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High population connectivity across the Indo-Pacific: Congruent lack of phylogeographic structure in three reef fish congeners

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ABSTRACT

We used the mitochondrial control region and a comparative approach to study the genetic population structure of two surgeonfishes, *Naso brevirostris* and *Naso unicornis*, across their Indo-central Pacific ranges. Our purpose was to compare our results with those of a previous study of *Naso vlamingii* [Klanten, S.O., van Herwerden, L., Choat J.H., 2007. Extreme genetic diversity and temporal rather than spatial partitioning in a widely distributed coral reef fish. Mar. Biol. 150, 659–670] another widely distributed Indo-central Pacific *Naso* species. We found no evidence of a barrier to gene flow between the Indian and Pacific Oceans for either species, consistent with what was shown for *N. vlamingii*. Overall, both target species lacked spatial population partitions and probably have complex patterns of gene flow on several spatial scales. Despite the lack of geographic population structure distinct clades were observed in *N. brevirostris*, similar to those found in *N. vlamingii*. Coalescence times for intraspecific clades of *N. brevirostris* and *N. vlamingii* approximate each other, suggesting parallel evolutionary histories. A bimodal mismatch distribution in *N. brevirostris* indicates that a biogeographic barrier separated *N. brevirostris* populations sometime during its species history. *Naso unicornis*, in contrast, lacked genetic structure of any kind, although it has what could represent a single surviving clade. Congruent lack of spatial population structure among all three species suggest that such patterns are not due to stochastic processes of DNA mutation and are most likely driven by ecological and environmental factors.

1. Introduction

Reef fish species tend to have broad geographic distributions comprising patchily distributed adult populations connected by pelagic larvae (Sale, 1991). Adult distributions reflect the spatial patterning of suitable reef habitats, which are often separated by hundreds or even thousands of kilometers of deep open Ocean. The application of molecular markers has provided the means to assess the extent to which widespread reef fish species may be partitioned into distinct populations, as well as the spatial scale of partitioning and the temporal pattern of population connectivity between isolated adult populations. An important finding of reef fish phylogeographic studies has been the identification of a degree of spatial structuring among populations greater than might be expected for species with large species ranges and high larval dispersal potential (Swearer et al. 2002; Planes and Fauvelot, 2002; Bernardi et al. 2003; Taylor and Hellberg 2003; Thacker et al 2007). This degree of spatial structuring has promoted interest in the nature, types, abundance and effectiveness of barriers that prevent or limit larval dispersal (Rocha et al., 2007; Hellberg 2007; Floeter et al. 2008) and the behavior of dispersing larvae (Leis 2007; Leis et al. 2007). In contrast, however, an increasing number of phylogeographic studies are also providing evidence of widespread reef fishes in which geographic population structure is minimal or altogether lacking. Some examples of reef fishes with little genetic population structure include holocentrids (Craig et al. 2007), scarine labrids (Bay et al. 2004), aulostomids (Bowen et al. 2001) and acanthurids (Klanten et al., 2007). Such studies suggest that the larvae of some reef fishes readily cross very large open ocean barriers that impede dispersal in other species (Lessios and Robertson 2006) and that larval movement across oceanic barriers may be highly variable among different reef fish taxa.

The molecular approach is an effective method for studying connectivity between discontinuous populations provided that the genetic diversity of the populations is effectively represented in the sample. However, it can be difficult to capture the molecular signal of populations and assess their connectivity, particularly when the effective population size is large and genetic diversity is high, as is the case with many benthic marine organisms (Palumbi 2003; Hellberg, 2007; Hedgecock et al. 2007). When much of a population's diversity remains unsampled, the molecular method is less effective. Thus, species with strong geographic structural

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differences between populations and low genetic diversity are ideal for this type of research, while those that lack structure and have high genetic diversity yield results that are more difficult to interpret. Moreover, in the absence of strong population structure stochasticity, such as homoplasy, may confound true population patterns. Therefore, weak genetic population structure must be interpreted cautiously, particularly in studies where only one molecular marker is used.

Because weak population structure obtained from a single molecular marker may be confounded by stochastic variation, validation of population patterns through an assessment of their congruence is often called for. In most cases the extent of congruence can be assessed using independent data sets, such as additional unlinked molecular markers within a single species or through comparative phylogeography. The second approach traces independent species histories through a single molecular marker. Comparative phylogeography has a number of applications (Bermingham and Moritz, 1998) and can help determine the extent to which homoplasy and other stochastic signals are confounding genetic population structure in certain reef fish species.

One of the most extreme examples of a reef fish with minimal population structure is *Naso vlamingii*. The population genetics of this species, inferred from the mitochondrial control region, is characterized by: (i) an extremely high nucleotide and haplotype diversity, (ii) a lack of geographic population structure resembling panmixia on a very large spatial scale and (iii) the presence of a temporal genetic structure presumed to have arisen from episodic isolation during a long species history (Klanten et al., 2007). Notwithstanding the biological interest of the *N. vlamingii* findings, because this study was based on a single marker there is the possibility that stochastic variation rather than a biologically relevant process is causing the observed genetic patterns. The purpose of the current study was to evaluate the biological relevance of these unusual genetic patterns by studying the same mitochondrial marker in two other equally widespread members of the genus *Naso*.

The first species chosen for this study was *Naso brevirostris*, which is sister to *N. vlamingii* (Klanten et al., 2004) and similar in terms of its geographic distribution and foraging ecology (Randall, 2001; Choat et al. 2002). The second species was *Naso unicornis*, which is also widespread but belongs to a separate clade from *N. vlamingii* (Klanten et al., 2004). *Naso unicornis* also differs from *N. brevirostris* and *N. vlamingii*, in being a benthic rather than a pelagic forager (Randall, 2001; Choat et al. 2002). All three species have similar life histories and have large pelagic larvae that persist in the pelagic environment for approximately 90 days (B. Victor, personal communication), giving all three species a high larval dispersal potential. In addition, the phylogenetic lineages of our two target species are comparable in age to that of *N. vlamingii* (Klanten et al., 2004). Ergo, these two species were chosen because they and *N. vlamingii* potentially share parallel evolutionary histories and might therefore have congruent population structures.

In light of the possibility that these three species may have similar population structures, the primary questions posed in this study were: (i) Do other *Naso* species exhibit elevated levels of genetic diversity as observed in *N. vlamingii?* (ii) Are other widely distributed *Naso* species characterized by a lack of geographical population partitioning across their equally wide species ranges? (iii) Do either of the target species in this study have a temporal rather than spatial population structure suggesting historical isolation across an ancient biogeographic barrier? Using a comparative approach, this study addresses the uncertainty surrounding the intriguing genetic partitioning observed in *N. vlamingii*, highlights the usefulness of comparative phylogeography as a tool for studying reef fish populations and provides insight into the population connectivity of these widespread reef fishes.

2. Methods

2.1. Sample collection and storage

Naso brevirostris and *N. unicornis* samples were collected at various locations in the tropical Indo-Pacific across their species distributions between March 2000 and February 2004 (Fig. 1). The majority of our samples originated at sites from the Seychelles Islands, Cocos Keeling, Christmas Island, Western Australia, the Great Barrier Reef, Solomon Islands and the Society Islands of French Polynesia. However, the *N. unicornis* data set was supplemented by a small number of samples from: Oman, Rodriguez Island, Reunion Island, Taiwan and Kimbe bay in Papua New Guinea. These supplementary samples were included only in the phylogenetic analysis and median joining haplotype network but due to the small sample size, these samples were excluded from population level analyses. Samples were either captured in the field by spearing or purchased at local fish markets. Fin clippings were stored in 80% ethanol or in salt-saturated dimethyl sulfoxide. One hundred and two *N. brevirostris* and 107 *N. unicornis* individuals were included in the final analysis.

2.2. Laboratory procedures

Total genomic DNA was extracted from fin clippings via proteinase K digestion followed by a standard salt-chloroform, DNA precipitation (Sambrook et al. 1989). The non-coding mitochondrial control region was PCR amplified using *Naso* specific, NA1 primers: **NA1-F** 5′-AGC ATT CTG AAC TAA ACT AC-3′ and **NA1-R** 5′-TGT CCC TTG ACT CTC AAT A-3′ (Klanten et al., 2007). DNA was amplified in 20 μL PCR reactions containing 2.5 mM Tris–Cl (pH 8.7), 5 mM KCL, 5 mM $(NH_4)_2SO_4$, 200 μM each dNTP, 3.5 mM $MgCl_2$, 10 μM each primer, 1 U of Taq Polymerase (Qiagen Ltd.) and 10 ng of template DNA. Thermocycling was carried out with an initial denaturation of 94 °C for 2 min, 35 cycles of denaturation, annealing and extension (94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s) and a final extension of 72°C for 10 min. PCR products were confirmed by gel electrophoresis on 1.5% agarose gels and purified by standard isopropanol purification. PCR products were sequenced with the NA1 forward primer using ABI (Applied Biosystems Incorporated) technologies at Macrogen sequencing service Seoul, South Korea. Sequences from this study can be obtained from GenBank Accession Nos. FJ216727-FJ216935.

2.3. Phylogenetic analysis

Sequences were first aligned using a Clustal W alignment (Higgins et al., 1994) implemented in BioEdit version 7.0.9.0 (Hall, 1999). Sequences were then further aligned and edited visually in BioEdit. Appropriate substitution models, chosen according to log likelihood score, were selected for both species using mrModeltest, version 2.2 (Nylander, 2004) in PAUP* (Swofford, 1999). PAUP* was also used for maximum likelihood analysis. Further, phylogenetic analysis was carried out in MrBayes, version 3.1 (Huelsenbeck and Ronquist, 2001). A Markov chain Monte Carlo (MCMC) with four chains for one million generations was performed, recording trees once every 100 generations. The first 26,000 and 170,000 trees were discarded as post-burn-ins for *N. brevirostris* and *N. unicornis*, respectively, when stationary was reached. *Naso unicornis* trees were rooted with *N. brevirostris* as out group and *N. brevirostris* trees were rooted with *N. vlamingii* as out group. Trees were sorted according to likelihood scores and majority rule consensus trees, for each species, were generated from the best 500 trees.

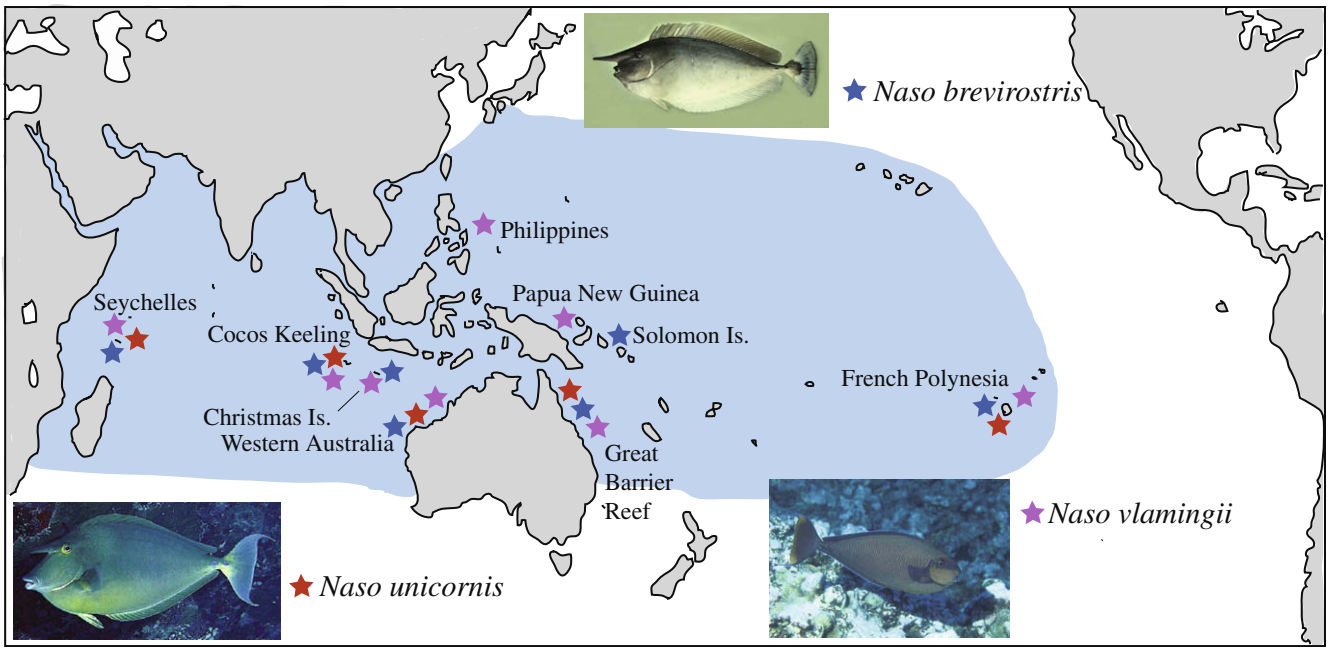

Fig. 1. Map illustrating the Indo-Pacific distribution of *N. brevirostris*, *N. unicornis* and *N. vlamingii*. Highlighted area generalizes the distribution of all three species. Blue stars, red stars and magenta stars indicate locations from which *N. brevirostris*, *N. unicornis* and *N. vlamingii* were, respectively, sampled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Molecular diversity and genetic partitioning

Arlequin version 3.1 (Excoffier et al. 2005) was used to calculate molecular diversity for all population level analyses. Median joining haplotype networks were drawn using Network version 4.5.0.1 (copyright 2004–2008, Fluxus Technologies Ltd.) for each species to visually illustrate haplotype variability and population geneology. Network calculations did not include gaps in the sequence alignment. Haplotype diversity (h) and nucleotide diversity (π) were used as measures of genetic diversity (Nei, 1987) for all sampled populations. Genetic distance between populations was calculated as a pairwise F_{st} matrix, plotted against geographic distance and tested for Isolation by distance with a Mantel test correlation executed by IBD web service (Jensen et al. 2005). Genetic population structure, was further explored among populations and clades with analysis of molecular variance (AMOVA), also implemented in Arlequin 3.1 (Excoffier et al; 1992; Weir, 1996). When temporal clades were present, i.e. haplotypes that grouped together but not according to the geographic areas where they were sampled, χ^2 goodness-of-fit tests determined if individuals from one ocean basin significantly dominated clades. The null hypothesis for this test was that clade membership of an individual was independent of the ocean basin where it came from. Additional AMOVA was performed to test for small-scale genetic structure between the north, central and southern Great Barrier Reef (GBR) but only the *N. unicornis* data set had sufficient representation from different areas of the GBR to justify this analysis.

2.5. Coalescence

Female generation times for *N. brevirostris* and *N. unicornis* were calculated using the formula $T = (\alpha + \omega)/2$, where α = the age at first reproduction and ω = the age at last reproduction (Pianka, 1978). This is the best proxy for generation time available in the absence of data regarding age specific mortality and fecundity, which cannot be acquired by normal sampling programs for fish that have extended life spans and multiple spawning episodes within the annual cycle. We calculated α and ω for both species using previously published otolith age estimates (Choat and Axe, 1996), which give some indication of transitions between stages in life history. Female coalescence time for both species was calculated with the formula: $t = \tau/2u$ and $u = 2\mu k$ (Rogers and Harpending, 1992), where u = the mutation rate for the entire sequence, t = generations since coalescence, μ is the mutation rate at each nucleotide position and k = the sequence length. Mutation rate (μ) for *N. brevirostris* and *N. unicornis* were calculated from divergence rate estimates of mitochondrial control region from swordfish (*Xiphias gladius*) (Alvarado Bremer et al. 1995). The proportional combination of 12.9% divergence rate for variable sites and 1.1% for conserved sites provided the overall sequence mutation rate. These divergence rates were as employed by Klanten et al. (2007) to determine female coalescence age in *N. vlamingii*. Mismatch distributions were produced in Arlequin for each species (Li, 1977; Rogers, 1995; Harpending, 1994; Schneider and Excoffier 1999; Excoffier, 2004). Mismatch distributions provided τ (tau), the final variable in the coalescence formula. The mismatch distributions also provide θ_0 and θ_1, which are values that, respectively, represent effective female population size (N_{ef}) at the time of the last common ancestor and the current effective female population size. Coalescence analysis estimated the time to most recent common ancestor for both *N. brevirostris* and *N. unicornis* data sets, as well as for individual non-geographic clades. Clade coalescence times were also evaluated in BEAST (Drummond and Rambaut, 2007) but consistent results could not be obtained from this analysis. Fu's test of selective neutrality was used to evaluate neutrality in our markers (Fu, 1997). Significantly negative Fu's *Fs* values are indicative of an expanding population.

3. Results

A total of 249 and 279 base pairs of the mitochondrial control region (HVR-1) were analyzed for *N. brevirostris* and *N. unicornis*, respectively. The difference in base pair length between species is due to a 30 bp indel in the *N. brevirostris* mitochondrial control

region, relative to *N. unicornis*. There was a contrast in the number of parsimony informative sites between data sets. *Naso unicornis* had 129 parsimony informative sites while *N. brevirostris* only had 78. However, in terms of uninformative, singleton sites *N. brevirostris* had 34 compared to 20 for *N. unicornis*. The ratio of transitions to transversions was approximately 5 to 1 and 4 to 1 in *N. brevirostris* and *N. unicornis*, respectively. Notwithstanding the high number of variable sites, sequences for both species aligned easily without uncertainty. Overall, haplotype and nucleotide diversities were high for both species, $h = 0.98$, $\%\pi = 7.32$ for *N. brevirostris* and $h = 0.99$, $\%\pi = 8.04$ for *N. unicornis*. Genetic diversity was somewhat higher for *N. vlamingii* ($h = 1.0$, $\%\pi = 13.6$). This is the highest genetic diversity reported from this molecular marker for any fish (Klanten et al., 2007).

3.1. Phylogenetic analysis

The best substitution model for *N. brevirostris* was T I M + I + G, with I and G values of 0.3524 and 0.8261 and for *N. unicornis* it was TVM + I + G, with I and G values of 0.3294 and 1.2501. Bayesian analysis placed *N. brevirostris* samples into three distinct lineages that were not arranged according to geography (see Fig. 2). χ^2 tests revealed that none of these lineages were significantly dominated by individuals from either ocean basin (χ^2 goodness-of-fit test, Yate's correction, df = 1, $\chi^2 = 0$; 0.21052; 3.5119, $p > 0.05$). Although similar, the three lineages differed slightly in support and overall structure. The lineage designated as clade 2 appears to be well supported, while clade 3 appears to be an unstructured conglomeration of haplotypes and clade 1, which has less support, may most appropriately be considered a sub-clade that branches off from clade 3. However, they were treated as three distinct non-geographic clades in AMOVA and coalescence analyses. *Naso vlamingii* exhibited four well supported non-geographic clades (Klanten et al., 2007). In contrast, the *N. unicornis* data set did not partition into any clades, geographic or otherwise. No suitable tree topology could be identified for *N. unicornis*, as all branches were poorly supported polytomies (Fig. 3). Removing transitions, which are more prone ot homoplasy, from the analysis, which are more prone to homoplasy, did not significantly alter tree topology in either *N. brevirostris* or *N. unicornis*, as was also shown for *N. vlamingii* (Klanten et al., 2007), suggesting that homoplasy is not driving the lack of geographic partitioning in the data. It is likely that some homoplasy is present in the data (see Fig. 2 for example) but the underlying phylogenetic pattern is not destroyed by it.

3.2. Population structure

Pairwise F_{st} comparisons showed genetic differentiation between a subset of populations of *N. brevirostris* but not *N. unicornis* (Tables 1a and 1b). For *N. brevirostris*, the Seychelles population was significantly different from Cocos Keeling, the GBR and French Polynesia. Pair wise F_{st} values increased gradually from west to

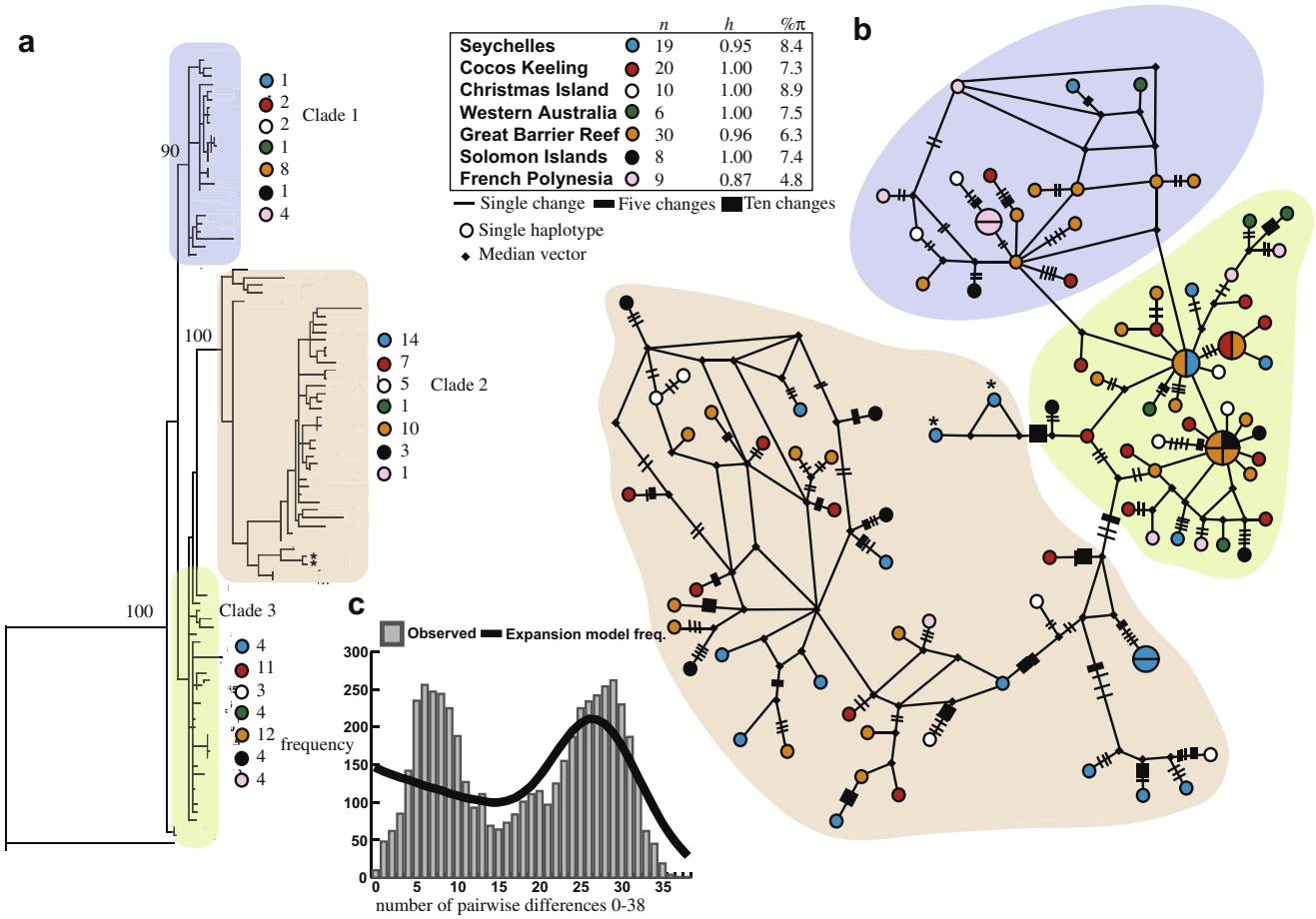

Fig. 2. (a) Rooted phylogram of 102 *N. brevirostris* mitochondrial control region sequences from Bayesian inference. Bootstrap values indicate three clades in which there is no geographic pattern. (b) Median joining network of *N. brevirostris* haplotypes. Sample size (n), haplotype diversity (h) and nucleotide diversity ($\%\pi$) are given. (c) Mismatch distribution of *N. brevirostris* haplotypes and Fu's *Fs* value. A significantly negative Fu's *Fs* value indicates an expanding population. A bimodal mismatch distribution indicates isolation across a geographical barrier, probably reflecting isolation across an ancient barrier sometime during this species' history. Haplotypes marked with asterisks indicate sequences that are peculiarly arranged in the network and may be affected by homoplasy.

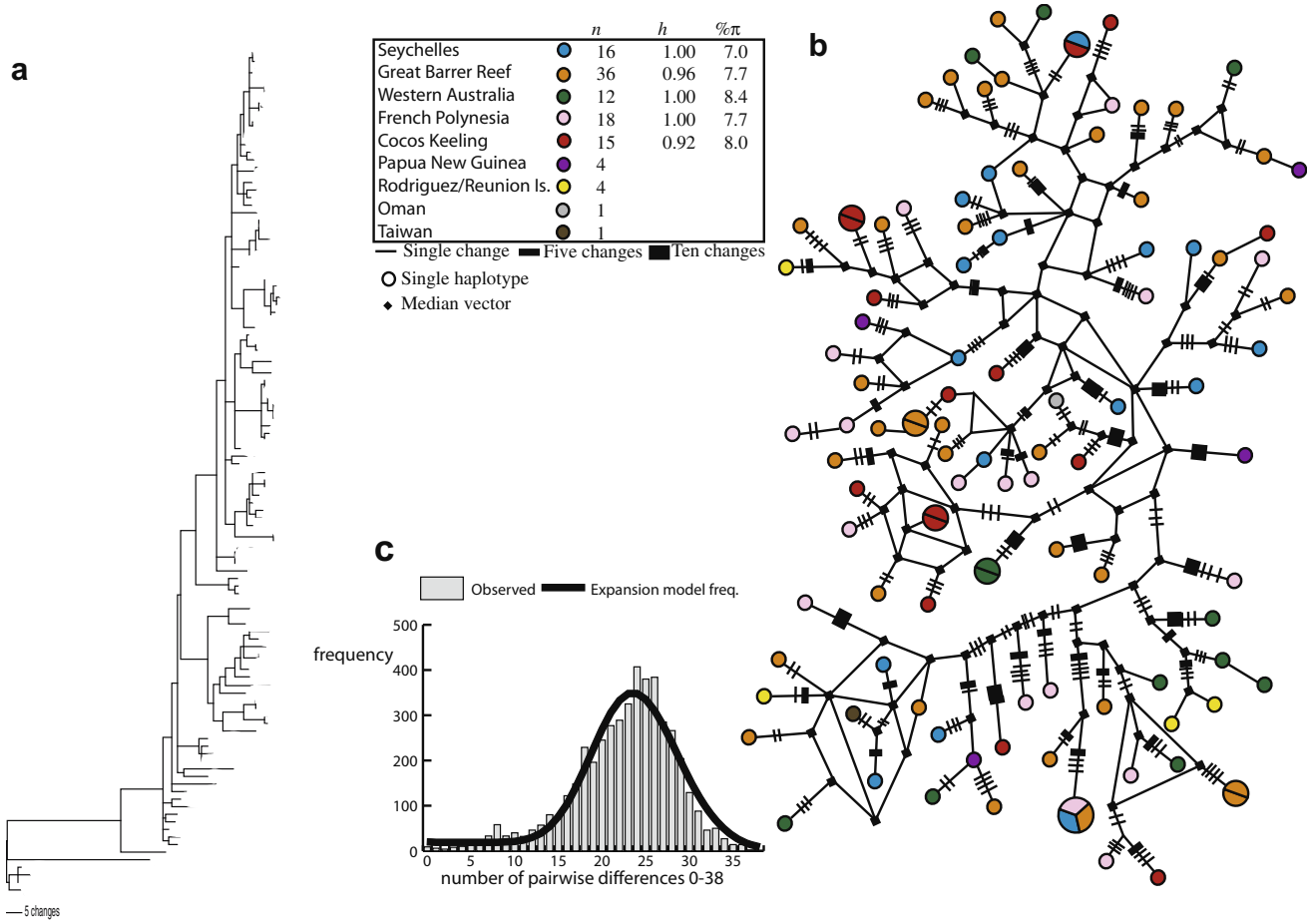

Fig. 3. (a) Rooted phylogram of 107 *N. unicornis* mitochondrial control region sequences from Bayesian inference. Bootstrap support values are not shown due to lack of significance. Actual topology for the tree is undefined. (b) Median joining network of *N. unicornis* haplotypes. Sample size (n), haplotype diversity (h) and nucleotide diversity ($\%\pi$) are given. (c) Mismatch distribution of *N. unicornis* haplotypes is unimodal and Fu's *Fs* value is significantly negative indicating an expanding population.

Table 1a
Population pairwise F_{st} for sampled *N. brevirostris* populations (below) and corresponding p values (above)

	S	CK	CI	WA	GBR	SI	FP
S		**0.03604**	0.44144	0.18919	**0.01802**	0.20721	**0.00001**
CK	**0.04260**		0.54955	0.36036	0.80180	0.84685	0.11712
CI	−0.00881	−0.01905		0.58559	0.27027	0.53153	0.14414
WA	0.06367	−0.00380	−0.03435		0.16216	0.45946	0.70270
GBR	**0.07449**	−0.02115	0.01244	0.02368		0.62162	0.07207
SI	0.01952	−0.04369	−0.02404	−0.02306	−0.03308		0.10811
FP	**0.16276**	0.05919	0.04213	−0.04402	0.06886	0.07197	

Symbols equal: S, Seychelles; CK, Cocos Keeling; CI, Christmas Island; WA, Western Australia; GBR, Great Barrier Reef; SI, Solomon Islands; FP, French Polynesia. Significant values appear in bold. Only the Seychelles population showed structure with any other population. Structure becomes stronger as the distance grows greater suggesting that remote populations are isolated by distance.

Table 1b
Population pairwise F_{st} for sampled *N. unicornis* populations (below) and corresponding p values (above)

	S	CK	WA	L	GBR	FP
S		0.07207	0.10811	0.46847	0.61261	0.36937
CK	0.02363		0.07207	0.12613	0.36937	0.25225
WA	0.01827	0.03348		0.07207	0.18018	0.09910
L	−0.0035	0.01757	0.02926		0.08108	0.37838
GBR	−0.0079	0.00456	0.01933	0.02448		0.09910
FP	0.00257	0.00846	0.02123	0.00071	0.02448	

Symbols equal: S, Seychelles; CK, Cocos Keeling; WA, Western Australia; L, Lizard Island; GBR, Great Barrier Reef; FP, French Polynesia. None of these values are significant suggesting no population structure between any two populations.

east. Mantel test showed a significant and positive correlation between pairwise F_{st} values and pairwise geographic distance for *N. brevirostris*, indicating that isolation by distance is a factor in the gene flow of this species ($Z = -348{,}455.8093$, $r = 0.4757$, $p = 0.046$). For *N. unicornis* there were no significant pairwise F_{st} values and no indication of population structure between any two geographic locations. Isolation by distance analysis for *N. unicornis* showed an extremely weak, negative correlation between pairwise F_{st} values and pairwise geographic distances but was statistically non-significant ($Z = 497.8586$, $r = -0.0472$, $p = 0.827$). Log transformation of either variable did not significantly alter the outcome of the tests in either *N. brevirostris* or *N. unicornis*. Significant structure was found for many pairwise population comparisons for

N. vlamingii (Klanten, personal communication). In particular, French Polynesia had significant structural differences with all other populations except Cocos Keeling (Table 1c). Isolation by distance was not significant for *N. vlamingii* ($r = 0.458$, $p = 0.078$) (Klanten et al. 2007).

All AMOVA fixation indices for this study are reported in Table 2. There was no evidence of population structure for *N. brevirostris* between the Indian and Pacific Oceans ($\Phi_{st} = 0.0299$, $p = 0.081$) whilst *N. unicornis* did have a significant population structure between the Indian and Pacific Ocean basins ($\Phi_{st} = 0.0184$, $p = 0.02$). However this Φ_{st} value is very small and when Φ_{st} values fall into this range it is difficult to distinguish between reduced gene flow and uninhibited gene flow (Palumbi, 2003). Therefore, any constraint on inter-oceanic gene flow in *N. unicornis* may be only light or negligible. For these analyses, the boundary between ocean basins was experimentally placed in three different locations: (i) between eastern and western Australia, (ii) between western Australia and Christmas Island and (iii) between Christmas Island and Cocos Keeling. Moving the boundary in this way did not significantly change the AMOVA result. Hence probably no barrier to gene flow exists between the Indian and Pacific Ocean basins for either of these species. Likewise, there was no evidence of population structure for *N. brevirostris* between the east and west Indian Ocean, or western and central Pacific Ocean ($\Phi_{st} = 0.0268$, $p = 0.083$). *Naso unicornis* again showed significant but negligible population structure at this spatial scale ($\Phi_{st} = 0.0182$, $p = 0.014$). When samples were analyzed for population structure at the smaller spatial scale of our sampling locations (i.e. the GBR, Seychelles, etc.), no population structure was detected for *N. unicornis* ($\Phi_{st} = 0.0089$, $p = 0.233$). However, shallow population structure was detected for *N. brevirostris* at this scale ($\Phi_{st} = 0.0773$, $p = 0.006$). When *N. unicornis* samples from the northern, central and southern GBR were segregated no evidence of population structure was found at this intraregional scale ($\Phi_{st} = 0.0256$, $p = 0.151$). In contrast to geographic tests of population structure, when the non-geographic clades of *N. brevirostris* were tested, deep population structure was found ($\Phi_{st} = 0.4996$, $p = 0.001$). AMOVA analysis of *N. vlamingii* populations revealed shallow structure between sampling locations and ocean basins ($\Phi_{st} = 0.065$, $p < 0.05$) and ($\Phi_{st} = 0.076$, $p < 0.05$), respectively (Klanten et al., 2007). Unfortunately Klanten et al. (2007) did not perform AMOVA on the non-geographic clades of *N. vlamingii* therefore no comparison can be made with *N. brevirostris*.

Table 2
Φ_{st} values for all three *Naso* species obtained from AMOVA analysis

N. unicornis	IO-PO	II-PP	5	N-C-S GBR
Φ_{st}	0.0184	0.0182	0.0089	0.0256
p	0.02	0.014	0.233	0.151
N. brevirostris	IO-PO	II-PP	7	C
Φ_{st}	0.0299	0.0268	0.0773	0.4996
p	0.081	0.083	0.006	0.001
N. vlamingii	IO-PO	II-PP	8	
Φ_{st}	0.0766	0.079	0.065	
p	<0.05	<0.05	<0.05	

Three principle geographic scales were tested for population structure: (1) when the entire species range was divided in half, i.e. by Indian and Pacific Ocean basins (IO-PO). (2) When the entire range was divided in quarters, i.e. East and West Indian and Pacific Oceans (II-PP). (3) When samples were grouped according to the geographic locations where they were collected i.e. Seychelles, French Polynesia, etc. *Naso unicornis* was primarily represented by five locations: Seychelles, Cocos Keeling, Western Australia, GBR and French Polynesia (5). *Naso brevirostris* was collected from all these locations as well as from Christmas Island and the Solomon Islands (7). *Naso vlamingii* was collected from all the previous locations plus the Philippines (8). An additional geographic scale was used in our *N. unicornis* data, in which samples from the GBR were divided according to North, Central and South areas of the GBR (N-C-S GBR). We also obtained a Φ_{st} value for our *N. brevirostris* data set to assess the amount of population structure between clades on a non-geographic scale (C). Φ_{st} values for *N. vlamingii* were taken from Klanten et al. (2007).

3.3. Coalescence

All coalescent data for this study are reported in Table 3. Nucleotide substitution rates differed greatly for all three *Naso* species. *Naso vlamingii* had the highest mutation rate nearly double that of its sister species *N. brevirostris* and substantially larger than that of *N. unicornis* as well. Hence, not surprisingly, *N. vlamingii* is also the most genetically diverse in both haplotype and nucleotide diversity. *Naso brevirostris* had the oldest mean coalescence time of 11.1 MY, followed by *N. vlamingii*, which had a mean coalescent time of 8.1 MY. *Naso unicornis* was the youngest of the three with a mean coalescence time of 6.2 MY. Mean coalescent ages for the clades of both *N. brevirostris* and *N. vlamingii* ranged between 2 and 5 million years ago. Both *N. brevirostris* and *N. unicornis* had equal θ_0 values, while *N. vlamingii* had a larger θ_0 value. *Naso unicornis* had a relatively large θ_1 value, much larger than the other two species, approximately 60 times larger than that of *N. brevirostris*, and over 100 times larger than *N. vlamingii*, which suggests a disproportionately large current effective population size for *N. unicornis*.

The mismatch distribution for *N. unicornis* was unimodal (Fig. 3c), the same as reported for *N. vlamingii* (Klanten et al. 2007). However, the mismatch distribution for *N. brevirostris* was clearly bimodal (Fig. 2c). Additionally Fu's *Fs* was significantly negative for all species, therefore, all three species probably represent global populations that have undergone expansion.

4. Discussion

4.1. Geographic population structure and gene flow

There is continuous water between the tropical Indian and Pacific Oceans that would seemingly permit passage for marine organisms and allow inter-oceanic gene flow to occur. Nevertheless, there appears to be a biogeographic barrier between the two

Table 1c
Population Pairwise F_{st} for sampled *N. vlamingii* populations (below) and corresponding *p* values (above)

	S	CK	CI	WA	PNG	PP	GBR	FP
S		**0.02734**	**0.00293**	0.11914	0.16504	**0.02734**	0.13379	**0.00879**
CK	**0.05849**		0.22656	0.09863	**0.03516**	**0.04004**	**0.02832**	0.06250
CI	**0.13285**	0.01808		0.06055	**0.01074**	**0.02539**	**0.00586**	**0.01074**
WA	0.05012	0.06427	0.10477		0.94531	0.34180	0.44531	**0.01074**
PNG	0.02235	**0.06063**	**0.12117**	−0.06473		0.38574	0.66113	**0.00488**
PP	**0.06137**	**0.07580**	**0.13385**	0.00689	0.00161		0.41895	**0.02441**
GBR	0.01899	**0.06019**	**0.13834**	−0.00596	−0.01314	−0.00411		**0.00781**
FP	**0.15666**	0.10021	**0.18820**	**0.24644**	**0.18933**	**0.18588**	**0.16680**	

Symbols equal: S, Seychelles; CK, Cocos Keeling; CI, Christmas Island; WA, Western Australia; PNG, Papua New Guinea; PP, Philippines; GBR, Great Barrier Reef; FP, French Polynesia. Significant values appear in bold (Klanten, personal communication).

Table 3
Coalescence times for each species and temporal clade

Coalescence	τ	θ_0	θ_1	μ	t(MY)		
					Lower	Mean	Upper
N. unicornis	**22.8 (18–25.8)**	**0.7**	**22518**	**6.6**	**4.9**	**6.2**	**7.1**
N. brevirostris	**25.5 (10.9–35.3)**	**0.7**	**357.1**	**4.8**	**4.7**	**11.1**	**15.4**
Clade I	6.1 (3.8–7.9)	0.31	73.8		1.6	2.7	3.5
Clade II	11.6 (7.2–25.8)	11.8	999.9		1.9	5	11.3
Clade III	5.6 (2.8–9.9)	1.671	42.2		1.2	2.4	4.3
N. vlamingii	**41.6 (31.4–65.3)**	**11.2**	**219.5**	**8.1**		**8.1**	
Clade I	17 (13.5–32.6)	13.7	1164			3.4	
Clade II	28.1 (19.8–52.6)	13.8	232.5			5.5	
Clade III	25.67 (19.4–40.7)	11.2	670.2			5	
Clade IV	14.95 (9.8–24.3)	6.8	2830			2.9	

Data regarding *N. vlamingii* were taken from Klanten et al. (2007). τ (tau) with 95% confidence interval. θ_0 Effective female population size (N_{ef}) at the time of coalescence, θ_1 N_{ef} at the present time, μ mutation rate per million years and t divergence time given in millions of years. Lower, mean and upper coalescence times given for *N. brevirostris* and *N. unicornis*.

oceans that obstructs the movement of many tropical marine species. The Indo-Pacific barrier most likely corresponds to the Indonesian Sunda Shelf (Rocha et al. 2007) and probably increases in potency during times of low sea level (Randall, 1998). It is also likely that the physical oceanography of the Indo-Australian Archipelago contributes to the effect of the Indo-Pacific barrier (Barber et al. 2006). The existence of many geminate (sibling) reef fish species allocated to either the Indian or Pacific Ocean indicate that the Indo-Pacific barrier has been instrumental to the speciation and biodiversity of shore fishes in the Indo-Pacific (Randall, 1998; Marie et al. 2007).

Phylogeographic studies show that the Indo-Pacific barrier also has an effect on genetic populations within species. Many tropical marine species found in both the Indian and Pacific Oceans have genetic population partitions that geographically coincide with a barrier somewhere between the Indian and Pacific Oceans. Specific examples include echinoderms (Williams and Benzie, 1998; Lessios et al. 2001), crabs (Lavery et al. 1996), shrimps (Duda and Palumbi, 1999) and reef fishes (Bay et al. 2004; Planes and Fauvelot, 2002). Even some pelagic fishes, which should be less limited by oceanic barriers than demersal reef fishes, show significant population partitions between the Indian and Pacific oceans (Menezes et al. 2006).

Notwithstanding a well-documented biogeographic barrier dividing the tropical Indian and Pacific oceans, some studies suggest that certain species regularly exchange migrants between the two ocean basins (Lessios et al. 2003; Klanten et al. 2007). The results of our study add to the number of species that have genetically continuous populations between the tropical Indian and Pacific oceans. In general, results for both *N. brevirostris* and *N. unicornis* are consistent with those found for *N. vlamingii* (Klanten et al. 2007). There was no evidence of a persistent barrier to gene flow across the Indo-Pacific in all three species and minimal geographic population structure was found in all three species, even at the largest spatial scales. It is possible that high within-population variation is artificially deflating Φ_{st} values, falsely indicating higher connectivity than actually exists. But high within-population variation cannot account for all of the observed patterns in the data, such as non-geographic clades and shared haplotypes found in both oceans. The fact that all three *Naso* species are congruent in lacking geographic structure makes it probable that a high amount of inter-oceanic gene flow occurs in these taxa.

4.2. Shared haplotypes

The presence of shared haplotypes at distant locations also suggests gene flow across large spatial scales. Unlike *N. vlamingii*, in which all samples had unique haplotypes, in both *N. unicornis* and *N. brevirostris* a minority of individuals at different locations shared haplotypes (Figs. 2b and 3b). The most extreme example of this was found in *N. unicornis*, in which individuals from the Seychelles, the GBR and French Polynesia, shared a single haplotype. In *N. brevirostris* one haplotype was shared between the Seychelles and One Tree Island in the southern GBR.

The existence of haplotypes shared across vast distances can be interpreted in two different ways. Because the mitochondrial control region is one of the fastest evolving and hyper variable gene regions known (Moritz et al. 1987), the presence of shared haplotypes may indicate that gene flow between distant populations has occurred on a relatively recent evolutionary time scale. Alternatively, the most frequently occurring haplotypes, as well as those found at the largest geographic scales, can also be interpreted as being the oldest (Posada and Crandall, 2001). In the latter scenario the shared haplotypes in *N. brevirostris* and *N. unicornis*, separated by vast distances, may actually be much older than other haplotypes in our data and may have had considerably more time to traverse long distances through many successive generations. In the *N. brevirostris* data, set the shared haplotypes are predominately found at or near the center of small star shaped phylogenies within the haplotype network (Fig. 2b). This central position, with a high number of connections to other haplotypes is usually occupied by the oldest haplotypes (Castelloe and Templeton, 1994). Contrastingly, in *N. unicornis* the majority of the most frequent haplotypes (those that occur more than once) are found on the periphery of the haplotype network and have only single connections to the main body of haplotypes (Fig. 3b). The small number of connections to these shared haplotypes indicates that they are much less likely to be the oldest (Castelloe and Templeton, 1994). Thus, although both these *Naso* species show similar geographic population structure, it is possible that they differ greatly in temporal patterns of gene flow and expansion. Because of the positioning of shared haplotypes in the haplotype networks, distant *N. unicornis* populations may have been connected much more recently than equivalent populations of *N. brevirostris*.

4.3. Temporal rather than spatial genetic structure

Like *N. valmingii*, *N. brevirostris* has strong population partitions that do not correspond to the geographic distribution of our samples for this species. Klanten et al. (2007) posited that, in *N. vlamingii* this non-geographic pattern constituted a temporal population structure that resulted from episodic isolation events sometime during its long species history. Our data for *N. brevirostris* are consistent with this suggestion. Population structure between the clades of *N. brevirostris* is deep (Table 2), similar to what might be expected for populations separated across a barrier.

Also, the mismatch distribution of *N. brevirostris* was distinctly bimodal (Fig. 2c), which is normally characteristic of species where two populations are separated by a geographic barrier (Miller et al. 2006; Mora et al. 2007). The bimodal mismatch distribution of *N. brevirostris* seems to contradict our AMOVA analysis and Fu's *Fs* values for this species, which do not indicate a contemporary geographic barrier to gene flow. This could be explained by the mismatch distribution reflecting the effects of an ancient barrier rather than of a contemporary one. Coalescence times calculated for the temporal clades of both *N. brevirostris* and *N. vlamingii* approximate each other. Notwithstanding the fact that these coalescence dates are heuristic explorations rather than precise ages, such congruence suggests that the same factors may be driving the temporal population structure in both species.

The Indo-Pacific barrier provides a potential mechanism for the non-geographical genetic partitioning of *N. brevirostris* and *N. vlamingii*. If the barrier has been more potent in the past during periods of low sea level, as has been suggested by several authors (Springer and Williams, 1990; Randall, 1998), then populations of these two *Naso* species could have historically been separated by the barrier long enough for genetic differentiation to occur. In other words, the Indo-Pacific barrier has affected *N. brevirostris* and *N. vlamingii* in a similar way as other species. However, because the clades within *N. brevirostris* and *N. vlamingii* (Klanten et al., 2007) are found in statistically equal numbers in both oceans, genetic exchange between the tropical Indian and Pacific Oceans would have to have been substantial since the last isolating low in sea level.

If the same factors are driving the temporal population structure in both *Naso* species then the fact that *N. vlamingii* does not have a multimodal mismatch distribution like *N. brevirostris* is difficult to reconcile. In reality, *N. brevirostris* and *N. vlamingii* could represent two different genetic stages of the speciation process. The stronger genetic subdivision manifested in the bimodial mismatch distribution of *N. brevirostris* could indicate that lineages within this species were more divergent than those of its sister species when a biogeographic barrier was lifted and introgression halted the speciation process. If the demographics of the two species during isolation were not the same then differences in effective population size would have had an effect on the time required for populations to reach reciprocal monophyly (Birky et al., 1989; Hellberg et al. 2002). Conversely, there is the possibility that the non-geographic clades in either species are in fact cryptic species with currently sympatric distributions. Perhaps both *N. brevirostris* and *N. vlamingii* deserve a closer scrutiny of taxonomy but the issue of cryptic speciation will not be discussed further in this paper.

Naso unicornis did not show a temporal population structure as *N. brevirostris* and *N. vlamingii* did. One explanation for this is that *N. unicorinis* was not affected by a barrier to gene flow as its two congeners appear to have been. However, the unimodal mismatch distribution and significantly negative Fu's *Fs* value of *N. unicornis* (Fig. 3c), both indicate a population that has undergone expansion. This is not what would be expected of a species with ongoing gene flow over long periods of time or a population that has remained stable for much of its evolutionary history. An alternative possibility is that *N. unicornis* represents a single extant temporal clade, that has survived while all other clades of this species became extinct before expansion became possible. The fact that the single *N. unicornis* clade has the shallowest coalescence time of the three *Naso* species is consistent with such an explanation.

4.4. Isolation by distance

Isolation by distance was not significant for *N. unicornis* or *N. vlamingii* but was significant for *N. brevirostris*. Pairwise F_{st} values for this last species indicated that population structure existed between the Seychelles and Cocos Keeling, between the Seychelles and the GBR, and between the Seychelles and French Polynesia. Why the Seychelles alone had structural differences with any other population is unknown; a larger sample size may reveal a greater number of pairwise structural differences between locations. Also, unsampled ghost populations (Hellberg, 2007) located between our sampling locations could be softening F_{st} values. More importantly, the depth of the structural differences increased from west to east and suggests that distance is an important factor in determining the population structure of this species.

Because *N. brevirostris* lacks genetic population structure at the largest spatial scale the fact that isolation by distance is significant in this species suggests that the most distantly separated populations are exchanging genes via intermediate stepping stone populations (Hellberg et al. 2002). However, *N. brevirostris* also has significant population structure at the smaller spatial scale of our sampling locations, indicating that gene flow between these locations is reduced. It may seem contradictory to have population structure on a small-scale but not on a large one but it is possible that this species employs both long and short-distance dispersal strategies in order to maintain populations. If the majority of *N. brevirostris* larvae do not stray far from their natal reefs then moderate population structure at a relatively small spatial scale would be expected. Simultaneously, a minority of individuals with a tendency to disperse long distances would provide sufficient gene flow to genetically homogenize distant populations and prevent genetic population partitions from developing on a global scale (Slatkin, 1987; Palumbi, 2003).

Genetic distance was negatively, although non-significantly, related to geographic distance in *N. unicornis*. This pattern seems opposite of what might be expected for a marine species with a huge geographic range. However, a similar relationship between genetic and geographic distance occurs in another widespread Indo-Pacific reef fish *Myripristis berndti*, in which peripheral populations at the Indian Ocean and Pacific Ocean extremes of its range are most similar (Craig et al. 2007). Chenoweth and Hughes (2003) also report a negative relationship between genetic and geographic distance in an estuarine polynemid with a pelagic larval stage, although at a much smaller scale. This unusual pattern may result from the founding of isolated pocket populations (sensu Ibrahim et al. 1995), on the periphery of the species range, by a few long distance migrants during the early stages of expansion, well ahead of the main expansion wave. Such pocket populations are expected to maintain their genetic signature long after they have been reintroduced into the general population (Ibrahim et al. 1995). In such a scenario populations in the center of a species distribution would accumulate more mutations while fringing populations at the extremes of the distribution would remain genetically similar to each other.

5. Conclusion

The congruent lack of phylogeographic partitioning found among these three *Naso* species illustrates the usefulness of comparative phylogeography in resolving complicated patterns of population structure. The lack of phylogeographic patterns exhibited by *N. brevirostris* and *N. unicornis* were, overall, congruent with the findings of Klanten et al. (2007) for *N. vlamingii*. All three species show no evidence of being affected by a current biogeographic barrier to dispersal between the Indian and Pacific Oceans, although the tempo and pattern of gene flow likely has varied from species to species. The unique temporal rather than spatial genetic structure reported by Klanten et al. (2007) for *N. vlamingii* was also found in its sister species *N. brevirostris*, indicating that this is not a

stochastic pattern but rather the result of largely congruent species histories and life histories.

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