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Mechanisms of speciation and faunal enrichment in Atlantic parrotfishes

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Abstract

Relationships based on mtDNA and nDNA sequences were used to assess effects of two major geographic barriers (the >30 myo Atlantic ocean and the ~11 myo Amazon–Orinoco outflow) on speciation among Atlantic parrotfishes (*Sparisoma* and *Nicholsina*). Allopatric distributions of sister taxa implicate isolating actions of both barriers in all recent speciation in these fishes, with no clear indications that any speciation resulted from other mechanisms. Molecular clock estimates of the timing of lineage splits indicate that both barriers acted by limiting dispersal well after they formed, although the Amazon barrier also may have been a vicariance agent. Fluctuations in sealevel, climate, and ocean-current dynamics over the past ~10 my likely produced marked variation in the effectiveness of both barriers, but particularly the Amazon barrier, allowing intermittent dispersal leading to establishment and allopatric speciation. A dynamic Amazon barrier represents a major engine of West Atlantic faunal enrichment that has repeatedly facilitated bidirectional dispersal, allopatric speciation, and remixing of the Caribbean and Brazilian faunas.
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1. Introduction

The importance of geographic barriers that limit connections between populations for the speciation of both terrestrial and marine shore organisms was well recognized by Darwin (1872). He identified two major barriers that affect tropical marine shore organisms: the Isthmus of Panama and the wide expanse of deep ocean separating the tropical Americas from the central Pacific. The consequences of the divisions of biotas by the final closure of the Isthmus of Panama 2–3.5 mya (Coates and Obando, 1996) proposed by Rosenblatt (1967) and Briggs (1974) have become well documented through genetic studies over the past decade or so (e.g. Knowlton et al., 1993; Bermingham et al., 1997; Lessios, 1998; Lessios et al., 1999, 2001, 2003; Tringali et al., 1999; Banford et al., 1999, 2004; Bernardi et al., 2003; Bowen et al., 2001; Muss et al., 2001). Speciation in reef fishes and other tropical shore organisms has been linked to the emergence of these and other historical land and marine barriers to dispersal, including the closing of the Tethys Sea (12–18 mya) that separated the Atlantic from the Indian Ocean, and the formation of the Atlantic Ocean (for review, see Bellwood and Wainwright, 2002).

Two major habitat disjunctions have affected the evolution of marine shore organisms within the tropical Atlantic: the >3500 km wide Atlantic itself, which gradually became established as a deep ocean barrier following the separation of Africa and South America ~84 mya

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(Pittman et al., 1993), and the Amazon barrier, the ~11 my old (Hoorn, 1996; Hoorn et al., 1995) freshwater outflow from the Orinoco and Amazon Rivers that spans 2300 km of the NE coast of South America and separates Brazilian and Caribbean reef habitats. Both these geographic features have long been regarded as the primary biogeographic barriers responsible for speciation that has produced marked differences in the composition of regional faunas (Ekman, 1953; Scheltema, 1968; Briggs, 1974, 1995; Floeter and Gasparini, 2000; Joyeux et al., 2001; Rocha, 2003). However, newer models of speciation among tropical West Atlantic fishes invoke mechanisms of faunal enrichment operating within regions (Streelman et al., 2002; Taylor and Hellberg, 2003; Rocha et al., 2005a,b), as distinct from between-region isolating effects of those two barriers.

The Atlantic and Amazon barriers may have acted in two ways that led to allopatric speciation: (i) as vicariance agents that, as they formed, subdivided once continuous populations (Rosen, 1975) and (ii) by restricting dispersal after they developed to levels that allow the occasional establishment of new, isolated populations (Briggs, 1974). Phylogeographic analyses, the interpretation of gene genealogies within a geographical context (Avise, 2000; Grismer, 2000; Arbogast and Kenagy, 2001), have been used to examine the historical dynamics of large-scale biogeographic patterns in relation to dispersal barriers in a range of Atlantic shore-fishes and invertebrates (Banford et al., 1999, 2004; Lessios et al., 1999, 2001, 2003; Bernardi et al., 2000; Muss et al., 2001; Bowen et al., 2001; Rocha et al., 2005a,b). Here we use such an analysis of the evolution of two closely related genera of parrotfishes, *Sparisoma* and *Nicholsina*, to assess effects of the Amazon and Atlantic barriers on speciation and faunal enrichment in these tropical reef-associated fishes.

We examine the following expectations about potential effects of the Amazon and Atlantic barriers on the evolution of these fishes: (1) If faunal enrichment resulted from allopatric speciation induced by those barriers, then sister taxa should be separated by those barriers. That pattern should be most evident among more recently diverged sisters, as geographic signals of older divergences may become obscured by signals of more recent divergences within the same lineage. If those barriers acted as vicariance agents then (2) there should be similar levels of genetic separation among different pairs of sister species separated by the same barrier; (3) there should be larger divergences between trans-Atlantic sister taxa than between trans-Amazon sisters; and (4) the calculated ages of divergences across a barrier should be similar to the barrier's estimated age. More recent divergences across a barrier than the age of that barrier, however, would indicate that dispersal across the barrier and subsequent isolation led to the production of new species. In that case, similarity in the timing of divergence among multiple sister-species pairs would suggest the effect of some major oceanographic event on dispersal, while dissimilarity could reflect some combination of variation in species dispersal characteristics, intermittent instability of the barrier, and effects of chance on which taxa participate in rare dispersal events. In contrast, if the most recently separated sisters within a variety of lineages are restricted to the same region, then it is more likely that enrichment has resulted primarily from intra-regional mechanisms than from effects of inter-regional barriers.

To examine this set of expectations, we used a group of nuclear and mitochondrial markers that should provide a reasonably complete phylogenetic picture. That combination also allows the use of a range of externally calibrated substitution rates, an important factor for parrotfishes because internal calibration is difficult due to a poor fossil record and a paucity of geminate parrotfish species separated by the rise of the isthmus of Panama, 3 mya. We used one nuclear (RAG1) and three mitochondrial (12S rRNA, 16S rRNA, Cytochrome *b*) molecular markers to evaluate historical and geographical patterns of genetic divergence: (1) across the Amazon barrier between four presumed species pairs of *Sparisoma*, and between presumed conspecific populations of one other member of that genus; and (2) between eastern and western Atlantic sisters and presumed conspecific populations of *Sparisoma* and *Nicholsina*. In addition, we evaluate the relative magnitude of (1) and (2); and the estimated ages (derived from a molecular clock) of those and other divergences in *Sparisoma* relative to the ages of the two geographic barriers.

2. Materials and methods

2.1. Study species and their systematic relationships

The parrotfish genus *Sparisoma* is restricted to the Atlantic and Mediterranean. Prior to 2001, it was thought to comprise nine species. Those included six west Atlantic species (*S. atomarium*, *S. aurofrenatum*, *S. chrysopterum*, *S. radians*, *S. rubripinne*, and *S. viride*) that were all thought to occur in both the Greater Caribbean (the Caribbean and adjacent tropical areas to the north) and Brazil, with *S. rubripinne* also being recorded in the East Atlantic (Randall, 1990; Afonso et al., 1999). One other species, *S. griseorubra*, is known only from islands in the large upwelling area on the SE Caribbean coast of Venezuela (Cervigón, 1982). In addition, there is one East Atlantic/Mediterranean species, *S. cretense*, and one species, *S. strigatum*, that occurs only at the south-central Atlantic islands of Ascension and St Helena (Bernardi et al., 2000).

Recent morphological reappraisals, however, have led to three of the five species found in Brazil (contrary to indications in the older literature, *S. aurofrenatum* evidently does not occur there) being revalidated as separate species: *S. amplum*, *S. axillare*, and *S. frondosum* (Moura et al., 2001), and one other described as a new species, *S. tuiupiranga*, by Gasparini et al. (2003). Based on their morphological similarities, Moura et al. (2001) considered the Caribbean and Brazilian populations of the fifth species, *S. radians*, to be conspecific. Likely sister-pairs of Brazilian

and Caribbean species are thought to be *S. amplum*/*S. viride*, *S. axillare*/*S. rubripinne*, *S. frondosum*/*S. chrysopterum* (Moura et al., 2001), and *S. tuiupiranga*/*S. atomarium* (Gasparini et al., 2003). However, neither Moura et al. (2001) nor Cervigón (1982) compared specimens of *S. griseorubra* and *S. frondosum* or considered the relationship between them. As is typical for parrotfishes, supposed Caribbean/Brazil sister species of *Sparisoma* have been distinguished largely through differences in the bright color patterns of terminal-phase (TP) males, which typically include the largest males in a population.

The Atlantic genus *Nicholsina*, which is closely related to *Sparisoma* (Bernardi et al., 2000; Westneat and Alfaro, 2005), currently comprises two species: *N. denticulata* from the eastern Pacific and *N. usta* from both sides of the Atlantic. Schultz (1968) defined two allopatric subspecies of the latter, *N. usta collettei* from the East Atlantic and *N. usta usta* from the West Atlantic, in both the Greater Caribbean and Brazil. However, detailed morphological analyses, particularly of TP color patterns, of those two West Atlantic populations of *N. usta* is lacking.

2.2. Sampling sites

Table 1 and Fig. 1 provide information on where we collected the different species. Bernardi et al. (2000) conducted a preliminary analysis of relationships within *Sparisoma* using samples collected in the East Atlantic at Sao Tome island (*S. rubripinne*), Ascension and St Helena islands in the South-central Atlantic (*S. strigatum*), Lampedusa Island in the Mediterranean (*S. cretense*), and Panama in the SW Caribbean (*S. atomarium*, *S. aurofrenatum*, *S. chrysopterum*, *S. radians*, *S. rubripinne*, and *S. viride*). Here we add data from fishes collected at 10 other East and West Atlantic sites (see Table 1). In addition, Bernardi et al.'s (2000) analysis included data on *Nicholsina denticulata* (East Pacific), and *N. u. collettei* from Sao Tomé, to which we add information on *N. u. usta* from both Florida (collected by S. Karl) and Margarita Island, NE Venezuela.

Table 1
Samples of *Sparisoma* and *Nicholsina* species from different localities that were used in this study

Species	Locality	*n*
Sparisoma amplum	Brazil (Fernando de Noronha)	2
Sparisoma atomarium	Bermuda	1
	Panama	1
	Venezuela (Margarita Island)	4
Sparisoma aurofrenatum	Bermuda	1
	Bahamas	2
	Panama	1
Sparisoma axillare	Brazil (Cabo Frio)	1
	(Arquipelago dos Abrolhos)	1
	Venezuela (Margarita Island)	2
Sparisoma chrysopterum	Bermuda	1
	Bahamas	2
	Belize	2
Sparisoma cretense	Italy	3
Sparisoma frondosu	Brazil (Arquipelago dos Abrolhos)	3
Sparisoma griseorubra	Venezuela (Margarita Island)	8
Sparisoma radians	Bermuda	1
	Panama	1
	Brazil (Fernando de Noronha)	6
	(Cabo Frio)	2
	(Parcel Manuel Luis)	2
Sparisoma rubripinne	Bermuda	1
	Bahamas	2
	Belize	1
	Panama	2
	Cape Verde	2
	Sao Tomé (Sao Tomé)	3
	Sao Tomé (Bombom)	2
Sparisoma strigatum	Ascencion Island	2
	Saint Helena	1
Sparisoma tuiupiranga	Brazil (Cabo Frio)	2
Sparisoma viride	Bermuda	1
	Bahamas	2
	Panama	1
	Venezuela (Margarita Island)	2
Nicholsina denticulata	Pacific Mexico	2
Nicholsina usta usta	Florida	2
	Venezuela (Margarita Island)	2
Nicholsina usta collettei	Sao Tomé	2

2.3. DNA extraction and polymerase chain reaction (PCR) amplification

Gill tissue and pectoral fin clips were taken from specimens speared in the field and preserved in a DMSO buffer solution (see Amos and Hoelzel, 1991). These samples were stored at ambient temperature during the collecting work, then at 4 °C in the laboratory. Tissues were digested overnight at 55 °C in 500 ml of extraction buffer (NaCl 400 mM, Tris 10 mM, EDTA 2 mM, SDS 1%). We then purified the DNA by standard chloroform extraction and isopropanol precipitation (Sambrook et al., 1989).

Amplification of the mitochondrially encoded 12S and 16S ribosomal gene regions was accomplished with the following primers: 12SAL and 12SBH, and 16SAR and 16SBR (Kocher et al., 1989). Amplification of mitochondrial Cytochrome *b* used GLUDG-L and CB3H primers (Palumbi, 1996). The nuclear RAG1 gene was amplified using primers RAG1F and RAG9R from Quenouille et al. (2004). Each 100 µl reaction contained 10–100 ng of DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT), 150 mM of each dNTP, and 0.3 mM of each primer, and was amplified with a cycling profile of 45 s at 94 °C, 45 s at 48 °C, 1 min at 72 °C, for 35 cycles. After purification (following the manufacturer's protocol; ABI, Perkin-Elmer), sequencing was performed in both directions with the primers used in the PCR amplification on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA).

2.4. Sequence analysis

Ribosomal RNA sequences used here from individuals of all *Sparisoma* species collected in Panama, as well as those from *Sparisoma cretense*, *S. strigatum*, *S. rubripinne* from Sao Tome, *N. denticulata* and *N. usta collettei* are

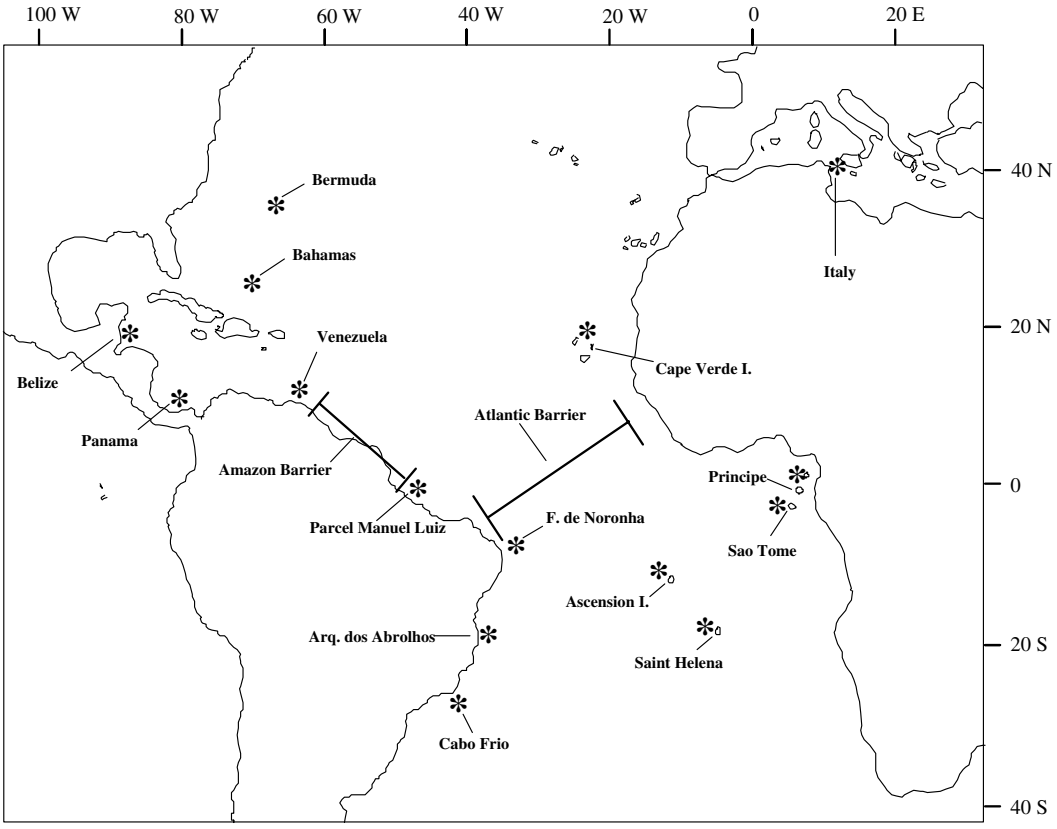

Fig. 1. Map showing sampling locations mentioned in Table 1, and the location of the two major oceanic barriers to dispersal by marine shore organisms in the tropical Atlantic.

from Bernardi et al. (2000). Sequences were aligned using the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems). Phylogenetic relationships were assessed by maximum parsimony (MP), neighbor-joining (NJ), and Bayesian methods implemented by the Software package PAUP (Phylogenetic Analyses Using Parsimony, version 4.0; Swofford, 2002) and MrBayes (version 2.1, Huelsenbeck and Ronquist, 2001). Most parsimonious trees were obtained using a Branch and Bound Search. Neighbor-joining reconstructions were based on a Kimura 2 parameter model. Statistical confidence in nodes was evaluated using 2000 non-parametric bootstrap replicates (Felsenstein, 1985). MrBayes default settings for the likelihood analysis were adopted including the GTR model (unequal base frequencies and six substitution rates). Stationarity of tree likelihood, sampled every 100 cycles, was consistently achieved after 3000 generations, and all sampled trees preceding stationarity were discarded.

2.5. The tempo of divergence and faunal enrichment

Estimation of the timing of divergences between species using genetic divergences assumes that mutations proceed randomly and uniformly (a molecular clock). Molecular clock enforcements were tested using a likelihood ratio test (Huelsenbeck and Rannala, 1997). Likelihoods, with or without an enforced molecular clock, were estimated using PAUP (version 4.0; Swofford, 2002). Likelihood ratios were then compared with a χ^2 distribution with s-2 degrees of freedom, where s is the number of taxa. Molecular clocks were only used for two mitochondrial markers 16S rRNA and Cytochrome *b*, because they have been frequently used in fish phylogenies and have derived, relatively well-known substitution rates (Meyer, 1993; McCune and Lovejoy, 1998). Genetic divergence was estimated using distances based on Kimura 2 substitution models. Divergence was estimated as the average pairwise distance between species minus the average pairwise distance within species.

For our molecular clocks, we used both Cytochrome *b* and 16S rRNA sequence data, employing the most frequently used ranges of rates of divergence: 1–2.5% sequence divergence per million years for the former (Banford et al., 2004; Perdices et al., 2005; Rocha et al., 2005b; Bowen et al., 2006) and 0.2–0.44% per million years for the latter (Banford et al., 2004; Bellwood et al., 2004; Read et al., 2005). These ranges of rates of divergence are based largely on levels of divergence between geminate species that are presumed to have been separated by the closure of the isthmus of Panama, ~3 mya. However, there is only one such geminate pair of parrotfishes, the two *Nicholsina* species we consider here, and sequence divergence between that pair is sufficiently large (see Results) that they probably separated well before that isthmus closed (see Discussion).

3. Results

3.1. DNA sequences

Sequences were obtained for all individuals investigated. These are deposited at GenBank under the Accession Nos. DQ457021–DQ457051. Tree topologies produced by either mitochondrial or nuclear data alone were identical. As homogeneity tests indicated that the mitochondrial and nuclear data sets were congruent, the two were combined for the remainder of the analyses.

A total of 2030 bp were compared among the 18 taxa investigated: 395 bp for the 12S region, 520 bp for the 16S region, 619 bp for the Cytb, and 496 bp for RAG1. Of these 2030 bases, 452 were variable and 359 were phylogenetically informative. Few insertions or deletions (indels) were observed (a total of 24 bp). These indels were included in our analysis and were counted as one single step each, regardless of size. Their removal or inclusion in the analysis did not change the topology of the resulting phylogenetic tree. A plot of transitions and transversions versus genetic distance indicated that saturation was not reached. When various weighting schemes were attempted (1:2, 1:3, 3:1), topologies remained unchanged, thus the following phylogenetic analysis was performed without weighting characters.

3.2. Phylogenetic analysis

A single-most parsimonious tree, which was identical to both the neighbor-joining and Bayesian trees, was obtained (tree length = 1034 steps, consistency index = 0.60; Fig. 2). The general phylogenetic relationships presented by Bernardi et al. (2000) for the non-Brazilian species of *Sparisoma* were based on 12S rRNA and 16S rRNA sequences alone. Those relationships, which are statistically consistent with the results presented here, were strongly supported in some, but not all, areas of the tree described by Bernardi et al. (2000). This issue of weakly supported areas of the tree was largely resolved here by the addition of two additional molecular markers (Cytb and RAG1). Since the focus of this paper is not phylogenetic, we will only briefly consider the main differences in the structure of the tree of Bernardi et al. (2000) and that in Fig. 2 here: the positions of *S. atomarium* and *S. radians*. In the former tree, *S. atomarium* was the sister to the remaining members of the genus, and *S. radians* the sister of *S. chrysopterum*. Both these relationships were only weakly supported. In the tree presented in Fig. 2 here, *S. atomarium* and *S. radians* are sister lineages and the East/Central Atlantic lineage of *S. cretense*/*S. strigatum* is the sister to all other members of the genus. The relationships in Bernardi et al.'s (2000) tree that involved *S. atomarium* and *S. radians* intuitively seemed questionable to us because those two species are much more similar to each other in terms of their general morphology, color patterns, and ecology than either is to any other congener. Thus the new mitochondrial and nuclear data added here produced a tree that is more consistent with our a priori (morphology- and ecology-based) estimations of likely sister relationships.

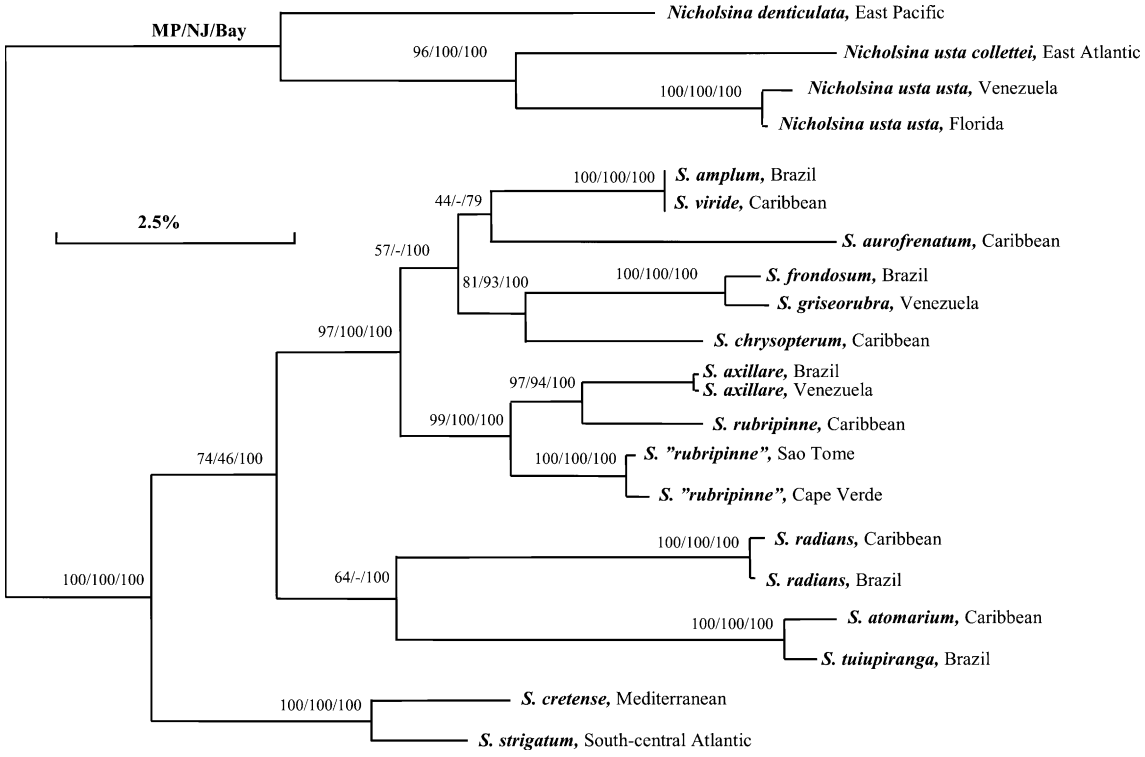

Fig. 2. Most parsimonious phylogenetic tree based on the nuclear (RAG1) and mitochondrial (12S, 16S, and Cytochrome *b*) markers. Numbers on nodes indicate results of bootstrapping (2000 replicates) with results for MP, NJ, and Bayesian methods. Only values above 40% are indicated.

3.3. Sister relationships

Three of the four predicted Caribbean—Brazilian sister pairs (Moura et al., 2001; Gasparini et al., 2003) clustered in monophyletic clades (Fig. 2): (1) *S. amplum*/*S. viride*, (2) *S. axillare*/*S. rubripinne*, and (3) *S. tuiupiranga*/*S. atomarium*. *S. frondosum* and *S. chrysopterum* are members of a clade that also includes *S. griseorubra*, which is the Caribbean sister of *S. frondosum*. Brazilian and Caribbean populations of *S. radians* also grouped together, as did East and West Atlantic populations of the *S. rubripinne* lineage. These groupings were in all cases well supported by bootstrap analysis (100%, Fig. 1) except for the *S. frondosum*/*S. griseorubra*/*S. chrysopterum* clade, which was at best supported by the Bayesian method (90% of the replicates). Both Cytochrome *b* and 16S rRNA data (see Table 2, group II) indicate that the likely West Atlantic sister of the East Atlantic *S. rubripinne* is *S. axillare* rather than *S. rubripinne*. However, relationships among these three were not well resolved as there is less than 50% bootstrap support for the *rubripinne*/*axillare* pairing (Fig. 2). The East and West Atlantic subspecies *N. usta collettei* and *N. usta usta* formed a robust clade (100% bootstrap support), with *N denticulata* as its sister clade.

The "most recently diverged" sisters in the various lineages were separated by the Amazon barrier in four cases (*S. amplum*/*S. viride*, *S. frondosum*/*S. griseorubra*, *S. axillare*/*S. rubripinne*, and *S. tuiupiranga*/*S. atomarium*), by the Atlantic barrier in two cases (*N. usta colletteil*/*N. usta usta*, and *S. rubripinne*/*S. rubripinne*–*S. axillare*), and by an equivalent barrier (several thousand kilometers of open ocean between the Cape Verde islands and Ascension/St Helena) in the remaining case (*S. cretense*/*S. strigatum*). No most recently diverged sister pairs essentially restricted to the same biogeographic region, with one possible exception: the significance of divergence between populations of *N. usta usta* in the northern and southern limits of the Greater Caribbean remains unresolved until samples of this species become available from Brazil.

3.4. Genetic divergence within species

Genetic diversity within a species within the same locality and between localities in the same biogeographic region typically was low, which is not surprising given the conservative nature of the molecular markers we used (Meyer, 1993) and the small sample sizes. Intraregional sequence divergence in Cytochrome *b* ranged from 0.0 to 0.2% within nine of the 13 species for which appropriate data are available. In only one case, there is a suggestion of distinct divergence between populations in the same region: Cytochrome *b* sequences of *N. usta usta* from the opposite ends of the Greater Caribbean (Florida and Venezuela) diverged by 1.0%.

3.5. Genetic divergences between sister-species pairs

The phylogenetic trees produced by Cytochrome *b* (Fig. 3) and 12S/16S data differed somewhat in the ordering of lineage formation, with the latter data providing an

Table 2
Levels of Cytochrome *b* and 16S rRNA sequence divergence (%SD) and timing (million years ago, mya) of splits of different lineages among the study species based on rates of sequence divergence varying between 1 and 2.5%/my for Cytochrome *b*, and 0.2 and 0.44%/my for 16S rRNA

Divergence type			Cytochrome *b*			16S rRNA		
			%SD	Splitting (mya)		%SD	Splitting (mya)	
				2.5%/my	1%/my		0.44%/my	0.2%/my
I. Between sisters in different West Atlantic regions	Brazil	Caribbean						
	S. amplum	*S. viride*	0.0	0?	0?	0.0	0?	0?
	S. axillare	*S. rubripinne*	5.6	2.2	5.6	0.2	0.5	1.1
	S. frondosum	*S. griseorubra*	4.2	1.7	4.2	0.1	0.2	0.9
	S. tuiupiranga	*S. atomarium*	3.0	1.2	3.0	1.1	2.5	5.5
	S. radians	*S. radians*	0.1	0?	0?	0.0	0	0
II. Between sisters across the Atlantic	West Atlantic	East Atlantic						
	S. axillare	*S. rubripinne*	5.6	2.2	5.6	1.0	2.3	5.1
	S. rubripinne	*S. rubripinne*	7.3	2.9	4.8	2.1	4.1	10.3
	N. usta usta	*N. usta collettei*	15.8	6.3	15.8	2.7	6.1	13.5
	Sparisoma	*S. cretense*/*strigatum*	16.7	6.7	16.7	5.9	13.4	29.5
III. Between Central and Eastern Atlantic sisters	Central Atlantic	Eastern Atlantic						
	S. strigatum	*S. cretense*	4.0	2.7	4.0	2.7	6.1	13.5
IV. Among *Sparisoma* lineages in the West Atlantic	Node 3		11.4	4.6	11.4	4.7	10.7	23.5
	Node 5		11.7	4.7	11.7	3.3	7.5	16.5
	Node 6		12.3	4.9	12.3	1.6	3.6	8.0
	Node 7		11.8	4.7	11.8	4.1	9.2	20.3
	Node 9		7.3	2.9	7.3	2.1	4.7	10.3
	Node 10		18.3	7.3	18.3	5.8	13.1	28.9
	Node 14		17.7	7.1	17.7	5.0	11.4	25.0
V. E Pacific/Atlantic *Nicholsina*species	Pacific	Atlantic						
	N. denticulata	*N. usta*	18.8	7.5	18.8	4.4	10.0	22.1

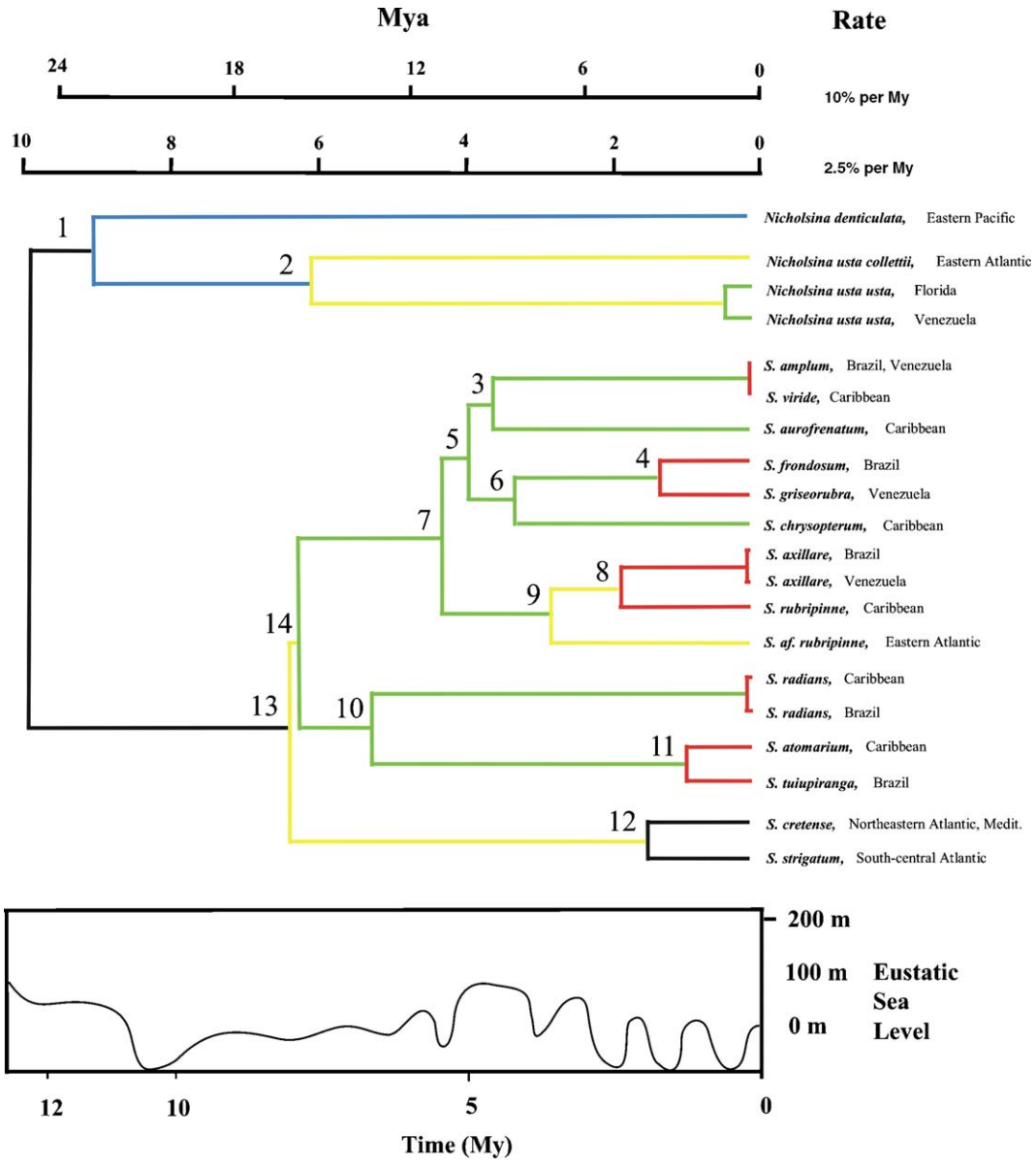

Fig. 3. The tempo of faunal enrichment in *Sparisoma* and *Nicholsina*. Maximum likelihood tree with enforced molecular clock based on mitochondrial Cytochrome *b* sequences. Color coding of divergence events: blue = Eastern Pacific/Atlantic event; yellow = trans-Atlantic splits; green = West Atlantic splits of unknown geography; red = trans-Amazon splits; black (node 12) = split between the East and Central Atlantic. Time scales based on rates of divergence span generally accepted ranges (1–2.5% per my) for fish Cytochrome *b* sequences (see text). Numbers identify different nodes referred to in Table 2. Representation of sealevel fluctuations is derived from Hallam (1984) and Haq et al. (1987), with zero indicating present day level.

ordering similar to that in Fig. 2. Levels of sequence divergence between the four Caribbean/Brazilian sister-pairs of *Sparisoma* ranged from 5.6 to 0% for Cytochrome *b* and 1.1 to 0% for 16S rRNA (Table 2). While there were strong divergences in three pairs (*S. axillare*/*S. rubripinne*, *S. frondosum*/*S. griseorubra* and *S. tuiupiranga*/*S. atomarium*), neither marker showed any detectable genetic divergence between *S. amplum*/*S. viride*, and there was negligible divergence between Caribbean and Brazilian populations of *S. radians* (Table 2). Sequence divergence between East Atlantic *S. rubripinne* and its West Atlantic sisters were larger than divergences in all four trans-Amazon sister-pairs of *Sparisoma* (Table 2) and broadly similar to those between the East and Central Atlantic sisters *S. cretense*/*S. strigatum*. Levels of divergence sister subspecies of *Nicholsina* across the Atlantic were greater than those among *Sparisoma* sisters described above, and similar to the early divergences within *Sparisoma* (Group IV in Table 2).

3.6. The tempo of diversification

The likelihood ratio test indicated that our data are consistent with a molecular clock. Fig. 3 displays the pattern of estimated times of lineage generation in both *Sparisoma* and *Nicholsina*, based on varying rates of sequence

divergence in Cytochrome *b*, while Table 2 presents such data from both that marker and 16S rRNA.

The range of rates of sequence divergence we used to place the separation of Pacific and Atlantic populations of *Nicholsina* (Group V in Table 2) at 7.5–21.7 mya, with Cytochrome *b* and 16S rRNA yielding similar estimates.

Times of three trans-Atlantic divergences (nodes 2, 13, and 9 in Table 2 and Fig. 3) ranged from 6.1 to 15.8 mya for the two subspecies of *N. usta* to 2.7 to 13.5 mya for that leading to the *S. cretense*/*S. strigatum* lineage, and 5.9 to 29.5 mya for the *S. rubripinne* lineage.

Six splits within the West Atlantic (group IV in Table 2, green in Fig. 3) that led to the formation of the seven main lineages of *Sparisoma* found there now, and that lack any geographic association with the Amazon barrier, were scattered throughout the same period as the trans-Atlantic divergences, ~3–29 mya. Cytochrome *b* and 16S rRNA indicate differences in the timing of some those events, with the latter producing distinctly older estimates in three of six cases.

The Cytochrome *b* divergence data suggest a cluster of three near-simultaneous splits near the beginning of diversification within the Atlantic (nodes 2, 13, and 14 in Fig. 3, Table 2). However, the 12S/16S estimates are concordant for only two of those cases (nodes 13 and 14) and suggest that another (node 10) was roughly simultaneous to those. The two events with consistent dates produced by both markers include one trans-Atlantic divergence (node 13), and one (node 14) involving the divergence of two major ecological lineages within the West Atlantic—the *S. atomarium*/*S. radians* lineage, which is restricted to vegetated bottoms (seagrasses and macroalgal beds), and the remaining, basically reef-living lineage.

Splits associated with the production of (allopatric) trans-Amazon geminate pairs (nodes 3, 4, 8, and 11 in Fig. 3) and the sole split that led to two (allopatric) sister species in the East/Central Atlantic (node 12 in Fig. 3) all occurred relatively recently in the sequence of diversification: 0–5.6 mya for the former and, somewhere between 2.7 and 13.5 mya for the latter (Table 2, Fig. 3). However, the two markers produced different orderings of the trans-Amazon events (Table 2, group I), and substantial differences in the estimated timing of the East/Central Atlantic event (Table 2, group III).

4. Discussion

4.1. Mechanisms of speciation in Sparisoma and Nicholsina

Five mechanisms have been proposed for speciation in the Atlantic shore organisms: (i) the formation of major barriers that acted as vicariance agents, dividing regional faunas and creating major biogeographic provinces (Rosen, 1975); (ii) inter-province dispersal and subsequent isolation across such major barriers (Briggs, 1974); and the generation of species within a province, either through (iii) within-region vicariance (e.g. Colin, 1975), (iv) ecological differentiation in different parts of a region in the absence of dispersal barriers (Rocha et al., 2005a), or (v) small-scale (same reef) mating-habitat segregation in the absence of dispersal barriers (Streelman et al., 2002; Taylor and Hellberg, 2005).

Our data provide strong support for allopatric speciation arising from effects of large-scale barriers being the primary agent of diversification in *Sparisoma* and *Nicholsina* in the Atlantic. First, all of the most recently diverged pairs of sister species are separated by the Amazon or Atlantic barriers or by the thousands of kilometers of deep ocean separating the East and Central Atlantic. There are no clear geographic indications of whether the Amazon barrier was involved in producing the remaining seven older divergences in West Atlantic lineages of *Sparisoma*. However, that could simply be due to the geographic signal of such events being obscured by the signals of more recent trans-Amazon dispersal and divergences.

Allopatric speciation within the Brazilian and Greater Caribbean provinces may also have contributed to faunal enrichment in *Sparisoma* and *Nicholsina*. Barriers that arise in the geographically complex Greater Caribbean during low sealevel stands probably resulted in allopatric speciation through vicariance in some reef-fishes (Colin, 1975). There also are distinct island/continent differences in faunal structure within both the Greater Caribbean (Robins, 1971) and Brazil (Rocha et al., 2005a; Lima et al., 2005) that could provide the basis for ecological speciation in different parts of either region. Although Brazil is geographically much simpler than the Greater Caribbean, strong differences between the shore-fish faunas of the continent and the offshore islands indicate that insular isolation and large-scale (island vs. continent, or latitudinal) ecological divergence likely contribute to speciation within that region (Rocha, 2003; Rocha et al., 2005a).

Streelman et al. (2002) proposed that speciation in parrotfishes has occurred through strong effects of sexual selection on TP male color patterns reinforcing the isolating effects of small-scale (within reef) habitat segregation of mating activities. Taylor and Hellberg's (2005) model for speciation in reef dwelling gobies is also based on spatial segregation of mating activities, but, unlike the former model, it also incorporates the development of distinct ecological differences between sub-populations living in different habitats. Ecological differentiation of populations in different habitats has been implicated in sympatric speciation in both plants and fishes (Barluenga et al., 2006; Savolainen et al., 2006), and reproductive isolation between sister taxa of different types of organisms generally is linked to ecological differentiation (Funk et al., 2006).

Parrotfishes and other Caribbean labroid fishes tend to migrate to and congregate at the same types of habitats for mating, and do so during the same times of day and seasons (e.g. Robertson and Warner, 1978; Warner and Robertson, 1978; Colin and Clavijo, 1988; Robertson, 1991; DRR, pers. obs.). Such behavior would reduce the likelihood of reproductive isolation developing in these organisms in the

absence of ecological differentiation coupled with habitat segregation. The divergence of the vegetated-bottom lineage (*S. atomarium*/*S. radians*/*S. tiuipiranga*) from the remaining reef-associated lineages represents the most obvious potential candidate for sympatric speciation in *Sparisoma* based on ecological differentiation and habitat segregation. Differing environmental conditions in adjacent reef habitats can lead to local populations of the same parrotfish species spawning at very different times of the year (Clifton, 1995), and ecological differences of this order conceivably could provide part of the basis for sympatric speciation in parrotfishes. Support for a sympatric speciation model in these fishes would come from a situation equivalent to those described by Barluenga et al. (2006) and Savolainen et al. (2006): recently diverged sister species that are endemic to the same reef have strong habitat segregation and other ecological differences. We know of nothing suggestive of such a situation among the parrotfishes we examined here.

4.2. The tempo of speciation in Sparisoma and Nicholsina

Three patterns are evident in the tempo of lineage formation in *Sparisoma* and *Nicholsina*: (i) Faunal enrichment has been an ongoing process within *Sparisoma* since it began somewhere between 10 and 30 mya (depending on the speed of the molecular clock), and continues up to the (in evolutionary time) present. While diversification of *Nicholsina* began at about the same time as that of *Sparisoma*, there has been relatively little activity since that start. Analyses of samples of *N. usta* from Brazil are needed to clarify the extent to which speciation activity has continued to the recent past in *Nicholsina*. (ii) Multiple trans-Atlantic divergences were scattered throughout most of the period since diversification began, with few indications of clusters of simultaneous activity. (iii) The most recent set of divergences are those that produced trans-Amazon geminate pairs. That pattern likely simply is due to these look-alike species being the most recent products of an ongoing process, among which the geographic signal of effects of the Amazon barrier is still evident.

Molecular clocks based on both Cytochrome *b* and 16S rRNA indicate that all the four trans-Amazon splits occurred well after that barrier formed. The Atlantic barrier began to be established with the separation of Africa and South America ~84 mya (Pittman et al., 1993). As it is now 3500 km wide it must have been broad and effective when trans-Atlantic divergences began in *Sparisoma* and *Nicholsina*, 16–30 mya. Thus both barriers clearly have acted by restricting dispersal well after they were established to levels that occasionally allow establishment of new populations that become new species. These recent divergences account for half the diversification in *Sparisoma*. The spread of possible ages of older divergences within *Sparisoma* in the West Atlantic is such that they all could have resulted from either vicariance or dispersal-limitation effects of the Amazon barrier. As tropical reef habitats existed in both the Caribbean and Brazil long before the Amazon barrier formed (e.g. Veron, 1995) and fossils demonstrate that parrotfishes evolved long before that barrier (Bellwood, 1994), it is quite possible that Amazon vicariance played a substantial role in the early diversification of *Sparisoma*.

What factors may have contributed to this pattern of frequent divergences in *Sparisoma* since the development of the Amazon barrier 11 mya? Effects of variation in oceanographic conditions on long-distance dispersal potential likely are involved. The basic circulation of the Atlantic apparently has remained stable since the closure of the Tethys 12–18 mya, although the final closure of the Isthmus of Panama likely had an affect on circulation dynamics (Maier-Reimer et al., 1990; Haug and Tiedemann, 1998). However, ocean-current dynamics, which are likely to affect inter-regional dispersal of pelagic larvae across both the Atlantic and Amazon barriers, have varied considerably with changing sealevels (e.g. Kaneps, 1979), with considerable fluctuations over the past 10 my (Fig. 3). The effectiveness of the Amazon barrier, in particular, is likely influenced by changing sealevels not only through variation in current speeds, which affect larval transit times, but also variation in the salinity of that barrier and the availability of habitat beneath the freshwater plume that can support populations within that barrier (Rocha, 2003). Fluctuations in the effectiveness of the Amazon barrier could be linked to all of the recent diversification of *Sparisoma* in the West Atlantic. Climate-driven fluctuations in effectiveness of the Atlantic barrier and the isolation of the central Atlantic islands could also have produced trans-Atlantic and East/Central Atlantic allopatric speciation through long-distance dispersal. However, while the time frames of variation in sealevel and recent diversification of *Sparisoma* on both sides of the Atlantic are coincident in a general sense, our inability to put precise figures on the timing of divergences means that we cannot test for a detailed association between the two.

Our results indicating a major role in speciation for inter-regional dispersal well after the formation of the Atlantic and Amazon barriers are consistent with those obtained for other widely distributed Atlantic shore-fish and invertebrate taxa (e.g. Muss et al., 2001; Lessios et al., 2001; Banford et al., 2004). Rocha (2003) estimated that only 12.7% of Brazilian reef fishes are restricted entirely to that region (see also Carpenter, 2002 for information on other shore-fishes) and pointed out that the occurrence of "Brazilian" species in the SE Caribbean near the northern edge of the Amazon barrier (e.g. *S. axillare*, as we show here) and "Caribbean" species in NE Brazil near the southern edge of that barrier indicates recent dispersal in both directions across that barrier. He concluded that the Amazon barrier is quite porous in biogeographic terms. Our results reinforce his conclusions: the Amazon barrier evidently represents a major agent of faunal enrichment in the West Atlantic, one that has achieved its importance as an engine of allopatric speciation by being both relatively weak and strongly dynamic.

Geminate sister species of fishes and other shore-living marine organisms derived from a single population that was split by the rising central American isthmus have often used to estimate lineage-specific rates of sequence divergence and hence the tempo of speciation (see Banford et al., 2004; Bellwood et al., 2004; Bernardi and Lape, 2005; Read et al., 2005; Rocha et al., 2005b; Lessios and Robertson, in press). Geological evidence indicates that the developing Central American isthmus could have disrupted connections between Atlantic and Pacific populations of inshore organisms (such as parrotfishes) on 3–4 occasions over the past ~10 my (Coates et al., 1992; Coates and Obando, 1996; Banford et al., 2004), with the final closure of the isthmus occurring somewhere between 3.1 and 2 mya (Coates and Obando, 1996). The range in rates of sequence divergence we used here indicates that the Atlantic and Pacific lineages of *Nicholsina* diverged 7.5–22.1 mya, well before the final closure of the isthmus. However, as *Nicholsina* lives in very shallow water along the shoreline and currently occurs on both the Atlantic and Pacific coasts of Panama, there is no obvious reason why final separation of the Pacific and Atlantic lineages should not have been delayed till the isthmus finally closed. If so, rates of sequence divergence in this genus, and probably *Sparisoma*, would be much higher than those we used here, all of the speciation within *Sparisoma* and the Atlantic *Nicholsina* lineage would have occurred within the past 5 my, all trans-Amazon sister pairs would have been separated within the last 1 my, and both the Atlantic nor Amazon barriers would have acted as agents of speciation entirely by restricting dispersal well after their formation.

4.3. Pelagic dispersal capabilities of Sparisoma

Little is known about the pelagic dispersal abilities of larval parrotfishes and how these may have affected gene flow across the Amazon and Atlantic barriers. One of us (B.C.V.) has examined the daily increments on otoliths of juvenile *S. viride* and *S. radians* from Panama and found that both species appear to have relatively long pelagic larval durations (PLDs) for reef fishes. In a sample of 24 individuals of each species, the mean estimated PLD for *S. viride* was 57 days (range 47–80) while *S. radians* had a mean of 60 days (range 50–93). In addition, both species showed a pattern of variation in the widths of daily increments that is associated with an ability to delay metamorphosis in another labroid fish, the bluehead wrasse, *Thalassoma bifasciatum* (see Victor, 1986). It would be these individuals that would determine the dispersal abilities for the species. The relatively long estimates of PLD for these *Sparisoma* spp., extending to over 3 months in this small sample, may explain the propensity for the genus to cross the Amazon and trans-Alantic barriers as well as the negligible genetic divergence between Caribbean and Brazilian *S. radians* and *S. amplum*/*S. viride*. The marginally longer PLD estimate and greater upper limit for *S. radians* may reflect a greater degree of dispersal ability (in concordance with the phylogenetic patterns), but may also disappear with increased sampling over space and time. More resolution of the intra-specific variation in PLD and sampling of additional *Sparisoma* species would be required for any attempt to assess the role of dispersal abilities in forming the patterns of divergence found in this study.

4.4. Taxonomic significance of our results

4.4.1. Sparisoma

Our results are largely consistent with the current morphology-based taxonomy of this genus. We found that 11 of the 12 recognized morphospecies in this genus are genetically divergent, and have been isolated from their sister species for between 0.2 and 5 my. However, three anomalous situations exist:

1. We found no genetic differences between the sisters *S. amplum* and *S. viride*. The only morphological differences between that pair noted by Moura et al. (2001) are readily noticeable differences in details of the color patterns of TP males. Faster evolving markers than those we used here, such as the mitochondrial control region, and nuclear microsatellites may indicate the age of separation of this sister pair, and help determine whether they represent valid biological species.
2. The trans-Atlantic divergence in the *S. rubripinne* lineage is substantially greater than that between any of the four trans-Amazon sister species pairs (including the sister pair within the *S. rubripinne* lineage—*S. frondosum*/*S. griseorubra*) and similar to that between the East/Central Atlantic sister pair *S. cretense*/*S. strigatum* (Figs. 2 and 3; Table 2). Hence the East Atlantic *S. rubripinne* clearly represents an as yet undescribed species, one with quite different TP color patterns to those of its West Atlantic sisters (DRR, pers. obs. at Cape Verde and Sao Tome).
3. In contrast to the situation with *S. amplum*/*S. viride*, there are striking similarities in the color patterns of each of two other trans-Amazon sister pairs of *Sparisoma*. Color patterns of *S. atomarium* in (some parts of) the Caribbean (see Humann and DeLoach, 2002; Reefnet, 2003) are virtually indistinguishable from those of *S. tuiupiranga* (DRR, pers. obs., and see Gasparini et al., 2001, www.Fishbase.org; Reefnet, 2003). The same situation applies with *S. griseorubra* and *S. frondosum* (DRR, pers. obs., and see www.Fishbase.org), to the extent that Rocha (2002) identified photos of *S. griseorubra* at its type locality as *S. frondosum*. Our genetic analyses have been pivotal in clarifying that these represent two pairs of valid sister species.
4. The occurrence of *S. axillare* in the SE Caribbean in the same habitats on the same reefs as its Caribbean sister *S. rubripinne* (DRR, pers. obs.) provides opportunities to test whether those sister species remain genetically isolated now that they have come into contact after more than 2 my of isolation. That situation and morphological similarities between other Brazilian and Caribbean

sister pairs of *Sparisoma* also raise the possibility that other Brazilian *Sparisomas* may occur unnoticed in the SE Caribbean.

4.4.2. Nicholsina

The East and West Atlantic populations of *N. usta* were designated as separate subspecies by Schultz (1968) on the basis of minor morphometric differences in preserved fishes. Our data show that these two forms are highly divergent genetically, more so than many of the *Sparisoma* species are from each other (Table 2, Figs. 2 and 3). Hence the East and West Atlantic forms represent a sister pair of species, *N. collettei* and *N. usta*, respectively. West Atlantic populations of *N. usta* at the northern and southern edges of the Greater Caribbean appear to be slightly divergent. The genetic and morphological status of *N. usta* from Brazil, and elsewhere in the Greater Caribbean need to be compared to resolve whether there are distinct Caribbean and Brazilian populations or species, perhaps with the latter also occurring in the SE Caribbean.

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