

Speciation on a round planet: phylogeography of the goatfish genus *Mulloidichthys*

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ABSTRACT

Aim The goatfish genus *Mulloidichthys* is abundant on reefs throughout the tropics. Characteristic of this genus is a long larval and pelagic juvenile phase, which could potentially confer large dispersal capacity. We sampled its mitochondrial DNA to answer the following questions: What speciation events have led to the formation of the extant species? How do they correlate with geological and oceanographic events? Are *M. dentatus* and *M. martinicus* geminate species formed by the rise of the Isthmus of Panama? Is there genetic structure between conspecific populations?

Location All tropical oceans.

Methods We constructed a phylogeny of *Mulloidichthys*, based on the *ATPase-8* and *ATPase-6* genes and the control region. We estimated degree of genetic structuring in four species.

Results The phylogeny revealed that the Indo-Pacific *M. pflugeri* diverged first, followed by *M. flavolineatus*, also from the Indo-Pacific, followed by the central Pacific *M. mimicus*. The most recent splitting event resulted in a tritomy composed of the Atlantic *M. martinicus*, the eastern Pacific *M. dentatus* and the Indo-Pacific *M. vanicolensis*. The differentiation between *M. martinicus* and *M. dentatus* was substantially smaller than divergence in the same DNA fragments in eight other fish genera likely to have been split by the rise of the Isthmus of Panama. Low genetic structuring was found between conspecific populations of *Mulloidichthys*, even across the entire Indo-Pacific. Only populations at Clipperton Atoll and at Ascension Island in the Atlantic were genetically isolated from other conspecific populations.

Main conclusions The oldest extant species of *Mulloidichthys* are found in the Indo-Pacific. Younger species probably maintained genetic contact between the Atlantic and the Indo-Pacific until the late Pleistocene. The low degree of genetic structuring and the unusual recent connections around the globe are likely to be the result of the large, highly mobile, and long-lived juvenile phase in this genus.

Keywords

ATP synthetase, control region, dispersal, gene flow, Isthmus of Panama, mitochondrial DNA, Mullidae, tropical reef fish, vicariance.

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INTRODUCTION

Classical zoogeographical studies have established that major provinces for tropical shallow-water marine animals broadly coincide with ocean basins (Briggs, 1974; Briggs & Bowen, 2012). Barriers that are presumed to have caused speciation

include the separation of the Atlantic Ocean from the Indo-Pacific by the closure of the Tethyan Sea in the north (Rögl & Steininger, 1984) and the establishment of the Benguela upwelling in the south (Shannon, 1985), both in the Miocene; the separation of the eastern Pacific from the rest of the Pacific by the 5000 km of open deep water, known as the 'Eastern

Pacific Barrier', which has been in place for the entire Cenozoic (Grigg & Hey, 1992); the completion of the Isthmus of Panama in the Pliocene (Coates & Obando, 1996); and the widening of the Atlantic Ocean starting in the Jurassic, which created the 3500 km wide Mid-Atlantic barrier (Larson & Ladd, 1973). Phylogeographical studies have sought to establish the order that these barriers have acted on pantropical taxa. Mitochondrial DNA (mtDNA) phylogenies have indicated that these major barriers affected most genera, but not necessarily at the same time, or in the same order. Often the deepest separation of extant species within genera was caused by the Eastern Pacific Barrier in the west and by the Old World barrier and the Benguela upwelling in the east, isolating the eastern Pacific and Atlantic inhabitants from the Indo-West Pacific (Lessios *et al.*, 1999, 2001; McCartney *et al.*, 2000; Colborn *et al.*, 2001). The rise of the Isthmus of Panama caused more recent separations, so that many sister clades of extant species are found today in the western Atlantic and eastern Pacific, respectively (reviewed in Lessios, 2008). This pattern, in turn, gave rise to the notion that if transisthmian so-called 'geminant' pairs differed in the degree of their divergence, then the pair with the least amount of differentiation within a taxon was formed by the final completion of the isthmus, and can thus be used to calibrate rates of evolution (Knowlton & Weight, 1998). More than 200 studies have used calibrations derived from this method to date molecular phylogenies (Lessios, 2008). For pantropical taxa, however, global sampling is needed to determine whether the Eastern Pacific Barrier and the Benguela upwelling had blocked circum-global gene flow prior to the emergence of the Isthmus of Panama; only under this condition would the genetic split between the geminate species date no later than the last direct water connection between the eastern Pacific and the Caribbean Sea. Several examples of genetic connectivity between Indian Ocean and eastern Atlantic populations that appears to post-date the rise of the Isthmus of Panama (Bowen *et al.*, 2001, 2006; Rocha *et al.*, 2005) suggest that the Benguela upwelling has not always acted as an absolute barrier. There is also a small number of 'transpacific' species breaching the East Pacific Barrier (Robertson *et al.*, 2004; Lessios & Robertson, 2006).

Among the fishes in which mtDNA has been sampled on either side of the Isthmus of Panama, there are seven pairs that show divergences much smaller than 14 others (Lessios, 2008). The eastern Pacific (EP) and western Atlantic (WA) members of three of these pairs (*Diodon holocanthus*, *Aluterus scriptus* and *Melichthys niger*) are also assigned to the same species, suggesting that morphological divergence has also been minimal. With surveys that only include the EP and WA samples it is not possible to determine whether the low divergence across the Isthmus of Panama is the result of slow differentiation between populations isolated for approximately 3 Myr, or whether genes have travelled over generations around the globe after the isthmus was closed. Here we present a global phylogeny of the goatfish genus *Mulloidichthys* (Mullidae), intended to help answer this question and

to establish the relationship and time of divergence of its species.

Mulloidichthys is a strictly marine genus of goatfish. The genus contains seven species. *Mulloidichthys martinicus* (Cuvier, 1829) is found on both sides of the tropical and subtropical Atlantic, from Cape Verde to the Gulf of Guinea and from Bermuda to Fernando de Noronha, Brazil, including the mid-Atlantic islands of Ascension and St. Helena. *Mulloidichthys dentatus* (Gill, 1862) ranges along the tropical West coast of the Americas from southern California to Peru, including the outer islands of Revillagigedo, Clipper-ton, Isla Coco and the Galápagos. *Mulloidichthys vanicolensis* (Valenciennes, 1831) was until recently believed to be limited to the Indo-West Pacific and the central Pacific (Randall, 2005), but it has now been confirmed as being spread all the way to the American coast (Robertson *et al.*, 2004; Lessios & Robertson, 2006). *Mulloidichthys mimicus* Randall & Guézé, 1980 is only known from the Marquesas, Kiritimati, and Baker and Howland Islands (Randall, 2005). *Mulloidichthys flavolineatus* (Lacepède, 1801) ranges throughout the tropical central and western Pacific and the Indian Ocean (Randall, 2005). *Mulloidichthys pflugeri* (Steindachner, 1900) has the same overall range, but is only found on a few oceanic islands (Randall, 2005). The recently described species, *Mulloidichthys ayliffe* Uiblein, 2011; morphologically similar to *M. mimicus*, is limited to the western and north-eastern Indian Ocean (Uiblein, 2011). *Mulloidichthys vanicolensis*, *M. dentatus* and *M. martinicus* are morphologically very similar (Stepien *et al.*, 1994). The latter two species have been assumed to be sister species formed by the emergence of the Isthmus of Panama (Meek & Hildebrand, 1923–1928; Thomson *et al.*, 1979; Vawter *et al.*, 1980; Stepien *et al.*, 1994; Bermingham *et al.*, 1997).

The life-history traits of the species of *Mulloidichthys* suggest that they are capable of dispersing widely. They spawn pelagic eggs, which after fertilization grow to larvae, then to elongate, fusiform, highly mobile pelagic juveniles (Clarke, 1995; Lo-Yat *et al.*, 2006) that settle on reefs at body lengths of approximately 5–10 cm (Munro, 1976; D.R.R., pers. obs.). The pelagic duration of *M. flavolineatus* is 45–50 days (Robertson *et al.*, 2004; Pothin *et al.*, 2006). Larvae and pelagic juveniles of Mullidae have been found 1100 km from the nearest shore between the central and eastern Pacific (Clarke, 1995). Shared haplotypes and very small F_{ST} values between populations of *M. vanicolensis* from these two oceanic regions indicate that there has been recent exchange of genes, almost certainly by early life stages that have crossed the Eastern Pacific Barrier (Lessios & Robertson, 2006).

Here we present a molecular phylogeny of the genus *Mulloidichthys* based on global sampling of three mitochondrial genes to address the following questions: What speciation events have led to the formation of the extant species? How do they correlate with geological and oceanographic events? Are *M. dentatus* and *M. martinicus* geminate species formed by the rise of the Isthmus of Panama? What is the degree of genetic structuring in conspecific populations?

MATERIALS AND METHODS

Collections

A total of 185 specimens belonging to all species of *Mulloidichthys*, except for the recently described *M. ayliffe* (Uiblein, 2011), were collected in 23 localities around the world (Fig. 1). One specimen of the spotted goatfish *Pseudupeneus maculatus* from Belize was used as the outgroup in phylogenetic analyses. A phylogeny based on 26 morphological characters has indicated that *Pseudupeneus* (along with *Parupeneus*) is the sister genus of *Mulloidichthys* (Kim, 2002). Genomic DNA was extracted from gill, skeletal muscle, or fin tissue either with direct digestion by Proteinase K (Lessios *et al.*, 1996) or with traditional phenol/chloroform extraction procedures.

DNA amplification and sequencing

Three regions of mtDNA were amplified: (1) the ATP synthetase 8 and ATP synthetase 6 (*ATPase-8* and *ATPase-6*) regions were amplified with primers ATP8.2 (5'-AAAGCRTYRGCCCTTAAAGC-3') and CO3.2 (5'-GTTAGTGGTCAKGGGCTTGGRTC-3') in a total volume of 50 μ L containing 0.2–0.5 μ L of template DNA, 2.5 μ L of each primer, 1 μ L of each dNTP, 5 units of Master Amp *Tfl* DNA Polymerase (Epicentre, Madison, WI, USA), 2.5 μ L of Master Amp *Tfl* 20 \times PCR buffer, and 5 μ L of 2.5 mM MgCl₂. Amplification reactions consisted of one initial cycle of heating to 94 $^{\circ}$ C for 5 s, followed by 39 cycles of denaturation at 94 $^{\circ}$ C for 30 s, primer annealing at 50 $^{\circ}$ C for 45 s, extension at 72 $^{\circ}$ C for 1 min, and ending with a 5-min incubation at 72 $^{\circ}$ C. Amplification products were separated in agarose minigels, then cycle-sequenced in both directions with the same primers as in their respective amplifi-

cations and electrophorezed in a model 373A or 377 automatic sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). The resulting sequences were trimmed to include only 842 bp between the initiation codon of *ATPase-8* and the stop codon of *ATPase-6*. (2) The control (D-loop) region was amplified with primers ProL (5'-TACCTCCAACCTCCCAAAGC-3') and SHA (5'-TCGACTGTTTACCAAAAACATA-3') in a mixture of the same composition as *ATPase*. Amplification reactions consisted of one cycle of heating to 94 $^{\circ}$ C for 2 min, followed by 5 cycles of denaturation at 94 $^{\circ}$ C for 30 s, primer annealing at 50 $^{\circ}$ C for 1 min, extension at 72 $^{\circ}$ C for 2.5 min, followed by 30 cycles of 94 $^{\circ}$ C for 30 s, 50 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 2.5 min, and ending with 5 min at 72 $^{\circ}$ C. After cleaning as above, the amplification products were cycle-sequenced in both directions using primer ProL and either primer ProLR1 (5'-TGGCAAGGARTGTGTACAC-3') or ProLR2 (5'-TGGARA GAAYGCTCGGCAT-3') to produce sequences up to 583 bp long from the highly variable (Lee *et al.*, 1995) 5' end of the control region, near the tRNA-Proline end. Sequences have been deposited in GenBank under accession numbers DQ111479–DQ111521 (*ATPase-8* and *ATPase-6* sequences of *Mulloidichthys vanicolensis* used by Lessios & Robertson, 2006), JX951432–JX951574 (the remaining *ATPase-8* and *ATPase-6* sequences) and JX951575–JX951760 (D-loop sequences).

Analysis of data

Phylogeny of the species

Sequences were aligned with CLUSTAL X 1.81 (Higgins *et al.*, 1996) and then adjusted manually. Gaps in the D-loop region were treated as missing data. The sequences from the two separate mtDNA regions were first examined for saturation

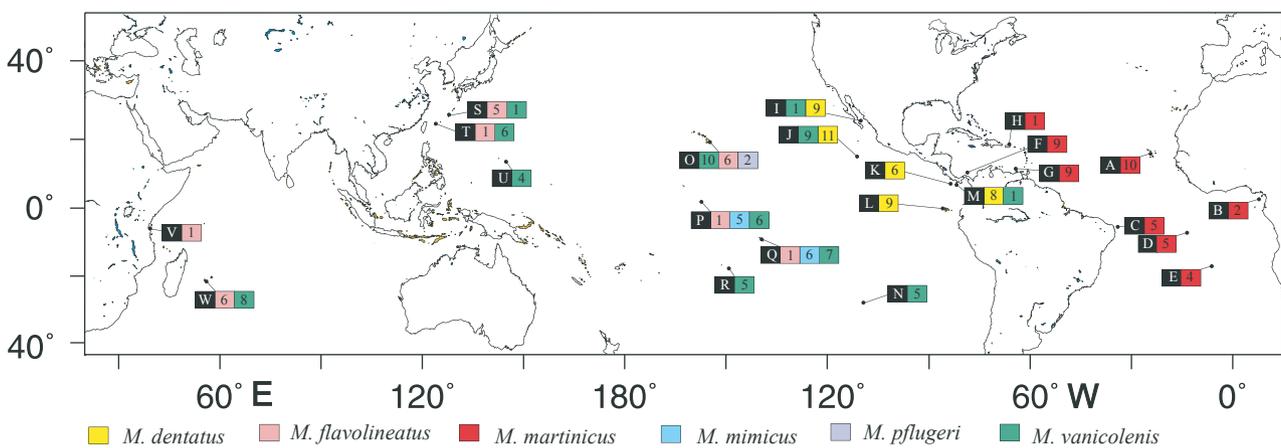


Figure 1 Collection localities and sample sizes of the genus *Mulloidichthys*. The box with the black background indicates the locality code; the rest of the boxes indicate the sample size of each species, colour-coded according to the legend. Locality codes: Eastern Atlantic: A: Cape Verde Islands; B: São Tomé, Brazil; Western Atlantic: C: Fernando de Noronha, Brazil; Central Atlantic: D: Ascension Island; E: St. Helena Island; Caribbean: F: San Blas Islands and Portobelo, Panama; G: Los Roques, Venezuela; H: Enrique, Puerto Rico; Eastern Pacific I: Los Islotes and San Ignacio Island, Sea of Cortez; J: Clipperton Atoll; K: Isla del Coco, Costa Rica; L: Isla Bartolomé and Isla Marchena, Galápagos; M: Isla Coiba and Isla Jicarón, Gulf of Chiriquí; N: Easter Island; Central Pacific: O: Oahu, Hawaii; P: Kiribati, Kiritimati; Q: Marquesas; R: Moorea; Western Pacific: S: Akajima Island and Sesoko Island, Japan; T: Ishigaki Island, Japan; U: Guam; Indian Ocean: V: Zanzibar Island; W: Réunion.

with Xia *et al.*'s (2003) entropy-based test. In both regions, the index of substitution saturation was significantly smaller than expected from a saturated symmetric or asymmetric tree ($P = 0$). Each DNA region (D-loop, *ATPase-8* and *ATPase-6*) was subjected separately to jMODELTEST v. 1.0 (Posada, 2008) to determine the best model of DNA substitution. According to the Akaike information criterion (AIC) these models were: for D-loop, the general time reversible model (Tavare, 1986) with a proportion 0.24 of invariable sites and a γ correction ($\alpha = 0.59$) (GTR+G+I); for *ATPase-8*, the Hasegawa *et al.* (1985) model with a transition/transversion ratio of 3.435 and a proportion 0.51 of invariable sites (HKY+I); and for *ATPase-6* the Hasegawa *et al.* (1985) model with a transition/transversion ratio of 3.115 and a γ correction ($\alpha = 0.25$) (HKY+G).

The sequences from the three regions were subjected to Zelwer *et al.*'s (2004) BIONJ test to determine whether they produced compatible phylogenetic trees. The test indicated that they contained significantly conflicting phylogenetic signals. A Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999) performed in PAUP* 4.0b10 (Swofford, 2002) with parameters estimated by maximum likelihood (ML) also suggested that the trees produced by the three DNA regions were significantly different. Examination of bootstrapped trees based on each sequenced region showed that the conflicts were the result of poorly supported clades. When clades with > 80% support were collapsed, there were no conflicts, but resolution in each tree was also poor.

Given the results regarding the compatibility of trees produced by D-loop, *ATPase-8* and *ATPase-6*, it was considered prudent to analyse concatenated sequences of 1425 bp in a manner that took into account rate and base composition heterogeneity between them. The data set was reduced to unique haplotypes and then two approaches were used, as follows:

1. The data were partitioned to the three DNA regions (D-loop, *ATPase-8* and *ATPase-6*), and the best model of DNA evolution as suggested by the AIC was applied to each unlinked partition of the data (Nylander *et al.*, 2004). The Bayesian phylogenetic reconstruction was performed in MRBAYES 3.2.1 (Ronquist & Huelsenbeck, 2003) with a heating parameter of $T = 0.01$. The parameter values for each model were estimated by the program, starting from Dirichlet priors. We calculated clade credibility values from 375,000 trees by sampling every 100th tree of a total of 50,000,000, after discarding the first 125,000 trees as burn-in. Convergence was determined from multiple runs, all of which produced the same topology, average split frequencies of < 0.01, and potential scale reduction factors equal to 1.00. We will refer to this reconstruction as the 'gene-partitioned tree'.
2. Instead of partitioning the data by gene, they were partitioned into four categories, one for the entire D-loop region and one for each of the codon positions of the ATPase genes. jMODELTEST was used to determine the model with the smallest AIC value for each of these partitions. To avoid assigning the same nucleotides to two separate categories, the 7 bp region at the 3' end of *ATPase-8* that overlaps with the

5' end of *ATPase-6* (invariant in all individuals of *Mulloidichthys*), was not used. Using these models for the four partitions, we reconstructed the phylogeny of *Mulloidichthys* in MRBAYES, letting the program estimate the parameters from Dirichlet priors. We calculated clade credibility values from 67,500 trees by sampling every 100th tree of a total of 9,000,000 after discarding the first 22,500 trees. Convergence was assessed in the same manner as above. We will refer to this reconstruction as the 'four-category tree'.

To estimate relative times of divergence between the species of *Mulloidichthys*, and their confidence limits we used PATHd8 1.0 (Britton *et al.*, 2002), using the topology and branch lengths of the gene-partitioned tree.

Comparison of transisthmian divergence with other genera

In order to ascertain whether divergence across the Isthmus of Panama in *Mulloidichthys* was compatible with that of other geminate pairs of fish, 15 presumed pairs were selected for sequencing of the same DNA regions as *Mulloidichthys*. In order to compare divergence values between this diverse array of genera, we calculated in MEGA 5 (Tamura *et al.*, 2011) maximum composite distances between the members of each pair, without applying any corrections for rate heterogeneity or invariable sites. Data from the 15 sister pairs have been deposited in GenBank under accession numbers KF021321–KF021425 (*ATPase-8* and *ATPase-6* sequences) and KF021426–KF021488 (D-loop sequences).

Intraspecific variation

To determine intraspecific genetic subdivisions among populations from different geographical localities, we calculated F_{ST} statistics (Wright, 1951) on the basis of the concatenated sequences, using ARLEQUIN 3.5.1.2 (Excoffier *et al.*, 2005). The best model of DNA evolution according to jMODELTEST for the entire concatenated sequences was GTR+G+I, but as ARLEQUIN does not provide this option, we based the Φ_{ST} statistics on Tamura & Nei (1993) genetic distances assuming a γ distribution with an α value of 0.449. Samples of populations with fewer than four individuals were excluded from the calculation of pairwise Φ_{ST} values. The probability of obtaining these values by chance was estimated from 10,000 random reshufflings of haplotypes between populations. To compare differentiation between major oceanic regions, we used analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) with the same number of reshufflings. Nucleotide diversity was also calculated in ARLEQUIN based on Tamura & Nei (1993) distances.

To find out whether any of the geographically widespread species with adequate sampling (*M. martinicus*, *M. dentatus*, *M. vanicolensis*) showed a pattern of isolation by distance, we used IBDWS 3.23 (Jensen *et al.*, 2005; <http://ibdws.sdsu.edu/~ibdws/>) to correlate Φ_{ST} values with the shortest geographical distance by sea between localities according to the procedure of Mantel (1967). For the purposes of this

analysis, negative values of Φ_{ST} were replaced by 0. Ten thousand reshufflings were used to generate null distributions for the Mantel test.

RESULTS

Species phylogeny

In *ATPase-8* and *ATPase-6*, the 186 sampled individuals (including the outgroup) had 80 unique haplotypes. In D-loop the same 186 individuals had 168 haplotypes. Four of these had differences in the *ATPase* genes, so that the concatenated sequences consisted of 172 unique haplotypes. The topologies of the gene-partitioned tree and the four-category tree agreed almost entirely, except for two terminal nodes joining individuals of the same species. The phylogenetic relationships between the haplotypes are shown in Fig. 2. Sequences of all recognized species of *Mulloidichthys*, except for those of *M. mimicus*, were monophyletic. The species found at a few oceanic islands of the Indo-Pacific, *M. pflugeri*, was the first to split from common stock, followed by the Indo-Pacific *M. flavolineatus*. *Mulloidichthys mimicus*, the putative snapper mimic from the central Pacific, split next. One individual from Kiritimati and one from the Marquesas identified by D.R.R. on the basis of colour pattern as belonging to this species formed a separate clade, sister to the remaining species of *Mulloidichthys*. This clade could be the result of incomplete sorting, or these individuals might indicate the presence of another undescribed species resembling *Lutjanus kasmira*. The sister clade to all other species of *Mulloidichthys* consists of a tritomy composed of the pan-Indo-Pacific *M. vanicolensis*, the eastern Pacific *M. dentatus*, and the Atlantic *M. martinicus*.

Timing of speciation events

The topology of the phylogenetic tree of *Mulloidichthys* indicates that the common ancestor of *M. vanicolensis*, *M. dentatus* and *M. martinicus* was present around the globe until the lineage leading to *M. martinicus* became isolated in the Atlantic, and the lineage leading to *M. dentatus* became isolated in the eastern Pacific. To determine the barriers that caused these speciation events it is necessary to constrain their timing. To do so, we sequenced the same mtDNA regions as those in *Mulloidichthys* in 15 other species pairs of fish with members on either side of the Isthmus of Panama, and we compared their divergence with that in cytochrome *c* oxidase subunit I (*COI*) determined by Bermingham *et al.* (1997). Table 1 shows these genetic distances, arranged in order of divergence in *ATPase-6*. As expected from linked mitochondrial genes, relative differentiation between members of each pair in *ATPase-6* and *COI* are in general agreement. Not surprisingly considering the small number of base pairs involved, the agreement is less tight in *ATPase-8*. The control region, on the other hand, shows a great deal of rate variation, out of step with relative rates in *ATPase-8*,

ATPase-6 and *COI*. Given the way this mitochondrial region is known to evolve, with a great many insertions and deletions and tandemly repeated motifs (Lee *et al.*, 1995), it would be risky to rely on it for estimating time since divergence between clades. Rates of differentiation in *ATPase-6* and *COI* appear much more reliable as rough molecular clocks. On the basis of data from these two regions, genetic distances between members of transisthmian species pairs other than *Mulloidichthys* fall in three groups: (1) those with values < 1% in both *ATPase-6* and *COI*; (2) those with values in the range of 1.59–6.79% in *ATPase-6* and 1.42–3.48% in *COI*; and (3) those with values > 12.83% in *ATPase-6* and > 9.37% in *COI*. All comparisons in Group 1 are between Atlantic and Pacific populations of species with circumtropical distributions. It is not unreasonable to assume that conspecific Atlantic and eastern Pacific populations in this group maintain genetic contact through stepping stones around the globe; that the members of the pairs in Group 2, with intermediate amounts of differentiation were split at some point close to the severance of water connections by the central American Isthmus; and that species in pairs with large divergences had speciated before the isthmus was completed. Thus, the cluster with intermediate distances (Group 2) can provide a rough estimate of substitution rates in fish *ATPase-6*, assuming that gene flow between members of the species pairs was interrupted approximately 3 Ma (Coates & Obando, 1996). The mean divergence in this group is 3.43%, corresponding to a rate of 1.14% per Myr. The genetic distance between *Mulloidichthys dentatus* and *M. martinicus* in *ATPase-6* and *COI* is the smallest among all transisthmian species pairs in which Atlantic and Pacific populations are sufficiently divergent in their morphology to be assigned to different species (Table 1). If the rate of divergence in *ATPase-6* is assumed to be 1.14% per Myr, then *M. dentatus*, *M. martinicus* and *M. vanicolensis* shared a common ancestor until 0.8–0.9 Ma. By entering this rate in the PATHd8 analysis, we estimate the split between these three species and *M. flavolineatus* as having occurred 1.31 ± 0.05 Ma (estimate \pm confidence interval, CI), the speciation event of *M. flavolineatus* as having occurred 3.73 ± 0.10 Ma, and that of *M. pflugeri* 5.66 ± 0.13 Ma. The alternative hypothesis, that *M. dentatus* and *M. martinicus* were isolated from each other by the rise of the Isthmus of Panama 3 Ma, would date these events as 3.6 times older.

Intraspecific variation

Although there are well-supported subclades within each species of *Mulloidichthys*, they contain haplotypes from different regions and distant localities (Fig. 2). Thus, there is no intraspecific phylogeographical structure. The high substitution rate of the control region has resulted in unique concatenated haplotypes of mtDNA in most individuals, which minimizes the probability that shared haplotypes are the result of convergence or parallel evolution. Thus, the few instances of shared haplotypes may be the result of migration. If so,

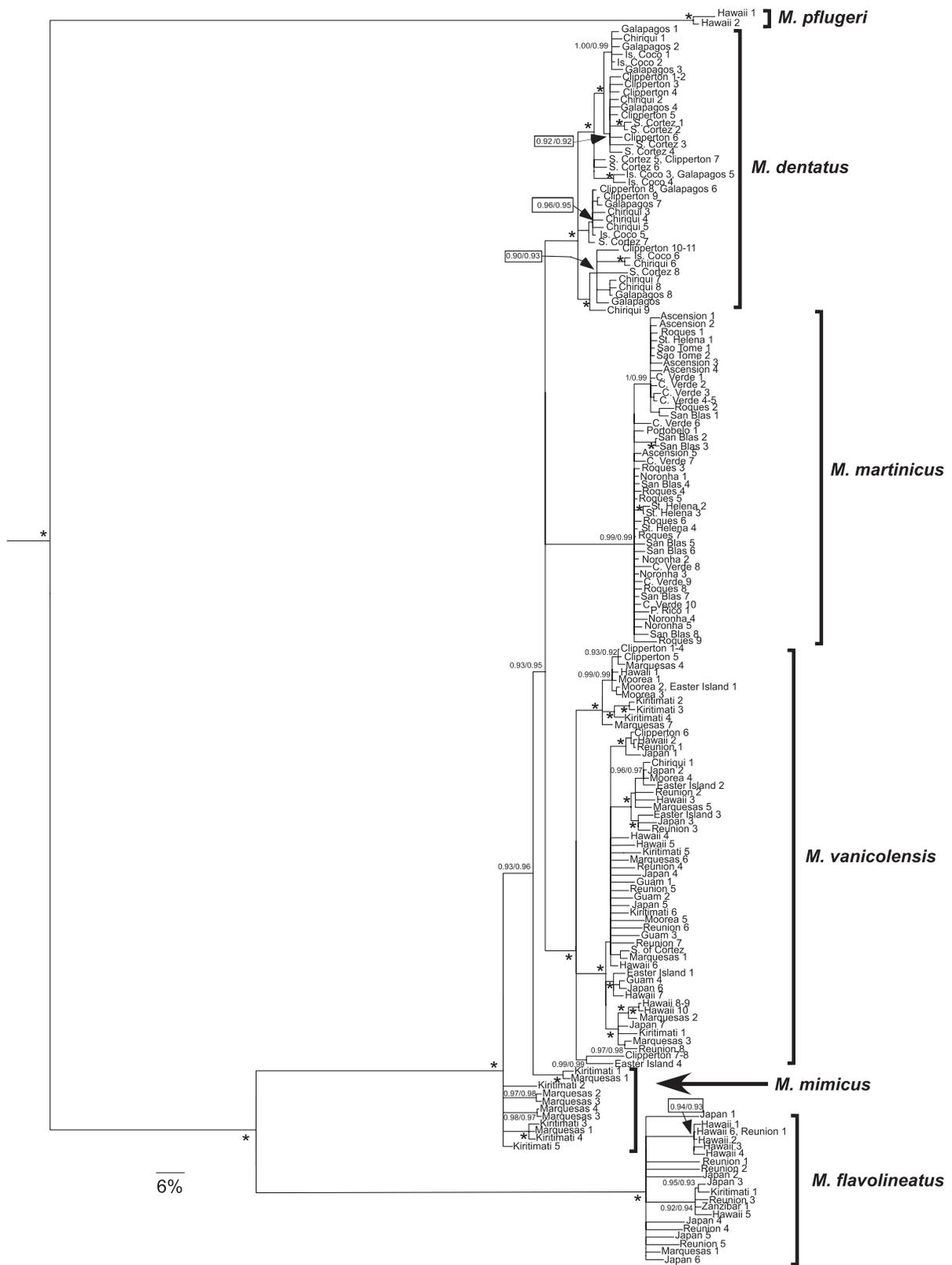


Figure 2 Phylogeny of concatenated sequences of the control region, *ATPase-8* and *ATPase-6* of *Mulloidichthys*, reconstructed using MRBAYES 3.2.1. The tree is rooted on one sequence of *Pseudupeneus maculatus* and is based on both the ‘gene partition’ and the ‘four-category’ model of DNA evolution (see text). Numbers next to nodes indicate Bayesian support from each of the two trees. Asterisks indicate nodes supported at a probability of 1 by both methods. Nodes with < 0.9 Bayesian support in the tree resulting from either model have been collapsed. Support values of some terminal nodes have been omitted for the sake of clarity. Numbers after locality names of terminal branches indicate the identity of individuals with indistinguishable haplotypes. Scale bar represents 6% genetic divergence.

Table 1 Number of sequences (NP: eastern Pacific species; NA: Atlantic species) and maximum composite likelihood distances (MCL; Tamura *et al.*, 2004) between pairs of species on either side of the Central American Isthmus in partial sequences of the control region, and in the complete *ATP8ase-8* and *ATP6ase-6* mitochondrial DNA genes. Genetic distances in *COI* are from Bermingham *et al.* (1997). The table is arranged in increasing divergence in *ATPase-6*. Values proposed as corresponding with the completion of the Isthmus of Panama (see text) are designated as Group 2.

Genus	Eastern Pacific	Western Atlantic	Control region			<i>ATPase-8</i>			<i>ATPase-6</i>			<i>COI</i>			Group
			NP	NA	MCL (%)	NP	NA	MCL (%)	NP	NA	MCL (%)	NP	NA	MCL (%)	
<i>Diodon</i>	<i>hystrix</i>	<i>hystrix</i>	3	1	0.40	3	2	0.00	3	2	0.00	5	3	0.58	1
<i>Aluterus</i>	<i>scriptus</i>	<i>scriptus</i>	2	2	1.23	2	2	0.30	2	2	0.29	2	4	0.87	1
<i>Melichthys</i>	<i>niger</i>	<i>niger</i>	2	2	6.12	2	2	0.60	2	2	0.44	4	2	0.18	1
<i>Diodon</i>	<i>holacanthus</i>	<i>holacanthus</i>	2	2	2.57	55	8	0.32	55	8	0.58	–	–	–	1
<i>Mulloidichthys</i>	<i>dentatus</i>	<i>martinicus</i>	43	45	10.24	43	45	0.07	43	45	0.94	5	5	1.26	
<i>Abudefduf</i>	<i>concolor</i>	<i>taurus</i>	1	2	9.83	3	3	0.60	2	3	1.59	3	2	1.42	2
<i>Anisotremus</i>	<i>interruptus</i>	<i>surinamensis</i>	2	2	11.17	2	2	1.21	2	2	1.94	5	2	1.62	2
<i>Holacanthus</i>	<i>passer</i>	<i>ciliaris</i>	1	2	21.87	2	2	1.21	2	2	2.22	2	1	4.87	2
<i>Abudefduf</i>	<i>trocheli</i>	<i>saxatilis</i>	2	2	19.89	6	5	0.00	6	5	2.50	3	4	4.49	2
<i>Anisotremus</i>	<i>taeniatus</i>	<i>virginicus</i>	1	2	11.11	2	2	3.74	3	2	3.79	2	2	4.36	2
<i>Gerres</i>	<i>cinereus</i>	<i>cinereus</i>	2	2	19.27	2	2	1.35	2	2	3.95	2	3	5.07	2
<i>Rypticus</i>	<i>bicolor</i>	<i>saponaceus</i>	9	10	9.70	12	11	5.41	12	11	4.66	3	2	3.17	2
<i>Lutjanus</i>	<i>argentiventris</i>	<i>apodus</i>	2	4	13.27	2	4	4.07	2	4	6.79	2	2	3.48	2
<i>Ophioblennius</i>	<i>steindachneri</i>	<i>macclurei</i>	1	1	15.95	1	1	5.64	1	1	12.83	3	3	12.37	3
<i>Chromis</i>	<i>atrilobata</i>	<i>multilineata</i>	5	5	16.02	7	7	4.34	7	7	15.47	3	2	9.37	3
<i>Chaetodon</i>	<i>humeralis</i>	<i>striatus</i>	2	2	38.87	2	2	14.40	2	2	19.88	3	3	10.40	3

migration events within a period of time shorter than would be required to accumulate base differences between distant populations are evident by indistinguishable haplotypes of *M. dentatus* at the Clipperton Atoll and the Sea of Cortez, as well as at Isla del Coco and the Galápagos Archipelago; of *M. vanicolensis* at Moorea and Easter Island (4300 km away); and (most impressively) of *M. flavolineatus* at Hawaii in the northern central Pacific and Réunion in the south-western Indian Ocean, a distance of 17,000 km (Fig. 2).

Lack of genetic differentiation between oceanic regions was also indicated by the AMOVA analyses of haplotypes within each species that spans more than one such region (Table 2). In all cases, the Φ_{CT} values between populations of different oceanic regions were not significant, and they explained very little of the total variation. Although there was little differentiation between regions, in *M. vanicolensis* and *M. martinicus* there was modest and significant differentiation between populations within a region. Pairwise F_{ST} statistics (Table 3)

indicated that these differences were caused by the isolation of the population of *M. vanicolensis* at Clipperton Atoll from six central Pacific populations and one Indian Ocean population, and by isolation of the *M. martinicus* population at Ascension Island from three of four western Atlantic populations. One striking difference between the species of *Mulloidichthys* was that nucleotide diversity within all *M. martinicus* populations was smaller than diversity in all other congeners, except for that of *M. flavolineatus* at Hawaii (Table 3).

Despite the wide range of geographical and genetic distances involved in the pairwise comparisons between samples, no pattern of isolation by distance was found. Coefficients of the Mantel correlations between Φ_{ST} and geographical distance were -0.31 for *M. martinicus*, 0.30 for *M. vanicolensis*, and 0.51 for *M. dentatus*, all of them not significant at the $P = 0.05$ level. Logarithmic transformations of each (or both) of the two variables produced similarly non-significant correlation coefficients. When the most

Table 2 Analysis of molecular variance (AMOVA) within species of *Mulloidichthys* found in different biogeographical regions. The analysis is based on Tamura & Nei (1993) distances with a γ correction ($\alpha = 0.449$) of the concatenated sequences. Significance was determined by randomly reshuffling haplotypes between populations 10,000 times.

Species	Regions	Between regions		Between populations		Within populations
		Variation (%)	Φ_{CT}	Variation (%)	Φ_{ST}	Variation (%)
<i>M. dentatus</i>	E. Pacific Islands, American mainland	-1.36	-0.014 NS	1.36	-0.000 NS	100.00
<i>M. flavolineatus</i>	Central Pacific, Western Pacific, Indian Ocean	-28.90	-0.289 NS	35.74	0.068 NS	93.16
<i>M. vanicolensis</i>	East Pacific, Central Pacific, Western Pacific, Indian Ocean	8.27	0.083 NS	6.49	0.148**	85.24
<i>M. martinicus</i>	East Atlantic, Central Atlantic, West Atlantic	6.04	0.064 NS	7.63	0.137**	86.33

NS, not significant; ** $P < 0.005$.

Table 3 Sample sizes, nucleotide diversity (in italics along the diagonal) and pairwise F_{ST} statistics between conspecific populations of *Mulloidichthys* with $n \geq 4$. The analysis is based on Tamura & Nei (1993) distances with a γ correction ($\alpha = 0.449$). Significance was determined by randomly reshuffling haplotypes between populations 10,000 times. Significant values are shown in bold ($P < 0.05$).

<i>M. dentatus</i>	<i>n</i>	Clipperton	Is. Coco	Galápagos	Sea of Cortez	Panama				
Clipperton	11	<i>0.0129</i>								
Is. Coco	6	0.004	<i>0.0160</i>							
Galápagos	9	-0.018	-0.082	<i>0.0154</i>						
Sea of Cortez	9	-0.033	0.008	-0.004	<i>0.0154</i>					
Panama	8	0.029	0.007	-0.031	0.057	<i>0.0159</i>				
<hr/>										
<i>M. flavolineatus</i>		Hawaii	Japan	Réunion						
Hawaii	6	<i>0.0072</i>								
Japan	6	0.179	<i>0.0168</i>							
Réunion	6	0.055	-0.073	<i>0.0147</i>						
<hr/>										
<i>M. vanicolensis</i>		Clipperton	Easter I.	Guam	Hawaii	Japan	Kiritimati	Marquesas	Moorea	Réunion
Clipperton	9	<i>0.0139</i>								
Easter I.	5	0.156	<i>0.0254</i>							
Guam	4	0.364	-0.001	<i>0.0125</i>						
Hawaii	10	0.307	0.022	-0.005	<i>0.0158</i>					
Japan	7	0.393	0.033	-0.061	0.011	<i>0.0152</i>				
Kiritimati	6	0.116	0.007	0.097	0.083	0.155	<i>0.0236</i>			
Marquesas	7	0.177	-0.067	-0.011	-0.044	0.036	-0.032	<i>0.0198</i>		
Moorea	5	0.026	-0.053	0.189	0.153	0.218	-0.013	0.004	<i>0.0204</i>	
Réunion	8	0.395	0.038	-0.052	0.015	-0.062	0.157	0.021	0.217	<i>0.0138</i>
<hr/>										
<i>M. martinicus</i>		Ascension I.	Brazil	Los Roques	Panama	St. Helena	C. Verde			
Ascension I.	5	<i>0.0070</i>								
Brazil	5	0.449	<i>0.0038</i>							
Los Roques	9	0.186	0.023	<i>0.0082</i>						
Panama	9	0.210	0.068	-0.012	<i>0.0086</i>					
St. Helena	4	0.200	0.161	-0.019	0.028	<i>0.0077</i>				
Cape Verde	10	0.054	0.188	0.040	0.049	0.042	<i>0.0083</i>			
<hr/>										
<i>M. mimicus</i>		Marquesas	Kiritimati							
Marquesas	6	<i>0.0138</i>								
Kiritimati	4	-0.043	<i>0.0161</i>							

divergent populations in each species were excluded, the correlation coefficients remained non-significant (*M. martinicus* without Ascension Island $r = -0.40$, $P = 0.946$; *M. vanicolensis* without Clipperton Atoll $r = 0.257$, $P = 0.142$).

DISCUSSION

The genealogy of the extant species of *Mulloidichthys* based on concatenated sequences of the control region, *ATPase-8* and *ATPase-6* indicates that haplotypes that belong to each species (except of *M. mimicus*) form monophyletic entities. Within the last 6 Myr, there were three speciation events in the Indo-Pacific before a final split, probably within the last 1 Myr, in which the most widespread species (west coast of America to east coast of Africa), *M. vanicolensis*, was separated at one end from the eastern Pacific *M. dentatus*

and – at about the same time – at the other end from the Atlantic *M. martinicus*.

How well does the mitochondrial tree reflect the history of speciation events? The reliance of phylogeographical inferences on mtDNA has been questioned, because similarity of mtDNA between sympatric species can be the result of introgression across species lines, and because the molecule can evolve under selection (Ballard & Whitlock, 2004). In *Mulloidichthys*, however, previously published protein electrophoresis results provide independent support that the mitochondrial tree is, in fact, the species tree, because relative interspecific divergences in mtDNA are reflected in isozymes. Shaklee *et al.* (1982) assayed 26 isozyme loci and found that the value of Nei's D between *M. pflugeri* and *M. flavolineatus* was 0.74, between *M. pflugeri* and *M. vanicolensis* was 0.61, and between *M. flavolineatus* and

M. vanicolensis was 0.34. Stepien *et al.* (1994) assayed 42 loci and found that the values of Nei's *D* between different populations of *M. vanicolensis* and *M. dentatus* ranged between 0.087 and 0.115. Vawter *et al.* (1980) assayed 23 loci and found that Nei's *D* between *M. dentatus* and *M. martinicus* was 0.168. Although it is dangerous to compare genetic distances obtained from different isozyme studies, because the determined values of divergence depend on the actual mix of loci, genetic distances in isozymes are in accordance with the mtDNA tree in showing that there is a distant relationship between *M. pflugeri* and *M. flavolineatus* on the one hand, and the other three species of *Mulloidichthys*, on the other.

The members of the *M. dentatus*–*M. vanicolensis*–*M. martinicus* tritomy are descendants of a common ancestor that maintained connections across the world's tropical oceans until recently. Such connections could be the result of a very widespread distribution sundered by vicariance events, or of dispersal events followed by isolation. How recently the three species separated from each other is not a straightforward question, but it does affect the interpretation of the entire history of the formation of all extant species of *Mulloidichthys*. Given the topology of the phylogenetic tree, and given that the divergence between the sister species across the Isthmus of Panama is so low relative to other – similarly separated – fish species, two possibilities exist. One is that *M. dentatus* and *M. martinicus* were actually separated by the formation of the Isthmus of Panama 3 Ma, and hence that the rate of evolution of *ATPase-8* and *ATPase-6*, as well as *COI*, in this genus is exceptionally slow. The other possibility is that there has been genetic exchange between what is now the Atlantic lineage and what is now the eastern Pacific lineage – via the Indian and Pacific Oceans – until well after the completion of the isthmus.

Some variation in the divergence values of different species of the same taxon across a barrier is expected, because of possible differences of the exact time of cessation of gene flow, and because of the vagaries of the coalescent process (Hickerson *et al.*, 2006). However, it is unlikely that these factors have caused the rate of *ATPase-8* and *ATPase-6* in *Mulloidichthys* to be 3.6 times slower than that of other species separated by the Isthmus of Panama. Similarly, the proposal that the isthmus might have been briefly breached at 2 Ma (Cronin & Dowsett, 1996) would not explain why *Mulloidichthys*, *Diodon*, *Melichthys* and *Aluterus* crossed the breach, whereas most other genera did not. An additional potential factor for low rates of evolution is that we might have accidentally amplified nuclear pseudogenes instead of the mitochondrial genes. Such nuclear pseudogenes tend to evolve at slower rates than their mitochondrial counterparts (Zischler *et al.*, 1995). The possibility that we sequenced pseudogenes in *Mulloidichthys* is remote. All of our *ATPase-8* and *ATPase-6* sequences were translated into amino acids without stop codons, which would rule out anciently incorporated pseudogenes. Pseudogenes incorporated so recently that they have not acquired mutations interrupting the open reading frame would also not substantially affect substitution rates. What is

more, *COI* also shows similarly reduced amounts of divergence in *Mulloidichthys*. It is highly unlikely that recently incorporated pseudogenes were sequenced in both cases.

The hypothesis that the most recent speciation event among extant species of *Mulloidichthys* was contemporaneous with the completion of the Isthmus of Panama becomes even more remote if one were to accept the proposal of Montes *et al.* (2012) that there was uninterrupted land between North and South America no later than 12 Ma. This hypothesis, however, is not likely to be correct. The interpretation offered by Montes *et al.*, based on their geological data from land, conflicts with extensive palaeoceanographic data which indicate that temperature, salinity and productivity patterns did not become different between the eastern Pacific and the Caribbean until approximately 4 Ma (e.g. Collins, 1996; Collins *et al.*, 1996a,b; Haug & Tiedemann, 1998; O'Dea *et al.*, 2007). If the eastern Pacific and the Caribbean were separated 12 Ma, the values of genetic divergence of fish mtDNA regions shown in Table 1, as well as differentiation in both mitochondrial and nuclear genes of sea urchins, crustaceans and molluscs (Lessios, 2008), would require that there was either an extreme slow-down in rates of molecular evolution in all of these geminate species, or else that they all maintained connections around the world in more recent times. In five of the genera included in our transisthmian comparisons (*Anisotremus*, *Holacanthus*, *Gerres*, *Rypticus* and *Ophioblennius*) the latter case is even more unlikely, because they lack extant species in the Indo-Pacific.

Although it is highly unlikely that the members of every single geminate pair of fishes on either side of the Isthmus of Panama were connected by gene flow through the Indo-Pacific after the rise of the Isthmus of Panama, it appears that this was the case in *Mulloidichthys*. If the divergence between *M. martinicus*, *M. dentatus* and *M. vanicolensis* is calibrated by the rate of substitution in *ATPase-8* and *ATPase-6* in other genera, gene flow between these three species was probably interrupted in the Pleistocene *c.* 0.8–0.9 Ma. Whether genetic connections consisted of a widespread common ancestor sundered by vicariance events, or of single pulses of colonization that were subsequently cut off from the source population cannot be inferred from the phylogeny. That molecular diversity of all *M. martinicus* populations is so much smaller than that of populations of all other species of *Mulloidichthys* suggests that the Atlantic was, in fact, colonized from the Indian Ocean in a single pulse. Such post-Isthmian connections between these two oceans have also been inferred from genetic data in other fishes (*Aulostomus*, Bowen *et al.*, 2001; *Gnatholepis*, Rocha *et al.*, 2005; and *Centropyge*, Bowen *et al.*, 2006).

The oceanographic obstacle to migration between *M. vanicolensis* and *M. dentatus* would be the Eastern Pacific Barrier. This 5000 km expanse of water is difficult to cross within a single larval duration and, as a result, most genera are represented by different species on either side. It is, however, still being breached by some 'transpacific' species of fishes (Robertson *et al.*, 2004), some of them frequently, others

only occasionally (Lessios & Robertson, 2006). *Mulloidichthys vanicolensis* is one of these species, but presumably there was a period of separation between eastern and central Pacific populations that allowed a speciation event before *M. vanicolensis* recolonized the eastern Pacific. The barrier that separated the westernmost populations of *M. vanicolensis* from *M. martinicus* was probably the Benguela upwelling off the coast of Southeast Africa (Shannon, 1985). The cold water of this upwelling area is a barrier for most tropical species, although its intensity has fluctuated since the beginning of the Pleistocene (Marlow *et al.*, 2000). The Agulhas Current, which carries warm water south along the east coast of South Africa, but is then retroflected to the south-east into the central Indian Ocean, has also been fluctuating (Hutson, 1980). This current often produces intrusions of warm water into the Atlantic (Shannon *et al.*, 1990). It is difficult to propose likely barriers in the central Pacific and the Indo-West Pacific responsible for the speciation events that led to *M. pflugeri*, *M. flavolineatus* and *M. mimicus*. If the separation of these species was due to vicariance, the signature of the event may have been erased by subsequent range expansions. Alternatively, these species may have originated through some form of sympatric or parapatric speciation (Rocha & Bowen, 2008).

Lack of phylogeographical structure within each species of *Mulloidichthys* supports the hypothesis of long-range past genetic connections, resulting in the continuity of ancestral populations until the Pleistocene. The only instances of apparent genetic isolation among populations of the same species of *Mulloidichthys* are those at some, but not all, of the remote islands. The population of *M. vanicolensis* at Clipperton Atoll is cut off from most localities in the central and west Pacific, although that of *M. dentatus* at the same atoll receives high genetic influx from the American mainland, 1000 km away. The population of *M. martinicus* at Ascension Island has recently exchanged few genes with populations on the American coast, although it is connected to Cape Verde off the African coast. Interestingly, however, other localities that, on the basis of their geography, might be expected to be equally isolated are not, and for this reason there is no signal of isolation by distance in any of the species. *Mulloidichthys vanicolensis* at Easter Island, 1900 km from the nearest shallow reef, is not differentiated from other localities we sampled, not even from Réunion in the Indian Ocean, 23,000 km away. Both *M. flavolineatus* and *M. vanicolensis* at Hawaii are (with few exceptions) connected to populations in the central and west Pacific as well as Réunion, 17,000 km away. *Mulloidichthys martinicus* at St. Helena is not genetically different from populations in the Caribbean despite its location, 1200 km more distant from the Americas than Ascension Island. Unlike Brazilian populations of surgeonfish that are blocked from exchanging genes with Caribbean ones by the outflows of the Orinoco and the Amazon (Rocha *et al.*, 2002), the population of *M. martinicus* at Fernando de Noronha in Brazil is connected to Caribbean populations. Other species of fishes with long-

lived planktonic stages, such as surgeonfishes (Horne *et al.*, 2008) and moray eels (Reece *et al.*, 2011) also show genetic connections over long oceanic distances.

In conclusion, extant species of the pantropical genus *Mulloidichthys* have speciated from each other within the last 6 Myr. They show signs of high capacity of gene flow, imparted by large, highly mobile and long-lived pelagic juveniles. This capacity has apparently permitted trans-global connections until the last 0.8–0.9 Myr. Among the four most widespread species, the general pattern is one of long-distance intraspecific connectivity, with sporadic isolation of some remote islands.

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