LACK OF GENETIC DIFFERENTIATION AMONG FOUR SYMPATRIC SOUTHEAST AFRICAN INTERTIDAL LIMPETS (SIPHONARIIDAE): PHENOTYPIC PLASTICITY IN A SINGLE SPECIES?

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ABSTRACT

Specimens of four sympatric intertidal limpet species (Siphonaria dayi, S. tenuicostulata, S. anneae and S. nigerrima) were collected from four localities on the east coast of South Africa and southern Mozambique. Their phylogenetic relationships were investigated using sequences of the mitochondrial COI gene and the intron-containing nuclear ATPSβ gene. Two closely related lineages were recovered, which grouped specimens on the basis of geography rather than morphology. One lineage was associated with the subtropical coastline of South Africa’s east coast and the other with the tropical coastline of northeastern South Africa and southern Mozambique. This genetic discontinuity coincides with a biogeographic boundary located in the vicinity of Cape St Lucia. Combined genetic diversity of the four species was lower than that of three other southern African congeners, and fell within the range determined for single southern African marine mollusc species. We suggest that the four limpet species are in fact different morphotypes of a single species.

INTRODUCTION

Siphonariid limpets (Gastropoda: Pulmonata) occur on temperate and tropical intertidal rocky shores throughout the world (Hodgson, 1999). In southern Africa, a total of nine species have been described, but their taxonomic status remains confused (Chambers & McQuaid, 1994). Four small-bodied species with largely sympatric distribution ranges occur on the high shore of South Africa’s subtropical east coast. Morphologically, these species are mainly distinguished on the basis of shell colour and structure. Siphonaria nigerrima Smith, 1903, is small (7–12 mm), with a thin, fragile shell that is dark brown-black, and with fused ribs. Siphonaria anneae Tomlin, 1944 and S. tenuicostulata Smith, 1903 are larger (10–15 mm) and have paler shells, with 30–40 distinct ribs in the former and 50–60 in the latter. Siphonaria dayi Allanson, 1959, is still larger (15–20 mm) and pale cream-white with 40–50 ill-defined ribs (Chambers & McQuaid, 1994). The shells of the four species also differ in their internal shell markings. Despite fairly clear differences in shell morphology, they appear to have similar microhabitat preferences and distribution ranges. Their taxonomic history is chequered; S. nigerrima was considered to be a synonym of S. carbo by Hubendick (1946), and S. tenuicostulata a synonym of S. anneae by Allanson (1959). All four species have direct development, with fully metamorphosed crawling larvae that emerge from gelatinous benthic egg masses (Chambers & McQuaid, 1994). Direct development presumably increases the possibility of local adaptation, and Chambers & McQuaid (1994) concluded, on the basis of shell morphology, that the four species were closely related, but valid species belonging to the subgenus Patellopis.

In the present study, we reexamine the taxonomic status of these species using two neutral genetic markers, one of them a mitochondrial gene, and the other a nuclear gene containing an intron.

MATERIAL AND METHODS

Twenty-four ingroup specimens were analysed morphologically and genetically. Specimens were collected at four localities throughout the ranges of the species and were identified based on shell morphology as described in Chambers & McQuaid (1994). Three of the sampling sites (Umhlanga Rocks, Blythedale Beach and Sodwana Bay) are in South Africa, and the fourth (Inhaca Island) is in southern Mozambique (Fig. 1). The specimens from both Umhlanga and Inhaca were found in adjacent shallow rock pools, whereas those from both Blythedale and Sodwana were collected from single rocks that were surrounded by sand and that became completely exposed during low tide. We also obtained samples of two other southern African congeners, namely Siphonaria capensis (from Cape Agulhas), and S. concinna (from Port Elizabeth). These two species were selected as outgroup taxa because COI sequence data from 15 Siphonaria species from southern Africa, Australasia and Southeast Asia revealed that they were most closely related to the ingroup (Teske, Barker & McQuaid unpublished). Samples were immediately preserved in 70% ethanol. Prior to DNA extraction, small tissue samples were removed from the foot of each specimen, air dried and then soaked in distilled water for at least 3 h. Genomic DNA was isolated using the Chelex extraction protocol (Walsh, Metzger & Higuchi, 1991). We sequenced a portion of the mitochondrial COI gene using universal primers (Folmer et al., 1994) and an intron-containing region of the nuclear ATPSβ gene. For the latter, we designed a genus-specific forward primer (SiphonariaATPSβ f: 5’-TGR ATT CCC TGA TGT TTT TGT GAG-3’), which was used in conjunction with a universal reverse primer (ATPSβr1: 5’-CGG GCA CGG GCR CCD GGN GGT TCG T-3’; Jarman, Ward & Elliot, 2002). PCR amplification for COI followed protocols in Zardi et al. (2007). PCR reactions of ATPSβ included 2.5 μl of BIOTAQ™ DNA Polymerase (5 units/μl, Bioline) and 1 μl of DNA extracts

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reverse direction using BigDye System (Promega), cycle sequenced both in the forward and
were deleted in a pairwise fashion and nodal support was
specified, missing sites
default settings in MEGA version 3.1 (Kumar, Tamura &
were reconstructed using Minimum Evolution and Neighbour-Joining (Saitou & Nei, 1987) using
times to ensure consistency of results. Phylogenies of combined
was a consensus based on all alignments recovered excluding a
thousand iterations were performed, and the final alignment
model; Tamura & Nei, 1993), including a gamma distribution
presently implemented in the programme (the Tamura-Nei
gramme estimates alignment and phylogeny simultaneously in
in a total of 25 µl. The PCR profile comprised an initial
denaturation step (2 min at 94°C), 35 cycles of denaturation
(30 s at 94°C), annealing (45 s at 60°C) and extension (45 s
at 72°C), and a final extension step (10 min at 72°C). PCR pro-
ducts were purified with the Wizard® SV Gel and PCR Clean-Up
System (Promega), cycle sequenced both in the forward and
reverse direction using BigDye® Terminator v3.1 Cycle Sequen-
cing Kit (Applied Biosystems) and visualized on an ABI 3100
As the ATPSβ sequences of ingroup and outgroup samples
were characterized by length differences, sequences were aligned
in BALI-PHY (Redeling & Suchard, 2005). This pro-
gramme estimates alignment and phylogeny simultaneously in
a Bayesian framework. We specified the most complex model
presently implemented in the programme (the Tamura-Nei
model; Tamura & Nei, 1993), including a gamma distribution
parameter and an assumed proportion of invariable sites. One
thousand iterations were performed, and the final alignment
was a consensus based on all alignments recovered excluding a
burn-in of 100 iterations. The procedure was repeated five
times to ensure consistency of results. Phylogenies of combined
sequence data were then reconstructed using Minimum Evolution
and Neighbour-Joining (Saitou & Nei, 1987) using
default settings in MEGA version 3.1 (Kumar, Tamura &
Nei, 2004). The Tamura-Nei model was specified, missing sites
were deleted in a pairwise fashion and nodal support was

RESULTS
Specimens with pale shells (Siphonaria dayi, S. anneae or S. tenuicos-
tulata) were found at all four localities, whereas S. nigerrima was
only found at Blythedale Beach and Sodwana Bay (Fig. 2).
Although the shells of most specimens could readily be assigned
to one of the four species, there were considerable morphological
differences between localities. For example, specimens of S. dayi
from Inhaca Island were small and oval, with finely ribbed
white ventral ridges, whereas those from Sodwana Bay were
larger, had thicker shells, more irregular outlines and broader
white ventral ridges with coarser ribs. Specimens of S. anneae
from Umhlanga Rocks were flatter and lighter both dorsally and
ventrally than those from Blythedale Beach and Sodwana
Bay. One specimen from Sodwana Bay (SO6) could not be
assigned to any species with confidence, while another (SO5),
although tentatively identified as S. anneae, was distinctly
darker than other such specimens.

Partial COI and ATPSβ sequences were 570 and 289
nucleotides in length, respectively. Six unique haplotypes were
recovered for COI and four for ATPSβ. These have been de-
posited in GenBank (Accession no. EF418589 – EF418602). The
mean and maximum number of base differences among
ingroup specimens were 3.26 and 7 (0.6% and 1.2%) for COI,
and 1.46 and 4 (0.4% and 1%) for ATPSβ. All variable sites
in the COI sequences were in third codon positions, and no het-
erozygotes were identified for ATPSβ. Phylogenetic reconstruc-
tion using combined COI and ATPSβ data recovered two major
lineages (Fig. 3). A northern lineage included specimens from
Inhaca Island and Sodwana Bay, and a southern lineage com-
prised specimens from Blythedale Beach and Umhlanga
Rocks. The COI sequences of all specimens from the southern
group were identical, whereas two ATPSβ haplotypes were
recovered from this region. Samples collected from the two
sites north of Cape St Lucia (Fig. 1) were characterized by
two distinct but closely related clusters, each confined to a
single sampling locality (Sodwana Bay or Inhaca Island).
Samples from Inhaca Island were all identical for both COI
and ATPSβ, whereas those from Sodwana Bay had four COI
haplotypes and one ATPSβ haplotype. None of the four
species studied were recovered as monophyletic lineages.

Combined genetic diversity indices of COI sequences of the
four Siphonaria species investigated in this study not only fall
within the range for single marine mollusc species, but are
even lower than those of three other southern African Siphonaria
species (S. capensis, S. concinna and S. serrata; Table 1).

DISCUSSION
Discrepancies between shell morphology, protein expression
and genetics
The question of how useful shell morphology is in resolving
phylogenetic relationships among closely related marine gastro-
pods is a matter of debate. Vermeij & Carlson (2000) suggested
that while anatomical soft tissue characters are too conserved,
shell morphology tends to be sufficiently variable to resolve
such relationships. However, like the limpets investigated in
the present study, many marine gastropods have simple shells that lack readily identifiable morphological features, and the problem is exacerbated by plasticity in shell morphology (Collin, 2003). While the inclusion of shell morphology characters into data-sets comprising anatomical and/or genetic data may result in better-resolved phylogenetic trees (Schander & Sundberg, 2001; Collin, 2003), the use of shell morphology characters on their own can be highly misleading. Intraspécific phenotypic plasticity of morphological characters is common in many marine molluscs and is generally related to environmental conditions. Examples include the development of larger apertural teeth or increased shell thickness in the presence of shell crushing predators (Appleton & Palmer, 1988; Trussell & Smith, 2000), a larger body size at sites with high wave exposure as compared to sheltered sites (Savini et al., 2004), increase in shell size at lower temperatures (Atkinson, 1994; Trussell, 2000), as well as lighter pigmentation and greater shell height in individuals living higher in the intertidal zone (Branch, 1975; Etter, 1988; Rugh, 1997; Sokolova & Berger, 2000). Because of the sensitivity of mollusan shell morphology to environmental conditions, the number of genetic studies that have synonymized species is likely to be higher than for most other marine invertebrate phyla (Knowlton, 2000).

Our analyses based on sequence data did not recover the four *Siphonaria* species as distinct genetic clusters. This contrasts with the results of a study using polyacrylamide gel electrophoresis of total soluble proteins (PAGE; Chambers et al., 1996). This previous study identified each species as being monophyletic, except that a single specimen of the planktonic disperser *S. capensis* was in a derived position within the *S. tenuicostulata* cluster. Moreover, most of the specimens of the planktonic disperser *S. concinna* were recovered as the sister taxon of the direct developer *S. tenuicostulata*. In the present study, both planktonic dispersers were identified as being distinct from the ingroup specimens by both molecular markers, and for that reason were used as outgroup taxa. The discrepancy between the two studies may be explained by the fact that the same genotype may express different phenotypes depending on its biotic or abiotic environment (Agrawal, 2001), and this manifests itself in the synthesis of different proteins as well as the expression of different morphological characters. Species that live in the intertidal zone are strongly affected by extremes in temperature and desiccation stress and exhibit physiological and behavioural adaptations that allow them to deal with the stresses caused by prolonged emersion (Johannesson, 1989).

The distribution of colour morphs is difficult to explain, but this should perhaps not be unexpected. Shell colour can be particularly variable as pigmentation is controlled by different factors in different taxa. For example, the shell pigments of archaeogastropods are thought to be formed as digestive residues...
(Cole, 1975), so that colour and patterning can be determined by the nature of the primary food source (Underwood & Creese, 1976). In contrast, shell colour in the meso- and neogastropods has a strong genetic basis (Reimchen, 1979; Atkinson & Warwick, 1983) and morph-dependent selection can be driven by factors such as thermal effects and predation (McQuaid, 1996; Parsonage & Hughes, 2002). In fact, the land snail *Cepaea* has been used as an example of the complexity of the control of shell colour in the Pulmonata by Jones et al. (1977), who concluded that at least eight evolutionary forces act on shell colour and that one factor alone rarely explains the frequencies of colour morphs fully. We found no obvious spatial separation between light and dark shells at localities where both types were found (Sodwana Bay and Blythedale Beach). Moreover, shells were found that could be classified as being intermediate between the light and dark species in terms of their shell coloration (Fig. 2, specimens SO5 and SO6). At both of these sites, specimens were more exposed to desiccation stress than at the sites where only light shells were found in rock pools (Inhaca Island and Umhlanga Rocks), suggesting that the hypothesis that shells with lighter pigmentation occur higher on the shore (where desiccation stress is greater; e.g. Branch, 1975; Etter, 1988) does not hold in this case.

Although some specimens were difficult to allocate to species, we are left with the problem that individuals that were apparently very clear examples of different species occurring within the same habitat were grouped together in the genetic analysis.

**Table 1.** Genetic diversity indices (+ SD) of the COI sequences of various southern African marine molluscs. The four *Siphonaria* species investigated in this study were treated as a single species (first row).

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>No. haplotypes</th>
<th>Haplotype diversity</th>
<th>Nucleotide diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Siphonaria</em> (ingroup spp. combined)</td>
<td>24</td>
<td>6</td>
<td>0.74 ± 0.06</td>
<td>0.006 ± 0.003</td>
</tr>
<tr>
<td><em>S. capensis</em>†</td>
<td>12</td>
<td>8</td>
<td>0.92 ± 0.06</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td><em>S. concinna</em>†</td>
<td>17</td>
<td>16</td>
<td>0.99 ± 0.02</td>
<td>0.025 ± 0.013</td>
</tr>
<tr>
<td><em>S. serrata</em>†</td>
<td>16</td>
<td>11</td>
<td>0.93 ± 0.05</td>
<td>0.031 ± 0.017</td>
</tr>
<tr>
<td><em>Nassarius kraussianus</em>‡</td>
<td>80</td>
<td>17</td>
<td>0.72 ± 0.04</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td><em>Perna perna</em> (western lineage)‡</td>
<td>82</td>
<td>41</td>
<td>0.87 ± 0.02</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td><em>P. perna</em> (eastern lineage)‡</td>
<td>58</td>
<td>10</td>
<td>0.49 ± 0.08</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td><em>P. perna</em> (combined)‡</td>
<td>140</td>
<td>51</td>
<td>0.87 ± 0.02</td>
<td>0.020 ± 0.009</td>
</tr>
<tr>
<td><em>Octopus vulgaris</em>§</td>
<td>13</td>
<td>5</td>
<td>0.54 ± 0.16</td>
<td>0.003 ± 0.002</td>
</tr>
</tbody>
</table>

†Teske, Barker & McQuaid unpubl.
‡Zardi et al., 2007.
§Teske et al., in press.
For example, most of the limpets from Umhlanga Rocks and Blythedale Beach were genetically indistinguishable, but included typical examples of both S. annaeae and S. nigerrima. Such specimens differed strongly not only in shell colour, but also in aspects of shell shape such as apex height, presence/absence of ribbing and robustness. Closely related molluscs can show rapid intergenerational convergence of shell form when grown under laboratory conditions (Wulkschegler & Jokela, 2002), but this situation is the reverse. The problem becomes one of explaining why animals that share the same habitat on the same shore differ so dramatically in shell morphology when we cannot invoke a genotypic explanation.

Biogeographic patterns

Explaining the genetic structure of these populations proves easier than explaining their morphologies. Recent phylogeographic studies of southern African coastal invertebrates identified a number of species that are subdivided into genetically distinct geographic units. In most cases, these are associated with different marine biogeographic provinces (e.g. Teske et al., 2006; Zardi et al., 2007), with the boundary between the cool-temperate west coast and the warm-temperate south coast, as well as the boundary between the south coast and the subtropical east coast, being of particular importance. Bolton et al. (2004) identified a discontinuity in the composition of benthic shallow-water macroalgae in the vicinity of St Lucia (Fig. 1), a region that may be considered the south-western boundary of the tropical Indian Ocean flora. The genetic discontinuity between the two main lineages of the Siphonaria species studied coincides with this boundary, and this result represents the first indication that the St Lucia biogeographic boundary could be important at what appears to be an intraspecific level. However, as the differentiation between lineages was small and there were large sampling gaps between populations, additional samples from the region between Blythedale Beach and Sodwana Bay are required to assess the importance of the St Lucia biogeographic boundary to the phylogeography of the Siphonaria species.

Conclusion

In this study on four southeast African intertidal limpets, a northern and a southern genetic lineage of what is apparently a single species were recovered. Each lineage contains at least twomorphs that are almost identical genetically, but different in terms of shell morphology. Apparent conflict between genetic and morphological data is itself is not problematic, but the fact that morphs are shared between the lineages is difficult to interpret. One possible explanation is that these morphs arose prior to genetic divergence of the two genetic lineages. This apparent morphological stasis and the fact that different morphs were found adjacent to each other in the same habitat suggests that there is no directional selection on shell morphology. However, this seems highly unlikely in terms of the stresses of desiccation and temperature that are characteristic of the species’ habitat.

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