marine invertebrates offering a good level of genetic variation suitable for analyses at different scales (Tarjuelo et al. 2001; Pérez-Portela and Turon 2008), from phylogenetic to population genetic analyses (Avise 2000), and being able to retain the signature of both past and present demographic events that affected the populations and species (Duran et al. 2004). Moreover, this gene has successfully been used in echinoderm species making our results comparable with those previously observed for other species (see e.g. Baric and Sturmbauer 1999; Lessios et al. 2003; Uthicke and Benzie 2003; Muths et al. 2009).

The main objectives of the present study are the following: (1) to explore the level of genetic structure of *Marthasterias glacialis* along the Southwest European coast, (2) to analyse the distribution of the genetic diversity in order to determine the effects of contemporary events versus historical demography and (3) to investigate the possible existence of barriers to gene flow between the Atlantic and Mediterranean basins for a species with high dispersal larva.

Materials and methods

Sampling

Samples of *Marthasterias glacialis* were collected between 2006 and 2009 from 10 different localities (Fig. 1, Table 1); nine of them from the Northeast Atlantic coast (Plymouth, Mutriku, Santander, Ares, Cascais, San Pedro, São Viçente, Sagres and São Miguel) and one from the Northwest Mediterranean coast (Costa Brava), covering a significant range of the species' distribution in Europe. Specimens were sampled either by SCUBA diving (depth between 5 and 25 m) or were directly handed from rocky shore intertidal. Between 13 and 31 sea stars were collected per population, with the exception of San Pedro where only 3 individuals could be sampled due to the low population density of the species in this particular locality. Regarding the Costa Brava samples, specimens of *M. glacialis* were collected from 10 different localities (separated between 3 and 10 km) since densities of the species are so low that no more than nine individuals could be collected per location (see Table 2).

Tube feet of each individual were removed with forceps, fixed in absolute ethanol and preserved at −20°C until processing. Animals were immediately released after tissue collection.

DNA analysis

Total DNA was extracted using a REDExtract-N-Amp kit (Sigma–Aldrich, www.sigma.com) from one or two tube feet. A fragment of the COI gene was amplified and sequenced. Universal primers described in Folmer et al. (1994) were initially used for the amplification of a fragment of the COI. In order to increase the quality of the sequencing reaction, from the first sequences available, we designed specific primers with the software PRIMER vs. 3.0 (http://www.fokker.wi.mit.edu/primer3/input.htm) as follows: MgCOI_F 5′ TC TCATATTTGGAGCTTGAG 3′ and MgCOI_R 5′ TAGG TGTTGAAAGAGAATGG 3′. PCR amplification reactions were performed in a 20 µl total-reaction volume with 10 µl of REDExtract-N-ampl PCR reaction mix (Sigma–Aldrich), 0.8 µl of each primer (10 µM), 4.4 µl of ultrapure water (Sigma–Aldrich) and 4 µl of template DNA. A single soak at 94°C for 7 min was followed by 35 cycles (denaturation at 94°C for 30 s, annealing at 48° for 30 s and extension at 72°C for 35 s) and a final extension at 72°C for 7 min on a thermal cycler (BioRad Mycycler, www.biorad.com). The same primers were used for the sequencing reaction, and the PCR products were purified and sequenced in STABVIDA (http://www.stabvida.net/).

All the sequences were edited and aligned using CodonCode vs. 2.0 software (http://www.codoncode.com/),

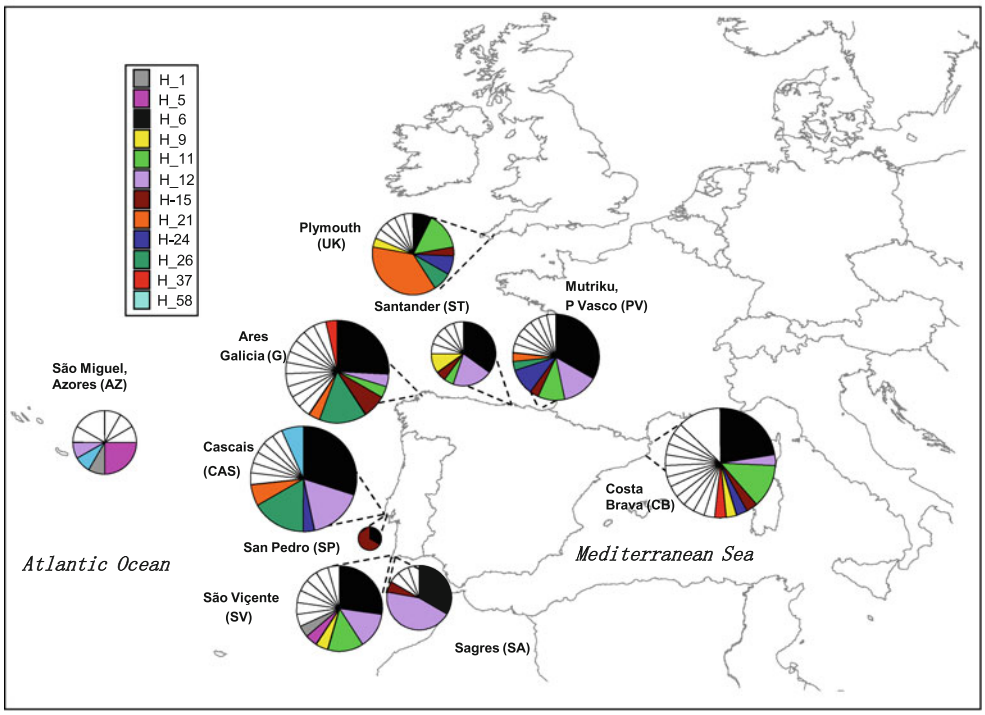

Fig. 1 Map of the sampling scheme for *Marthasterias glacialis*. Pie charts represent haplotype frequencies for each population, and their size is proportional to sample size. Private haplotypes for each population are represented in white colour

Table 1 Diversity measures for populations of *Marthasterias glacialis* from COI

Population	Code	Coordinates	*n*	*Nh*	*Np*	% *Np*	*Hd*	π
Plymouth, UK	UK	50°18′51.54″N 4°9′15.71″W	28	12	5	41.6	0.854 (±0.055)	0.00829 (±0.00070)
Mutriku, Basque country	PV	43°19′51.19″N 2°21′45″W	30	14	7	50	0.869 (±0.049)	0.00494 (±0.00079)
Bahía, Santander	ST	43°28′41.23″N 3°4934″W	20	10	5	50	0.853 (±0.063)	0.00642 (±0.00119)
Ares, Galicia	G	43°25′2.34″N 8°14′29.51″W	28	17	10	58.8	0.923 (±0.037)	0.00794 (±0.00073)
Cabo Raso, Cascais	CAS	38°41′29.93″N 9°25′49.13″W	31	12	6	50	0.873 (±0.039)	0.00715 (±0.00082)
São Viçente	SV	37°1′17.37″N 8°59′37.82″W	22	13	7	53.8	0.909 (±0.045)	0.00593 (±0.00132)
Sagres	SA	36°59′53.85″N 8°56′54.92″W	19	7	3	42.8	0.749 (±0.075)	0.00342 (±0.00079)
São Miguel, Azores	AZ	37°43′44.77″N 25°31′38.13″W	13	9	5	55.5	0.910 (±0.068)	0.00754 (±0.00137)
San Pedro de Estoril	SP	38°41′11.23″N 9°21′20.71″W	3	2	0		0.667 (±0.314)	0.00639 (±0.00301)
Atlantic basin			194	61	50	81.9	0.904 (±0.014)	0.00703 (±0.00015)
Mediterranean basin								
Costa Brava	CB		31	19	12	63.17	0.929 (±0.031)	0.01359 (±0.00184)
Only Lineage I			25	16	9	56.25	0.910 (±0.002)	0.00708 (±0.00102)
Total			225	73	61	83.5	0.911 (±0.013)	0.00811 (±0.00053)

Population, population code, coordinates of sampling location, number of individuals (*n*), number of haplotypes (*Nh*), number of private haplotypes (*Np*), percentage of private haplotypes (*% Np*), haplotype diversity (*Hd*) and nucleotide diversity (π) are shown. Numbers in brackets are standard deviations

Table 2 Localities from Costa Brava, positions, total number of individuals (*n*), number of individuals belonging to Lineage I and number of individuals belonging to Lineage II

Locality	Position	*n*	Lineage I	Lineage II
Blanes	41°40′25.62″N 2°48′10.68″E	9	7	2
Cala Sant Francesc	41°40′42.73″N 2°48′25.94″E	1	1	0
Pared Bisbe	42°14′19.26″N 3°15′21.44″E	7	6	1
Els Caials	42°17′00.80″N 3°17′15.44″E	3	3	0
ŚArnella	42°20′11.73″N 3°12′13.28″E	3	2	1
Cap Norfeu	42°14′37.54″N 3°15′57.40″E	2	2	0
El Gat	42°14′13.54″N 3°15′50.59″E	2	0	2
Montgrí	42°3′40.33″N 3°13′0.83″E	2	2	0
Rostella	42°14′39.01″N 3°15′32.25″E	1	1	0
Sant Feliú Guixols	41°46′43.43″N 3°02′44.37″E	1	1	0

and results from the alignment were verified by eye. Sequences of the haplotypes found in this study have been deposited in GenBank (accession numbers from HM107700 to HM107772) (http://www.genbank.com).

Phylogeography and phylogeny

The complete dataset was used to construct an unrooted network, under the null hypothesis of no genetic differentiation among populations. We used the Network vs. 4.5.1.0 program (http://www.fluxus-engineering.com/sharenet.htm), which assumes the median-joining network method in the absence of recombination (Bandelt et al. 1999). This method begins by combining the minimum spanning trees within a single network. With a parsimony criterion, median vectors ("mv", which represent missing intermediates haplotypes) are added to the network. The loops observed in the networks were solved using the criteria derived from coalescent theory (Templeton et al. 1987; Templeton and Sing 1993).

For phylogenetic analysis of the haplotypes obtained, we included a sequence of *Urasterias licki* from GenBank (Acc number DQ077934.1) as an outgroup. The best-fit model of nucleotide substitution for the data was selected by statistical comparison of 56 different models of evolution with Modeltest vs. 3.0 (Posada and Crandall 1998) using the Akaike Information Criterion (AIC). Values of the evolution model selected were then fed into MrBayes software (Huelsenbeck and Ronquist 2001), and the haplotype tree under the Bayesian Inference (BI) criterion was estimated after 1 million generations with a sample frequency of 100 (10,000 final trees). After verifying that stationarity had been reached, the first 1,000 trees were discarded and an independent majority-rule consensus tree was generated from the remaining trees (9,000 trees).

Genetic divergence within and between lineages of *M. glacialis* were calculated by a Kimura two parameters distance in MEGA vs. 3.0, and divergence times were inferred applying a molecular clock.

Population genetic analyses

Numbers of haplotypes (*Nh*), haplotype diversity (*Hd*) and nucleotide diversity (π) values were computed with DnaSP vs. 4.10.3 (Rozas et al. 2003). Samples from San Pedro were omitted from population genetics analyses because of the low sample number. The existence of different population groupings was tested with the software SAMOVA vs. 1.0 (Dupanloup et al. 2002) (http://www.web.unife.it/progetti/genetica/Isabelle/samova.html). An analysis of molecular variance (AMOVA) was performed to examine population structure, and its significance was tested running 16,000 permutations in Arlequin vs. 3.1 (Excoffier et al. 2005). Populations were grouped within Atlantic and Mediterranean basins following our prior expectation of a genetic division caused by the "basin boundary". For further analyses of population genetic structure, pairwise genetic distances (F_{st}) between populations was assessed by performing 10,000 permutations with the same software. A multidimensional scaling performed on Primer vs. 6 (Clarke and Warwick 2001) was used to graphically visualize these *Fst* results.

Finally, in order to detect the effect of isolation by geographical distance, we compared the correlation of genetic distances (F_{st}/1-F_{st}) with log-transformed geographical distances. Isolation by distance was tested for all pairs of populations with the Mantel test procedure as proposed by Rousset (1997), and 10,000 permutations were executed in Arlequin vs. 3.1.

Demographic analysis

The history of effective population size was tested by the mismatch distribution as the distribution of pairwise differences among all haplotypes in a sample following the models of Rogers and Harpending (1992) and Rogers (1995) in Arlequin vs. 3.1. The mismatch distribution method is based on the assumption that stable population sizes present a multimodal distribution of growths and declines, leaving a characteristic signature in the DNA sequences, so recent population growth is expected to generate a unimodal distribution of pairwise differences between sequences of a given sample. We assessed the fit of mismatch distributions to the theoretical distribution in an expansion scenario by simulation. The sum of squared deviations between observed and expected mismatch distributions was used as a test statistic, and its *P*-value represents the probability of obtaining a simulated sum of squared deviations larger or equal to the observed one.

Since the mismatch distribution approach has been considered very conservative in inferring population expansion events (Ramos-Onsins and Rozas 2002), the history of effective population size was also assessed by means of other statistics such as Tajima's *D* test (Tajima 1989), Fu's F_s test (Fu 1997) and R^2 test (Ramos-Onsins and Rozas 2002) using DnaSP vs. 4.20 (Rozas and Rozas 1999). These tests have been used to detect changes in population size. Analyses were performed for all populations as well as for the populations pooled into two regions, the Atlantic and Mediterranean.

In order to estimate the approximate time of demographic expansion in generations (t) of Atlantic and Mediterranean populations of *Marthasterias glacialis* from coalescence methods, we used the equation $T = 2ut$ (Rogers and Harpending 1992), where T is the date of the growth or decline measured in units of mutational time and u is the mutation rate per sequence and per generation. The value of u was calculated from $u = 2\mu k$, where μ is the mutation rate per nucleotide and k is the number of nucleotides of the analysed fragment. The general mutation rates applied for echinoid COI have been reported to range from 1.6 to 3.5% per million years (Lessios et al. 1999; McCartney et al. 2000), but estimates in Asterinidae sea star species fixed this value around 5% per million years (Hart et al. 1997). We used both mutation rates to calculate demographic and divergence events in order to represent the estimated range in all the Echinodermata.

Results

Genetic diversity

We obtained 626 bp of the mitochondrial gene COI from 225 individuals of *Marthasterias glacialis* from nine Atlantic localities and one Mediterranean locality (see Fig. 1). From the 75 polymorphic sites (12%), only eight of them corresponded to non-synonymous substitutions. Four of the non-synonymous substitutions were only found in São Vicente population (SV). A total of 73 haplotypes were obtained from all the sequences, and 60 of them were private haplotypes (82%). The number of haplotypes per location ranged between two in San Pedro (where only three specimens were sequenced) and 19 in the Mediterranean area. Haplotype diversity (*Hd*) and nucleotide diversity (π) assessed for the whole geographical range were 0.911 (±0.013 SD) and 0.0081 (±0.000 SD), respectively (Table 1). Both, *Hd* and π were much higher for the Mediterranean area, when Lineage I and II were

pooled together (0.929 and 0.014, respectively), than Atlantic ones (average for Atlantic populations $Hd = 0.845$, $\pi = 0.006$). Moreover, the Mediterranean basin showed the highest number of haplotypes and level of private haplotypes. We observed that H_6 was the most common haplotype and was the only one represented within most of the main populations (see Table 6). The next most frequent haplotype, H_12, was represented all along the Iberian Peninsula coast and Azores, but it did not appear in the UK population.

Phylogeny and phylogeography

The network obtained for COI presented six loops that could be unambiguously resolved (Fig. 2a). The results demonstrated the existence of two sharp divergent lineages within the Mediterranean basin lacking intermediate haplotypes that connect both networks. Distance between both lineages was 13 mutations steps.

Whereas Lineage I had an Atlanto–Mediterranean distribution and was represented in every population, Lineage II was represented by only three Mediterranean haplotypes (six individuals). The BI tree reconstructed from the haplotypes showed a shallow phylogeny where relationships near tips were not strongly supported and largely unresolved. Separation between lineages, of being mutually monophyletic groups, was supported by 0.72 of posterior probability (Fig. 2b). Divergence of lineages was also consistent with a bimodal mismatch distribution observed into the Mediterranean basin (see full explanation in *Demographic events*). According with the network results, H_6 and H_12 may be the ancestral haplotypes within Lineage I due to their high frequency, wide geographical distribution and central position within the network.

Genetic distance between lineages was estimated as 2.9% (Kimura 2 parameters), whereas intra-lineage distances were much lower reaching values of 0.92 and 0.32% for Lineage I and II, respectively. Applying a molecular clock and assuming constant rates of mutation, estimations of divergence between Lineages I and II of *Marthasterias glacialis* happened around 580,000 and 830,000 years ago (confidence interval of ±120,000 years), according to a molecular clock of 5% -My calibrated for COI in Asterinidae or 3.5% -My for sea urchins, respectively.

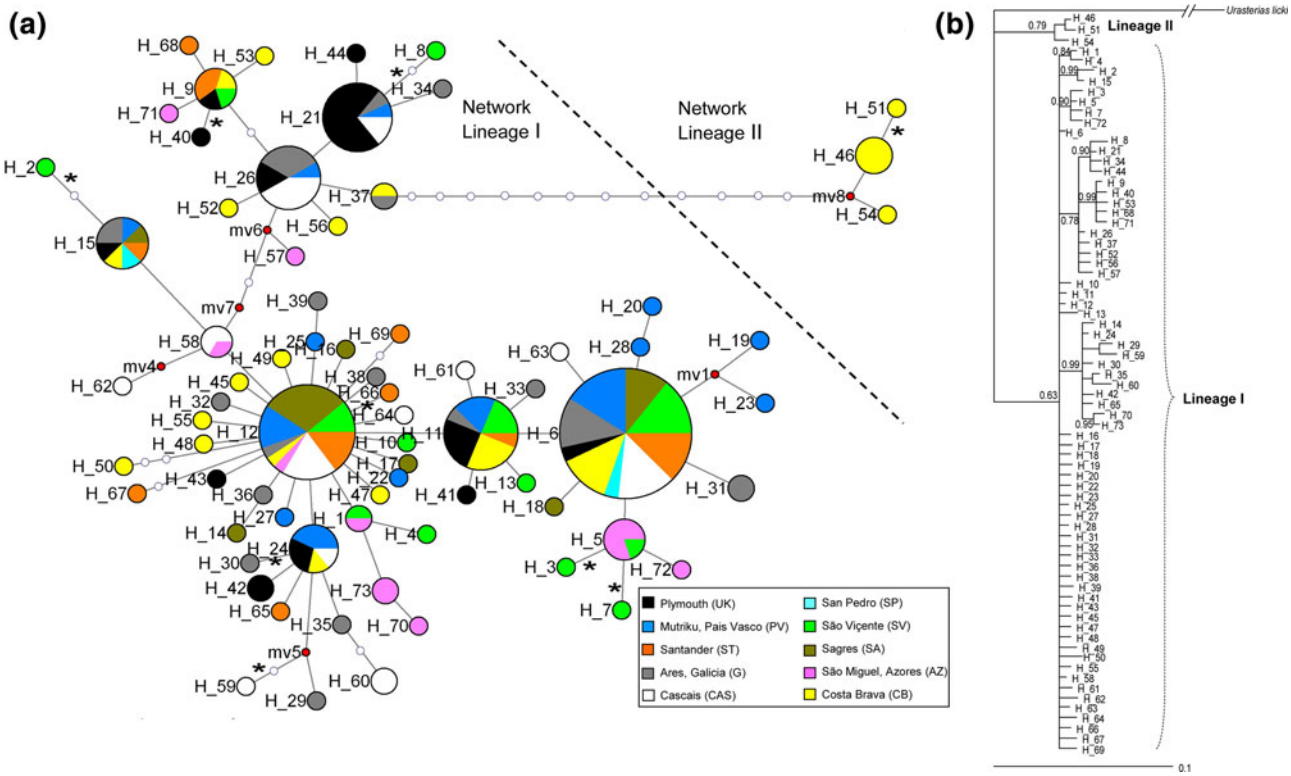

Fig. 2 **a** Median-joining haplotype network for *Marthasterias glacialis* from COI. Area of the *circles* is proportional to the number of individuals found for each haplotype. Partitions inside the *circles* represent the proportion of each population within each haplotype. *Red dots* ("mv") represent missing, probably unsampled haplotypes or extinct sequences. *Lines between circles* represent one mutational step and * non-synonymous substitutions; **b** Bayesian Inference phylogenetic tree from the haplotypes rooting the tree with a sequences of the species *Urasterias licki*; values of posterior probabilities are indicated when >0.5

Table 3 Analysis of the molecular variance (AMOVA) for the COI from populations of *Marthasterias glacialis*

Source of variation	d.f.	Sum of squares	Variance components	% Variation	*P* value	Fixation index
AMOVA between basins						
Among groups	1	0.754	−0.00631	−1.39	0.443	Fct: −0.0139
Among populations within groups	7	6.648	0.02159	4.75*	0.000	Fsc: 0.04688
Within populations	213	93.476	0.43885	96.64*	0.000	Fst: 0.03364
AMOVA without grouping						
Among populations	8	7.402	0.01987	4.33*	0.000	Fst: 0.04331
Within populations	213	93.47	0.43888	95.67		
Total	221	100.878	0.45872			

Analyses are presented pooling population from Mediterranean and Atlantic basins and for the whole area without grouping

Table 4 Population pairwise genetic differentiation (F_{st}) between sampled populations of *Marthasterias glacialis*

	UK	PV	ST	G	CAS	SV	SA	AZ
PV	0.23771**							
ST	0.15645 **	−0.00152						
G	0.06652*	0.03158	−0.00103					
CAS	0.09201**	0.02177	−0.00594	−0.02378				
SV	0.22309**	0.00561	0.00267	0.043591	0.04142			
SA	0.2754**	−0.00683	0.00200	0.05915**	0.04722	0.01882		
AZ	0.21992**	0.13125**	0.10036*	0.09945*	0.09904*	0.06028*	0.16250**	
CB (Lineage I)	0.12208**	0.01085	−0.02987	−0.01516	−0.01451	0.01110	0.02307*	0.10283*
CB (lineage I and II)	0.11238**	0.10244**	0.05153*	0.04845*	0.05740*	0.08751*	0.10016*	0.07736**

* Signification at $P < 0.05$ and ** highly significant when $P < 0.01$

Population structure

The SAMOVA for K = 2 clustered the Mediterranean (CB) and the Atlantic Iberian Peninsula within a group and the Azores and UK population in other two group.

The AMOVA grouping populations in the Atlantic and Mediterranean groups revealed that most of the genetic diversity was due to variability within populations (96.6%, $P = 0.000$) and between populations within basins (4.75%, $P = 0.000$), but no significant differences in genetic structure were detected between Atlantic and Mediterranean basins ($P = 0.443$; Table 3).

Further analyses based on pairwise comparisons (*F*st) showed significant differences between UK (Plymouth) and all the other populations and between AZ (Azores) and all the populations except SV (São Vicente; Table 4). Also, CB (Costa Brava) revealed significant differences with most of the populations when both lineages, Lineage I and II, were included into the analyses. Due to the high genetic divergence between Lineage I and II and in order to avoid grouping together two different genetic pools with different demographic histories, *F*st calculations were also performed for CB including only Lineage I. When the CB was analysed excluding individuals belonging to Lineage II, non-significant differences with Atlantic populations of the Iberian Peninsula were detected but differences with AZ and UK populations remained significant, reflecting the lack of strong genetic structure within the Iberian Peninsula.

The MDS showed populations around the Iberian Peninsula grouped together and the AZ and UK populations separated from the rest (Fig. 3). Despite these differences in genetic structure between populations, they did not fit to the isolation by distance model; the Mantel test did not show a significant correlation between genetic differentiation and geographical distance ($r = 0.046$; $P = 0.123$). This suggests that the differentiation is not directly related to geographical distance and that other hydrological or historical demographic events might have played an important role in cutting off the connectivity between AZ, UK and other areas.

Demographic events

The parameters of the mismatch distribution for each population independently, for Atlantic basin populations and for all populations, pooled together were not significantly different from a sudden expansion model (Fig. 4). The Costa Brava displayed a distinctive bimodal pattern with distant peaks, coherent with the presence of two

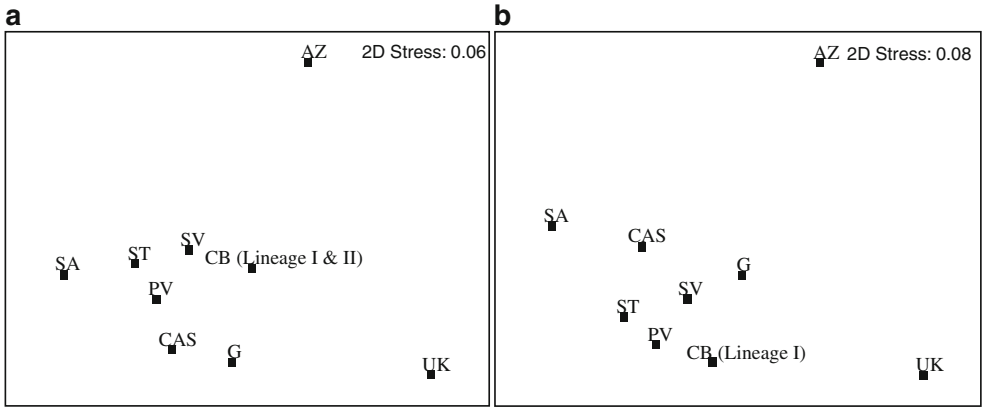

Fig. 3 Multidimensional scaling (MDS) for the COI of *Marthasterias glacialis* from the F_{st} values. **a** from F_{st} including Lineage I and II and **b** from F_{st} including only Lineage I

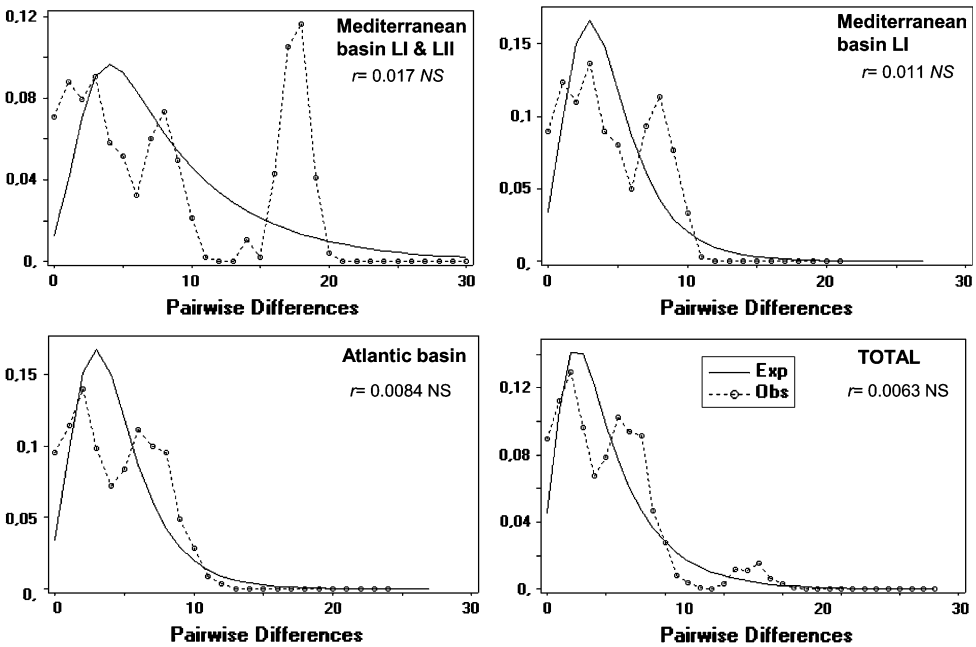

Fig. 4 Mismatch distribution. Graphs represent the mismatch distribution for Mediterranean basin including and excluding Lineage II, Atlantic basin and the whole dataset

different lineages or genetic pools. This fact justified performing analyses separately (including and excluding Lineage II), and in both cases, we could not reject the hypothesis of sudden expansion in the Mediterranean.

The three neutrality tests statistics, Tajima's D, Fu's F_s and Rozas's R^2, were significant for the whole dataset. Moreover, Fu's F_s and Rozas's R^2 were significant when Atlantic populations were pooled together, and for the Costa Brava when only Lineage I was considered. The Tajima's D test was not significant for the Atlantic basin and Lineage I in Costa Brava, but Ramos-Onsins and Rozas (2002) suggested that the F_s and R^2 tests are more powerful than Tajima's D in detecting population changes. Negative values of Tajima's D and Fu's F_s tests have been interpreted as signatures of population expansion (Table 5).

We estimated an approximate time of expansion (t) for the Atlantic geographical region and Lineage I from the Mediterranean basin. From our estimations, the expansion might have taken place approximately between 81,400 and 57,000 generations ago in the Atlantic side and between 87,800 and 61,400 generations ago in the Mediterranean (mutation rates of 3.5 and 5% per my, respectively). Lacking information on *M. glacialis* sexual maturation, we inferred these data from other sea stars in which full maturity is reached in a period of 2–3 years (Yamaguchi and Lucas 1984; Bos et al. 2008). Using this information as an estimate of the generation time, then the expansion events occurred around 162,800–114,000 years ago in the Atlantic Ocean and 175,600–122,800 years ago into the Mediterranean basin for Lineage I.

Table 5 Neutrality tests for each population of *Marthasterias glacialis* and for Atlantic and Mediterranean basin including and excluding Lineage II

	Tajima's D	Fu's Fs	Rozas' R^2
Atlantic basin	−1.66772	−51.747**	0.0376*
UK	0.03324	−1.04921	0.116
PV	−0.95613	−5.2132**	0.084
ST	−1.08945	−1.62230	0.090
G	−0.69720	−6.09772*	0.091
CAS	−0.51871	−1.29340	0.100
SV	−1.44166	−4.72095*	0.0711**
SA	−1.56076*	−1.10765	0.102
AZ	−0.35691	−1.9480	0.1298
Mediterranean basin			
Lineage I & II	−0.55056	−3.73360	0.0936
Lineage I	−1.4101	−6.633**	0.1245**
Total	−1.85632 *	−62.928**	0.0319**

* Signification at $P < 0.05$ and ** highly significant when $P < 0.01$

Discussion

Lineage divergence

One of the most distinctive findings from our results was the existence of two highly divergent lineages of *Marthasterias glacialis* within the Mediterranean basin. Estimation of lineage split goes back to 830,000–580,000 years ago (±120,000) when both Mediterranean and Atlantic basins underwent important climatic and sea level fluctuations due to the Pleistocene glaciations. Glacial periods were associated with successive sea level drops (up to 120 m lower than currently), which dramatically affected the circulation between Atlantic and Mediterranean water masses and the coastal marine fauna (Patarnello et al. 2007; Maggs et al. 2008). Marine currents in the Gibraltar Strait likely became restricted limiting gene flow between basins, which might have promoted local adaptation and lineage divergence in *M. glacialis* at both sides of the Strait. Reestablishment of marine currents during interglacial periods might have allowed secondary contacts between populations from both basins with a recolonization of the Mediterranean by the Atlantic lineage. Contemporary water circulation between basins along the Gibraltar Strait, dominated by a strong surface inflow from the Atlantic into the Mediterranean as far as the Alboran Sea, may assist in maintaining a pattern of unidirectional gene flow from the Atlantic to the Mediterranean. The hypothesis of allopatric divergence in *M. glacialis* is reinforced by a strong genetic divergence lacking intermediate haplotypes between lineages and consistent with data obtained by Baric and Sturmbauer (1999) for the brittlestar *Ophiothrix fragilis*. These authors found two mitochondrial lineages in the species, one of them being endemic to the Mediterranean and the other with Atlanto–Mediterranean distribution, caused by recent recolonization from the Atlantic to the Mediterranean basin. Allopatric divergence by vicariance events has been invoked as one of the most likely models of genetic differentiation and speciation process promoting genetic discontinuities (Palumbi 1994; Cunningham and Collins 1998). A number of studies on Echinodermata have already addressed this particular matter (see examples in Lessios et al. 2001; Wares 2001; Waters et al. 2004; Addison and Hart 2005).

Whether lineages of *M. glacialis* belong to two different taxonomic entities is a problematic question lacking biological, reproductive and ecological information from both lineages. Applying molecular markers has demonstrated prevalence of cryptic and sibling species in marine environments otherwise undetectable from classical techniques of morphological analysis (Palumbi 1994; Knowlton 2000; Feral 2002), but the criteria to distinguish species from molecular data are not standing yet (Avise 1994; Lee 2004). The degree of divergence to define taxonomic units is variable between animal groups, and the comparison with closely related taxa is necessary before reaching consistent conclusions (Hebert et al. 2003). Within the Echinodermata, there is a wide range of intraspecific and interspecific variability for the COI; for instance, a 7% of divergence separated cryptic species of *Holothuria nobilis*, which was also consistent with colour patterns (Uthicke and Benzie 2003). Deep divergence into lineages of the sea star *Coscinasterias muricata* between North and South Australian populations (7.3% for COI) and the correlation between mitochondrial and nuclear markers demonstrated that reproductive isolation had evolved for both forms of the species (Waters and Roy 2003). Within the Asteroidea, genetic distances for COI ranged from 2.5 to 24% among nominal species of Asterinidae (Waters et al. 2004; Hart et al. 1997). However, large databases have recently showed that average of conspecific divergence in Echinodermata is around 2% for COI (Ward et al. 2008; Uthicke et al. 2010), with the highest levels of intraspecific variability reaching maximum values of 2.23% for some of the species studied. The 2.9% of divergence obtained between the sympatrically distributed lineages of *M. glacialis* is higher than those intraspecific values observed in other Echinodermata and might reflect the existence of two cryptic species. The extent of reproductive isolation for both lineages should be further tested by nuclear markers.

Demographic history and population genetics of lineage I

Sequences of COI from *Marthasterias glacialis* provided insight into the historical demography of the species. This

mitochondrial marker was characterized by a relatively low nucleotide diversity and high haplotype diversity, comparable to those of other echinoderms (McCartney et al. 2000; Uthicke and Benzie 2003; Addison and Hart 2005) and probably due to a recent expansion of the species along the Atlantic and Mediterranean Sea. Several points from our data supported the hypothesis of historical expansion; (1) a star-shaped network with a few high-frequency central haplotypes connected with many low-frequency haplotypes (Templeton et al. 1995), (2) significant negative Tajimas' *D* and Fu *Fs* tests and (3) a unimodal mismatch distribution, for Atlantic pool and Mediterranean lineage I, fitting within the expected expansion model, which has frequently been attributed to a population expansion after periods of small effective population size (Rogers and Harpending 1992). A bimodal distribution detected for the Mediterranean area emerges from grouping together two genetic pools holding different demographic history. Unimodal distributions in other marine invertebrates have been related to episodes of sea level oscillations during the Pleistocene and prior to the last glacial maximum 18,000 years ago (Zulliger et al. 2009; Couceiro et al. 2007). According to our results, a rapid population expansion after a glacial period would explain the present diversity of the species (Avise et al. 1984). From a more detailed analysis of the mismatch distribution test at both basins, we detected a double peak pattern that probably corresponds to two successive population increases in size. Based on a mutation rate of 5%-My and the frequencies of pairwise differences, we estimate that the first and second expansions happened in the Mediterranean sea, around 127,000 and 48,000 years ago, respectively, and 96,000 and 31,000 years ago in the Atlantic basin, which may correspond to two interglacial periods before the last Pleistocene glaciation. However, our estimations of expansion times may be biased concerning the Mediterranean due to the reduced effective population size and the different numbers of generations per year reported for Atlantic and Mediterranean populations.

Marthasterias glacialis was also characterized by an absence of differentiation along the Atlantic Iberian Peninsula and the Mediterranean coast within Lineage I, and only Plymouth and Azores were significantly different from other populations. The absence of population subdivision at both sides of the Gibraltar Strait strongly contrasts with population differentiation patterns across the Atlanto–Mediterranean transition described for a number of marine invertebrate species (see Borsa et al. 1997; Patarnello et al. 2007). Although discrepancies between population genetic patterns for high dispersal echinoderms between Atlantic and Mediterranean sea suggest that similar biological features do not necessarily imply similar population structure (Calderon et al. 2008; Muths et al. 2009; Zulliger et al. 2009), a number of the marine invertebrate species analysed demonstrated either a common pattern of "genetic divergence" at both sides of this Strait or isolation by distance (see e.g. Quesada et al. 1995; Zane et al. 2000; Pérez-Losada et al. 2002). Hence, biogeographic barriers may be differentially permeable even for species with similar dispersal abilities (Addison and Hart 2005), and genetic patterns likely result from the stochastic nature of larval dispersal, colonization events and historical demography. Panmixia of *M. glacialis* along the Atlantic Iberian Peninsula and Mediterranean basin may be explained by either (A) a contemporaneous unidirectional gene flow from the Atlantic to the Mediterranean basin or (B) a past intense gene flow implying that the current gene frequencies have not already returned to equilibrium for mitochondrial DNA (Avise 1994; Palumbi 1994; Benzie 1999). Recolonization of the Mediterranean by members of the Atlantic lineage might have resulted in a genetic homogenization over the entire distribution range of the species, which could explain the lack of genetic subdivision. Since patterns of haplotype frequencies and distribution may be difficult to interpret in terms of contemporary gene flow and given that hypotheses A and B are not mutually exclusive, any of them can be discarded. Future analyses including markers evolving at higher rate to infer recent processes will be necessary in order to estimate the present levels of connectivity in this species.

Despite the apparent panmixia of *M. glacialis* along the Iberian Peninsula, the populations of UK (Plymouth) and Azores were different from all the other populations. Such a distinctive genetic structure of both populations may be explained by the geographic distribution of the species. This sea star is widely distributed along the northeast Atlantic and Mediterranean Sea but absent in the English Channel, Plymouth being the limit of its distribution within the Channel (Savy 1987; Ellis and Rogers 2000). On the other hand, the Azores islands are located at the western limit of distribution of the species. Although the UK population had haplotypes represented within all the main clades, a closer approximation highlights the lowest percentage of private haplotypes and the lack of one of the main ancestral haplotypes (H12). Populations in the distribution limits may be submitted to periodical extinction events. Survival of the UK population might happen by sporadic recruitment events from northern sources whose population structure is unknown. This would explain the absence of one of the most widespread haplotypes, the lowest occurrence of private haplotypes and the fact that the Plymouth population is the only one with no evidence of demographic expansion, positive values of Tajima's *D* and the highest ones for Fu's *Fs* and R^2 tests. On the other hand, the remote population of Azores, with some private haplotypes, seems to be relatively isolated with certain

recruitment from southern Portugal (Cascais and São Vicente).

Conclusions

From the data obtained in this study, we interpret that sea level fluctuations during Pleistocene glaciations promoted allopatric differentiation of lineages and speciation in *M. glacialis* and played an important role in shaping the genetic diversity of this species. In addition to a recolonization of the Mediterranean from the Atlantic lineage, demographic expansions might have erased previous population genetic structure. Therefore, the genetic patterns may be far from the equilibrium, and they may not necessary reflect the contemporary genetic exchange. The distinct structure of Plymouth population and the lack of correlation between genetic differentiation and geographical distance suggest that historical factors, such as haphazard arrival of larvae and local extinctions, rather than contemporary gene flow are responsible for this peculiar pattern. On the other hand, the remoteness of Azores islands might explain the structure of this population.

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Appendix

See Table 6.

Table 6 Haplotype frequencies of *Marthasterias glacialis* per population. Numbers in bold are private haplotypes

	UK	PV	ST	G	CAS	SV	SA	CB	SP	AZ
H_1	0	0	0	0	0	0.0455	0	0	0	0.0767
H_2	0	0	0	0	0	**0.0455**	0	0	0	0
H_3	0	0	0	0	0	**0.0455**	0	0	0	0
H_4	0	0	0	0	0	**0.0455**	0	0	0	0
H_5	0	0	0	0	0	0.0455	0	0	0	0.3076
H_6	0.0174	0.333	0.35	0.250	0.290	0.273	0.316	0.226	0.333	0
H_7	0	0	0	0	0	**0.0455**	0	0	0	0
H_8	0	0	0	0	0	**0.0455**	0	0	0	0
H_9	0.0357	0	0.1	0	0	0.455	0	0.0323	0	0
H_10	0	0	0	0	0	**0.0455**	0	0	0	0
H_11	0.143	0.1	0.5	0.0357	0	0.136	0	0.129	0	0
H_12	0	0.133	0.2	0.0357	0.161	0.136	0.421	0.0323	0	0.0769
H_13	0	0	0	0	0	**0.0455**	0	0	0	0
H_14	0	0	0	0	0	0	**0.0526**	0	0	0
H_15	0.0357	0.0333	0.05	0.0714	0	0	0.0526	0.0323	0.666	0
H_16	0	0	0	0	0	0	**0.0526**	0	0	0
H_17	0	0	0	0	0	0	**0.0526**	0	0	0
H_18	0	0	0	0	0	0	**0.0526**	0	0	0
H_19	0	**0.0333**	0	0	0	0	0	0	0	0
H_20	0	**0.0333**	0	0	0	0	0	0	0	0
H_21	0.0357	0.0333	0	0.0357	0.0645	0	0	0	0	0
H_22	0	**0.0333**	0	0	0	0	0	0	0	0
H_23	0	**0.0333**	0	0	0	0	0	0	0	0
H_24	0.0714	0.1	0	0	0.0323	0	0	0.0323	0	0
H_25	0	**0.0333**	0	0	0	0	0	0	0	0
H_26	0.0714	0.0333	0	0.143	0.161	0	0	0	0	0
H_27	0	**0.0333**	0	0	0	0	0	0	0	0
H_28	0	**0.0333**	0	0	0	0	0	0	0	0

Table 6 continued

	UK	PV	ST	G	CAS	SV	SA	CB	SP	AZ
H_29	0	0	0	**0.0357**	0	0	0	0	0	0
H_30	0	0	0	**0.0357**	0	0	0	0	0	0
H_31	0	0	0	**0.0714**	0	0	0	0	0	0
H_32	0	0	0	**0.0357**	0	0	0	0	0	0
H_33	0	0	0	**0.0357**	0	0	0	0	0	0
H_34	0	0	0	**0.0357**	0	0	0	0	0	0
H_35	0	0	0	**0.0357**	0	0	0	0	0	0
H_36	0	0	0	**0.0357**	0	0	0	0	0	0
H_37	0	0	0	0.0357	0	0	0	0.0323	0	0
H_38	0	0	0	**0.0357**	0	0	0	0	0	0
H_39	0	0	0	**0.0357**	0	0	0	0	0	0
H_40	**0.0357**	0	0	0	0	0	0	0	0	0
H_41	**0.0357**	0	0	0	0	0	0	0	0	0
H_42	**0.0714**	0	0	0	0	0	0	0	0	0
H_43	**0.0357**	0	0	0	0	0	0	0	0	0
H_44	**0.0357**	0	0	0	0	0	0	0	0	0
H_45	0	0	0	0	0	0	0	**0.0323**	0	0
H_46	0	0	0	0	0	0	0	**0.0129**	0	0
H_47	0	0	0	0	0	0	0	**0.0323**	0	0
H_48	0	0	0	0	0	0	0	**0.0323**	0	0
H_49	0	0	0	0	0	0	0	**0.0323**	0	0
H_50	0	0	0	0	0	0	0	**0.0323**	0	0
H_51	0	0	0	0	0	0	0	**0.0323**	0	0
H_52	0	0	0	0	0	0	0	**0.0323**	0	0
H_53	0	0	0	0	0	0	0	**0.0323**	0	0
H_54	0	0	0	0	0	0	0	**0.0323**	0	0
H_55	0	0	0	0	0	0	0	**0.0323**	0	0
H_56	0	0	0	0	0	0	0	**0.0323**	0	0
H_57	0	0	0	0	0	0	0	0	0	**0.0769**
H_58	0	0	0	0	0.0645	0	0	0	0	0.0769
H_59	0	0	0	0	**0.0323**	0	0	0	0	0
H_60	0	0	0	0	**0.0645**	0	0	0	0	0
H_61	0	0	0	0	**0.0323**	0	0	0	0	0
H_62	0	0	0	0	**0.0323**	0	0	0	0	0
H_63	0	0	0	0	**0.0323**	0	0	0	0	0
H_64	0	0	0	0	**0.0323**	0	0	0	0	0
H_65	0	0	**0.05**	0	0	0	0	0	0	0
H_66	0	0	**0.05**	0	0	0	0	0	0	0
H_67	0	0	**0.05**	0	0	0	0	0	0	0
H_68	0	0	**0.05**	0	0	0	0	0	0	0
H_69	0	0	**0.05**	0	0	0	0	0	0	0
H_70	0	0	0	0	0	0	0	0	0	**0.0769**
H_71	0	0	0	0	0	0	0	0	0	**0.0769**
H_72	0	0	0	0	0	0	0	0	0	**0.0769**
H_73	0	0	0	0	0	0	0	0	0	**0.1538**

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