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## Evolution of coral reef fish *Thalassoma* spp. (Labridae). 2. Evolution of the eastern Atlantic species

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**Abstract** The genetic relationships within and among congeneric species of marine fish from the Atlantic and the Mediterranean are poorly known. Relationships among all five species of the wrasse genus *Thalassoma* present in the Atlantic and the Mediterranean were examined using sequence data from the mitochondrial control region. Sampling was focused on the mid-Atlantic *T. sanctaehelenae* (Valenciennes, 1839) and *T. ascensionis* (Quoy & Gaimard, 1834), the eastern Atlantic *T. newtoni* (Osório, 1891) from Sao Tome, and the eastern Atlantic/Mediterranean *T. pavo* (Linnaeus, 1758). Two western Atlantic species *T. bifasciatum* (Bloch, 1791) from the Caribbean and *T. noronhanum* (Boulenger, 1890) from Brazil served as outgroups. Tissues from a total of 132 individuals were sequenced.

*T. newtoni* from Sao Tome preferentially grouped with the central Atlantic *T. sanctaehelenae* and *T. ascensionis*. *T. pavo* exhibits two distinct coloration patterns, one in the Cape Verde Islands and one in the eastern Atlantic Islands and Mediterranean. However, no genetic discontinuities between the Cape Verde Islands and the remaining samples or between Atlantic and Mediterranean individuals were found. Within Mediterranean populations of *T. pavo*, our data suggested the presence of a genetic break between eastern and western regions.

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### Introduction

The genetic relationships among eastern, central, and southern Atlantic fish populations, either with each other or with Mediterranean conspecifics or congenics are poorly known (Kotoulas et al. 1995; Borsa et al. 1997; Chikhi et al. 1997; Naciri et al. 1999). A molecular phylogeny of 26 of 27 recognized wrasses in the genus *Thalassoma* has provided insights on the relationships among six Atlantic and Mediterranean species (Bernardi et al. 2003). Western Atlantic species included the sister species *T. bifasciatum*, found from Bermuda to the Caribbean, and *T. noronhanum*, found along the Brazilian coast and at offshore islands such as Fernando de Noronha. In the South Atlantic, there are two species, endemic to each of the oceanic islands of Ascension and Saint Helena, *T. ascensionis* and *T. sanctaehelenae*, respectively. In the eastern Atlantic there were two species, *T. newtoni*, found from Sao Tome to Angola, and *T. pavo*, found in the southern Mediterranean (with recent advances into its northern parts due to warming events, Guidetti et al. 2002) to the eastern Atlantic, all the way south to the Gulf of Guinea including the Macaronesian archipelago (herein Azores, Canaries, Madeira) and the Cape Verde Islands. While that study provided some information about the relationships among species (Bernardi et al. 2003), a more in depth

study was necessary to evaluate the details of the relationships, including those within species.

Specifically, the phylogenetic relationships between the mid-oceanic island endemics from Ascension Island and Saint Helena, *T. ascensionis* and *T. sanctaehelena*, with each other and with the eastern Atlantic and Mediterranean species *T. pavo* and *T. newtoni* are poorly known.

Relationships based on the DNA sequences of cytochrome *b*, 16S rRNA, and one intron of the S7 ribosomal protein of *T. pavo* from the Mediterranean, of *T. newtoni* from Sao Tome, of *T. ascensionis* and *T. sanctaehelena* from the southern Atlantic, and of the western Atlantic species *T. bifasciatum* and *T. noronhanum* were examined in the accompanying paper (Bernardi et al. 2003). In the present study, we used a more rapidly evolving molecular marker, the mitochondrial control region, and a larger sample size with a total of 132 individuals, including additional Atlantic and Mediterranean populations.

The specific goals were to answer questions at different phylogenetic levels: (1) What are the relationships among the oceanic island species (*T. sanctaehelena*, *T. ascensionis*) and the mainland species (*T. newtoni*, *T. pavo*)? (2) What is the relationship between Atlantic and Mediterranean populations of *T. pavo*? (3) Are the populations of *T. pavo* from the Mediterranean genetically structured?

To address these questions, we established a molecular phylogeny based on the mitochondrial control region for *Thalassoma* spp. individuals collected in

Brazil, the Caribbean, Ascension Island, Saint Helena Island, Sao Tome, Cape Verde Islands, the Azores, Madeira, and 11 locations in the Mediterranean.

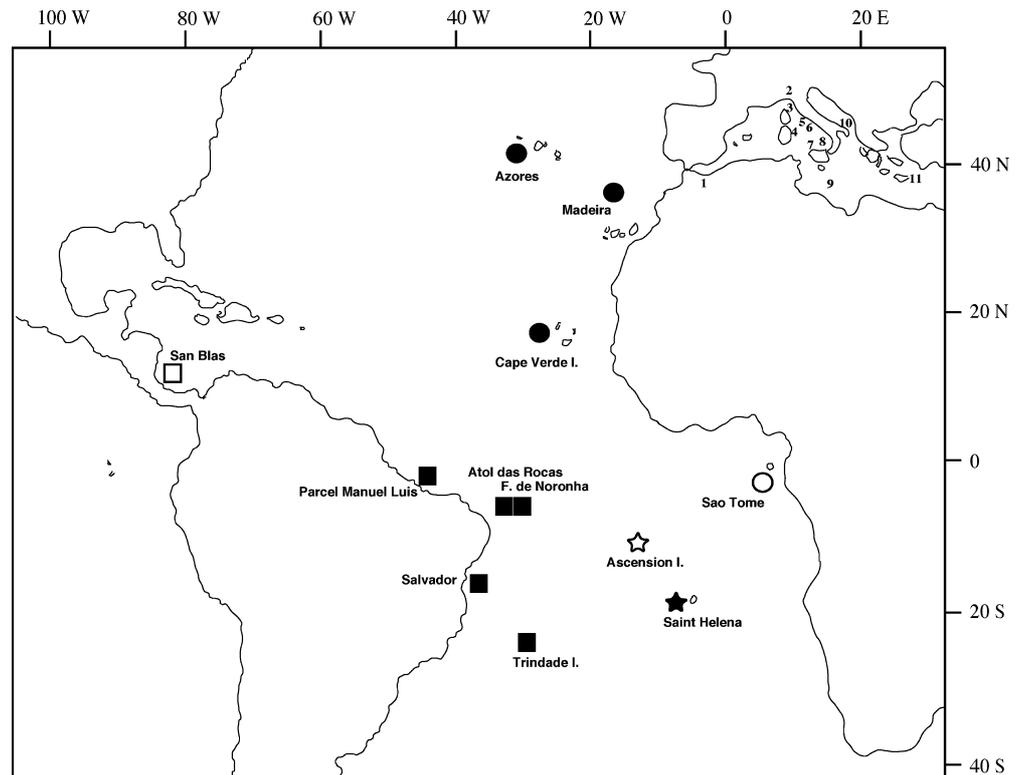
## Materials and methods

### Tissue samples and DNA extraction

All six species of Atlantic *Thalassoma* spp. were used in this study. We focused especially on *Thalassoma pavo* (Linnaeus, 1758), *T. newtoni* (Osório, 1891), *T. sanctaehelena* (Valenciennes, 1839), and *T. ascensionis* (Quoy & Gaimard, 1834). We used *T. bifasciatum* (Bloch, 1791) and *T. noronhanum* (Boulenger, 1890) as outgroups following Bernardi et al. (2003). Fish were sampled between April 1996 and November 2002. Sampling localities are shown in Fig. 1. The number of individuals sampled and sequenced per locality are detailed in Table 1. Samples of *T. newtoni* were collected on two separate occasions: in October 2000 by D.R. Robertson and in September 2002 by P. Wirtz. These samples were labeled ST and SAO, respectively. We obtained samples of *T. noronhanum* from several mainland and island locations and samples of *T. pavo* from several locations in the Atlantic and 11 locations in the Mediterranean. Eight of these locations were in the western basin of the Mediterranean, while three (Otranto, Linosa, and Rhodes) were from the Adriatic, Ionian, and Aegean Seas, respectively, sub-regions of the eastern basin. One location was on an artificial platform used for aquaculture. At present, this platform is just north of the Bay of Naples at Pozzuoli, but it reached its present location after being towed from the Adriatic Sea where it could have gathered recruits. All fish sampled there were adults.

Liver, fin, or muscle tissue was extracted immediately upon capture of individuals and preserved in 95% ethanol in the field, then stored at 4°C in the laboratory. Tissues were digested overnight at 55°C in 500 µl of extraction buffer (NaCl 400 mM, Tris

**Fig. 1** Map of sampling locations mentioned in Table 1. Symbols correspond to different species: open square, *Thalassoma bifasciatum*; filled squares, *T. noronhanum*; open star, *T. ascensionis*; filled star, *T. sanctaehelena*; open circle, *T. newtoni*; filled circles, *T. pavo*. Sampling localities in the Mediterranean are numbered 1–11. They correspond to: 1, Al-Hoceima; 2, Genoa; 3, Calvi; 4, Olbia; 5, Platform (Pozzuoli); 6, Naples (Baia); 7, Ustica; 8, Lipari; 9, Linosa; 10, Otranto; 11, Rhodes (see Table 1 for sample numbers)



**Table 1** *Thalassoma* spp. Species names, collection localities, and numbers of sequenced individuals of Atlantic and Mediterranean *Thalassoma* spp. used in this study. One sequence of *T. bifasciatum* was obtained from GenBank. Its geographic origin was not reported

Species	Locality	<i>n</i>
<i>T. bifasciatum</i>	San Blas, Panama	2
	GenBank	1
<i>T. noronhanum</i>	Parcel Manuel Luis, Brazil	2
	Fernando de Noronha, Brazil	2
	Atol das Rocas, Brazil	3
	Salvador, Brazil	1
	Trindade Island, Brazil	1
<i>T. sanctaehelenae</i>	Saint Helena	2
<i>T. ascensionis</i>	Ascension Island	2
<i>T. newtoni</i>	Sao Tome	15
<i>T. pavo</i>	Cape Verde Islands	2
	Madeira, Portugal	12
	Azores, Portugal	11
Mediterranean W.	1. Al-Hoceima, Morocco	3
	2. Genoa, Italy	10
	3. Calvi, Corsica, France	3
	4. Olbia, Sardinia, Italy	10
	5. Pozzuoli, Italy	11
	6. Naples, Italy	3
	7. Ustica, Italy	2
	8. Lipari, Italy	7
Mediterranean E.	9. Linosa, Italy	3
	10. Otranto, Italy	9
	11. Rhodes, Greece	15

10 mM, EDTA 2 mM, SDS 1%). We purified the DNA by standard chloroform extraction and isopropanol precipitation (Sambrook et al. 1989).

#### Polymerase chain reaction (PCR) amplification

Amplification of a 5' portion of the mitochondrial control region was accomplished using the primers CR-A and CR-E of Lee et al. (1995). All amplifications (25 µl) contained 10–100 ng of DNA, 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 150 mM of each dNTP, and 0.3 mM of each primer, and used a cycling profile of 45 s at 94°C, 45 s at 48°C, and 1 min at 72°C for 35 cycles. Automated sequencing was performed in both directions with the primers used in the amplification utilizing an ABI 3100 automated sequencer (Applied Biosystems, Foster City, Calif.).

#### Phylogenetic analyses

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the mitochondrial sequences. Phylogenetic relationships were assessed using the

neighbor-joining (Kimura-2-distances) method and the maximum-parsimony method implemented by the software package PAUP (phylogenetic analyses using parsimony, vers. 4.0, Swofford 1998). Topological confidence was evaluated with 1000 bootstrap replicates (Felsenstein 1985). In both neighbor-joining and maximum-parsimony methods, bootstrapping analysis was performed with equal weighting of transitions and transversions. All statistical tests were performed using the PAUP package. Population structure was calculated by an analysis of molecular variance (AMOVA, Excoffier et al. 1992) based on pairwise distances as implemented by Arlequin (vers. 1.1, Schneider et al. 1997). Gene flow ( $F_{st}$ ) was calculated using the software package DNAsp (vers. 3, Rozas and Rozas 1999) following Hudson et al. (1992).

## Results

### Sequence characteristics

Amplifications of the mitochondrial control region from 132 individuals resulted in 321 aligned base pairs (GenBank accession numbers AY329670–AY329800). A 2-bp insertion (positions 300, 301) was observed in *Thalassoma noronhanum*, and a 1-bp insertion (position 5) was observed in the Island of Rhodes population of *T. pavo*. No other insertions or deletions were observed in our data sets. Out of these 321 bases, 113 were variable, and 100 were phylogenetically informative. As expected, transitions were more frequent than transversions (ratio trs/trv = 3.75) and saturation was not present (plots not shown). Thus, we did not weigh transitions and transversions differently. Genetic divergence within and among species is shown in Table 2. Genetic divergence within a species was never > 1.5% (for *T. newtoni*), while genetic divergence between species was never < 3.2% (between *T. sanctaehelenae* and *T. ascensionis*). These data are in agreement with other reported genetic divergences for the mitochondrial control region in fishes (McCune and Lovejoy 1998).

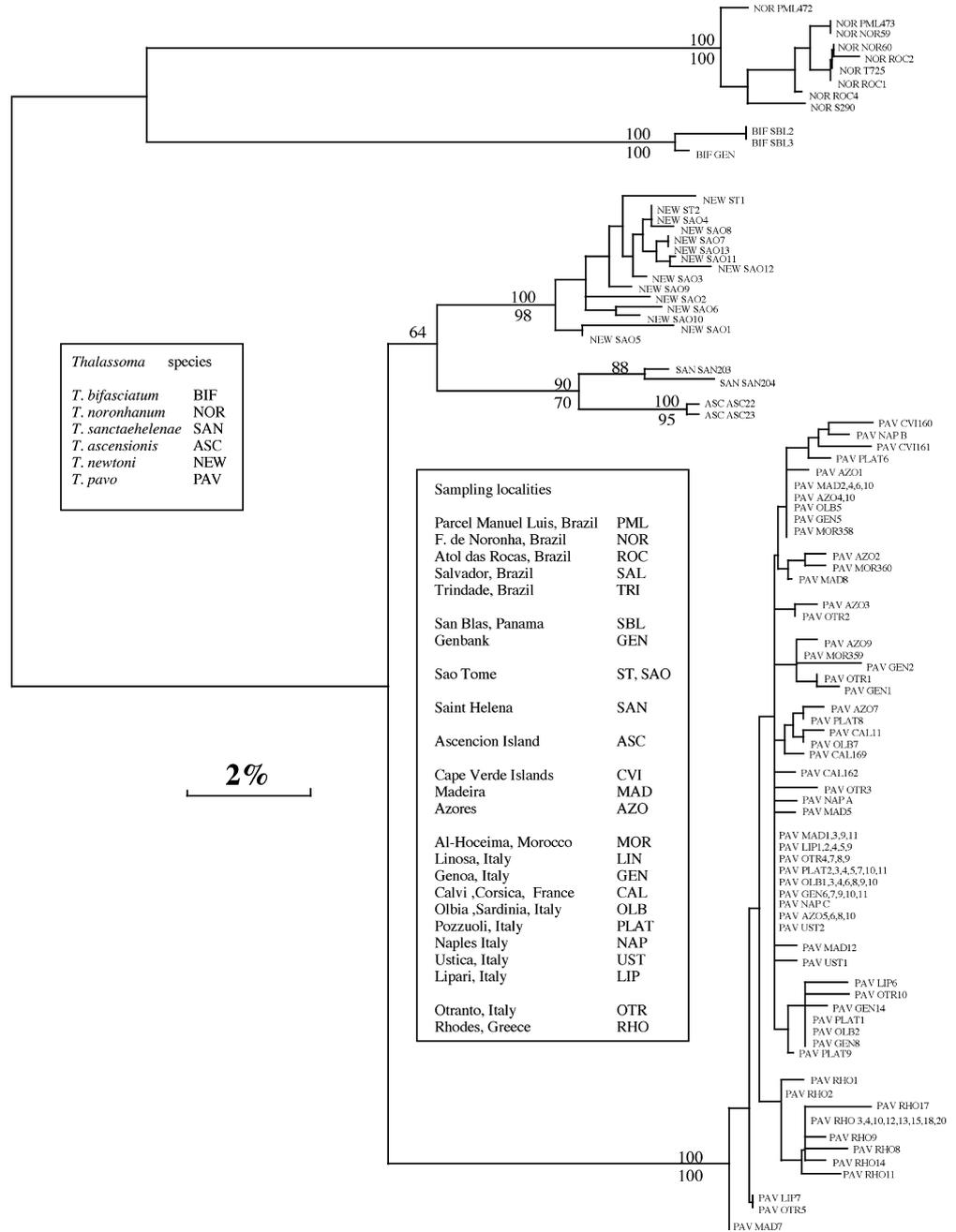
### Phylogenetic relationships

The seven most-parsimonious trees were obtained with the maximum-parsimony method, one of them being identical to the neighbor-joining tree. As expected, differences among the seven trees were all located in regions that were weakly supported (see details below). The seven most-parsimonious trees were 2252 steps long (consistency index = 0.586). One of these

**Table 2** Average pairwise genetic (Kimura-2) distances (% sequence divergence) between species, and within species (**bold numbers on diagonal**)

	<i>T. bifasciatum</i>	<i>T. noronhanum</i>	<i>T. sanctaehelenae</i>	<i>T. ascensionis</i>	<i>T. newtoni</i>	<i>T. pavo</i>
<i>T. bifasciatum</i>	<b>0.84</b>					
<i>T. noronhanum</i>	18.6	<b>0.93</b>				
<i>T. sanctaehelena</i>	21.0	21.2	<b>1.30</b>			
<i>T. ascensionis</i>	20.0	20.8	3.20	<b>0.31</b>		
<i>T. newtoni</i>	21.3	21.3	5.90	7.50	<b>1.50</b>	
<i>T. pavo</i>	22.3	23.7	10.2	10.2	9.8	<b>0.72</b>

**Fig. 2** *Thalassoma* spp. Molecular phylogeny based on the mitochondrial control region of the eastern Atlantic *Thalassoma* species using western Atlantic species as outgroups. A neighbor-joining (above the nodes) and maximum-parsimony (below the nodes) bootstrap support indicated at the major nodes. Labels for species and localities are shown in keys in the figure. Scale bar: 2% Kimura-2 genetic distance



most-parsimonious trees and the bootstrap supports for both maximum-parsimony and neighbor-joining methods are shown in Fig. 2.

At the species level, the phylogenetic relationships obtained with the mitochondrial control region were identical to the ones previously obtained using the mitochondrial cytochrome *b*, 16S rRNA, and the nuclear first intron of the *S7* protein (accompanying paper Bernardi et al. 2003). *T. bifasciatum* and *T. noronhanum* individuals were used as outgroups. Individuals grouped into two distinct clades according to their species identification (Fig. 2), confirming that *T. noronhanum* is indeed a bona fide species (Rocha et al. 2001). Within

*T. noronhanum*, we found no evidence of genetic differentiation between mainland and island populations ( $F_{st} = 0.0$ ).

Ingroup individuals were grouped into four distinct clades (Fig. 2). Individuals from Ascension Island and Saint Helena were in two well-supported sister clades. Individuals from Sao Tome grouped in a robust clade that was a sister clade to Ascension Island and Saint Helena, but this relationship was not very robust as only 64% of the neighbor-joining bootstrap replicates supported this relationship. All the *T. pavo* samples from the Cape Verde Islands, Madeira, Azores, and the Mediterranean grouped in one robust clade (100% bootstrap support).

## Population structure

The population structure of *T. pavo* was assessed by separating the samples into two regions, the Atlantic populations and the Mediterranean populations, using an analysis of molecular variance (AMOVA). We found that 85% of the variance in the data derived from within-population variance, while only 4% of the variance was attributable to the separation of Atlantic and Mediterranean populations. This result indicates little genetic difference between Atlantic and Mediterranean regions. This was confirmed by the estimated high levels of gene flow between the Atlantic and the Mediterranean indicated by the low  $F_{st}$  levels ( $F_{st}=0.053$ ,  $N_m=8.9$ ). We further analyzed our data by looking at possible population structure within the Mediterranean. The Mediterranean Sea is divided into western and eastern basins by a shallow saddle between Sicily and Tunisia. This saddle has been proposed to be a major historical barrier to gene flow during periods of low sea water levels (Nikula and Väinölä 2003). In the case of *T. pavo*, estimated gene flow between the eastern basin (Linosa, Otranto, and Rhodes) and the other samples was found to be fairly high ( $F_{st}=0.24$ ,  $N_m=1.60$ ). These values however were magnified by the single population east of the Peloponnesus (Island of Rhodes). Indeed gene flow between the Island of Rhodes population and the remaining *T. pavo* samples was very restricted ( $F_{st}=0.52$ ,  $N_m=0.47$ ). In contrast gene flow between Linosa + Otranto populations and western Mediterranean populations was very high ( $F_{st}$  not significantly different than zero). More samples from the eastern basin are needed to confirm this finding. We also tested for possible genetic differences between the eastern (Naples, Pozzuoli) and western (Calvi, Olbia) Tyrrhenian Sea as well as between the Tyrrhenian and the newly populated Ligurian Sea (Genoa), as this region includes a minor biogeographic barrier (Astraldi et al. 1995). In none of these cases did we find any signature of gene flow restriction ( $F_{st}=0.0$  in all pairwise comparisons).

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## Discussion

The genus *Thalassoma* includes species that are morphologically very similar, and therefore color patterns have traditionally been relied on for purposes of systematics (Bernardi et al. 2003). In the present study, as for any molecular study on the genus, it is interesting to compare the color patterns of the individuals and the genetic groupings observed. In the case of the western Atlantic species, individuals grouped into two well-separated clades corresponding to *T. bifasciatum* and *T. noronhanum*. These clades were well supported and almost as distant from each other as they were from the eastern Atlantic species (18.6% vs. 21.0% sequence divergence), thus confirming the specific status of *T. noronhanum* (Rocha et al. 2001).

In the case of the mid- and eastern Atlantic individuals, the situation was more complex. Individuals from the mid-Atlantic species (*T. ascensionis* and *T. sanctaehelenae*) formed two separate clades, but these clades showed little sequence divergence (3.2%), possibly indicating a recent split. Indeed, Gomon and Forsyth (1990) consider them as synonymous. More samples from these two species are needed to fully understand the role of historical factors in their separation.

Interesting relationships between geographical distribution and coloration patterns were seen in *T. pavo*. There are two main coloration patterns for terminal phase (TP) fish that were observed. In the Mediterranean (+Azores) TP fish have two bands behind the head, one blue and one red, while in the Cape Verde Islands, the TP looks like the Mediterranean TP, but with no bands behind the head (Wirtz 2000). The initial phase (IP) also displays two main coloration patterns. In the Mediterranean, IP fish have solid colored bodies (light green-yellow), with usually four thin green bands, while in Cape Verde Island (and Sao Tome *T. newtoni*), IP fish have large black stripes on a tan body. From these patterns, it could be assumed that there are at least two main genetic groups, one in the Mediterranean and Azores and one in the Cape Verde Islands.

While for a long time *T. newtoni* was synonymized with *T. pavo*, mostly due to the IP color pattern described above, large genetic divergence was observed between Sao Tome and Mediterranean individuals. Furthermore *T. ascensionis* and *T. sanctaehelenae* were more closely related to *T. newtoni* than *T. newtoni* individuals were to *T. pavo* (Fig. 2). This adds to the evidence (Bernardi et al. 2003) that the color differences in *T. newtoni* parallel the genetic differences, and the original description of these individuals as *T. newtoni* is valid.

In contrast, the Cape Verde Islands *T. pavo*, with a unique set of color patterns in the initial and terminal phases, did not form a separate clade, but were indistinguishable from the Mediterranean samples. In this case, color pattern was a poor predictor of genetic isolation, as evidenced by mitochondrial control region sequences. The decoupling of color pattern and genetic isolation is not confined to the genus *Thalassoma*, but also occurs in the *Dascyllus trimaculatus* species complex (Bernardi et al. 2002). Thus, a sharp distribution boundary appears to exist between Cape Verde and Sao Tome, but not between Cape Verde and the Mediterranean.

Lastly, the lack of population structure between Atlantic and Mediterranean populations of *T. pavo* is surprising. Several studies on fish populations have indeed found a genetic break between Atlantic and Mediterranean populations (Kotoulas et al. 1995; Borsa et al. 1997; Chikhi et al. 1997; Naciri et al. 1999). Furthermore, one other wrasse species, *Coris julis*, showed evidence of genetic differentiation between eastern Atlantic and Mediterranean populations (Guillemaud et al. 2000;

Aurette et al., submitted). A true sibling species (i.e. not a population variant) was also identified in the Cape Verde Islands, *Coris atlantica* (Guillemaud et al. 2000; Aurette et al., submitted). Unlike the *C. julis* situation, we did not find a genetic separation of Atlantic and Mediterranean individuals. In addition, within the Mediterranean, unlike the genetic discontinuity observed between sand goby (*Pomatoschistus minutus*) individuals from the eastern and western basins (Stefanni and Thorley 2003), we did not find population structure either. We did, however, find a strong genetic break between the Island of Rhodes population and the other populations (to the west). This is in agreement with other studies on marine populations that uncovered a genetic break at the Peloponnesus, and not at the Sicily/Tunisia shoulder (Magoulas et al. 1996; Borsa et al. 1997; Mamuris et al. 1999; Bahri-Sfar et al. 2000; Nikula and Väinölä 2003). The presence of a possible genetic break between eastern and western regions of the Peloponnesus is particularly interesting in light of the relatively recent colonization of the Mediterranean from the Atlantic. The Mediterranean was completely desiccated during the Messinian period, approximately 5–6 million years ago (Duggen et al. 2003). Thus, either eastern and western populations have diverged since then, and experienced little gene flow between them, or the eastern population was originally founded by a few invading western individuals that carried original genetic divergence (founding effects). These two hypotheses predict small and large genetic divergences between eastern and western populations, respectively. Genetic divergence between the Island of Rhodes population and western Mediterranean populations was only 1.08%, which corresponds to approximately 0.5–0.1 million years since divergence (using a molecular clock of 2.5–10% per million years, Terry et al. 2000). Although molecular clocks should be used cautiously, this divergence is well within the time of existence of the Mediterranean Sea in its present form.

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