4. MOLECULAR ANALYSES OF SIX COWRIE SPECIES FROM THE INDIAN SUBCONTINENT AND THEIR RELATIVE PHYLOGEOGRAPHY

Introduction

Molecular sequence data from six cowrie species were analyzed to determine the biogeographic affinities of the Indian marine biota. Mt.DNA sequences were generated from the Cytochrome Oxidase subunit I for the following species: Mauritia arabica (Cypraea arabica), Palmadusta lentiginosa (Cypraea lentiginosa), Purpuradusta gracilis (Cypraea gracilis), Adusta onyx (Cypraea onyx), Erronea caurica (Cypraea caurica) and Erronea errones (Cypraea errones). Each species is more widely distributed cowrie with described geographically partitioned subspecies. Indian populations contained a mixture of species with geographic ties to the West (P. lentiginosa), to the East (M. arabica, P. gracilis, A. onyx and E. errones) and a mixed assemblage (E. caurica) but so far the distributions of Indian populations were not uniquely discovered. Biogeographic affinities of individuals from India for each of the six lineages examined indicate that the Indian marine fauna is composed of a mixture of taxa with varied biogeographic relationships. Comparative genetic studies are revealing highly
structured marine populations, even in the absence of apparent oceanographic or biogeographic barriers. Additionally, the study of widely distributed Indo-West Pacific species indicate that most lineages are structured across this broad geographic area, showing either isolation by distance, regional affinities or deeply divergent, basinally-structured subclades.

Phylogenetic diversity measures are based on the observation that more distantly related species tend to be more distinct in terms of their morphological or other characters. They therefore are relatedness as a criterion for assessing what weighting each species should be given. In cladistics, sister groups are two clades, separated by a single speciation event. The phylogenetic diversity measure based on equal weighting for sister groups. Historically, resolved phylogenies of such diverse marine groups have been difficult to construct. Traditional morphological characters do not provide sufficient information for comprehensive cladistic analyses and interspecific relationships often are left unresolved. However the advent of molecular sequence data furnishes a potentially vast amount of appropriate phylogenetic signal to resolve the relationships within diverse groups at all levels of divergence.
A brief analysis of Indian assemblages indicates that the fauna is composed of species that show three general biogeographic patterns. First, many species are taxa with a wide Indo-Pacific distribution and can be found from the shores of the African continent in the Western Indian Ocean clear across the Indo-West Pacific (IWP) to French Polynesia in the central Pacific. Second, many species are restricted to the (North Western) NW Indian Ocean and Arabian Sea and are found from the Arabian Peninsula to Sri Lanka. Third, other taxa have distributions stretching from the Indian subcontinent eastward to the Andaman Sea.

We set out to test the faunal affinities of Indian reef taxa using six cowrie species as target taxa to explore genetic connectivity of Indian populations. The six taxa were chosen because species contains described subspecies, indicating that there might be some geographic structuring for these species throughout their range.

Mitochondrial genes

Mitochondrial DNA sequences have been used successfully as phylogenetic hypotheses at almost all levels of diversification from the ordinal level to populations and at various ages of divergence.
Two commonly used genes are the large subunit ribosomal RNA (16s) and the Cytochrome oxidase subunit I (COI). While considerable information concerning the evolution of these genes exists in other major groups, little work on their utility and evolution within gastropods has been done. This study addresses the usefulness of these two genes to reconstruct phylogenetic history at different levels of divergence. Extensive taxonomic sampling and phylogenetic breadth provide the opportunity to investigate phylogenetic signal in these genes from the origin of cowries in the Late Jurassic to the present.

Material and Methods

Individual specimens were collected from throughout their range and stored in alcohol prior to DNA extraction. Molecular analyses were performed at the Florida Museum of Natural History, University of Florida. DNAzol was used to extract DNA from foot tissue following the manufacturer’s recommended protocol. Tissue digestion occurred overnight and was supplemented with 5-μl proteinase K (20 mg/ml). PCR amplification of the mtDNA COI gene followed the following parameters: 95°C for 2 min., then 94°C for 40 s, 42°C for 40 s, and
72°C for 1 min, for 37 cycles. The following primers (Folmer et al., 1994) were used for amplification: LCO-1490 (5'-3') GGT CAA CAA ATC ATA AAG ATA TTG G, and HCO-2198 (5'-3') TAA ACT TCA GGG TGA CCA AAA ATC A, and Palumbi's (1996) 16Sar and 16Sbr primers were used for 16S: 16Sar (5'-3') CGC CTG TTT ATC AAA AAC AT, and 16Sbr (5'-3') CCG GTC TGA ACT CAG ATC ACG T. Products were cleaned with Promega Wizard columns and were cycle sequenced using BigDye terminator kits following the manufacturer's (Perkin-Elmer) protocol in one-quarter volumes and further sequenced using an ABI377.

Sequences were verified using Sequencher and easily aligned by hand. Within taxa there were no amino acid substitutions. Molecular analyses were performed using PAUP* (v.4.0b10). Topologies were constructed using Neighbor-Joining (K2P) and parsimony methods. Topological support was measured by bootstrap procedure (1000 replicates – NJ).

Results

*Mauritia arabica*

*Mauritia arabica* contains three described subspecies that are distributed allopatrically across the Indo-West Pacific. *M. arabica*
arabica is distributed predominantly in the Pacific Ocean, extending from American Samoa in the East to Taiwan, the Philippines, Indonesia and northwestern Australia. M. arabica asiatica is distributed in from the western Pacific around Southeast Asia and into the Northern reaches of the Indian Ocean, ending its distribution in the Northwest Indian Ocean around Oman. M. arabica immanis is found in the remaining areas of the western Indian Ocean including the offshore Mascarene islands, Madagascar and East African coast (Fig. 1). Representative samples collected from throughout the entire range confirm the existence of these three lineages. Based on outgroup comparison with M. maculifera (not shown), M. a. immanis shares a more recent common ancestor with M. a. asiatica than with M. a. arabica. These relationships are strongly supported with bootstrap values greater than 90. While strong geographic structure is evident on a basinal level, little structure exists within each of the subspecies. Some overlap occurs along a contact zone between M. a. asiatica and M. a. arabica that runs from Taiwan southward through the Philippines, Palau and Indonesia. Individuals from both subspecies can be found living together on the same reef and even under the
same piece of coral rubble. Individuals of *M. arabica* from India are members of the *M. arabica asiatica* subspecies.

**Palmadusta lentiginosa**

*Palmadusta lentiginosa* is a species distributed throughout the Northern reaches of the Indian Ocean. Two subspecies are recognized: *P. lentiginosa lentiginosa* from around India and *P. lentiginosa dancalica* from the Arabian Peninsula. The division between these two subspecies occurs around the Gulf of Kutch. Two specimens from Oman (*P. l. dancalica*) and two specimens from Tuticorin (*P. l. lentiginosa*) were sequenced. Reciprocal monophyly was not recovered between the two subspecies, indicating that, unlike the subspecies of *M. arabica*, they are not clearly distinct evolutionary lineages (Fig.2).

**Erronea errones**

The species *Erronea errones* is one of the most conchologically variable species of cowries. As such, many names have been ascribed to the species and all levels of description: subspecies, races, variations, forms, etc. The most recent interpretation of the species by Lorenz and Hubert (1993)
recognized five geographically structured taxa that were recognized mostly as significant forms or variations, only one of which was raised to subspecies based on a purported unique mutation to the lineage: *C. errones azurea* from Broome in NW Australia. While these variants are described by Lorenz and Hubert to have geographical affinities, conchological forms can vary remarkably within their ascribed regions and multiple forms may exist within a single large geographic range.

We tested whether there was any geographic substructure throughout the extant range of *Erronea erronea* by sequencing individuals found throughout the species range. No clear geographic structure is evident from the molecular results (Fig.3).

**Erronea caurica**

The *C. caurica* are the members of the Oman and Andaman clade. The sequenced nuclear marker for *C. errones* and *C. caurica* hybrids reveals that the proportion of hybrids within the *errones* population was more than >50%. The sequenced nuclear marker after cloning appears to be *errones*. And also it was found that no remnant *caurica* ITS in them which indicates that these hybrids are not sterile "mules" or F1s, but have since interbreed with other
errones and erased any caurica nuclear traces in the ITS marker (Fig.4).

Purpuradusta gracilis

*Purpuradusta gracilis* is a species found throughout most of the IWP, from the east coast of Africa eastward out to Fiji and Marshall Islands in the central Pacific. The species has been reported from Hawaii, but its presence as a permanent member of the community is uncertain. Lorenz and Hubert (1993) recognized up to three geographically structured subspecies: *P. gracilis gracilis* in the northern Pacific Ocean through to Indian and perhaps the Maldives, *P. gracilis macula* from the Southern Pacific Ocean and around the Australian continent, and finally *P. gracilis notata*, a subspecies restricted to the Western Indian Ocean.

Molecular sequence data from individuals collected throughout the described ranges of these three subspecies indicate that two distinct evolutionary lineages exist and generally correspond to a split between the Western Indian Ocean and remaining IWP. Sequence data do not support the distinction between *P. gracilis gracilis* and *P. gracilis macula*, but instead recognize members from these geographic regions as a single
subspecies: *P. gracilis gracilis*. The Western Indian Ocean subspecies, *P. gracilis notata*, is clearly distinct from *P. gracilis gracilis*. Individuals of *P. gracilis* from India are members of *P. gracilis gracilis* (Fig. 5).

**Adusta onyx**

The taxonomic history of the onyx-complex is complicated. Linnaeus’s original descriptions (1758) of cowrie species included *Cypraea adusta*, *Cypraea succincta* and *Cypraea onyx*. These three names all correspond to members of this clade. Subsequent authors have synonymized (Burgess, 1985) or split the clade into three independent taxa (Lorenz and Hubert, 1993). Even the lumpers recognize these distinct geographic subspecies.

We tested the evolutionary relationships of these taxa by sequencing individuals found throughout the described ranges of the various taxa. As expected, the Western Indian Ocean taxon appears as a distinct evolutionary lineage and corresponds to the species *Adusta adusta*. However, contrary to expectations, no distinction was found between individuals sampled between the Indian and Pacific ranges, usually considered to be at least different subspecies (Lorenz and Hubert, 1993) consider the Indian
Ocean subspecies as *A. succincta succincta* and the Pacific Ocean taxon to be *A. succincta onyx*. If the sequence data correctly elucidate the evolutionary relationships among these geographically disparate populations, then we must conclude that a single taxon exists throughout the entire range. While both names *succincta* and *onyx* correspond to this clade, *A. onyx* has priority over *A. succincta* by first reviser's choice of Hanley (1855). Individuals from India are members of *Adusta onyx* (Fig.6).

**Discussion**

Phylogenetic results from sequence analyses are by and large in agreement with previous cowrie classifications (Schilder & Schilder, 1971; Lorenz & Hubert, 1993). Most generic and many suprageneric names are supported with the molecular data. This phylogeny is the first in a series of papers that uses cowries to test speciation mechanism and diversification throughout the world's tropical regions. Because cowries exhibit similar diversity profiles to other diverse reef-associated groups, patterns and processes documented in cowries are likely applicable and should be tested in other groups. Phylogenetic results are for the most part consistent with previous cowrie classifications. Most previously recognized
genera or subgenera are strongly supported as monophyletic. In this present study, the *C. errones* contains mitochondrial DNA of *C. caurica* and *C. errones* looks very interesting as it has haplotypes from regular Pacific *errones* and from 2 *caurica* lineages so there must be some sort of hybridization going on. The *C. caurica* are the members of the Oman and Andaman clade. The sequenced nuclear marker for *C. errones* and *C. caurica* hybrids reveals that the proportion of hybrids within the *errones* population was more than >50%. The sequenced nuclear marker after cloning appears to be *errones*. And also it was found that no remnant *caurica* ITS in them which indicates that these hybrids are not sterile "mules" or F1s, but have since interbreed with other *errones* and erased any *caurica* nuclear traces in the ITS marker. This result was compared with sequenced results of *C. errones* collected from Singapore and none are hybrids. The *C. lentiginosa* is not different from Oman, *C. gracilis* is the member of Pacific clade not Western Indian Ocean and the same with *C. onyx*. To date, more than 1300 individual animals have been sequenced from over 150 species of cowries (Mayer, 1998). This large database reveals that the geographic signal to the speciation process is generally maintained today. The largest biogeographic barrier identified in
the database is that between the Indian and Pacific Ocean Barrier. This phylogenetic study lays the foundation for further, more detailed analyses of diversification throughout the world’s tropical oceans. The phylogeny can also be used to test the relative roles that the competing Center of Origin (Briggs, 1999), Center of accumulation (Ladd, 1960) and Center of Overlap (Woodland, 1983) theories contribute. Additionally, this overall phylogeny, tested against clock-like behaviour in the molecular markers, will be a valuable tool to assess the reliability of fossil taxa and rate constancy in the future. Based on the phylogeographic structure found in cowries, particularly the prominence of allopartic subspecies, geographic patterning needs to be tested for other wide-ranging species groups before one can conclusively claim lack of geographic signal based on recognized species. In conclusion, the persistence of geographic signal of divergence events is remarkable in cowries, and geographic isolation contributes prominently to Indo-West Pacific diversification.
FIGURE CAPTIONS

Fig. 1. *Mauritia arabica*. Relationship among *M. arabica arabica*, *M. a. asiatica* and *M. a. immanis* based on COI mt. DNA. Tuticorin individuals (indicated by the stars on both the map and phylogeny) fall within the *M. a. asiatica* clade. Bootstrap values (1000 reps, K2P) indicated below branches.

Fig. 2. *Palmadusta lentiginosa*. Molecular results based on COI mt. DNA indicate little structuring within *Palmadusta lentiginosa* despite described subspecific allopatry.

Fig 3. *Erronea erronea*. Intraspecific haplotype relationships among *Erronea erronea* individuals sampled throughout the taxon's range based on COI mt. DNA. Tuticorin individuals are noted by a star on both the map and phylogram.

Fig. 4. *Erronea caurica*. Relationship among *Erronea caurica* subspecies based on COI mt. DNA. Tuticorin individuals (indicated by the stars on both the map and phylogeny) fall within either the *E. caurica derosa* clade (Eastern Indian Ocean) or an unnamed lineage with geographic affinities to the Arabian Sea. Bootstrap values (1000 reps, K2P) indicated below branches.

Fig. 5. *Purpuradusta gracilis*. Relationship between *P. gracilis notata* and *P. gracilis gracilis* based on COI mt. DNA. Tuticorin individuals (indicated by the stars on both the map and phylogeny) fall within the *P. gracilis gracilis* clade. Bootstrap values (1000 reps, K2P) indicated below branches.

Fig. 6. *Adusta onyx* and *Adusta adusta*. Relationship between *Audusta adusta* and *Adusta onyx* based on COI mt. DNA. Tuticorin individuals (indicated by the stars on both the map and phylogeny) fall within the *Adusta onyx* clade. Bootstrap values (1000 reps, K2P) indicated below branches.
Fig. 1
Fig. 4

- E. ovum chrysostoma
- E. caurica caurica
- E. errones
- E. caurica quinquefasciata
- E. caurica elongata
- E. caurica dracaena
- E. caurica derosa
- E. caurica (new ssp.?)

1% change
Adusta adusta

Adusta onyx

P. gracilis notata

P. gracilis gracilis

1% change

Fig. 5

Adusta adusta

Adusta onyx

1% change

Fig. 6