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Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea

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Abstract

The phylogeographical patterns of a small marine fish, the common goby, *Pomatoschistus microps*, were assessed at 12 sites along the northeastern Atlantic coasts and the western Mediterranean Sea. A combination of two genetic markers was employed: cellulose acetate allozyme electrophoresis (CAGE) and sequence analysis of a 289 bp fragment of the mitochondrial locus cytochrome *b*. Both markers were congruent in revealing significant differences between samples (global $F_{ST} = 0.247$ for the allozymes and $\Phi_{ST} = 0.437$ for the mitochondrial DNA data) and a pattern of isolation-by-distance. Phylogeographical analyses yielded a shallow branching structure with four groups. Three of those were confined to the Atlantic basin and showed a star-like pattern. The fourth group contained a central haplotype occurring at the edges of the species' distribution, accompanied by a few more rare variants, which were restricted to the Mediterranean Sea. A genetic break was observed around the British Isles, with distinct haplotypes dominating at either side of the English Channel. A significantly negative correlation between the degree of genetic diversity and latitude was recorded both for mitochondrial DNA (mtDNA) and allozymes in the Atlantic basin. Gene flow analysis suggested that recolonization of the North Sea and the coasts of western Scotland and Ireland may have taken place from a glacial refugium in the Southern Bight of the North Sea. These results are discussed in the perspective of possible postglacial migration routes of marine fish along the northeastern Atlantic coasts.

Keywords: allozymes, glacial refugium, mitochondrial DNA, northeastern Atlantic Ocean, phylogeography, *Pomatoschistus microps*

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Introduction

Although allozyme-based surveys are particularly suitable for drawing conclusions about the degree of intra- and interspecific genetic differentiation, the interpretation of how and when this differentiation arose remains limited due to the low historical resolution of these markers. This has changed with the application of mitochondrial DNA (mtDNA) analysis, which is appropriate for addressing historical issues because of its maternal inheritance, lack of recombination and faster rate of evolution compared with coding nuclear DNA. Thus, there has been an exponential increase in the number of phylogeographical studies based on mtDNA analysis in recent years (Avise 2000). However, to date, most studies have focused on terrestrial and freshwater species (reviewed in Taberlet *et al.* 1998 and Hewitt 2000). This is not surprising, as the generally high degree of genetic structuring in these groups makes it relatively straightforward to correlate patterns of genetic divergence with past palaeoclimatological and vicariance events. For example, the Pleistocene glaciations have left a distinct genetic imprint on the structure of many freshwater and terrestrial species, which can be interpreted as a postglacial range expansion from one or multiple ice-free refugia in the south (Bernatchez & Wilson 1998; Taberlet *et al.* 1998). A rich literature on phylogeographical patterns of anadromous fish such as salmon and brown trout also

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points to the drastic impact of climate (e.g. García-Marín *et al.* 1999; Verspoor *et al.* 1999; Consuegra *et al.* 2002).

Phylogeographical patterns in genuine marine species have been studied to a lesser degree. Compared with the formidable barriers for dispersal posed to terrestrial and freshwater species, the marine environment is much more homogeneous. Hence, in general, marine fish show a low degree of genetic differentiation (see Ward *et al.* 1994; for a review). This makes it more difficult to detect historical and ongoing processes of population divergence. A number of studies have dealt with oceanic species for resolving biogeographical issues (Palumbi 1996; Lessios *et al.* 1999); other studies have targeted reef fishes (Planes & Fauvelot 2002; Rocha *et al.* 2002). While phylogeographical patterns of a number of fish and invertebrate species along the Pacific American coast have been elucidated, the evolutionary history of the northeastern Atlantic marine fauna remains less documented (but see Wilke & Davis 2000; Wilke & Pfenniger 2002). Exceptions are commercially exploited fish species such as mackerel (Nesbø *et al.* 1999), sea bass (García de León *et al.* 1997), flounder (Borsa *et al.* 1997) and cod (Nielsen *et al.* 2003). Yet, phylogeographical patterns are less easily resolved in these species because of the high levels of gene flow. Highly vagile fish species often show no mtDNA heterogeneity on a scale of hundreds of kilometres, which necessitates the use of highly variable markers. Because of their extremely high variability and mutation rate, these markers are often less appropriate for inferring historical processes. Thus, for elucidating phylogeographical patterns of the marine fauna along the European coasts we need to employ a model organism showing reduced levels of gene flow.

The common goby, *Pomatoschistus microps* (Krøyer 1838) (Gobiidae, Teleostei), is one of the most abundant estuarine and coastal fish species along the European coasts (Healey 1972). Its range of distribution extends along the Atlantic coast from central Norway (Trondheimsfjorden), around the British and Irish coasts to Morocco and the Baltic Sea (Miller 1986). In the Mediterranean Sea its occurrence is limited to lagoons and estuaries in the Gulf of Lion and Corsica (Bouchereau & Guelorget 1997). Adults are considered poor swimmers because the pelvic fins are fused in a suction disc (Miller 1986), suggesting that most large-scale dispersal is likely to depend on the pelagic larval stage, which lasts between 6 and 9 weeks (Jones & Miller 1966). Mediterranean *P. microps* complete their entire life cycle, including reproduction, in estuaries and lagoons (Pampoulie *et al.* 2000), whereas Atlantic common gobies perform spawning and thermal migrations (Jones & Miller 1966). Adult *P. microps* are rarely encountered offshore and their habitat is limited to very shallow tidal pools and estuaries. Thus, despite its pelagic larval phase, *P. microps* is probably limited in its dispersal due to ecological constraints, and might reveal a clear phylogeographical structure. Hence, in this study we use the common goby as a model for gaining a better knowledge of the evolutionary patterns of marine fish along the European coasts. More specifically we aim to test the following predictions:

1. The Pleistocene glaciations influenced not only freshwater and terrestrial organisms, but marine species were also heavily affected. Ice-covered areas to the north were unsuitable habitats, forcing a southward migration. Thus, in the northeastern Atlantic Ocean we expect the genetic imprint of a postglacial northward range expansion from a southern source population, probably located along the Iberian Peninsula. We predict a loss of genetic diversity in *P. microps* populations at higher latitudes (Hewitt 2000). However, the effect of natural selection might also be responsible for the loss of genetic variation commonly observed at higher latitudes (Mishmar *et al.* 2003). Hence, we employ two independent and complementary markers, allozyme electrophoresis and sequence analysis of the mtDNA.
2. Glacial refugia along the margins of the Scandinavian ice sheet in the English Channel or in the southern North Sea have been proposed for anadromous salmonids (García-Marín *et al.* 1999; Verspoor *et al.* 1999). The euryhaline *P. microps* is capable of surviving in brackish water. Thus, if a glacial refugium existed in the area, it is not unlikely that an isolated population of *P. microps* managed to survive in the area as well. Analysis of the geographical distribution of the various haplotypes and past gene flow events (Beerli & Felsenstein 2001) may shed more light on the existence and location of these putative refugia.
3. During Pleistocene decreases in sea level, the connection between the Atlantic Ocean and the Mediterranean Sea was either severed or severely restricted (Flores *et al.* 1997), leading to the long-lasting isolation of populations, which evolved in allopatry. Moreover, considering the differences in migratory behaviour between Atlantic and Mediterranean *P. microps*, we hypothesize that recurrent genetic exchange between them must be limited. Thus, Mediterranean populations of the common goby have probably been evolving in allopatry for a long time and will most likely show a significant differentiation from their Atlantic conspecifics.

Materials and methods

Samples were taken by fishing using a hand net in tidal pools at low tide, with the exception of the samples from the Schelde estuary (Doel), which were taken at the cooling water intake of the nuclear power plant. Sampling sites with the number of fish screened for allozyme electrophoresis and mtDNA are listed in Table 1. Fish were frozen in dry ice or liquid nitrogen immediately after capture and stored in a –80 °C freezer until analysis.

Table 1 Sampling sites of *Pomatoschistus microps* along the European coasts including the numbers of fish screened with allozyme electrophoresis (AE) and SSCP analysis of mtDNA, respectively

Sampling site	Geographic location	Latitude	Longitude	Code	Period	AE	mtDNA
Trondheim (Norway)	Norwegian Sea	63°24′ N	10°24′ E	Tro	Jun ′00	50	26
Oban (West Scotland)	Northeastern Atlantic Ocean	56°24′ N	5°28′ W	Ob	Aug ′99	40	14
Galway (West Ireland)	Northeastern Atlantic Ocean	53°16′ N	9°03′ W	Ga	Aug ′99	54	27
Texel (The Netherlands)	Southern North Sea	53°00′ N	4°46′ E	Tex	Nov ′01	61	25
Dale (South Wales)	Celtic Sea (Northeastern Atlantic Ocean)	51°42′ N	5°10′ W	Da	May ′97	—	22
Doel (Belgium)	Southern North Sea	51°19′ N	4°16′ E	Do	March ′98	57	16
Bray-Dunes (Northwest France)	Southern North Sea	51°04′ N	2°31′ E	Bdu	Sept ′01	—	26
Plymouth (Southwest England)	English Channel	50°22′ N	4°09′ W	Ply	Nov ′96	—	14
La Tremblade (West France)	Gulf of Biscay (Northeastern Atlantic Ocean)	45°46′ N	1°08′ W	LT	Oct ′00	53	32
Pérols (South France)	Gulf of Lion (Mediterranean Sea)	43°34′ N	3°57′ E	Per	Jan ′99	53	20
Vaccarès Lagoon (South France)	Gulf of Lion (Mediterranean Sea)	43°33′ N	4°31′ E	Vac	Aug ′00	—	20
Faro (South Portugal)	Northeastern Atlantic Ocean	37°04′ N	7°55′ W	Fa	Dec ′98	56	29

Allozyme electrophoresis

A total of 364 individuals of *Pomatoschistus microps* from eight sampling sites was genotyped using cellulose acetate allozyme electrophoresis (CAGE) (Richardson *et al.* 1986) at 13 putative allozyme loci: *AK**, *AAT**, *CK-A**, *LDH-A**, *LDH-B**, *LDH-C**, *MDH-A*, *MDH-B**, *GPI-A**, *GPI-B**, *PGM-1**, *PGM-2** and *FH**. Tris–maleate (pH = 7.8) and Tris–glycine (pH = 8.8) buffer systems were used as described by Hebert & Beaton (1989). Loci were stained using methods given by Hebert & Beaton (1989) and Richardson *et al.* (1986). The slowest migrating locus was designated 1 or A according to the nomenclature of Shaklee *et al.* (1990). The most common allele was called ′100′ and other alleles were classified according to their mobility relative to allele 100 for the locus under study.

Sequence and single-strand conformation polymorphism analysis of mtDNA

A total of 270 *P. microps* collected at 12 sites was screened for a 289 bp fragment from the cytochrome *b* locus in the mtDNA (Table 1). A fin clip was taken from each fish specimen and stored in 100% ethanol. DNA extraction was carried out with the DNEasy tissue kit (Qiagen International). Specific primers (Kocher *et al.* 1989) yielded a 300 bp fragment of the cytochrome *b* locus. Polymerase chain reaction (PCR) conditions were as follows: denaturation at 95 °C for 3 min, annealing at 56 °C for 45 s, elongation at 72 °C for 45 s with a total of 35 cycli and a final elongation at 72 °C for 7 min. The $MgCl_2$ concentration was 2 mM. Visible bands were cut out under UV, purified with the ′GFX PCR DNA and Gel Band Purification′ kit (Amersham Biosciences), eluted in 25 μL double-distilled water and cloned into the pCR 2.1-TOPO clining vector (TA Cloning kit, Invitorgen) vector. A second PCR was carried out on the PCR products and both strands were sequenced with standard M13 primers using the ′SequiTherm Excell II′ kit (Epicentres Technologies). The reaction yielded a 289 bp fragment, which was analysed on a LI-COR 4200 automated sequencer (LI-COR GeneReadIR DNA system) with the ALIGNIR software (LI-COR). A search in BLAST confirmed that the right fragment had been amplified. Based on the sequences thus obtained from four different specimens of *P. microps* specific internal primers were designed for screening populations using single-strand conformation polymorphism (SSCP) analysis (Orita *et al.* 1989). Primer sequences for the SSCP are: Pmic-cyt*b*F 5′-ATTTTGGCT-CCTTACTAGGC-3′ and Pmic-cyt*b*R 5′-TCAGGGAAGAA-CGTAGCC-3′. Conditions for the PCR were: denaturation at 97 °C for 3 min, denaturation at 95 °C for 45 s, annealing at 54 °C for 45 s, elongation at 72 °C for 45 s and final

elongation at 72 °C for 7 min with a total of 35 cycles at a concentration of 2 mM $MgCl_2$. Pre-made gels were employed (Excel gel DNA analysis kit of Amersham Biosciences, cat. no. 17-1198-07). In the SSCP analysis each individual was run several times under identical conditions. A few individuals from each mobility group (depending on the SSCP mobility class frequency) were randomly chosen and their nucleotide sequence was determined on an automated DNA sequencer. The SSCP was carried out at a temperature of 4 °C with a run time of 2 h at 600 V. Bands were visualized using the DNA silver staining kit of Amersham Biosciences (cat. no. 17-6000-30). The various haplotypes, which were visualized by the SSCP analysis, were subsequently sequenced. PCR conditions for the sequencing reactions were initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s, annealing at 54 °C for 15 s, elongation at 70 °C for 1 min with a total of 35 cycles, and final elongation at 70 °C for 7 min with a $MgCl_2$ concentration of 2 mM.

Data analysis

Genetic diversity was measured as the level of polymorphism, observed and expected heterozygosity and haplotype diversity. Allele frequencies and the observed and unbiased expected heterozygosity were calculated in GENETIX v. 4.02 (Belkhir *et al.* 2001). Linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) were tested for significance in GENEPOP v. 3.1 (Raymond & Rousset 1995). Single-locus *F*-statistics were calculated from allele frequencies of all loci examined for each population according to Weir & Cockerham (1984) in GENETIX. Standard deviations of single-locus F_{ST} values were obtained by jackknifing over all populations according to Weir (1990) and the significance of the multilocus F_{ST} was assessed with permutation tests (1000 replicates).

Sequences were aligned in CLUSTAL W v. 1.7 (Thompson *et al.* 1994). Genetic diversity, measured as haplotype diversity (h) and nucleotide diversity (π) (Nei 1987) was computed in DNASP v. 3.51 (Rozas & Rozas 1999). A linear regression between $\log(x + 1)$ transformed data on the heterozygosity, respectively, haplotype diversity against latitude was computed in STATISTICA v. 6.0 (Statsoft Inc. 2001). Because visual inspection of the sequences revealed a strongly unequal distribution of mutation sites throughout the cytochrome *b* fragment, the program TREE-PUZZLE (Strimmer & von Haeseler 1996) was used to calculate the parameter α of the gamma distribution. Pair-wise genetic distances were computed in ARLEQUIN v. 2.0 (Schneider *et al.* 2000) according to the model of Tamura & Nei (1993), which takes into account unequal nucleotide frequencies and unequal mutation rates within the fragment. Significance was assessed with permutation tests (1000 replicates). The distribution of pair-wise differences between haplotypes (mismatch distribution) (Rogers & Harpending 1992) with its parameters τ, θ_0 and θ_1 and Tajima's D-statistic (Tajima 1989a) were also computed in ARLEQUIN and tested for significance with permutation tests (1000 replicates). To assess whether any indications for group structure could be observed, we performed a multidimensional scaling analysis (MDSA) on the pair-wise Tamura–Nei genetic distances in STATISTICA. Subsequently, samples were grouped and subjected to a hierarchical analysis of variance (AMOVA) (Excoffier *et al.* 1992) in ARLEQUIN in order to ascertain whether the group structure as observed in the MDSA was significant. Global Φ_{ST} value across all samples based on sequences was also computed in ARLEQUIN. A minimum spanning network was calculated in ARLEQUIN to assess whether any structuring of haplotypes could be recorded. To assess directions of past gene flow between populations we employed a coalescence-based method using the program MIGRATE v. 1.5 (Beerli 2002). This program calculates maximum likelihood estimates for migration rates and effective population sizes based on coalescence theory, allowing for asymmetric migration rates and different subpopulation sizes (Beerli & Felsenstein 2001). Parameter values were 10 short chains with 500 steps and 10 000 sampled genealogies, and three long chains with 5000 steps and 100 000 sampled genealogies.

Results

Genetic diversity analysis

Allele frequencies at the various loci are listed in the Appendix. Table 2 presents results on observed and expected heterozygosity, mean number of alleles per locus and the proportion of polymorphic loci. Loci *AAT**, *GPI-B**, *PGM-1** and *PGM-2** are polymorphic in all samples. Loci *AK**, *CK-A**, *LDH-C** and *FH** are monomorphic for the same allele in all samples. The lowest and highest levels of heterozygosity and average number of alleles per locus are found in the samples at the northern- and southernmost sampling sites (respectively Trondheim and Faro), with the sample from Faro showing a level of heterozygosity an order of magnitude higher than in Trondheim. The samples from Doel, Texel, Galway and Oban show similar values for the average number of alleles per locus. No significant departures from Hardy–Weinberg proportions were observed with the exception of locus *PGM-2** in the sample from La Tremblade ($P < 0.001$) and locus *MDH-B** ($P < 0.001$) in Galway. *MDH-B** is fixed for the same allele in all samples except for Galway, where three individuals were found to be heterozygous for two alleles other than the common one. No significant linkage disequilibrium was observed with the exception of loci *GPI-A** and **AAT* in the sample from Faro ($P = 0.006$).

Thirty-eight mtDNA haplotypes were detected among all samples. Sequences of the various haplotypes have been deposited at the EMBL sequence database and Accession

Table 2 Observed (H_O) and unbiased (H_E) expected heterozygosity (Nei 1987) with SD, mean number of alleles per locus (MNA), number of mtDNA haplotypes (N_h), number of unique haplotypes (N_h unique), haplotype (h) and nuceotide (π) diversity with SD and percentage of occurrence of the most common haplotype (% MCH) of *Pomatoschistus microps*. For sampling site abbreviations see Table 1

Site	H_E	H_O	MNA	h	N_h	N_h unique		% MCH
Tro	0.075 (0.152)	0.066 (0.138)	1.231	0	1	0	0	H14-100%
Ob	0.106 (0.185)	0.097 (0.169)	1.385	0.143 (0.119)	2	1	0.0010 (0.0008)	H4-93%
Ga	0.108 (0.161)	0.117 (0.183)	1.692	0.484 (0.061)	3	2	0.0017 (0.0004)	H4-70%
Tex	0.121 (0.200)	0.114 (0.186)	1.615	0.357 (0.115)	4	2	0.0022 (0.0011)	H4-80%
Da	—	—	—	0.177 (0.106)	3	2	0.0012 (0.0007)	H1-91%
Do	0.124 (0.201)	0.125 (0.207)	1.539	0.608 (0.130)	5	1	0.0060 (0.0015)	H4-63%
Bdu	—	—	—	0.557 (0.109)	5	2	0.0059 (0.0016)	H4-65%
Ply	—	—	—	0.791 (0.089)	6	3	0.0081 (0.0015)	H1-40%
LT	0.120 (0.195)	0.098 (0.156)	0.308	0.633 (0.090)	7	3	0.0048 (0.0008)	H1-58%
Per	0.081 (0.157)	0.076 (0.155)	1.539	0.653 (0.076)	5	2	0.0032 (0.0007)	H14-50%
Vac	—	—	—	0.606 (0.062)	4	0	0.0028 (0.0007)	H14-50%
Fa	0.139 (0.203)	0.135 (0.193)	1.769	0.857 (0.062)	16	13	0.0081 (0.0013)	H6-38%

numbers of the various haplotypes are presented in Table 3. All differences between haplotypes were due to substitutions (transition/transversion ratio = 6.03) and no indels were recorded. Compared with allozymes, a similar geographical difference in haplotype diversity is observed, with the Faro sample showing the highest level of haplotype diversity and number of haplotypes (h = 0.857, N_h = 16), while in the sample from Trondheim a single haplotype was recorded (h = 0, N_h = 1). The sample from Faro is not only the most diverse, it also has a very high number of unique haplotypes (13 of 16 among 29 fish) (Table 2).

Genetic diversity and latitude

Allozyme data show a negative, although not significant, correlation between latitude and level of heterozygosity ($R^2 = 0.231$, $P = 0.26$). An outlier in the regression is the sample from Pérols, revealing a much lower degree of heterozygosity than expected for its latitude. When only the Atlantic samples are taken into account, the regression between heterozygosity and latitude approaches significance ($R^2 = 0.56$, $P = 0.052$) (Fig. 1a). No latitudinal trend in allele frequencies was observed at any locus.

The mtDNA data reveal a similar negative correlation between haplotype diversity and latitude, but in this case the regression is significant ($R^2 = 0.69$, $P = 0.0008$) with a haplotype diversity ranging from 0 (only 1 haplotype in the sample from Trondheim) to 0.857 (16 haplotypes in the Portuguese sample) (Fig. 1b).

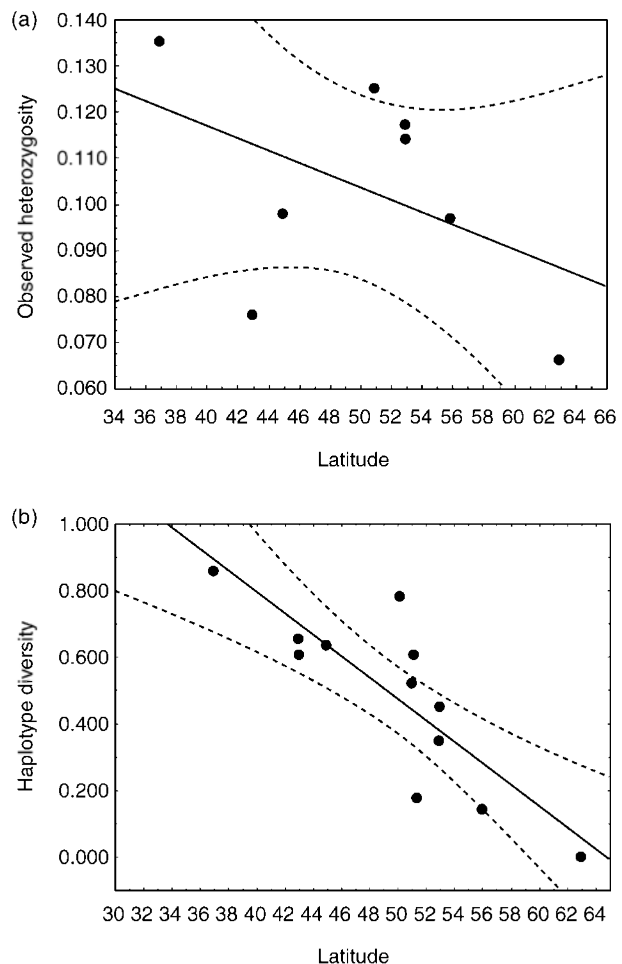

Fig. 1 Linear regression of (a) heterozygosity based on alloymes ($R^2 = 0.231$, $P = 0.26$, NS) and (b) haplotype diversity based on a 289 bp fragment of the mtDNA cytochrome *b* ($R^2 = 0.69$, $P = 0.0008$) against latitude for all samples of *Pomatoschistus microps*.

Genetic structure

The multilocus F_{ST} and Φ_{ST} values across all samples amount to 0.247 for allozymes and to 0.437 for the mtDNA data, respectively. Permutation tests on single-locus F_{ST}

Table 3 *Pomatoschistus microps*: distribution of haplotypes per sampling area. 'H' refers to the haplotype. AN: EMBL Accession nos of the haplotypes. For sample abbreviations see Table 1

AN	H	Tro	Ob	Ga	Tex	Da	Do	Bdu	Ply	LT	Per	Vac	Fa
AJ550471	H1				1	20	2	4	6	19			1
AJ550472	H2						1						
AJ550473	H3						1		1	4			1
AJ550481	H4		13	19	20		10	17	2				
AJ550482	H5		1										
AJ550507	H6					1		2		2			11
AJ550480	H7												1
AJ550479	H8												1
AJ550486	H9												1
AJ550504	H10												1
AJ550495	H11												2
AJ550476	H12										7	8	
AJ550475	H13										1	1	
AJ550477	H14	27					2				10	10	
AJ550478	H15										1	1	
AJ550487	H16			1									
AJ550491	H17			7									
AJ550485	H18								1				
AJ550501	H19								3				
AJ550502	H20									3			
AJ550493	H21									1			
AJ550499	H22									2			
AJ550489	H23								1				
AJ550490	H24				1								
AJ550488	H25												1
AJ550494	H26												1
AJ550484	H27							2					
AJ550474	H28										1		
AJ550506	H29												2
AJ550492	H30							1					
AJ550500	H31												1
AJ550505	H32												2
AJ550503	H33									1			
AJ550498	H34												1
AJ550508	H35												1
AJ550497	H36												1
AJ550483	H37				3								
AJ550496	H38					1							
Total		27	14	27	25	22	16	26	14	32	20	20	29

values showed that samples are significantly differentiated at four out of five polymorphic loci: *PGM-2**, *GPI-A**, *GPI-B** and *AAT** (Appendix). The highest degree of differentiation is found at locus *AAT**. Pair-wise F_{ST} values were significant for all samples except between Texel and Doel. All pair-wise mtDNA distances were highly significant, with the exception of Pérols–Vaccarès, Doel–Bray-Dunes, Texel–Oban and Oban–Galway.

Although less samples were analysed for allozymes than for mtDNA, the MDSA clearly revealed a similar group structure for both markers (Fig. 2a,b). The samples from the southern North Sea (Texel, Doel and Bray-Dunes) grouped together with those from western Scotland and Ireland (Oban and Galway). A second group comprised the English Channel, the Celtic Sea, the Gulf of Biscay and southern Portugal (Dale, Plymouth, La Tremblade and Faro). The third group consisted of the western Mediterranean Sea (Gulf of Lion: Pérols and Vaccarès), including also the sample from Trondheim (Fig. 2a), which is attributed to the high frequency of occurrence of haplotype H14 in Mediterranean and Norwegian common gobies (ranging from 50 to 100%) (Table 2). The grouping of the western Mediterranean Sea and Trondheim for the allozymes (Fig. 2b) results from similarities in frequencies at loci *GPI-A** and *GPI-B**; both

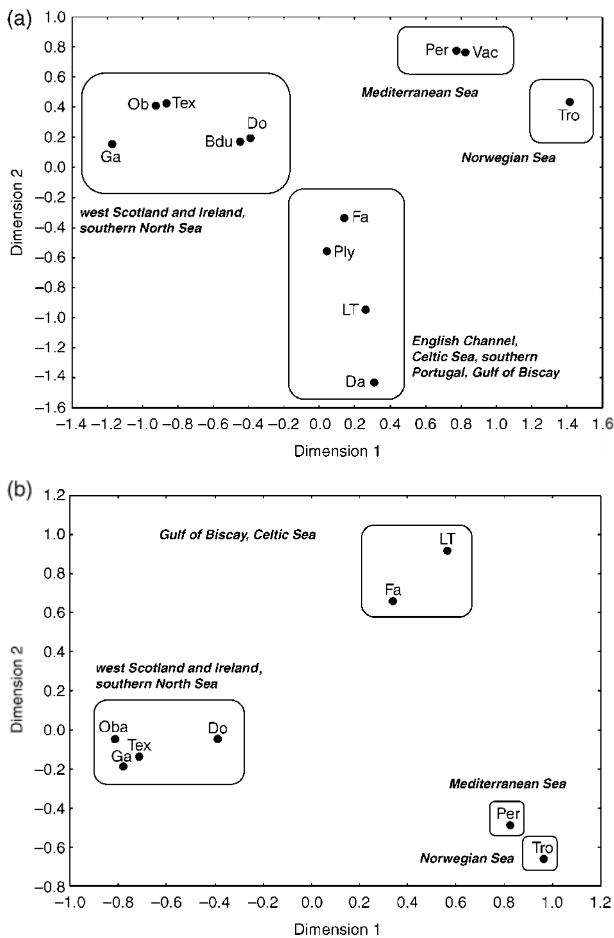

Fig. 2 *Pomatoschistus microps*: MDSA based on (a) the matrix of pair-wise Tamura–Nei genetic distances of cytochrome *b* samples (Stress value = 0.0002) and (b) pair-wise F_{ST} values of allozyme samples (Stress value = 0.0000). For sample abbreviations, see Table 1.

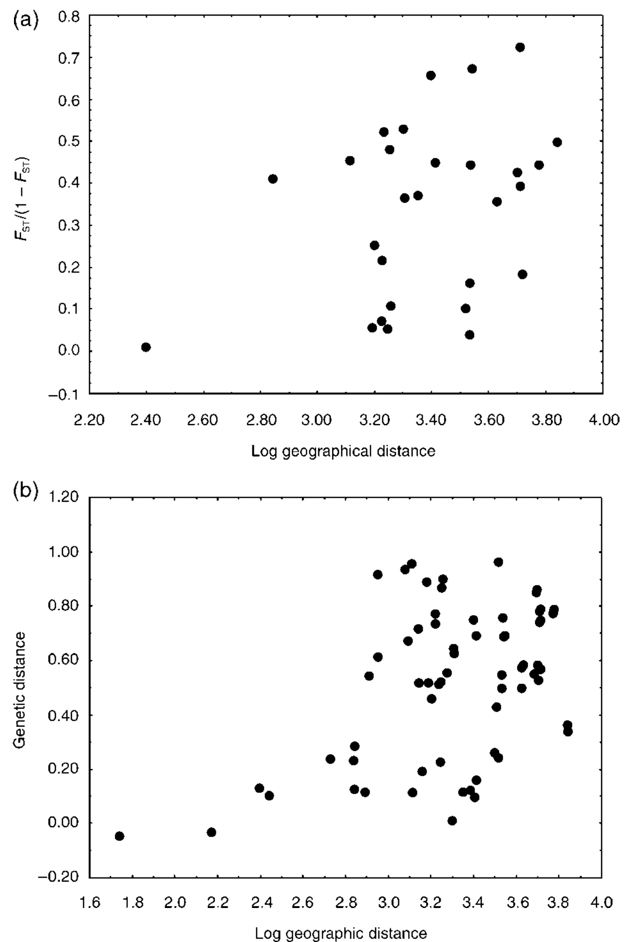

Fig. 3 Allozyme-based pair-wise F_{ST} values (a) and pair-wise genetic distances based on mtDNA sequences (b) vs. log-transformed coastal distance for all samples of *Pomatoschistus microps*.

samples are fixed for *GPI-A*100* and have higher frequencies of at *GPI-B*90* than the other samples (Appendix).

The mtDNA samples were grouped according to the structure observed in the MDSA to perform a hierarchical analysis of variance (AMOVA) between two groups: Atlantic vs. Mediterranean. The largest amount of variation was due to differences between populations within groups (40%) but 27% was explained by the differences between Atlantic and Mediterranean samples. All values were highly significant. An AMOVA was also performed on Atlantic samples only, to assess the significance of the differences between the groups observed in the MDSA. The sample from Trondheim was excluded from this hierarchical analysis because of its large differentiation from all other Atlantic samples. In total, 54.3% of the variation is due to the differences between the two geographical groups (respectively the H1- and H4-group), whereas only 8.38% is explained by differences between populations within the groups.

Isolation-by-distance

A plot of (i) allozyme-based pair-wise F_{ST} and (ii) Tamura–Nei genetic distances (mtDNA) vs. the geographical distance shows a clear pattern of isolation-by-distance (Fig. 3a,b). However, a Mantel test performed on pair-wise $F_{ST}/(1 - F_{ST})$ for all allozyme samples was not significant ($r = 0.32$, $P = 0.081$). However, a Mantel test based on the pair-wise mtDNA distances was significant ($r = 0.33$, $P = 0.037$).

Geographic distribution of the haplotypes

Pooling of all samples revealed three common haplotypes (H1, 19.5%; H4, 29.9% and H14, 18.0% frequency occurrence in the total sample size), and accompanied by a fairly high number of rare to very rare variants (Table 3). Only one haplotype (H14) is shared between the Mediterranean and Atlantic samples. Surprisingly, this is also the only

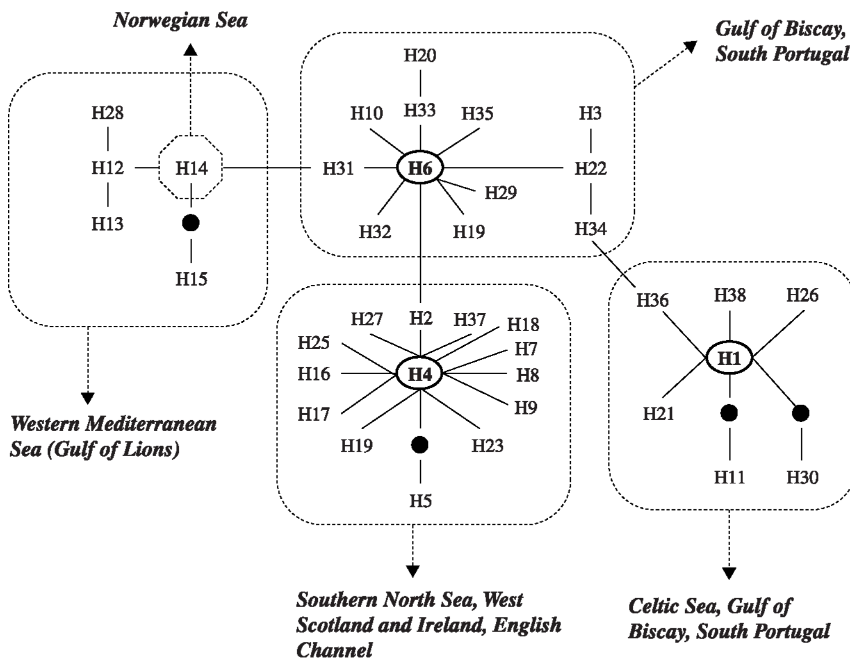

Fig. 4 Minimum Spanning network for the *Pomatoschistus microps* haplotypes with the geographical location where the haplotypes of the various clusters mainly occur. Filled circle represents a missing haplotype in the network. Each branch represents a single mutation between haplotypes.

haplotype occurring in the Trondheim sample. Only two other individuals carrying H14 were found in the Atlantic basin (Doel). Haplotype H4 seems to be restricted to the samples from the English Channel and the southern North Sea (Plymouth, Bray-Dunes, Doel and Texel) and the northwestern British coasts (Galway and Oban); it was not recorded either in Faro or La Tremblade. Haplotype H1 is the most common haplotype in La Tremblade and Dale. It also occurs in Texel, Doel and Plymouth, albeit at a low frequency (Table 3).

Phylogeographical analysis

Because of the small distances between the *Pomatoschistus microps* haplotypes, the relationships between haplotypes are better visualized with a network than with a phylogenetic tree (Posada & Crandall 2001). A Minimum Spanning Tree revealed four geographically separate clusters (Fig. 4), each with a common haplotype in the centre and a number of rare variants radiating. One cluster, containing only four haplotypes, comprises the Mediterranean and Trondheim haplotypes. It is connected with the Atlantic group through haplotype H14. Within the Atlantic, three clusters are recorded with, respectively, H1, H4 and H6 as the central common haplotype. We notice that the haplotypes radiating from these central haplotypes are also confined to the same geographical region.

Tests of the goodness-of-fit revealed that the model of a sudden population expansion for the pair-wise distribution of the *P. microps* haplotypes could not be rejected. The value for τ amounted to 3.10, with $\theta_0 = 0.133$ and $\theta_1 = 566.95$. Assuming a mtDNA mutation rate of 2%/Ma, this corresponds to a time of expansion of 536 000 years BP, employing the formula $\tau = \mu T$, with μ representing the mutation rate and T the number of generations since time of expansion. Tajima's D-statistic amounted to –1.40, approaching significance ($P = 0.054$), further supporting the model of a sudden population expansion after a bottleneck (Tajima 1989b).

Gene flow analysis

Maximum likelihood gene flow analysis on the Atlantic samples (excluding Trondheim because of its isolation) showed (i) a northward gene flow from the Faro population as far north as the Schelde estuary, (ii) a northward gene flow through the Southern Bight, and (iii) a northward gene flow from the Southern Bight towards Scotland and Ireland (Fig. 5).

Discussion

Most striking in the general results is the congruence between the mitochondrial and allozyme genotypes. There is (i) a significant degree of population differentiation in *Pomatoschistus microps* throughout its distributional range, (ii) differentiation between northern and southern *P. microps* populations in the northeastern Atlantic basin, (iii) a strong correlation between the degree of genetic variation and latitude, and (iv) a pattern of isolation-by-distance. The mtDNA data yield a finer resolution, showing (i) the different histories for Atlantic and Mediterranean *P. microps*

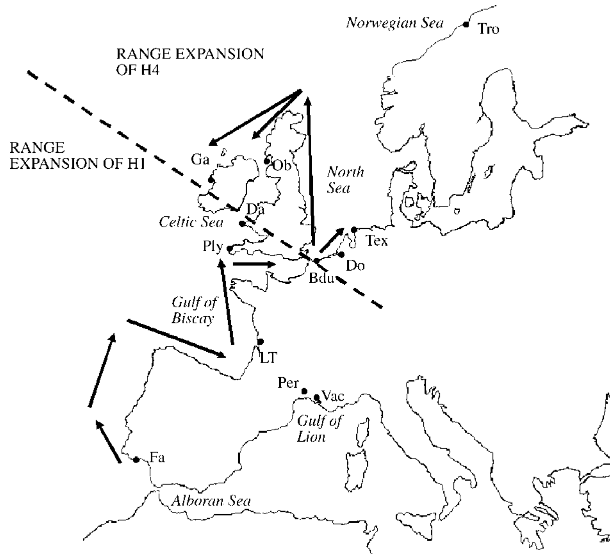

Fig. 5 *Pomatoschistus microps*: past migration events as inferred from the maximum likelihood gene flow analysis. For sample abbreviations we refer to Table 1.

in the Minimum Spanning Tree, and (ii) the striking genetic discontinuities around the British Isles.

Loss of variation during postglacial range expansion or natural selection?

The common goby shows reduced diversity with latitude, which represents a pattern typical for terrestrial and freshwater populations inhabiting formerly glaciated areas (Merilä *et al.* 1996; Bernatchez & Wilson 1998; Hewitt 2000). Such a correlation is usually attributed to historical factors due to founder events (the loss of alleles) during range expansion following deglaciation (Hewitt 2000). By contrast, selection might play a role as well in maintaining latitudinal differences in the levels of genetic diversity (Mishmar *et al.* 2003). Given the unambiguous congruence of two independent markers in the common goby, we favour the hypothesis that neutral variation provides the main explanation and we suggest that genetic variation was lost during the postglacial expansion of *P. microps* into formerly glaciated areas.

Phylogeographical patterns in the western Mediterranean Sea

The origin of the Atlantic–Mediterranean sand gobies, to which the common goby belongs, lies within the Mediterranean Sea, where the oldest fossil remains have been found (Simonovic 1999). Moreover, the most closely related species to *P. microps* is *P. marmoratus*, which is endemic in the Mediterranean Sea (Wallis & Beardmore 1984; Huyse 2002). Thus, the origin of *P. microps* is likely to be found in the Mediterranean Sea. This implies that haplotype H14 might be an ancestral haplotype that dispersed into the Atlantic and gave rise to the Atlantic haplotypes. The *P. microps* that stayed behind diverged into the endemic Mediterranean haplotypes (H12, H13, H15, H28).

Haplotype H14 also occurs in a high frequency at the northernmost edge of *P. microps'* distributional range, while it is almost absent elsewhere. A possible explanation could be that its presence in Trondheim is due to migration with ballast water of ships, as has been suggested for the presence of Black Sea gobies in the Great Lakes of the US (Dougherty *et al.* 1995). Although this possibility cannot be excluded for certain, we do not believe that this is the case for the common goby. First, no shipping activity is present in the lagoons of Pérols or Vaccarès where our samples were taken. The species lives sedentary in these lagoons all year round. Second, as haplotypes H12 and H14 occur in equal frequencies in the Mediterranean Sea, we should record at least some H12 in the Trondheim sample as well, which is not the case. To explain the current distribution of H14 we propose two hypotheses. One possibility is that H14 used to have a much broader distribution, but was displaced by a more recent range expansion of H1 and H4. Such a scenario was proposed to explain phylogeographical patterns in *Hydrobia* sp. (Wilke & Pfenniger 2002), where two subspecies are geographically separated by another species, due to a range shift or expansion of the latter. Alternatively, individuals with haplotype H14 may correspond to long-distance dispersers in the northeastern Atlantic Ocean, as described by Ibrahim *et al.* (1996). Long-distance dispersers may have expanded rapidly and filled up available niches before others, leading to edge populations with reduced variability (Hewitt 2000). The edge population of *P. microps* in the Norwegian Sea has indeed a lower variability compared with all the others.

The northeastern Atlantic Ocean: a postglacial range expansion in two phases?

Evidence for recolonization events from multiple refugia. That the highest level of diversity was found in the southernmost population from Faro may reflect the older age of this population. It is concordant with the idea of a northward range expansion of *P. microps* from a refugium along the Atlantic coast of the Iberian peninsula as proposed for salmon (Consuegra *et al.* 2002) and brown trout (García-Marín *et al.* 1999). Because the common goby needs shallow water to breed, the ice-covered coasts of northwestern Europe proved unsuitable during glaciation. Although the current distribution of *P. microps* must be the result of its northward range expansion after the last ice age, which ended ~10 000 years BP, the mismatch analysis indicates

that the origin of the Atlantic haplotypes has to be considerably more ancient. With an estimate of 536 000 years BP, this might correspond with the onset of an earlier warm interglacial period during the Cromerian complex (Prokopenko *et al.* 2002). *P. microps* must have experienced similar range contractions and expansions during earlier glaciations and interglacials.

A glacial refugium in the southern North Sea? A similar grouping structure is indicated by both markers as revealed by the MDSA, clearly dividing the Atlantic samples in a northern (H4) and a southern (H1) group. This points to similar underlying mechanisms, suggesting historical factors rather than environmental effects. The gene flow analysis indicates the southern Portuguese coast as the source of the northward range expansion of H1, while the Gulf of Biscay may be the origin of the *P. microps* that have migrated to southwestern Britain. The Southern Bight of the North Sea is suggested as the origin of the *P. microps* that colonized Scotland and Ireland (H4). This implies that a population of H4-*P. microps* must have survived in a glacial refugium in the southern North Sea. A similar scenario has been suggested for its relative, the sand goby, *P. minutus* (Gysels 2003). García-Marín *et al.* (1999) proposed that the drainage systems of the major rivers (Gibbard 1988) acted as a glacial refugium for brown trout. Verspoor *et al.* (1999) suggested a glacial lake in the southern North Sea as a refugium for Atlantic salmon. The distribution of the *P. microps* haplotypes points to a refugium in the southern North Sea rather than in the English Channel. Hence, the English Channel and the southern North Sea probably consist of a zone of secondary contact between H1 and H4-dominated *P. microps*. The fact that a unique haplotype, H17, was found in the Irish sample in an appreciable frequency (29%) suggests that this population may also have been isolated from all the others for some time. Hynes *et al.* (1996) recorded distinct mtDNA haplotypes in Irish brown trout and suggested a complex recolonization process from multiple refugia in this area.

Population genetic differentiation and implications for recurrent levels of gene flow

The western Mediterranean Sea has been is connected to the Atlantic Ocean through the Strait of Gibraltar since the end of the Pliocene (Blanc 2002) with a net surface inflow of Atlantic water, while Mediterranean water flows out at depth. The real encounter between these two bodies of water takes place in the most western part of the Mediterranean Sea, the Alboran Sea, resulting in a frontal zone, the Almería-Oran Oceanic Front (AOOF) (Tintore *et al.* 1988). This front seems to pose a barrier to Atlantic–Mediterranean dispersal in many species (Naciri *et al.* 1999; Zane *et al.* 2000). Apparently, recurrent gene flow is also limited or even absent between Atlantic and Mediterranean *P. microps*: the differentiation between the common gobies from the Portuguese coast (Faro) and the western Mediterranean Sea is striking, considering that these geo-graphically close populations did not share a single haplotype. Allozyme allele frequencies also differ markedly between these two samples. Alternatively, lack of contemporary gene flow between Atlantic and Mediterranean *P. microps* may also be explained by the fact that Mediterranean *P. microps* complete their entire life cycle in the estuarine environment, in contrast to Atlantic *P. microps*, which carry out spawning and thermal migrations (reviewed in Bouchereau & Guelorget 1997). Thus, even if Atlantic *P. microps* manage to cross the AOOF, the probability of genetic exchange with resident Mediterranean populations is probably small.

Along the northeastern Atlantic coasts a pattern emerges of restricted gene flow and isolation-by-distance. Gene flow between the common gobies from Galway and Oban may be maintained by the North Atlantic Drift, which flows northwest along the Irish and Scottish coasts. Genetic exchange between the North Sea gobies and those from western Scotland and Ireland may be enhanced by the branching of the North Atlantic Drift into the North Sea (Turrell 1992). The lack of differentiation between the Gulf of Biscay and the Celtic Sea is congruent with the results for European hake (Lundy *et al.* 1999) and Dover sole (Exadactylos *et al.* 1998). The Shelf Edge Current, flowing from northwestern Africa to Norway along the edge of the Continental Shelf, provides a means for the dispersal of planktonic fish larvae between the Bay of Biscay and the western coasts of the British Isles (Bartsch & Coombs 1997). A northward residual current flows through the English Channel into the Southern Bight of the North Sea (Prandle *et al.* 1996), which might explain the limited amount of differentiation between the samples from the English Channel and the Southern Bight.

Conclusions

Pomatoschistus microps is significantly differentiated throughout its distributional range with the highest degree of differentiation found between Atlantic and Mediterranean populations. This may point to a barrier for dispersal across the AAOF. Alternatively, differences in migratory behaviour between Atlantic and Mediterranean gobies might restrict genetic exchange. The lack of genetic differentiation between samples from the North Sea, the English Channel and the northwestern coasts of Scotland and Ireland is consistent with larval dispersal via oceanic currents along the coasts of continental Europe and southern England, as recorded for other species along the European Atlantic coasts.

The congruence of allozymes and mtDNA points to the importance of historical factors for explaining recurrent patterns of population structure. The negative correlation between genetic diversity and latitude suggests a loss of diversity during postglacial range expansion due to a founder event, while the star-like phylogeny of the Atlantic haplotype clusters points to a population expansion following upon a contraction. Phylogeographical patterns in this species are complex but suggest that the source population, from where the species expanded its range northward, inhabited the Atlantic coast of the Iberian Peninsula. The North Sea and the coasts of western Scotland and Ireland were probably recolonized by a population surviving in a glacial refugium in the southern North Sea. Hence, phylogeographical structure represents an inherent component of the evolution of marine fish populations.

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This study forms part of the PhD of Els Gysels, whose interests include the population genetics and phylogeography of marine gobies. Bart Hellemans is specialized in molecular genetic techniques on marine and freshwater fish. Christophe Pampoulie is a goby ecologist whose main research interests are the relationships among genotypes, phenotypes and the environment. Filip Volckaert's research comprises the population genetics and evolutionary biology of marine and freshwater fishes and their parasites.

Appendix

Number of individuals (*N*), allele frequencies, observed and nonbiased expected heterozygosity (H_O and H_E) with SD of *Pomatoschistus microps*. MNA: mean number of alleles; *P*: proportion of polymorphic loci (0.99 level). F_{ST} values significant at the 0.05 level are indicated with an asterisk. For samplings site abbreviations we refer to Table 1

		Samples								
Allele	Locus	Tro	Ob	Ga	Tex	Do	LT	Per	Fa	F_{ST}
*AAT**	*90	0.000	0.000	0.000	0.000	0.000	0.012	0.010	0.020	0.374*
	*100	0.670	0.136	0.878	0.655	0.673	0.952	0.186	0.878	
	*150	0.330	0.864	0.132	0.345	0.327	0.036	0.804	0.122	
N		47	33	53	58	26	42	51	49	
H_E		0.447	0.239	0.231	0.456	0.449	0.093	0.322	0.253	
H_O		0.404	0.212	0.264	0.414	0.500	0.095	0.275	0.286	
*GPI-A**	*90	0.000	0.419	0.213	0.066	0.047	0.189	0.000	0.269	0.142*
	*100	1.000	0.581	0.787	0.934	0.953	0.802	1.000	0.694	
	*113	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.037	
N		49	37	54	61	53	53	52	54	
H_E		0.000	0.494	0.338	0.124	0.091	0.324	0.000	0.448	
H_O		0.000	0.351	0.426	0.131	0.094	0.321	0.000	0.426	
*GPI-B**	*65	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.009	0.148*
	*90	0.776	0.514	0.546	0.418	0.361	0.406	0.942	0.510	
	*100	0.224	0.486	0.454	0.574	0.639	0.566	0.058	0.462	
	*116	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.019	
N		49	36	54	61	54	53	52	52	
H_E		0.352	0.507	0.500	0.500	0.466	0.519	0.110	0.532	
H_O		0.327	0.528	0.537	0.443	0.574	0.491	0.077	0.500	
*LDH-A**	*65	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.012
	*100	1.000	1.000	1.000	1.000	0.979	1.000	1.000	1.000	
N		50	37	53	61	47	52	51	53	
H_E		0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	
H_O		0.000	0.000	0.000	0.000	0.043	0.000	0.000	0.000	
*LDH-B**	*100	1.000	1.000	1.000	1.000	0.989	1.000	1.000	1.000	0.001
	*105	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	
N		50	37	53	61	46	52	51	53	
H_E		0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	
H_O		0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	
*MDH-A**	*80	0.000	0.000	0.000	0.008	0.000	0.000	0.009	0.000	−0.003
	*100	1.000	1.000	1.000	0.992	1.000	0.990	0.991	1.000	
	*150	0.000	0.000	0.000	0.010	0.000	0.010	0.000	0.000	
N		49	40	52	61	57	52	53	56	
H_E		0.000	0.000	0.000	0.016	0.000	0.019	0.019	0.000	
H_O		0.000	0.000	0.000	0.016	0.000	0.019	0.019	0.000	
*MDH-B**	*75	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.030
	*100	1.000	1.000	0.942	1.000	1.000	1.000	1.000	1.000	
	*115	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	
N		49	40	52	61	57	56	52	53	
H_E		0.000	0.000	0.112	0.000	0.000	0.000	0.000	0.000	
H_O		0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	
*PGM-1**	*86	0.104	0.000	0.070	0.000	0.020	0.046	0.023	0.009	0.016
	*100	0.896	0.975	0.910	0.984	0.980	0.918	0.942	0.953	
	*106	0.000	0.000	0.000	0.008	0.000	0.000	0.035	0.000	
	*114	0.000	0.025	0.020	0.008	0.000	0.036	0.000	0.038	
N		48	40	50	61	51	55	43	53	
H_E		0.189	0.049	0.168	0.033	0.039	0.155	0.112	0.092	
H_O		0.125	0.050	0.180	0.033	0.039	0.164	0.116	0.094	

Appendix *Continued*

Allele	Locus	Samples								
		Tro	Ob	Ga	Tex	Do	LT	Per	Fa	F_{ST}
*PGM-2**	*90	0.000	0.000	0.000	0.027	0.000	0.000	0.000	0.000	0.357*
	*95	0.000	0.058	0.019	0.696	0.536	0.286	0.571	0.500	
	*100	1.000	0.942	0.972	0.277	0.464	0.714	0.429	0.500	
	*120	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	
N		47	26	53	56	28	42	35	46	
H_E		0.000	0.111	0.056	0.442	0.507	0.413	0.497	0.506	
H_O		0.000	0.115	0.057	0.446	0.357	0.381	0.229	0.522	
MULTI-LOCUS										0.247*
P		0.188	0.375	0.375	0.313	0.588	0.438	0.333	0.250	
H_E		0.062	0.150	0.088	0.098	0.148	0.150	0.104	0.066	
		(0.141)	(0.284)	(0.150)	(0.185)	(0.209)	(0.194)	(0.185)	(0.144)	
H_O		0.054	0.141	0.095	0.093	0.128	0.140	0.085	0.062	
		(0.127)	(0.279)	(0.172)	(0.174)	(0.190)	(0.185)	(0.153)	(0.143)	
MNA		1.19	1.38	1.56	1.56	1.59	1.81	1.67	1.44	

The following loci are fixed for the same allele: *AK**, *CK-A**, *FH**, *LDH-C**.