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Mitochondrial DNA differentiation between the antitropical blue whiting species *Micromesistius poutassou* and *Micromesistius australis*

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This study investigated the biogeography and genetic variation in the antitropically distributed *Micromesistius* genus. A 579 bp fragment of the mitochondrial *coI* gene was analysed in 279 individuals of *Micromesistius poutassou* and 163 of *Micromesistius australis*. The time since divergence was estimated to be *c*. 2 million years before present (Mb.p.) with an externally derived clock rate by Bayesian methods. Congruent estimates were obtained with an additional data set of cytochrome *b* sequences derived from GenBank utilizing a different clock rate. The divergence time of 2 Mb.p. was in disagreement with fossil findings in New Zealand and previous hypotheses which suggested the divergence to be much older. It, therefore, appears likely that *Micromesistius* has penetrated into the southern hemisphere at least two times. Paleoceanographic records indicate that conditions that would increase the likelihood for transequatorial dispersals were evident *c*. 2–1·6 Mb.p.. Haplotype frequency differences, along with pairwise F_{ST} values, indicated that Mediterranean *M. poutassou* is a genetically isolated population.

Key words: biogeography; dispersal; divergence time; Gadidae; vicariance.

INTRODUCTION

In a biogeographical sense, the term antitropical, or bipolar, distribution is applied to sister taxa found in both the northern and southern hemisphere, but separated by the warm tropics (Hubbs, 1952). Antitropical distributions are raising intriguing questions on how cold and temperate adapted taxa can be present in both hemispheres, since the high temperatures in the tropics appears to be a formidable barrier to gene flow (Briggs, 1987; Lindberg, 1991; Crame, 1993). Two mechanisms have been proposed to explain antitropical distributions: dispersal and vicariance. Dispersal can cause disjunct distributions through a founding event by random movements across unsuitable habitats or long distances. For marine species, the possibility of equatorial crossing can increase through an extension of temperate zones during glacial periods, or during other times with favourable oceanographic conditions, or crossing can take

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place in deeper, colder water masses at any time (Bowen & Grant, 1997; Burridge, 2002). The vicariance hypothesis assumes that the ancestor of the now isolated taxa occurred in a continuous distribution across the tropics. A climatic, oceanographic or geologic event could then have disrupted this distribution into genetically isolated populations (Lindberg, 1991; Burridge, 2002; Heads, 2005).

Although molecular dating is prone to errors for several reasons and should be interpreted with caution (Arbogast *et al.,* 2002), it can serve as a method for testing biogeographical hypotheses, such as vicariance *v*. dispersal (Bowen & Grant, 1997; Burridge, 2002). If vicariance is the underlying cause, divergence could be expected on a time scale of tens of millions of years where continental plate movements have been significant (Bowen & Grant, 1997). Several recent studies based on molecular dating, however, have indicated that dispersal is the more likely main cause of antitropical distributions in marine fishes (Stepien & Rosenblatt, 1996; Burridge, 2002; Grant *et al*., 2005). Many of these divergences have been found to have occurred within the Pliocene and Pleistocene periods [the last 5 million years], thus deeming vicariance based on tectonic forced ocean-basin evolution unlikely. On the other hand, the highly fluctuating high-latitude climate during these periods has enhanced the possibility for dispersals.

In the Gadinae subfamily Gadidae (the cod family), *Micromesistius* is the only genus with an antitropical distribution (Fig. 1). The blue whiting *Micromesistius poutassou* (Risso 1827) supports the seventh largest fishery in the world (FAO, 2010) and is found in the north-east Atlantic Ocean from the Barents Sea to the coast of Morocco and in the Mediterranean Sea, with the largest spawning aggregations found on the banks west of Ireland. It is also found sparsely in the north-west Atlantic Ocean (Bailey, 1982). The other species in the genus, southern blue whiting *Micromesistius australis* Norman 1937 supports large fisheries in two disjunct areas: off the southern South American coast and to the south of New Zealand. Despite the vast distance between them, *M. poutassou* and *M. australis* are morphologically almost indistinguishable, except for a clear difference in the number of gillrakers (Svetovidov, 1948). Their life cycles and ecologies are also strikingly similar; both species are mesopelagic with high dispersal capacities and feed predominantly on zooplankton (Bailey, 1982; Niklitschek *et al*., 2010). The high morphological and ecological resemblance between the two species might be an indicator of recent divergence, but because these attributes are subject to natural selection, neutral or nearly neutral genetic markers provide an alternative tool to test hypotheses on evolutionary models.

Whether the high similarities in morphology and ecology in *M. poutassou* and *M. australis* are mirrored at the molecular level was investigated by Dobrovolov *et al*. (2005). Using isozyme varation, they estimated the divergence between the two species to be at 3·24 million years before present (MB.P.). This is in conflict with a comprehensive study on Gadiform biogeography, where Howes (1990) concluded that gadoids, and among them *Micromesistius* 'have evolved along with evolution of the Atlantic shelves and their distribution (and bipolarity) is a consequence of the geological processes which have formed the Atlantic Ocean.' Howes (1990, 1991) maintained a vicariance approach to explain the disjunct New Zealand and South American populations of *M. australis*, and argued that their present distributions result from the disruption and drift of the South American and Australian plate away from Antarctica. Thus, Howes (1990, 1991) suggested that disjunctions observed

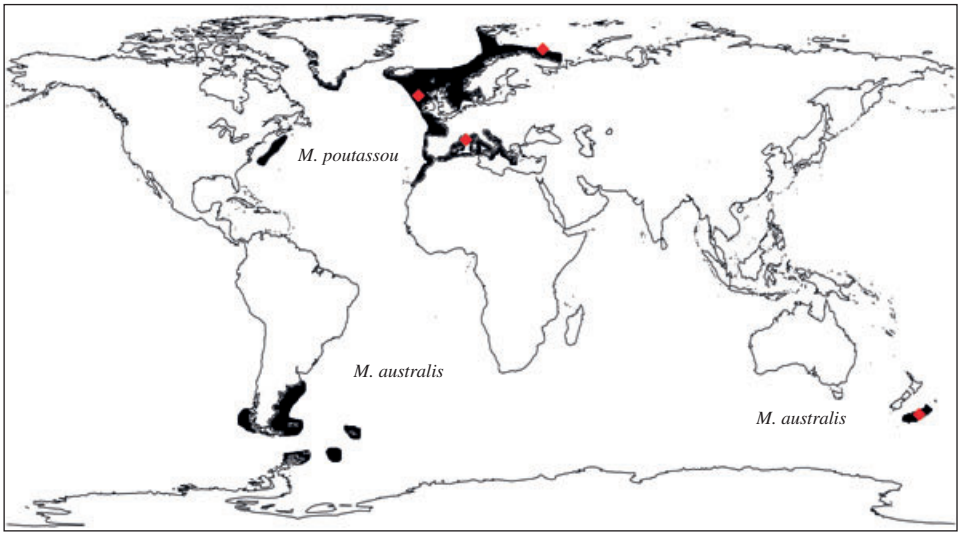

FIG. 1. Approximate distribution of *Micromesistius poutassou* and *Micromesistius australis* after Cohen *et al*., (1990). Areas sampled are indicated (◆) (see Table I. for accurate sampling site locations).

within *Micromesistius* are due to tectonic processes coupled with Miocene global warming and subsequent cooling [25–12 MB.P. (Crame, 1993)]. If this would be true, the high conservation of morphology and ecology in *Micromesistius* observed today would be remarkable and probably without comparison within marine fish taxa.

Regarding intraspecific genetic differentiation, several studies have found significant differentiation across the distribution range of *M. poutassou*. There is evidence for genetically isolated populations in the Barents Sea and in the Mediterranean Sea, indicated both by the use of allozyme (Mork & Giæver, 1995; Giæver & Stien, 1998) and by mini and microsatellite DNA markers (Ryan *et al.,* 2005). Evidence of genetic heterogeneity has also been reported among spawning localities west of the British Isles, probably due to different hydrographical patterns among the localities (Ryan *et al*., 2005; Was *et al*., 2008). In the Southern Ocean *M. australis*, Inada & Nakamura (1975) considered the South American and New Zealand populations to be distinct sub-species, *M. a. pallidus* and *M. a. australis* based on geographic isolation and meristic and morphometric differences. Ryan *et al*. (2002) reported population-level allele frequency differences at mini and microsatellite loci between *M. australis* from Falkland Island and New Zealand waters.

This study aimed to resolve some of the uncertainties concerning both the historical biogeography and the current population structures, using samples from most of the range of *M. poutassou* and the New Zealand population of *M. australis*. Variation in the 'barcode' region of *coI* was used for divergence time estimation. Cytochrome *b* (*cyt b*) sequences from Genbank from both species were also analysed, and allowed for inclusion of both *M. australis* populations. It was expected that the use of presumably neutral markers such as *coI* and *cyt b* would provide a more reliable estimate of time since divergence than the previous study by Dobrovolov *et al*. (2005) using

TABLE I. Sampling data. Note that the number of individuals from each location is the number of individuals sequenced (in total 442), not the number of individuals originally sampled (in total 598)

Species	Location	Group	*n*	Lat	Lon	Year	L_T (mm)	% M	A (years)
Micromesistius poutassou	Porcupine St	West of Ireland	44	55·13	10·18	2004	266	55	4
M. poutassou	Porcupine St	West of Ireland	48	55·29	10·04	2004	261	75	3
M. poutassou	Hebrides St	West of Ireland	11	56·30	9·29	2004	273	64	5
M. poutassou	Rockall St	West of Ireland	10	58·45	13·13	2004	271	80	3
M. poutassou	East of Corsica	Corsica	84	43·30	4·30	1997	257	30	2
M. poutassou	Barents Sea St	Barents Sea	82	71·12	28·30	1992	274	56	2
Micromesistius australis	New Zealand Bounty	New Zealand	75	−48·00	179·00	1997	385	63	6
M. australis	New Zealand Pukaki	New Zealand	88	−50·03	173·22	1997	418	72	8

n, sample size; Lat, latitude; Lon, longitude; Year, year of capture; L_T, average total length; % M, percentage of males; A, average age.

isozymes. This, in conjunction with an extensive literature study on climatic conditions in the plausible timeframes, provided a robust framework for investigating the biogeography of *Micromestistius.*

MATERIALS AND METHODS

SAMPLING

Specimens were sampled aboard research vessels, between 1992 and 2004. Samples of muscle tissue were dissected from 398 *M. poutassou* and 200 *M. australis* and stored in 96% ethanol. Details regarding the sampling are found in Table I. The four samples taken from the breeding areas west of Ireland were treated as one population, as no significant genetic differentiation among these localities were found in the following analysis. For similar reasons, the two New Zealand samples were treated as one group.

EXTRACTION, AMPLIFICATION AND SEQUENCING OF DNA

DNA extraction was done in a solution of Proteinase K, TE-buffer and 5% chelex (Bio-Rad; www.bio-rad.com) (Walsh *et al*., 1991), digested over-night and then boiled for 5 min, centrifuged and diluted 1:19 with pure water. DNA was amplified via polymerase chain reaction (PCR) using the primers FishF1- 5′-TCA ACC AAC CAC AAA GAC ATT GGC AC-3′ and FishR2- 5′-ACT TCA GGG TGA CCG AAG AAT CAG AA-3′, designed to amplify the 5′ end of the cytochrome *c* oxidase subunit I gene (*coI*) in fishes (Ward *et al*., 2005). The settings for the PCR conditions were as follows: 5 min of initial denaturation at 94° C, then the next steps were recycled 35 times: denaturation, 94° C for 1 min, annealing, 55° C for 30 s and elongation, 72° C for 1 min. A final elongation followed at 72° C for 7 min. The PCR-products were sequenced using the BigDye v 3.1 chain termination sequencing kit (Applied Biosystems; www.appliedbiosystems.com). The phred/phrap/consed

TABLE II. Mitochondrial DNA (mtDNA) sequences from GenBank included in this study. Sampling locations are given for the *Micromesistius* sequences

Species	Gene	Accession number	Sampling locality	Group
Micromesistius poutassou	*cytb*	EU264030	Northern Aegean	Mediterranean Sea
M. poutassou	*cytb*	EU264031	Northern Aegean	Mediterranean Sea
M. poutassou	*cytb*	EU264032	Northern Aegean	Mediterranean Sea
M. poutassou	*cytb*	EU264028	Northern Aegean	Mediterranean Sea
M. poutassou	*cytb*	EU264029	Northern Aegean	Mediterranean Sea
M. poutassou	*cytb*	EU492307	Baltic and Skagerak	North-east Atlantic Ocean
M. poutassou	*cytb*	EU492308	Baltic and Skagerak	North-east Atlantic Ocean
M. poutassou	*cytb*	EU224066	Bay of Biscay	North-east Atlantic Ocean
M. poutassou	*cytb*	EU224067	Bay of Biscay	North-east Atlantic Ocean
M. poutassou	*cytb*	EF427580	Cantabric Sea	North-east Atlantic Ocean
M. poutassou	*cytb*	EF427581	Cantabric Sea	North-east Atlantic Ocean
M. poutassou	*cytb*	EU492136	North Sea	North-east Atlantic Ocean
M. poutassou	*cytb*	EU492137	North Sea	North-east Atlantic Ocean
M. poutassou	*cytb*	EF439548	W. Mediterranean	Mediterranean Sea
M. poutassou	*cytb*	EF439549	W. Mediterranean	Mediterranean Sea
Micromesistius australis	*cytb*	AB490243	New Zealand	New Zealand
M. australis	*cytb*	AB490242	New Zealand	New Zealand
M. australis	*cytb*	AB490240	Argentina	Argentina
M. australis	*cytb*	AB490241	Argentina	Argentina
M. australis	*cytb*	AB490239	Argentina	Argentina
Outgroups				
Gadus morhua	*coxI*	NC_002081		
Gadiculus argenteus	*cytb*	EU224053		

suite of programmes (Gordon *et al*., 1998; Gordon, 2003; Ewing & Green, 1998; Ewing *et al*., 1998) and the polyphred software (Kwok *et al*., 1994; Nickerson *et al*., 1997) were used to assemble and inspect the trace files following sequencing. Sequences that had bases with a probability of error $>3{\cdot}16 \times 10^{-4}$ in the region of interest were discarded. After assessment of sequence quality, the dataset was reduced from 598 to 442 individuals (279 of *M. poutassou* and 163 of *M. australis*). Sequences were aligned using Muscle (Robert, 2004) and Clustal-W (Thompson *et al*., 1994) and inspected by eye. Unique sequences were deposited in GenBank with accession numbers HQ882605-HQ882700.

ADDITIONAL GENBANK SEQUENCES

Twenty *cytb* sequences, 15 *M. poutassou* and five *M. australis*, were obtained from GenBank to create an additional data set. Sequence alignment was carried out in the same manner as with the *coI* sequences. Sequences from GenBank allowed for inclusion of the South American population and outgroup sequences for *M. poutassou* and *M. australis* were also obtained from GenBank (Table II).

DATA ANALYSIS

The DNAsp 5 software (Librado & Rozas, 2009) was used to investigate descriptive statistics, to estimate haplotype diversity and nucleotide diversity, and to perform neutrality tests;

Tajima's (1989) D and Fu's (1997) F_S, based on the number of segregating sites. Statistical significance was assessed by comparing the test statistics with coalescence simulations in the DNAsp 5 software (10 000 replicates). When applied to presumably neutral evolving loci like *coI*, these test statistics has proven effective to test the null hypothesis of constant population size. Negative values are expected for a population that have experienced considerable growth in the past (Ramos-Onsins & Rozas, 2002). Pairwise F_{ST} values were also estimated with DNAsp 5. The significance was assessed with the permutation test (Hudson *et al*., 1992) (10 000 replicates). The best fit substitution model was found to be the Tamura–Nei model (TrN) (Tamura & Nei, 1993) with a proportion of invariable (p_{inv}) sites of 0·815 for *coI* and 0·819 for *cytb* determined by the Bayesian information criterion (Schwarz, 1978) in jModeltest 0.1 (Posada, 2009). To assess whether it was reasonable to apply the molecular clock to estimate divergence times, the likelihood-ratio test (LRT) was applied using PHYLIP (Felsenstein, 2005). For this test, the haplotype sequences excluding singletons (haplotypes represented by only one individual) were used for the *coI* dataset, with an Atlantic cod *Gadus morhua* L. 1758 sequence from GenBank (Accession number: NC_002081) as outgroup. Silvery pout *Gadiculus argenteus* Guichenot 1850 is probably the closest relative of *Micromesisitus* (Teletchea *et al*., 2006) but no *coI* sequence was available for this species. For the *cytb* dataset, the LRT was done with a *G. argenteus* sequence from GenBank (Accession number: EU224053) as outgroup. Genetic distances between species and localities were estimated with the TrN model in MEGA 4 (Tamura *et al*., 2007). Phylogenetic relationships among the haplotypes excluding singletons were investigated with a maximum parsimony median joining network constructed with Network 4.5 (Bandelt *et al*., 1999). The software-package BEAST 1.6.1 (Bayesian evolutionary analysis by sampling trees) (Drummond & Rambaut, 2007) was used to analyse the sequences in a Bayesian framework. The parameters of interest from this analysis were divergence time and time to most recent common ancestor (TMRCA) within each species. The software uses a Markov Chain Monte-Carlo (MCMC) algorithm to average over the tree space and to create posterior probability distributions for the parameters in the analysis. Based on strong indices of population growth from Tajima's D and Fu's F_S tests, the exponential growth model was selected as tree prior. To test the robustness of the exponential growth model to estimate the divergence time between *M. poutassou* and *M. australis*, a Yule tree prior, which is optimized for datasets consisting of sequences from different species, was also applied with a single sequence from each species and the out-groups. Uninformative, default priors were used on all other parameters, except for the p_{inv} sites, which was set according to the value suggested by jModeltest (Posada, 2009). The MCMC was run for 10 million generations, with 10% of the generations from the start of the run discarded as burn-in. Sampling was done every 1000th generation. For the *coI* analysis, the substitution rate was fixed to 0·006 substitutions site^{-1} M^{-1} years, a calibration obtained from sister taxa separated by the closure of the Isthmus of Panama (Bermingham *et al*., 1997). For the *cytb* dataset, a divergence rate of 0·00818 substitutions site^{-1} M^{-1} years was used, calibrated from the opening of the Bering Strait and the divergence of the Atlantic and Pacific Ocean *G. morhua* lineages (Bigg *et al*., 2008). The software TRACER 1.5 (Rambaut & Drummond, 2007) was used to analyse the output and to check convergence and mixing of the MCMC. In all MCMC runs, the effective sample size (ESS) for all parameters exceeded 200. Maximum clade credibility trees were produced by TreeAnnotator 1.6.1 provided by the BEAST package.

RESULTS

coI SEQUENCE VARIATION

The alignment of the 442 *coI* sequences, each 579 bp long, revealed 96 haplotypes, 52 private to *M. poutassou* and 44 private to *M australis.* The distribution of individuals to each haplotype with respect to sampling locality is shown in Table III.

The *coI* lineages were completely sorted between the two species, and there were four fixed differences between *M. australis* and *M. poutassou.* Out of a total of 71

TABLE III. Frequencies of haplotypes in the different localities. Singleton haplotypes are not shown, but their number is given for each locality. Relative frequency (%) in respect to the total number of haplotypes in each locality is given in parentheses

Haplotype	Barents Sea	Corsica	West of Ireland	*Micromesistius australis*	Sum
C1	44 (53·7%)	36 (42·9%)	60 (53·1%)	0	140
C2	0	0	0	82 (50·3%)	82
C3	4 (4·9%)	21 (25·0%)	9 (8·0%)	0	34
C4	7 (8·5%)	4 (4·8%)	5 (4·4%)	0	16
C5	0	0	0	14 (8·6%)	14
C6	7 (8·5%)	0	6 (5·3%)	0	13
C7	0	0	0	10 (6·1%)	10
C8	2 (2·4%)	2 (2·4%)	4 (0·35%)	0	8
C9	0	0	0	6 (3·7%)	6
C10	0	0	0	6 (3·7%)	6
C11	5 (6·1%)	0	0	0	5
C12	0	4 (4·8%)	0	0	4
C13	1 (1·2%)	0	3 (2·7%)	0	4
C14	2 (2%)	0	1 (0·9%)	0	3
C15	0	2 (2·4%)	1 (0·9%)	0	3
C16	0	0	0	3 (1·8%)	3
C17	0	0	2 (1·8%)	0	2
C18	1 (1·2%)	1 (1·2%)	0	0	2
C19	1 (1·2%)	0	1 (0·9%)	0	2
C20	0	0	2 (1·8%)	0	2
C21	0	0	2 (1·8%)	0	2
C22	0	2 (2·4%)	0	0	2
C23	0	0	2 (1·8%)	0	2
C24	0	0	0	2 (1·2%)	2
C25	0	0	0	2 (1·2%)	2
C26	0	0	0	2 (1·2%)	2
C27	0	0	0	2 (1·2%)	2
Singletons	8 (9·8%)	12 (14·3%)	15 (13·3%)	34 (20·9%)	69
Sum	82	84	113	163	442

mutations there were 67 segregating sites. Sixty nine of the mutations were synonymous and two were replacement changes, found in first and second positions at sites 17 and 250, respectively, both of these between *M. australis* haplotypes. There were 39 parsimony-informative sites. Twenty-one sites were polymorphic in *M. australis,* while monomorphic in *M. poutassou.* In contrast, 28 sites were polymorphic in *M. poutassou*, but monomorphic in *M. australis.* Eighteen sites were polymorphic in both species. By analysing haplotype networks with and without singletons, a considerable amount of homoplasy was detected. Of singletons, 45% of the mutations separating the haplotypes (the network link between the closest haplotype) were found at nucleotide sites defining more than one singleton haplotype, whereas between haplotypes with a frequency of two or more, the percentage was 30. Therefore, singletons were excluded the presented haplotype network construction and in phylogeny estimation to reduce the influence of homoplasy and to increase

TABLE IV. Haplotype diversity, nucleotide diversity, Tajima's D and Fu's F_S neutrality tests for detecting of population growth and net Tamura–Nei distance between *Micromesistius australis* and the other groups. The table shows sample size (n), number of haplotypes (m), estimated haplotype diversity ($\hat{h}$), estimated nucleotide diversity (π). Tajima's D and Fu's F_S statistics are given with significance: ***, $P < 0{\cdot}001$; **, $P < 0{\cdot}01$; *, $P < 0{\cdot}05$; NS, not significant at significance level $a = 0{\cdot}05$. Net Tamura–Nei distance (d_A) given as number of substitutions per site

Species	n	m	$\hat{h}$	π	D	F_S	d_A – *M. australis*
Micromesistius poutassou	279	52	0·73	0·0024	−2·29**	−74·92***	0·02122
M. australis	163	44	0·74	0·0022	−2·38**	−66·86***	–
M. poutassou localities							
Barents Sea	82	18	0·70	0·0023	−2·05*	−13·35**	0·02145
West of Ireland	113	28	0·71	0·0022	−2·19**	−31·50***	0·02159
Corsica	84	20	0·76	0·0028	−1·71 NS	−14·16***	0·02057

visual clarity in figures. Slightly higher genetic diversity was found in the Corsica sample (Table IV). The neutrality tests, Tajima's D and Fu's F_S, were significantly negative in all comparisons, except for the D test statistic of Corsica, suggesting that both the species and pooled samples have experienced growth in effective population size in the past.

CYTOCHROME B SEQUENCE VARIATION

The alignment of the 20 *cytb* sequences from GenBank consisted of 523 bp. Eight fixed differences separated the species. There were 34 mutations and 32 segregating sites, of these, one was a replacement change which separated the two sequences from the Cantabric Sea from the rest of the *M. poutassou* samples.

PHYLOGENY AND GENETIC DIFFERENTIATION

The *coI* haplotype network (Fig. 2) showed typical star-shaped clusters for both species with the most common haplotype occupying the central position in each cluster. The second most common haplotype in *M. poutassou*, *C3*, was dominated by individuals sampled in the Mediterranean Sea (relative frequency: 62%). It is also the haplotype closest to *M. australis*, separated by 10 substitutions. The Corsica sample also showed the lowest genetic distance to *M. australis* (Table IV.). F_{ST}-values were significantly different from zero between the Mediterranean Sea (0·042) and west of Ireland population, and between the Mediterranean Sea and Barents Sea (0·041). F_{ST}-values between west of Ireland and Barents Sea were negative. Excluding singletons, there were private haplotypes within all the sampling localities in *M. poutassou*: two in the Corsica sample (*C12*, four individuals and *C22*, two individuals), four in the west of Ireland sample (*C17*, *C20*, *C21* and *C23*, all with two individuals), and one in the Barents Sea sample (*C11*, five individuals). If the Barents Sea and west of Ireland samples are considered as taken from the same population, a common haplotype (*C6*, 13 individuals) was private to this pooled population. With the LRT test, none of the genes were found to violate the assumption of clock-like evolution, ($P > 0{\cdot}05$

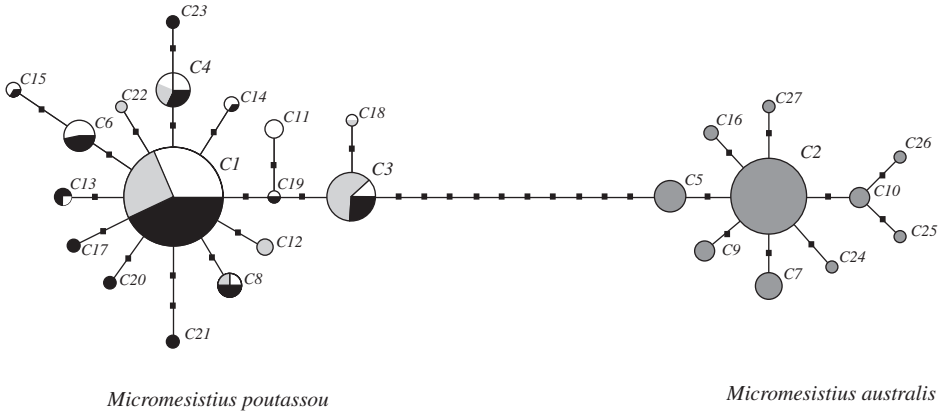

FIG. 2. Median-joining network of *coI* haplotypes excluding singletons. The size of the nodes is proportional to number of individuals sharing the haplotype. Shaded sectors indicate locality and size of sector is proportional to the relative frequency of the total individuals in the haplotype (▩, New Zealand; ■, west of Ireland; ▨, Mediterranean Sea (Corsica); □, Barents Sea). Substitutions separating haplotypes are indicated (■).

for both *coI* and *cytb*) and molecular dating could be done with a strict clock. The Bayesian phylogenetic tree of the main *coI* haplotypes (Fig. 3) supported the basal position of the *C3* and *C18* haplotypes, which formed a well-supported clade with the oldest split in the *M. poutassou* clade. In the Bayesian *cytb* tree (Fig. 4), the *M. australis* individuals sampled from Argentina and New Zealand did not form separate clades, but were clustered together with no apparent geographic structuring. The divergence time between the two species were estimated to be 1·69 million years before present (MB.P.) (95% highest posterior density interval (HDP): 0·79–2·77 MB.P.) for the *coI* sequences, and 2·00 MB.P. (95% HDP: 1·00–3·15 MB.P.) for the *cytb* sequences. The TMRCA's for *M. poutassou* were found to be 0·79 MB.P. (*coI*, 95% HDP: 0·41–1·22) and 0·94 MB.P. (*cytb*; 95% HDP: 0·47–1·45 MB.P.), and for *M. australis* the TMRCA's were 0·58 MB.P. (*coI*; 95% HDP: 0·28–0·99 MB.P.) and 0·43 MY (*cytb*; 95% HDP: 0·13–0·81 MB.P.).

DISCUSSION

HISTORICAL BIOGEOGRAPHY OF *MICROMESISTIUS*

Fossil findings in central Europe and Mediterranean from the Pliocene to the Miocene (Brzobohaty, 1994; Bannikov, 2009) have indicated a north-east Atlantic Ocean origin for *Micromesistius.* This is in agreement with the diversification in Gadidae, which is thought to have happened in the North Atlantic Ocean in the late Oligocene to early Miocene (Svetovidov, 1948; Nolf & Steurbaut, 1989; Howes, 1991; Bakke & Johansen, 2005). In the southern hemisphere, fossil findings of *Micromesistius* sp. otoliths in New Zealand have been dated to the early Pliocene (Schwarzhans, 1981). This implies that *Micromesistius* was already present in the southern hemisphere *c*. 4–5 MB.P. The early and mid-Pliocene (5–3·5 MB.P.) ocean

was significantly warmer and had a deeper thermocline (Crame, 1993; Philander & Fedorov, 2003; Haywood *et al.*, 2009), which suggests that the ancestor of this southern hemisphere *Micromesistius* must have crossed the equator at some time prior to this warm period (5 Mb.p.). Therefore, two different hypotheses are proposed to explain the discrepancy between the early Pliocene fossil records and the divergence time estimated in this study: (1) Speciation in *Micromesistius* took place in the early Pliocene or before, based on fossil findings of *Micromesistius* in early Pliocene strata. If so, the molecular clock calibration used in the present study is severely biased. (2) *Micromesistius* has invaded the southern hemisphere at least two times, the last one through dispersal in the late Pliocene and early Pleistocene, *c*. 2 Mb.p., suggested by the divergence time estimated in the present study.

If hypothesis 1 is correct, the molecular clock rates used in this study are much too fast. Based on the warm period recorded in the early to mid-Pliocene (see above), 5 Mb.p. might be used as a conservative calibration point. The substitution rate for *coI* would then be 0·0021 substitutions site^{-1} M^{-1} years, or 0·42% sequence divergence M^{-1} years (estimated from Tamura–Nei distance of 0·0212 between *M. poutassou* and *M. australis*), which is in high contrast to the universal rate of 1·2% sequence divergence M^{-1} years from Bermingham *et al.* (1997). A larger deviation from this rate, however, has previously been reported. Cárdenas *et al.* (2005) studied diversification in the *Trachurus* genus with similar methods as those used in this study. Based on fossil calibrations, they reported an mtDNA divergence rate of 0·13–0·15% per M^{-1} years, and that the initial diversification in the genus occurred 15–18 Mb.p. They disagreed with Karaiskou *et al.* (2003) who suggested this diversification to happen 2–5 Mb.p., based on the rate from Bermingham *et al.* (1997). If such a slow rate is true for *Micromesistius*, it will have consequences for previous and future molecular phylogenetic studies in the Gadinae and local rates may have to be calibrated for each genus or species. Carr *et al.* (1999), Bakke & Johansen (2005) and Coulson *et al.* (2006) all use the rates from Bermingham *et al.* (1997) to produce time-calibrated phylogenies for gadines. On the other hand, the double invasion hypothesis seems probable in the light of paleoclimatic records. After a mid-Pliocene warm peak, the North Atlantic Ocean rapidly cooled at high latitudes from 3·5 to 2·5 Mb.p., followed by a more modest cooling from 2·5 Mb.p. to present. This coincided with the onset of the northern hemisphere glaciations at *c*. 2·7 Mb.p., and a highly variable climate characterized by glacial cycles persisted throughout the Pleistocene (Lawrence *et al.*, 2009; Filippelli & Flores, 2009). In addition, a shoaling of the thermocline which occurred gradually through the Cenozoic made way for upwelling of cold water in tropical upwelling zones *c*. 3 Mb.p. (Philander & Fedorov, 2003). At present, the southern limit of *M. poutassou* is along the North African coast associated with the Canary Current, which is a rather cold east boundary current causing upwelling. In a paleoceanographic reconstruction of the Canary Current production and sea surface temperatures (SST) for the past 3 M years, a period of low SSTs and high seasonal variations up to 6° C took place *c*. 2·0–1·6 Mb.p., followed by a warmer interval lasting until *c*. 0·85 Mb.p. (Pflaumann *et al.*, 1998).

In southern South America, several glaciations have been recorded between 2·16 and 1·43 Mb.p. (Rabassa, 2008). The Falkland Current, where the northern limit of *M. australis* distribution is found today, is a branch of the Antarctic circumpolar current that flows northwards along South America, converging with the warm, southward-flowing Brazil Current outside the southern Brazilian coast. The glacial

periods in South America influenced the Falkland Current, and during glaciations, it probably reached much farther north than present (Rabassa, 2008). The coinciding of these events in the south-west Atlantic Ocean and the lower temperatures off North Africa suggests that the distance between suitable habitats where reduced, and therefore increases the probability of dispersal. The west part of the Equatorial Atlantic Ocean was as it is at present, warmer and with a deeper thermocline than the eastern part (Nikolaev *et al*., 1998). Taking in consideration today's sparse occurrence of *M. poutassou* in the north-east Atlantic Ocean, it is unlikely that the *Micromesistius* ancestor was extending its habitat southwards there. A strong correlation between southern limit and temperature observed at present in *M. poutassou*. Perry *et al*. (2005) studied the effect of warm and cold periods in modern times and distribution limits in 20 fish species, including four gadines, in the North Atlantic Ocean. *Micromesistius poutassou* was found to have the greatest movement of the southern boundary between warm and cold periods of the species in the study.

Howes (1990, 1991) vicariance hypothesis does not appear to be a plausible explanation for the present *M. australis* distribution in the light of the shallow genetic divergence reported in this study. If there have been two invasions to the southern hemisphere, however, the genetic lineage of the first invasion would now be extinct and the causes and timing of the first invasion remain unknown without more fossil data. Schwarzhans (1981) reported fossil findings of related *Gadiculus* genus dated to late Miocene south of New Zealand. *Gadiculus* is now only found in the north-east Atlantic Ocean, with a similar distribution to *M. poutassou*, and demonstrates that other pelagic gadines have occurred and become extinct in earlier epochs in the southern hemisphere. As shown above, the Pleistocene timeframe coincides with climatic and oceanographic events in the waters where the present day southern limit of *M. poutassou* and northern limit of *M. australis* are found. This supports the validity of the divergence time estimate, thus *Micromesistius* has most probably invaded the southern hemisphere two times. The divergence time of 3·24 M years estimated by Dobrovolov *et al*. (2005) with isozymes is also in support of a more recent divergence, and the uncertainties regarding the degree of neutrality of isozymes and the general accuracy of molecular clocks could explain the difference between the two estimates.

INTRASPECIFIC DIFFERENTIATION

The Corsica sample appears to represent a partially isolated Mediterranean Sea population of *M. poutassou*, indicated by the F_{ST} values and the presence of several private haplotypes found in the Corsica but not in the Barents Sea and west of Ireland samples. Presence of private haplotypes between populations is also an indicator of lack of gene flow (Slatkin, 1985). Singletons should not be considered in such evaluations, because it is not possible to distinguish true private haplotypes from widespread, but very low-frequency haplotypes (Árnason *et al*., 2000). Genetic differentiation between the Mediterranean Sea and North Atlantic Ocean samples is in agreement with a study by Ryan *et al*. (2005) who reported evidence for a partially genetic isolated population in the Mediterranean Sea using microsatellite DNA markers. Allozyme studies of *M. poutassou* also indicated a genetically distinct (although non-significant) Mediterranean Sea population (Mork & Giæver, 1995; Giæver & Stien, 1998). Interestingly, the earlier allozyme (Mork & Giæver, 1995; Giæver &

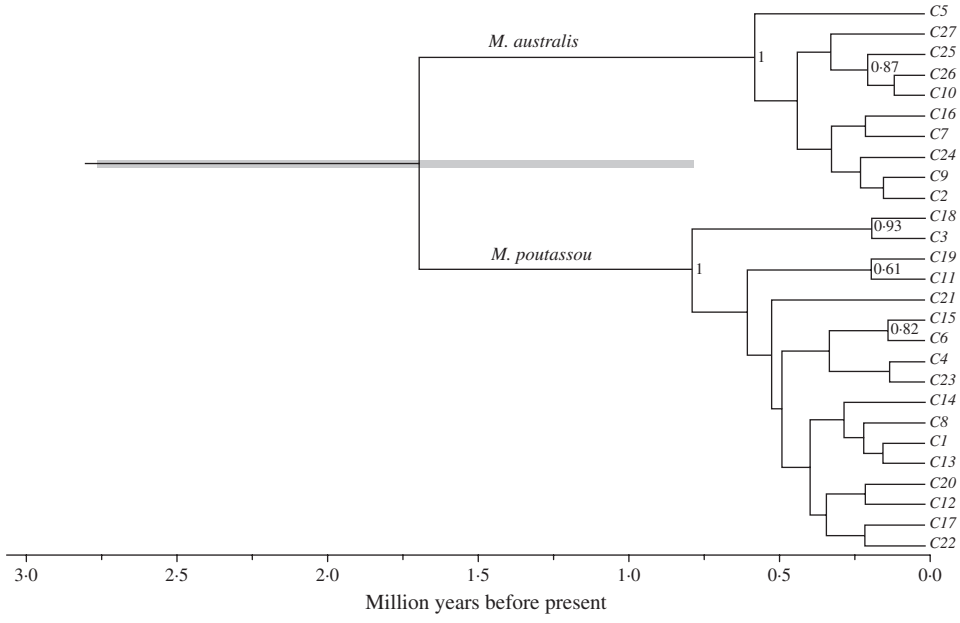

FIG. 3. Bayesian highest credibility phylogenetic tree of *coI* haplotypes (singletons excluded) in *Micromesistius poutassou* and *Micromesistius australis*. The tree is calibrated with a substitution rate of 0·006 substituitions site^{-1} M^{-1} years. Posterior probabilities are given for nodes when exceeding 0·5. The 95% highest posterior density interval for the root age is indicated (▭).

Stien, 1998) and microsatellite (Ryan *et al*., 2005) studies all found statistically significant evidence for a genetically isolated population in the Barents Sea, which was not detected with the mtDNA markers in this study. During the glacial periods after *c*. 0·45 MB.P., ice sheet during glacials extended to the English Channel and covered the North Sea (Toucanne *et al*., 2009), and covered the present feeding and breeding grounds of the northern *M. poutassou* populations. Considering the extensive migratory behaviour of *M. poutassou*, it is likely that the North Sea and Barents Sea populations were established during interglacial periods from southern refugees, as for instance the Mediterranean Sea population. This might explain the genetic patterns observed, with a genetically different population in the Mediterranean Sea, and with the slightly higher genetic diversity found in the Corsica sample reflecting a more stable environment in the Mediterranean Sea during the Pleistocene. The TMRCA for *M. poutassou* is older than for *M. australis* for both *coI* and *cytb* (Figs 3 and 4). In the *coI* tree, the Mediterranean-dominated *C3* and *C18* clade is the reason for this, and if these were not considered, the TMRCA for the two species would be similar. A similar pattern is seen in the *cytb* tree, where a Mediterranean Sea haplotype is rooting the *M. poutassou* clade. This further strengthens the hypothesis that the Mediterranean Sea has been a sheltered refuge for *M. poutassou.* The remarkable resemblance between *M. poutassou* and *M. australis* in morphology and biology is mirrored in genetic diversity and the similarity of TMRCA within both species (when not considering Mediterranean Sea haplotypes). This suggests that population structure and size fluctuations have been similar and could be due to an evolutionary strategy of having large population sizes and being able to respond

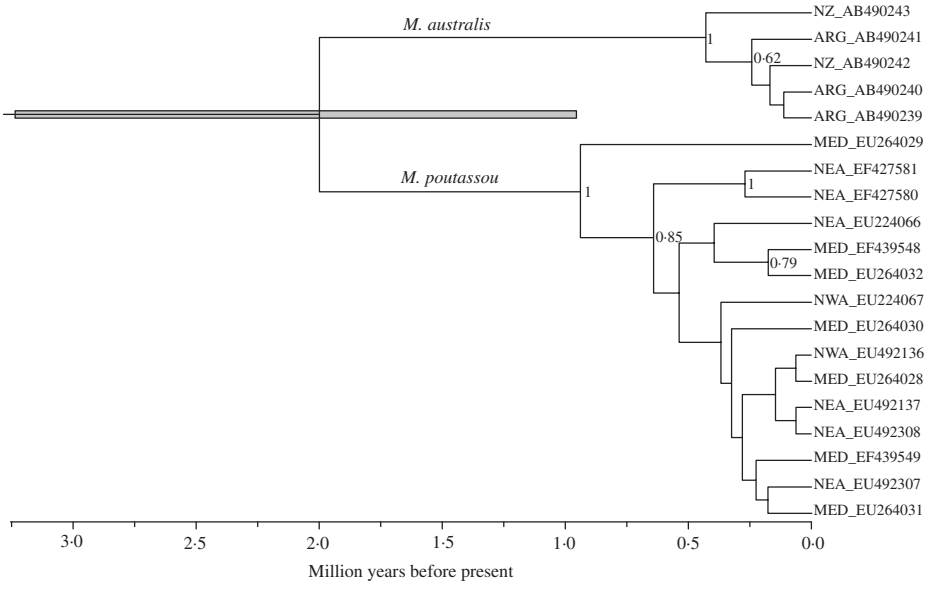

FIG. 4. Bayesian highest credibility phylogenetic tree of *Micromesistius poutassou* and *Micromesistius australis*. *cyt b* sequences from GenBank. Sequences are grouped after sampling localities: NZ, New Zealand; ARG, Argentina; MED, Mediterranean Sea; NEA, north-east Atlantic Ocean. The tree is calibrated with a substitution rate of 0·00818 substituitions site^{-1} M^{-1} years. Posterior probabilities are given for nodes when exceeding 0·5. The 95% highest posterior density interval for the root age is indicated (▭).

quickly to a changing climate and utilize new habitats as they open up. Both species are mesopelagic and generalist feeders (Bailey, 1982) occurring in habitats with similar physical environments, therefore the selection pressure has probably not been different enough to result in divergence in biological and morphological traits. A similar pattern has been reported among eastern Pacific temperate anchovies (genus *Engraulis*) and tropical anchovies (genus *Cetengraulis*) (Grant *et al*., 2010).

Regarding the two disjunct *M. australis* populations, the phylogenetic reconstruction of the five *cyt b* sequences from GenBank does not support Inada & Nakamura's (1975) suggestion of giving sub-species status to the two populations. The two New Zealand sequences did not form a monophyletic clade (Fig. 4), which one could expect if the populations had been separated on a sub-species time scale. The most likely explanation for the observed pattern seems to be partially separated population with gene flow through dispersals in the Antarctic circumpolar current during the interglacial periods. In the present interglacial, Shpak (1975) recorded *M. australis* in an intermediate area, the Bellinghausen Sea in the Pacific Ocean (Bailey, 1982). Whether gene flow happens through larvae drift or by migration of adult specimens (or both) is not known. A more complete sampling throughout the range of *M. australis* will be necessary to make any sound inference regarding the differentiation between the South American and New Zealand populations and possible causes. On the time scale of a glacial period, the isolation may be long enough to create significant genetic differentiation at genetic markers with higher rate of evolution, as found by Ryan *et al*. (2002) with microsatellites. The vicariance hypothesis of Howes (1990, 1991) regarding the disjunct *M. australis* distribution appears unlikely. The

findings in this study and in the one of Ryan *et al*. (2002) indicate that there must have been gene flow between the populations relatively recently, on time scales where continental drift is negligible.

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