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MORPHOLOGICAL AND GENETIC DIVERGENCE OF THE MANGROVE GASTROPOD *CERITHIDEA DECOLLATA* ALONG THE EASTERN COAST OF AFRICA

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MESTRADO EM ECOLOGIA MARINHA 2011

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RESUMO

Um dos principais focos da Biologia Evolutiva é compreender a origem e os processos pelos quais as populações de organismos divergem e se tornam distintas. A diversidade a nível intra-específico pode afectar os processos ecossistémicos, existindo evidências empíricas de que a estabilidade dos ecossistemas pode ser aumentada se existir uma elevada diversidade genética ou fenotípica das espécies. Como tal, a diversidade genética nas populações é importante, não apenas para o potencial evolutivo de uma espécie a longo prazo, mas também para a existência de flexibilidade face a alterações ambientais que afectam os processos ecológicos. Esta possibilidade tem implicações importantes em termos conservacionistas e merece um estudo mais aprofundado, sendo importante ter em conta que a análise da diversidade e diferenciação genética das populações irá providenciar informação fundamental para a conservação, permitindo a preservação da biodiversidade e uma melhor gestão dos recursos vivos.

Nos ambientes marinhos, a estrutura genética populacional é influenciada pelos requisitos ecológicos específicos e pelo padrão de história vital dos organismos. Assim sendo, a diferenciação genética em organismos marinhos é altamente influenciada pela sua capacidade de dispersão e, consequentemente, pelo seu modo de reprodução. Para a maioria dos invertebrados marinhos, a chave para a compreensão da estrutura populacional, baseia-se, em parte, no conhecimento da sua dispersão, que, em geral, é muito limitada quando adultos. Dessa forma, a presença ou ausência de uma fase larvar planctónica tem-se mostrado um factor importante na determinação do grau de estruturação espacial das populações nestes organismos. A dispersão de estados larvares permite a troca de indivíduos e genes ao longo da extensão geográfica natural das populações, que pode ser contínua se não houver restrições à dispersão. Uma população distribuída continuamente pode aproximar-se da panmixia, ou pode ser caracterizada por um isolamento por distância, onde a troca de indivíduos e genes ocorre localmente. No entanto, pouco se sabe sobre a prevalência de isolamento por distância em diferentes espécies marinhas, e de que forma é que a variação no fluxo genético e distâncias de dispersão podem afectar a sua expressão a diferentes escalas geográficas.

É necessário também ter em conta que factores ambientais podem influenciar a presença ou ausência de estruturação nas populações marinhas. Por exemplo, os padrões de circulação de massas de água, os regimes de temperatura e a topografia costeira afectam grandemente a estrutura populacional em ambientes costeiros, e podem oferecer muitas oportunidades de dispersão passiva. Os eventos de afloramento costeiro, regime de marés, e

fluxo estuarino também podem influenciar a dispersão, a uma menor escala espacial. No entanto, muitos destes factores ambientais podem ter um efeito duplo: podem ser responsáveis pela dispersão de larvas planctónicas, actuando como corredores ao fluxo genético ou, alternativamente, podem funcionar como barreiras físicas ao fluxo genético. Assim, um elevado potencial de dispersão nem sempre se traduz em elevados níveis de fluxo genético, e as espécies podem não atingir o seu potencial de dispersão devido a mecanismos de retenção local.

Pensa-se, actualmente, que é possível que o nível de estruturação genética se reflicta em diferenças a nível fenotípico. No entanto, a contribuição relativa de factores genéticos e ambientais para a expressão fenotípica não foi ainda resolvida. O mesmo se verifica em relação a factores físicos e biológicos que se pensa promoverem a diferenciação morfológica entre populações contíguas, e que são ainda pouco compreendidos. As espécies exibem, muito frequentemente, variações espaciais e temporais nas suas características morfológicas, e, uma vez que têm sido detectadas grandes diferenças entre populações que habitam locais e ambientes diferentes, a determinação das bases para a diferenciação fenotípica é fundamental para a compreensão da evolução dos organismos.

Neste sentido, neste estudo recorreu-se ao uso de dados genéticos e morfológicos, que permitem fazer a interpretação de padrões de variabilidade, permitindo investigar-se a fonte duma possível variação inter-populacional e, também, reconhecer (ou não) a existência de grupos discretos de indivíduos ao longo de áreas costeiras. Mais especificamente, pretendeu-se inferir sobre o fluxo genético na espécie *Cerithidea decollata*, nos mangais costeiros do Leste Africano, à luz dos padrões putativos de transporte pelas correntes marinhas. A análise separada destes dois tipos de dados irá ajudar a estabelecer até que ponto é que os factores ambientais influenciam a expressão de variabilidade fenotípica em *C. decollata*, assim como melhorar a compreensão da biogeografia da espécie. O foco da investigação resume-se na avaliação dos padrões filogeográficos da espécie através da análise de marcadores genéticos (sequências parciais do gene mitocondrial da citocromo oxidase I - COI) e de informação quantitativa morfométrica da concha, revelando os níveis de diversidade populacional. O estudo foi realizado ao longo de um gradiente geográfico latitudinal: toda a costa leste Africana (desde o Quénia à República da África do Sul). A informação sobre a estrutura das populações destes gastrópodes dos mangais irá contribuir para um progresso na área da biologia evolutiva, uma vez que os factores mais importantes na formação das estruturas filogeográficas actuais deste género são ainda pouco conhecidos.

Esta tese é constituída por 3 partes distintas: uma introdução geral (capítulo 1), uma investigação específica (capítulo 2) e as conclusões finais (capítulo 3). O trabalho científico

desenvolvido resultou num artigo científico, a submeter numa revista internacional indexada. No capítulo 2, averiguou-se as diferenças na forma da concha dos espécimes das 32 localidades amostradas através de técnicas de morfometria geométrica, recorrendo a análise de imagem e análises estatísticas. Os resultados indicam que existe uma convergência na forma da concha, e que os diversos morfotipos se sobrepõem ao longo do gradiente em estudo. No entanto foi revelada uma variação significativa entre localidades, indicando que, para a espécie em causa, existe maior variabilidade morfológica a mesoescalas espaciais do que a macroescalas espaciais. Este padrão é provavelmente uma consequência da similaridade de condições ambientais a que os indivíduos estão expostos ao longo do gradiente, levando a uma convergência de formas entre as diversas regiões geográficas – a uma macroescala geográfica (norte, centro e sul do gradiente), mas a acção de factores mais locais pode levar a alguma diferenciação entre localidades. Relativamente à análise genética, utilizaram-se sequências parciais do gene COI, sequenciadas a partir de 172 indivíduos distribuídos por 30 populações ao longo da costa. Uma primeira análise exploratória revelou elevada variação nas diversidades haplotípica e nucleotídica consoante a população. A rede de haplótipos revelou a existência de relações complexas entre haplótipos, resultando numa estruturação genética populacional moderada ao longo da costa, o que foi confirmado pelas análises de variância molecular (AMOVAs). Investigou-se também a história demográfica dos grupos filogeográficos definidos, através de testes de neutralidade e análises da distribuição das frequências das diferenças genéticas encontradas. Os resultados foram consistentes com a hipótese de expansão demográfica recente a norte do gradiente (Quénia, Tanzânia, Moçambique norte), o que tem sido verificado também para outras espécies do oceano Índico. Para as zonas centro (Moçambique centro) e sul do gradiente (Moçambique sul e República da África do Sul), os resultados sugerem vários eventos de colonização. Verificou-se ainda a existência de uma correlação significativa entre distâncias genéticas e geográficas. Visto que se prevê uma elevada capacidade de dispersão para esta espécie, por possuir estados larvares planctónicos de 2-3 semanas, estes resultados indicam a possibilidade de um isolamento por distância devido a barreiras físicas, havendo um factor de interacção entre a hidrologia costeira e a conectividade das populações.

Combinando os resultados provenientes da análise de morfometria geométrica e da análise genética, observa-se que estes apontam para a existência de uma certa independência entre a variabilidade genética e morfológica observadas. Na realidade, as diferenças genéticas obtidas não se traduziram em diferenças significativas na forma da concha, sendo que a variação desta última se pode dever à existência de plasticidade na expressão de fenótipos, ainda que isso não seja possível de demonstrar recorrendo apenas ao dados deste estudo.

A combinação de métodos morfométricos com a genética molecular mostrou ser uma ferramenta robusta para analisar a diferenciação das populações em diversos aspectos, permitindo aumentar o conhecimento sobre diferenciação populacional desta espécie marinha, que poderá eventualmente fornecer uma base para o desenvolvimento de outros estudos relacionados. Foram evidenciados os possíveis factores que levam à divergência genética e morfológica das populações, numa escala espacial muito abrangente, fornecendo informação sobre os padrões de diversidade na costa Leste Africana. Por fim, recomenda-se que este estudo, e outros estudos do mesmo tipo, possam ser aprofundados através de análises complementares que permitam estudar os níveis de diferenciação ecológica e adaptativa entre populações, para que se possa compreender melhor a influência de factores ambientais na evolução das espécies.

Palavras-chave: *Cerithidea decollata*, costa leste Africana, mtDNA COI, estrutura genética, morfometria geométrica

ABSTRACT

In this study I aimed to investigate the sources of a possible inter and intra population variation and the existence of discrete groups of individuals in the mangrove gastropod *Cerithidea decollata* along the eastern coast of Africa. The combined analysis of morphometric and genetic data helped to improve the comprehension of the population genetic structure and phylogeography of the species. The assessment of shell shape variation revealed a convergence of morphological characteristics with an overlap of shape across the latitudinal gradient in study. However, significant differences were found among sampling localities, showing that most variation in shape occurs at meso spatial scales but not at macro spatial scales. This pattern is most likely a consequence of the similarity of environmental conditions to which specimens are exposed along the coast, leading to a convergence of shell shape across the gradient. Nevertheless the action of specific local pressures may lead to some shape variation among locals. Regarding the genetic analysis, I used partial sequences of the mitochondrial gene cytochrome oxidase I (COI) sequenced from 172 individuals distributed by the populations along the coast. The network analysis showed the existence of a moderate population genetic differentiation along the coast, which was confirmed by the molecular variance analyses. The demographic history of the defined groups was also investigated using neutrality tests and mismatch distributions. The results were consistent with the sudden expansion hypothesis for the northern region of the gradient, which has also been observed for other species inhabiting in the Indian Ocean. A correlation between genetic and geographic distances was also observed. Once it was predicted a high dispersal ability for *C. decollata*, due to its planktonic larval stage of 2-3 weeks, these results indicate the possibility of isolation by distance as consequence of physical barriers with the existence of a correlation between coastal hydrology and population connectivity. The predominant mechanisms that lead to population differentiation include a combination of factors, such as dispersal ability of the species, ocean currents, habitat discontinuities, phenotypic plasticity, physical barriers and geographical distance. The information about the population structure of this mangrove gastropod will contribute to a progress in evolutionary biology, once the determinant factors in the formation of the actual phylogeographic structures of this kind still remain unsettled.

Keywords: *Cerithidea decollata*, estearn African coast, mtDNA COI gene, genetic structure, geometric morphometrics

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CHAPTER 1

GENERAL INTRODUCTION

The study on the relation biodiversity-functioning of the ecosystem initially focused on species and functional richness of ecological groups. More recently, researchers (e.g. Duffy and Stachowicz 2006) suggested a concept of biodiversity at a larger scale expanding both upward and downward in the taxonomic hierarchy, from genetic variation within each species, to variety of superior *taxa*. They present examples of how genetic diversity might affect oceanic ecosystem processes, considering that species- and population-diversity could lead to a reduced variation of communities in time, and increase their resilience to disturbances. Results from studies on marine invertebrate communities generally have shown that as species' diversity increases, the stability of individual species' abundances decreases, but stability of overall community biomass increases (Tilman 1999, Stachowicz et al. 2002). Species influence many aspects of ecosystem functioning, and particular functions are often most strongly influenced by different species (Eviner and Chapin 2003). Nevertheless, over the long term, the capacity of ecosystems to continue adapting to environmental change must ultimately be compromised by continuing extinctions of species (Duffy and Stachowicz 2006).

There is empirical evidence that the stability of the marine ecosystem can be improved when the genetic and phenotypic diversity of species is high (Duffy and Stachowicz 2006). In this way the genetic diversity of populations is important not only to the long term evolutionary potential of a species but also to the existence of flexibility when considering environmental changes in ecological time scales, safeguarding the normal structure and functioning of marine ecosystems. This possibility has important implications in terms of conservation and deserves further study, as the analysis of population diversity and differentiation will provide essential information to conservation biology and preservation of biodiversity (González-Tizón et al. 2008). Thereby, given the general scenario and the urgency of many marine conservation and management issues concerning biodiversity (Roberts and Hawkins 1999), we should not shy away from providing scientific insights to managers and policy makers.

The comprehension of divergence patterns in tropical marine organisms within biogeographic regions is necessary to hypothesize about the origin of biodiversity. A number of studies on the connectivity and genetic structure of populations in the tropical Pacific Ocean have provided vital information about the origins and maintenance of biodiversity in this region (Schleyer et al. 2008, Reid et al. 2008 and 2010, Lee and Boulding 2009, Duda and

Lessios 2009). The divergence patterns can be revealed through the comparison of distribution of genotypes within species with ample geographical distribution (Duda and Lessios 2009).

Information on the population structure of the tropical mangrove gastropod *Cerithidea decollata* will contribute to a progress in evolutionary history of tropical marine organisms, once the most important factors determining the development of their current phylogeographic structures still remains an open question (Kojima et al. 2006).

POPULATION GENETICS AND PHYLOGEOGRAPHIC STUDIES IN THE MARINE ENVIRONMENT

In recent years, several studies have addressed the taxonomy, phylogeny and phylogeography of gastropods using shell morphology as well as molecular approaches (e.g. Wilding et al. 2001, Rosenberg 2002, Kojima et al. 2004, 2005, 2006, 2008, Reid et al. 2008, Lee and Boulding 2009, Miura et al. 2010, Kamimura et al. 2010, Silva et al. submitted). These contributed to a progress in evolutionary biology, but the spatial scale of demographic connections between populations still remains a central issue in marine biology (Couceiro et al. 2007). Populations of many marine species often have enormous population sizes and a pelagic larval stage, and these traits have been assumed to connect populations demographically across a far broader spatial scale than most terrestrial species (Caley et al. 1996). A successful dispersal between populations leaves a genetic wake that can reveal historical and contemporary patterns of connectivity. Studies on genetic differentiation in sea organisms suggest the role of larval dispersal is often tempered by adult ecology. Also, changes in differentiation with geographic distance are limited by disequilibrium between drift and migration. The phylogeographic analysis of populations of marine organisms can also provide information about historical variations in oceanic environments, since the effects of major environmental changes result in unique and long-retained genetic structures, such as the geographic genetic heterogeneity that is caused by geographic isolation and the extremely low genetic diversity that is caused by population bottleneck effects (Avise 1994, 2000). The similar phylogeographic structures among co-distributed species suggest that common historical and/or contemporary oceanographic factors may have formed the structures.

Generally speaking, direct-developing species are expected to have lower dispersal abilities than species with a planktonic larval stage, and tend to be more geographically fragmented, although long-distance dispersal has been reported for some species without any pelagic stage (Martel and Chia 1991, Helmuth et al. 1994, Johannesson and Johannesson 1995). Inverse relationships between the level of genetic structuring among populations and the expected dispersal ability have been shown for many intertidal molluscs through the

comparison of genetic structures of related species with different larval modes (Hoskin 1997, Todd et al. 1998, Kyle and Boulding 2000, Wilke & Davis 2000, Collin 2001). However, a growing body of literature has demonstrated the existence of cryptic subdivision of populations over small distances in some organisms with a potentially high dispersal capacity (Grosberg and Cunningham 2001). Such barriers to dispersal have been found along hydrographical boundaries in the Atlantic, Pacific, and Indian oceans (e.g. Avise 1992; Star et al. 2003; Sotka et al. 2004) and across shifts in habitat (e.g. Burton and Feldman 1981; Johnson and Black 1995; Riginos and Nachman 2001) though they are not ubiquitous. When phylogeographic breaks are shared among disparate *taxa* with varying life histories, they provide a rational basis for guesses about where effective genetic exchange is lacking and thus should be considered critical information for the management of fisheries and the placement of marine protected areas (Hellberg 2009). Where these breaks occur also suggests the kinds of physical features that act as important barriers. In other places, barriers sit where sea level changes during the Pleistocene exposed shallow shelves (Crandall et al. 2008). Whereas such shared phylogeographic breaks generally coincide with places where many species' distributions end, the converse is not necessarily true, as evidenced by Burton (1998). For species sharing a phylogeographic break in the present day, however, that does not imply that multiple species had populations sundered by a common isolating event in the past. Shared geographical breaks often include species where lineages to either side of the break were isolated at different times (see Schulze et al. 2000, Taylor and Hellberg 2006). The underlying causes for divergence may thus vary among species with a common phylogeographic break; but what maintains the break is more likely to be shared (Hellberg 2009). Studies on species from Mozambique and South Africa coastal zones exploring mitochondrial DNA variation and gene flow between populations support the idea that individual species and populations have suffered recent demographic expansions (Tolley et al. 2005, Gopal et al. 2006, von der Heyden et al. 2007, Neethling et al. 2008). It is rather likely that changes in the sea level and temperature during this geological time led to such expansions (Silva et al. 2010b). Overall, the evidence seems to indicate that the spatial scales of demographic connection between marine populations will depend on a complex mix of behavior, oceanography, and interactions with co-occurring species and most certainly varies with geographic context (Cowen et al. 2006). As a consequence, population dynamics cannot be explained based on local samplings but it is necessary to invest in studies at a vast geographical scale (Armonies 2001).

Over the past decade, mark-recapture studies, chemical tagging studies, and detailed modeling of realistic currents and larval survival have all reinforced a view that successful

dispersers may travel far less than their apparent potential, even for species with quite long pelagic development. Genetic data have contributed to this view (Hellberg et al. 2001). On the one hand, gene flow may refer to the genetic realization of ongoing patterns of dispersal between populations. This is what ecologists are after when trying to assess the demographic independence or interdependence of populations. From this demographic perspective, gene flow only matters if the dispersal levels it heralds contribute significantly to the persistence of populations. The movements of a few odd larvae may homogenize populations genetically, but will fail to rescue a heavily harvested population with no other demographic inputs (Hellberg 2009). On the other hand, evolutionary biologists may be interested in genetic exchange between populations that may have been otherwise isolated for thousands of generations. Rare genetic exchange between populations that are demographically isolated can still introduce foreign alleles that can spread adaptive change, alter modes of speciation, and cloud our ability to discern historical changes in population size and connectivity (Hellberg 2009). Because so many benthic marine animals move little in adulthood, movements by larvae have been expected to be responsible for most dispersal and gene flow between populations. As an example, Johnson (2006) stated that benthic stages of marine invertebrates with an indirect life cycle, colonizing substrata such as rocky shores, are likely to be distributed in a complex spatial arrangement. Potential benthic habitat for these animals is often patchy, reflecting the discontinuous distribution of hard bottoms along coastline distances at which connectivity via larval dispersal is thought to take place. A source–sink metapopulation model could explain population dynamics in these systems (Roughgarden and Iwasa 1986). The location of sink populations would be determined by the interaction between the potential of larval dispersal, which depends on larval duration and behaviour, and the oceanographic conditions prevailing over the shelf and at the coast (Largier 2003, Aiken et al. 2007). Several other comparative population genetics studies of marine benthic invertebrates have also provided evidence for significantly higher genetic diversities for planktotrophic species than for direct-developing species (e.g. Wray and Raff 1991, Havenhand 1995, Hoskin 1997, Arndt and Smith 1998, Kyle and Boulding 2000). For levels of genetic subdivision, Collin (2001) found that *Crepidula* limpets with directly developing larvae showed greater phylogeographic differentiation (more breaks, more reciprocal monophyly between populations) than did planktonically developing congeners. Notwithstanding, estimates of the average distances by which marine larvae disperse are generally poorly described (Couceiro et al. 2007). Duda and Lessios (2009) suggest that the lack of concordance in the distributions of genotypes and patterns of gene flow of species in the western and central Pacific implies that differences in species traits (possibly

related to life history, dispersal ability, or other aspects of their biology) or unique demographic histories have affected their current genetic population structures.

Several studies have focused on the environmental factors influencing the presence or absence of a population structure in marine organisms. Researchers suggest that water masses circulation patterns, temperature regimes, coastal topography and hydrology greatly affect the structure of marine populations (Planes et al. 2001, Stepien et al. 2001, Rocha et al. 2005, Chambers et al. 2006, Lutjerharms 2006), offering opportunities for passive dispersal (Neethling et al. 2008). Thus, dispersal is expected to occur as a result of ocean currents and the admixture of larvae from source populations before recruitment to adult habitat (Avise, 1994). Given the speeds of oceanic currents, marine propagules could conceivably disperse hundreds or even thousands of kilometers in a single generation. As a result, marine populations were once seen as demographically open, with genetic isolation over the long term hard to come by. Nonetheless, as discussed above, accumulating evidence has been suggesting otherwise. Recurring complications include the presence of cryptic species, selection on markers, and a failure to account for differences in heterozygosity among markers and species (Hellberg 2009). A better understanding of effective population sizes is also needed.

There is a wide number of examples of statistically significant levels of population subdivision without isolation by distance. In these studies the genetic differences between neighbouring populations often exceed those between more distant populations. For several species with pelagic larvae (limpets: Johnson and Black 1984; barnacles: Hedgecock 1986, echinoids: Watts et al. 1990, Moberg and Burton 2000), adult populations show low levels of genetic subdivision, yet repeated sampling of recruits from the same site over time reveals that different cohorts are genetically differentiated (Hellberg et al. 2002). This pattern is termed chaotic genetic patchiness (Hellberg et al. 2002) and all these varying patterns reflect both the dispersal powers of the organisms and their population history. In addition, factors such as temperature, oxygen, salinity, phytoplankton availability and radiation can influence recruitment rates (O'Riordan et al. 2004) and larval migration (Morgan and Christy 1996). The influence of estuarine currents and brackish water can also limit the pattern of offshore larval dispersal and onshore larvae transport, favouring genetic differentiation from the rest of the populations (Silva et al. 2009).

Research on these issues gives an insight on speciation processes in the marine environment, which require the acquisition of reproductive isolation. If populations are separated either by a physical barrier or due to the weak ability to disperse, speciation may follow: the acquisition of intrinsic reproductive isolation is then a consequence of the accumulation of genetic differentiation (Mayr 1963). Increasingly, attention has shifted to the possibility that reproductive barriers might arise in populations not separated by major physical features (Bush and Howard 1986), i.e. that speciation might begin with genetic diversification in spite of some gene exchange between constituent populations (Wilding et al. 2001). Rice and Hostert (1993) concluded that laboratory experiments on the development of isolation strongly support the idea that reproductive isolation can evolve between sympatric or parapatric populations if divergent selection is strong relative to gene flow. Miura et al. (2010) also state that genetic divergence is a precursor for allopatric diversification, but it is ultimately reproductive isolation that determines speciation.

MORPHOLOGICAL VARIATION

Intertidal invertebrates are model organisms for examining the environmental control of life-history traits because of the sharp gradients in biological and physical factors found in their habitat, including wave action, temperature, food availability, desiccation and predation (Smith and Ruiz 2004). However, the effect of a particular factor on ecological and evolutionary processes is difficult to separate when multiple factors vary simultaneously (Pardo and Johnson 2005). In fact, the physical and biological factors promoting morphological differentiation between contiguous populations are still poorly understood (Dawson 2001, Waters et al. 2005). Species very frequently exhibit temporal and spatial variations in their morphological traits and once major differences have been detected between populations inhabiting different environments (Pigliucci 2001), the determination of the causes for phenotypic variation is fundamental for the comprehension of how organisms evolve (Roff 1992). In this way efforts have been made to evaluate the degree of interaction between genotype and environment in the expression of phenotypes in various species (Pardo and Johnson 2005) and to explore the mechanisms behind phenotypic variation (Struhsaker 1968, Janson 1982, Berven and Gill 1983, Travis 1983, Fletcher 1984, Brown 1985, Trussell 1997, Lively et al. 2000, Smith and Ruiz 2004).

In recent years, it has become evident that phenotypic plasticity, or the capacity of an organism to produce phenotypic changes in response to environmental cues, is more common

than initially expected (Smith and Ruiz 2004). The potential importance of plasticity as an adaptive response to environmental heterogeneity has been widely recognized. A plastic life history may allow an organism to respond to, and persist across, a wide spectrum of environmental conditions. More specifically, spatial or temporal changes in habitat conditions may favour phenotypes that can respond to these environmental conditions (Bradshaw 1965, Lively 1986, Schlichting 1986, Trussell 1997; see review by Tollrian and Harvell 1999).

In intertidal habitats, snails can exhibit large habitat-dependent phenotypic variability between populations separated by even a few meters (Johannesson 2003), suggesting that eco-phenotypic plasticity can be the result of specific ecological conditions (Monteiro et al. 2000, Brian et al. 2006). These habitats are often characterized by strong gradients in abiotic and biotic factors, such as predation, food availability, and physical factors (e.g. hydrodynamic forces, desiccation), which ultimately depend on intertidal height or exposure (Underwood 1984, Menge 2000, Addy and Johnson 2001, Somero 2002). When these factors act differentially (e.g. size-selective predation), they can produce clinal variation in life-history traits, and gradients in such factors have been invoked to explain pronounced vertical clines in size structure and other life-history traits (Vermeij 1972, Rochette et al. 2003). Therefore, stage-, shape-, or size- specific selective components add phenotypic variation to populations (Hollander et al. 2006). For example, Brown (1985) found population differences in growth and size at maturation between snails reared in vernal and permanent ponds, and suggested that phenotypic plasticity is an adaptive response to inhabiting such an unpredictable environment as vernal pools. Further, in a comparison of physiological traits between two gastropods with direct versus planktonic development, Parsons (1998) found that high dispersal ability was associated with increased plasticity, while restricted dispersal was associated with greater differentiation. Other authors such as Meyer (2003) suggest that several gastropod ‘species’ are morphologically uniform but genetically different between archipelagos, although the same environmental factors that justify the absence of shape differentiation between populations can, in return, contribute to a local morphological variation, when acting in opposite directions (Silva et al. 2010a). It is important to understand the basis of intraspecific variation (genotype vs phenotype) in order to have a better insight on processes like adaptation, speciation and geographic variation (Trussel 2000).

THE COMBINED USE OF MORPHOMETRIC AND MOLECULAR TOOLS

In present time, with all the advanced scientific and technological tools available, it is

important to combine various approaches to address biological issues in order to have a more extensive view on the subject. This allows us to integrate evidence from different types of data and analyses, enriching our research. In studies of population differentiation the use of genetic markers should, under certain conditions, be able to empirically estimate levels of gene exchange between marine populations. Such genetic markers can provide general answers to questions about marine connectivity, but much gets lost in the specifics (Hellberg 2009). So it is with genetic markers: no single marker is best for every question. The choices for inferring population connectivity and isolation can be grouped into two categories: frequency markers (microsatellites) and sequence markers. In this thesis I opted to use sequence markers, which power derives from the ability to infer relationships between alleles. Mitochondrial DNA (mtDNA) sequences have usually served as sequence markers to date, although single-copy nuclear sequences are emerging as another form of these markers (Hellberg 2009). Nevertheless, sequences from the mtDNA gene region of cytochrome oxidase I (COI) amplified by universal primers have proven fine markers for detecting phylogeographic structure and recognizing cryptic species in an array of marine species. In fact, mtDNA has been successfully used to resolve genetic population structure in several gastropods (Kyle and Boulding 2000, Wilke and Davis 2000, Collin 2001, Marko 2004, Waters et al. 2005). Specifically, the gene COI has been the marker of choice in most studies; its popularity being largely based on the existence of a robust, universal primer pair, which has proved successful for numerous invertebrate species (Folmer et al. 1994). For sequence analyses focused on resolving the history of divergence among populations, sample size of 10 or even three individuals may be adequate (Pluzhnikov and Donnelly 1996). In addition, mitochondrial loci also contain the signatures of historical demographic events that can play a major role in establishing contemporary biogeographic patterns and population structure (Couceiro et al. 2007).

However, mitochondrial genes are particularly prone to losing diversity after a situation of invasion of a new habitat by a small number of founders, because mtDNA is haploid with uniparental inheritance and thus has only one quarter the effective population size of nuclear genes (Avise 1994), and so introduced populations would have reduced genetic diversity because of founder events, bottlenecks and genetic drift (Nei et al. 1975). Kojima et al. 2004 also suggests that the use of faster evolving molecules than COI might give a more contemporary view of the genetic structure. Nevertheless mtDNA is a good genetic marker for studying the genetic variations and relationships at and bellow species level, because its mutation rate is indeed high (Hewitt 2001).

Interest in morphological variation has long been a driving force behind many biological studies. Understanding the nature of, and searching for an explanation for this variety is a major research focus, including fields as diverse as functional morphology, macroevolution, sexual selection, and evolutionary developmental biology (Rosenberg 2002). Understanding is made more difficult when sets of morphological characters have several functions (Berglund et al. 1996). Actually, differences in shape may signal different functional roles played by the same parts, differences to the same selective pressures, as well as differences in the processes of growth and morphogenesis (Zelditch et al. 2004).

The use of geometric morphometrics in this work is indispensable, once it is a very valuable tool to study the organismal phenotype and allows the quantitative assessment of its variations of form. This method offers precise and accurate description while serving for purposes of visualization, interpretation and communication of results for shape differences that have been mathematically analyzed (Zelditch et al. 2004). It allows the elucidation of patterns of shape variation (Adams et al. 2004). Capturing geometry by way of landmark data has become rather commonplace. Landmarks are precise locations on biological forms that hold some developmental, functional, structural, or evolutionary significance (Lele and Richtsmeier, 2001). Landmark locations are recorded as two- or three-dimensional coordinates resulting in a spatial map of the relative location of the chosen points. Variability is particularly difficult to characterize, because each data set is collected in a coordinate system specific to the orientation of the object during data collection. Therefore, operations of translation, rotation, and reflection are routinely used to transport all forms into a single coordinate system to estimate variability (Rohlf and Marcus 1993, Richtsmeier et al. 2002). Shape variation is described either as differences in coordinates of corresponding landmarks or is estimated using a thin-spline function to map the deformation in shape between specimens (Bookstein 1991). These approaches generate shape variables that are then used in statistical analysis. To specify a model in morphometrics, good statistical sense and solid knowledge of the phenomena under study are needed. In addition, geometric morphometrics also have some limitations such as the selection and number of landmarks, which can affect statistical power (Adams et al. 2004) and might be dependent on the good preservation of specimens. To our knowledge, precise guidelines on this matter have not yet been defined. Despite these problems, these methods represent a good approach to the challenging issue of shape variation.

All things considered, it is important to combine data from morphological and genetic analysis when studying populations, in order to obtain a vision that encompasses several perspectives giving a better insight on the causes of population differentiation.

AN EVOLUTIONARY ASSOCIATION BETWEEN MANGROVES AND *CERITHIDEA* GASTROPODS

Gathering data on the larval dispersal patterns and historical demography for all marine species is impossible. It has been proposed that model species (i.e., those organisms of limited economic and conservation importance) can serve as a proxy for species with comparable life-history characteristics (Palumbi et al. 2003), as their lack of commercial value lowers the risk that population structure is influenced by fishing depletion or aquaculture (Couceiro et al. 2007).

Gastropods have shown to be particularly adequate in population differentiation studies for several reasons: (1) they inhabit heterogeneous environments and exhibit conspicuous variation in morphology, life-history and behavior (Sutherland 1970, Roberts and Hughes 1980, Fletcher 1984, Brown 1985, Johannesson et al. 1993, Trussell and Smith 2000, Rolan et al. 2004); (2) their gene flow can be limited (Little 1989), and (3) both types of development, direct and indirect, occur in gastropods, making them good models for comparison between species with different life strategies.

There are about 20 living species in *Cerithidea* genus (Reid et al. 2008) and most of them are in critical need of a taxonomical revision (Houbrick 1984, Reid et al. 2008, Miura et al. 2010). In *Cerithidea* snails, some species are direct-developers while others have a larval pelagic stage (Houbrick 1984), although no publications with precise descriptions for the species in study, *Cerithidea decollata*, were found. Nevertheless Reid (personal communication) suggests a planktonic life cycle for this species, similar to *Cerithidea rhizophorarum*, which has a planktonic life of 12-20 days, but no more information was available (Kojima et al. 2006). *Cerithidea* snails inhabit shallow tropical and warm temperate seas in the Indian and eastern Pacific, western and eastern Atlantic (Brigs 1974, Reid et al. 2008). They show a close association with mangroves as they live in roots and trunks of mangrove trees, anchored by dried mucous, and migrate to the surrounding muddy platform during low tide to feed (*C. decollata*: Berry 1972, Cockcroft and Forbes 1981; other species: Ohtaki et al. 2002, Vannini et al. 2006). The trees provide the snails with shelter, protection from predators, a solid substrate and sometimes food as well (Reid et al. 2008). As tideland

organisms play an important role in the purification of coastal waters and in the cycling of matter within those ecosystems (Kamimura and Tsuchiya 2004), *Cerithidea* gastropods are thought to contribute to this ecological function in the ecosystem because of their high biomass and bioturbation (Kamimura and Tsuchiya 2004). Parsimonious reconstruction of ancestral habitats suggests that the living snails in Potamididae family (in which the genus *Cerithidea* is included) are an adaptive radiation that has always been closely associated with mangroves and that the specialized tree-climbing group *Cerithidea* derived from mud-dwelling ancestors (Reid et al. 2008). Three subgenera have been recognized within *Cerithidea sensu lato*, based on the morphology of the shell and radula, and this division corresponds to differences of habitat and biogeographic distribution (Houbrick 1984). At least 10 species can be distinguished by their shells (Brandt 1974, Wilson 1993, Brown 1994, Hasegawa 2000, Ma 2004), while the other species further needed molecular information (Reid et al. 2008).

In studies on population differentiation, one compounding factor may be the sampling sites employed for the populations to be compared. Because habitat distribution can have a marked effect on subdivision (Johnson and Black 2006), intraspecific comparisons need to take into consideration the differences in the physical isolation of sampling sites. In species with strong gene flow it is still unknown though, if larger mangroves contribute to the regional larval pool with disproportionately more larvae than smaller mangroves (Silva et al. 2010b). Future studies on congeneric snails from the eastern coast of Africa and other biogeographic regions elsewhere in the world would provide the data needed for higher resolution of these hypotheses.

OBJECTIVES OF THE THESIS

The general objective of the present thesis is to assess the patterns of genetic and phenotypic differentiation of *Cerithidea decollata* in coastal mangroves of eastern Africa, and infer about the evolutionary processes that have shaped them, in the light of the present knowledge on ocean currents and other oceanographic features, and on the biology and ecology of the species. It is the aim of this work to investigate the species phylogeographic patterns through the analysis of a specific molecular marker (partial sequence of mitochondrial gene COI) and the analysis of morphometric quantitative information gathered during shell shape comparisons.

The specific objectives of this thesis are defined bellow:

1) To examine and compare the levels of genetic diversity within each population;
2) To compare the genetic differentiation between populations and evaluate the degree of spatial structure at a linear geographical scale of approximately 3200km, considering the level of genetic isolation according to geographical distances;
3) To compare the morphological diversity and differentiation of the shells within and between populations;
4) To hypothesize about the occurrence of gene flow between populations and about the possibility of a directional tendency in the flow, in case it is shown to occur;
5) To analyse the phylogeography of this gastropod species, in view of the genetic and morphometric data combined, along a latitudinal gradient;
6) To evaluate the connectivity - between mangroves of the coastal areas in study;
7) To synthesize the above information.

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CHAPTER 2

Connectivity of populations and shell shape variation in the widespread mangrove whelk *Cerithidea decollata* along the eastern coast of Africa

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Abstract

The dispersal ability of a species seems to influence not only its geographical range, but also its population genetic structure and evolutionary change. In this study we aimed to assess the inter- and intrapopulation genetic and morphological variation of *Cerithidea decollata* along the eastern coast of Africa. The present population structure of *C. decollata* was examined by sequencing 420 base pairs of the mitochondrial COI gene in 172 snails from 30 sites, in a combined analysis with patterns of morphometric differentiation in 1799 snails from 32 sites. Analysis of pairwise Φ_{ST} and molecular variance showed a moderate and significant spatial population differentiation from Kenya to the Republic of South Africa, suggesting genetic divergence between northern, central and two southern regions of this latitudinal gradient which was further confirmed by the SAMOVA. A correlation between geographic distance and genetic divergence along the latitudinal gradient under study was also revealed. Haplotype network, Tajima's D, Fu's Fs tests and mismatch analysis suggest a recent population expansion in the northern region and several colonization events in the central and southern regions. The morphometric approach performed through shell shape image analysis suggests that morphological variations in shell shape are somewhat independent of the genetic divergence, revealing an overlap of shape across the latitudinal gradient but significant differences among-population at a local level.

Keywords *Cerithidea decollata*, estearn African coast, mtDNA COI gene, genetic structure, geometric morphometrics

Introduction

In the marine environment the population genetic structure of a species is mainly influenced by species-specific ecological requirements and life-history traits (Wilke and Davis 2000, Riginos and Nachman 2001). Genetic differentiation in marine organisms, invertebrates in particular, is, therefore, highly influenced by their dispersal capacity (Hedgecock 1986, Bagley and Geller 1999, Santos et al. 2006, Levin 2006) and consequently by their mode of reproduction (Palumbi 1995). Most marine invertebrates have a limited or reduced mobility when adults (gastropods: Thorpe et al. 2000, others: Janson 1983, Johnson et al. 2001): in this way, the presence or absence of planktotrophic larval stages has shown to be an important factor in the determination of the degree of populations' spatial structure (Avise 2004).

A number of population genetics comparative studies supports the hypothesis that marine invertebrate species with direct development have less potential for gene flow, showing higher levels of spatial structure when compared to species with planktonic larvae, and therefore indirect development (gastropods: Janson 1987, Hoskin 1997, Kyle and Boulding 2000, Collin 2001; bryozoans: Goldson et al. 2001, Watts and Thorpe 2006; holothurians: Arndt and Smith 1998; sea stars: Hunt 1993; solitary corals: Hellberg 1996). Larvae dispersal through oceanic currents connects populations geographically separated, resulting in a partial homogeneity of their genetic structure (Slatkin 1985, Waples 1998, Bohonak 1999), where most of the genetic differences are due to within population variation (Gooch et al. 1972, Gaines et al. 1974, Martel et al. 2004). On the contrary, in species with no larval stages, it is expected a substancial spatial structure even at geographical microscales (Snyder and Gooch 1973; Day and Bayne 1988, Johannesson et al. 1993, Tatarenkov and Johannesson 1994, Sokolova and Boulding 2004). Prior research on marine invertebrates supports the idea that population structure can be quite variable, including populations (1) genetically structured (*Littorina sitkana* and *Littorina subrotundata*, Lee and Boulding 2009; *Perisesarma guttatum*, Silva et al. 2010a); (2) with a chaotic genetic patchiness (*Uca annulipes*, Silva et al. 2010b) or genetic fragmentation (Ward 2006); (3) isolated by distance; and (4) genetically homogenous (*Littorina scutulata* and *Littorina plena*, Lee and Boulding 2009).

Several studies suggest that habitat size and continuity are factors possibly shaping the evolutionary trajectories of marine organisms (Colborn et al. 2001, Silva et al. 2009, 2010a and 2010b). When a species has a continuous distribution and there are no restrictions to dispersion along its natural geographical range, we might consider either one of the following: (1) panmixia (Cook et al. 2002) or (2) isolation by distance (Martel et al. 2004), where the exchange of genes and individuals preferentially occurs locally (Slatkin 1985, Cook et al. 2002), decreasing with increasing geographical distance. However, according to Pogson et al. (2001)

very little is known about the prevalence of isolation by distance in marine species and how variation in gene flow and dispersal distances might affect its expression at different geographic scales. When species have a patchy discontinuous distribution, a high potential for dispersion will lead to regular exchange of individuals between local subpopulations, connected in a metapopulation complex (Levins 1969, Hanski and Simberloff 1997, Hanski, 1999, Armonies 2001, Hixon et al. 2002). The estimation of parameters such as gene flow (Ebert et al. 1993) and levels of interdependence of local populations (Johnson et al. 2001) plays, therefore, an important role in the comprehension of population dynamics and evolution of these organisms.

It has been shown that the level of genetic structure might be reflected in phenotypic differences (Levins 1968, Hoffman and Parsons 1989, Crowl and Covich 1990, Palumbi 1995, Parsons 1998), although the relative contribution of genetic and environmental factors in phenotypic expression has not yet been solved (Smith and Ruiz 2004). Authors suggest that the occurrence of local morphological variation can also be explained by shape plasticity (Hoffman and Parsons 1989, Crowl and Covich 1990; Rosenberg 1997, Rufino et al. 2004, Miner et al. 2005, Silva and Paula 2008).

In this study we aim to assess the level of populations' morphological and genetic diversity and spatial structure of the mollusc gastropod *Cerithidea decollata* along the eastern coast of Africa. The modern diversity pattern of indo-pacific gastropods of the genus *Cerithidea* is identical to other mangrove species (see Ellison et al. 1999, Ellison 2002 and Reid 2008), as they are highly specialized to this type of habitat (Glaubrecht 1999). However very little is known about the biology of most of these tropical gastropods and even their systematic classification is still not settled, despite recent works clarifying phylogenetic relationships (Houbrick 1984, Kojima et al. 2006; Reid et al. 2008; Miura et al. 2010). To our knowledge there is no detailed information on the development and presence of larval stages in *C. decollata,* although Reid (personal communication) suggests that *Cerithidea rhizophorarum* life cycle is the best guess for *C. decollata*. Contrary to the suggestion by Houbrick (1984), it seems that *C. rhizophorarum* is in fact planktotrophic: Kojima et al. (2006) report that it has a planktonic life of 12-20 days, but no more information was available. In this way it can be expected a high dispersal ability for this species. Morphological variations in this and other mollusc genera can be possibly caused by genetic factors (Day and Bayne 1988) and environmental factors as well. These latter include physical and chemical water properties, desiccation, food availability and quality, and predatory pressures (Behrens-Yamada 1987, Trowbridge 1994) acting on ecophenotypic plasticity (Kemp and Bertness 1984). Parasitism might also be important, leading to changes in size, thickness and shape of shells (Rohde 1993,

Levri and Fisher 2000, Levri et al. 2005, 2007). These phenomena have been documented for *Cerithidea* gastropods (see Lafferty 1993, Miura et al. 2006a), however few studies have quantified the genetic and hereditary variance in phenotypic characteristics (Smith and Ruiz 2004).

The eastern coast of Africa offers a set of unique characteristics for studying the relative importance of oceanographic processes in the genetic structure of marine organisms (Silva et al. 2010b). There are three main current systems influencing this coast: (1) the Agulhas´ warm current; (2) the Mozambique channel eddy system and (3) the Equatorial convergent current (Lutjeharms 2006). The patterns of these currents influence temperature regimes, coastal topography, composition and distribution of species (Chambers et al. 2006; Neethling et al. 2008). In addition, the anti-cyclonic circulation (Sætre and da Silva 1984) facilitates an almost random dispersal of planktonic larvae (Silva et al. 2010b), contributing to the homogenization of populations along the coast. The mesoscale phenomena occurring in the continental platform such as eddies and counter currents (Lutjeharms and da Silva 1988) may also contribute to larvae dispersal or retention (Paula et al. 2001). Other events such as upwelling, tidal regime and estuarine flow also influence dispersal at a smaller spatial scale (Abelson and Denny 1997, Quinteiro et al. 2007). Most of these oceanographic factors can act in a double way: dispersing planktonic larvae and acting as corridors to gene flow or alternatively, acting as physical invisible barriers (Palumbi 1994, Crispo and Hendry 2005, Jolly et al. 2005, Quinteiro et al. 2007, Galarza et al. 2009, Silva et al. 2009, 2010b), corresponding, in most cases, to the limits of biogeographic regions (Barber et al. 2000, Patarnello et al. 2007).

Combining data from genetic and morphological approaches when studying population dynamics produces valuable information that allows a more complete and correct interpretation of variability patterns and recognition of the factors involved in population differentiation (Silva et al. 2010a). The specific objectives of this study, using *C. decollata* a study case, are to (i) examine and compare the levels of diversity within and among regions, areas and localities through a mitochondrial DNA (mtDNA) survey and shell shape analysis; (ii) assess the degree of population genetic structure at a 5km-3200km spatial scale; (iii) reveal patterns of differentiation using a phylogeographical approach.

Material and Methods

Sampling design and processing

Samples of *C. decollata* were collected from mangrove frests from Kenya to South Africa, ranging a linear geographical north-south gradient of 3200km (Figure 1).

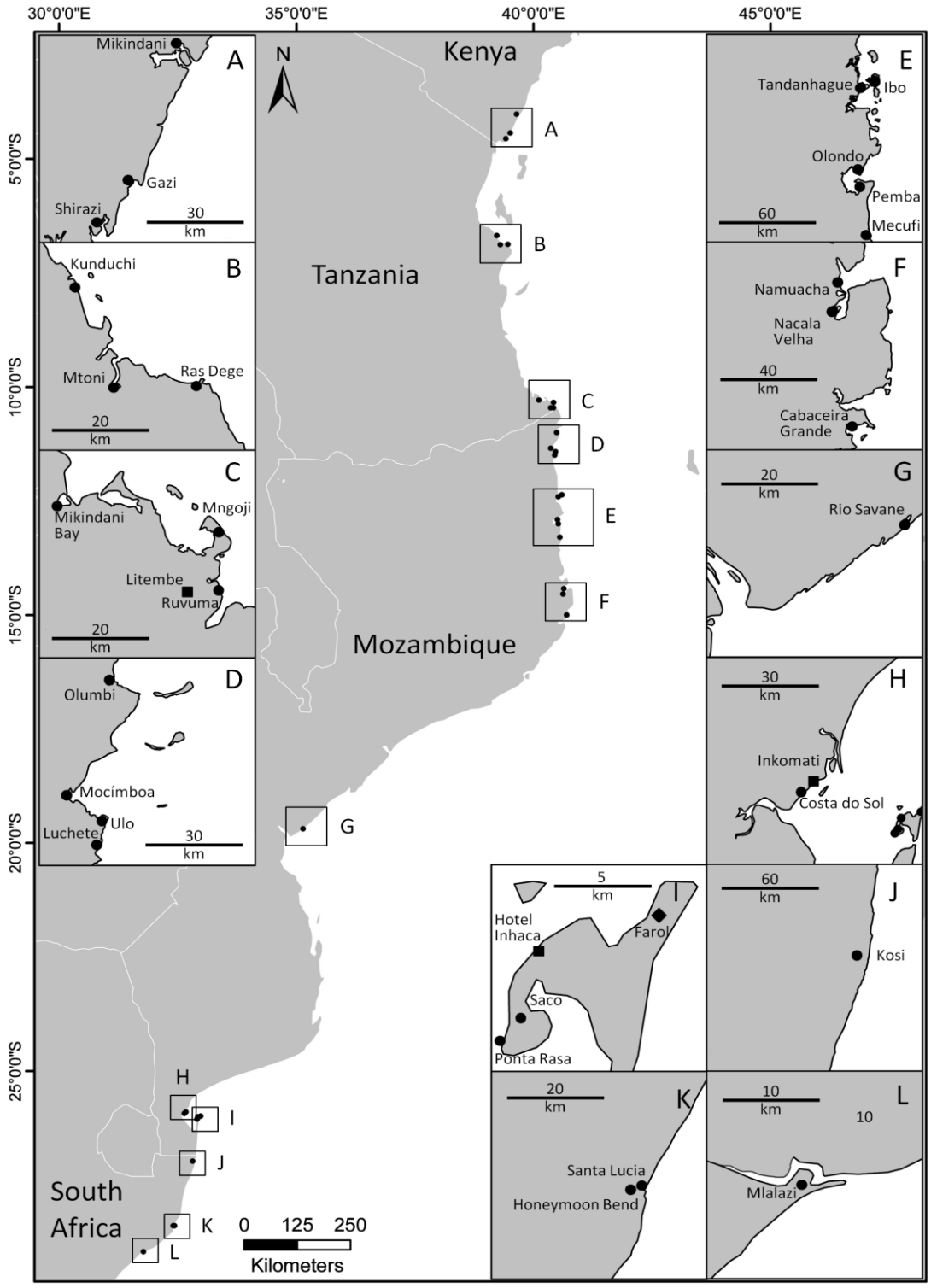

Figure 1 – Sampling locations for *C. decollata* in the east African coast. Areas: A – Mikindani (Mi), Gazi (G), Shirazi (Sh); B – Kunduchi (K), Mtoni Kijichi (MK), Ras Dege (RD); C – Mikindani Bay (MiB), Mngoji (Mn), Litembe Pwani (LP), Ruvuma estuary (RE); D – Olumbi (Ol), Mocimboa da Praia (MP), Ulo (U), Luchete (Lu); E – Tandanhangue (T), Ibo Kirimbas (IK), Olondo (O), Pemba (P), Mecúfi (Me); F – Nacala Velha (NV), Namuacha (N), Cabaceira Grande (CG); G – Rio Savane (RS); H – Inkomati (I), Costa do Sol (CS); I – Hotel Inhaca (HI), Mangal do Farol (MF), Saco (S), Ponta Rasa (PR); J – Kosi bay (KB); K – Santa Lucia (SL), Honeymoon bend (HB); L – Mlalazi (Mlz). • Samples used in both genetic and morphometric analyses; ▪ samples used exclusively in morphometric analysis; ◆ samples used exclusively in genetic analysis.

A spatially nested sampling design was adopted, consisting of four regions, 12 areas and 33 sites, following a hierarchical geographical scale, as follows: macrogeographical scale for the regions (> 500km), mesogeographical scale for the areas (> 50km < 100km) and microgeographical scale for the sites (> 1km < 50km). The design consisted of the four following major regions, comprising each one different sampling areas (adding up a total of 12): Kenya/Tanzania north (areas A and B); Tanzania south/Mozambique north (area C to F); Mozambique centre (area G); Mozambique south/Republic of South Africa (area H to L). Most sampling areas incuded several independent sampling sites (replicates; exceptions included areas G, J and L, which were represented by single point locations). The location of the sampling areas and sites, and correspondent abbreviations are presented in Figure 1. The geographical distances between sampling sites were measured by the shortest sea distance (km), using the software GOOGLE EARTH 6.1 (Google Inc).

Sampling was conducted during the year of 2006, between January and December. Upon collection by hand at each location, half of the captured specimens were immediately fixed in ethanol 70% for morphometric analysis, while the other half was preserved in absolute ethanol (shells were broken to allow adequate tissue penetration) for genetic analyses.

Genetic Analysis

A total of 172 individuals from 30 sites were used for the genetic analysis. Total DNA was isolated from a portion of mantle tissue, using E.Z.N.A. Mollusc DNA Isolation Kit (Omega Biotek), in accordance with the manufacturer´s instructions and yielding DNA suitable for downstream applications. Extracted DNA was visualized on 1% TBE agarose gels.

A 1000-1300 base pairs (bp) fragment of the of the mtDNA cytochrome *c* oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR) using as primer pairs COI-bf (5'-GGGGCTCCTGATATAGCTTTTCC-3', Miura et al. 2006b) and COI-6 (5'-GGRTARTCNSWRTANCGNCGNGGYAT-3', Shimayama et al. 1990) or LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3', Folmer et al. 1994) and COI-6. The 25µl PCR reaction mixture included (Silva et al. submitted): 3µl of total DNA as a template, 5µl of 5x Taq buffer (Fermentas), 2.5µl of a 25mM $MgCl_2$ solution, 2.5µl of a 2µg/µl BSA solution, 1µl of a 2mM dNTP solution, 1µl of a 25µM solution of each primer and 0.2µl of a 5U/µl Taq DNA polymerase solution (Fermentas), and 8.8µl of ddH_2O. The reaction mixtures were subjected to the following temperature conditions (Herbert et al. 2003): denaturation at 94ºC for 60 s; 5 cycles of denaturation at 94ºC for 30 s, annealing at 45ºC for 60 s and extension at 72ºC for 60 s; followed by 30 cycles of denaturation at 94ºC for 60 s, annealing at 50ºC for 90 s, extension at

72ºC for 60 s; and a final extension step at 72ºC for 5 min. PCR products were observed on 1.5% TBE agarose gels after electrophoresis. The PCR products of the samples were then purified by the Exo-SAP clean-up protocol (Werle et al. 1994) and sent to Stab-Vida (http://www.stabvida.com/) for sequencing in the forward and reverse directions (primers COI-bf or LCO1490, and COI-6) using a 3700 ABI DNA Sequencer (Applied Biosystems). Sequences analyzed in this study were deposited in GenBank (Accession Nos).

Chromatograms were analyzed and sequences were verified, edited and aligned using the software SEQUENCHER 4.8 (Gene Codes Corporation). A segment of 420 bp of the partial COI gene was recovered for all samples. A diversity analysis of the genetic data was performed using the software DNASP 5.0 (Librado and Rozas 2009): number of polymorphic sites, segregating sites, mutations, number of mtDNA haplotypes and average number of pairwise differences were calculated. Haplotype diversity (h) and nucleotide diversity (π) were calculated for each location, as well as for the entire pooled populations using ARLEQUIN 3.5 (Excoffier and Lischer 2010).

Phylogeographic relationships between haplotypes were explored by haplotypic network analysis and phylogenetic tree-building algorithms. Haplotype network was constructed with the program NETWORK 4.6.0.0 (Shareware Phylogenetic Network Software Website) using the median-joining method (Bandelt et al. 1995). The resulting network is a combination of minimum spanning trees with median vectors (consensus sequences) added by a parsimony criterion (Quinteiro et al. 2007). Phylogenetic trees were constructed using software PAUP* 4.0b10 (Swofford 2002), applying the neighbor-joining (NJ) and maximum-parsimony (MP) methods for most likely topology. To estimate pairwise genetic distances among haplotypes for NJ analysis, the most appropriate evolutionary model (using the Akaike Information Criterion – AIC) was identified using the likelihood-ratio tests implemented by jMODELTEST 0.1.1 (Posada 2008). MP analysis was performed using a fast heuristic search by random stepwise addition. For both NJ and MP analyses, the strength of the branches was tested by 10 000 bootstrap replicates and the species *Cerithidea valida* (GQ273837), *Cerithidea scalariformis* (GQ273843) and *Cerithidea costata* (GQ273851) were used as outgroups (sequences were obtained from molecular database NCBI Nucleotide BLAST).

Genetic differentiation among samples was assessed through pairwise Φ_{ST} as implemented in ARLEQUIN 3.5 (Excoffier and Lischer 2010) using pairwise differences, since the selected evolutionary model was not available in this program package. Significant deviations from the null hypothesis of no differentiation were assessed with 10 000 permutations of individuals among populations after Bonferroni correction. Differences in haplotype frequency

among samples were also analyzed using the exact test of population differentiation (Raymond and Rousset 1995).

In order to assess correlations between genetic and geographic distances and to test if genetic differentiation among samples could be explained by isolation by distance (IBD), values of $\Phi_{ST}/(1-\Phi_{ST})$ were plotted against geographical distances (obtained with GOOGLE EARTH, Google Inc) for each pair of samples. Further, the significance of the correlation achieved was tested by Mantel Z test (Mantel 1967) with 10 000 iterations, using software IBD 1.52 (Bohonak 2002).

Population genetic structure was explored through a spatial analysis of molecular variance (SAMOVA) approach, using the software SAMOVA 1.0 (Dupanloup et al. 2002), which defines groups of populations that are geographically homogeneous and maximally differentiated from each other in terms of genetic variation. The program uses a simulation approach that incorporates geographical information but without imposing any *a priori* group structure.

Partioning of mtDNA variation and correlation of haplotypes at different levels of hierarchical subdivision was investigated by analysis of molecular variance (AMOVA), considering different population structures and using ARLEQUIN 3.5 (Excoffier and Lischer 2010). In a first approach overall patterns of genetic differentiation among samples were assessed (AMOVA I). In a second approach, spatial population structure was evaluated considering the samples grouped based on pairwise Φ_{ST} values and on *a priori* expectation of spatial population differentiation (see Silva et al. 2010a) across the latitudinal gradient sampled (AMOVA II). In addition, the best structure obtained by SAMOVA was further tested for significance by Φ-statistics and hierarchical analyses of molecular partition (AMOVA III).

Mismatch distributions (Slatkin and Hudson 1991) were analyzed with ARLEQUIN 3.5 (Excoffier and Lischer 2010) to explore the demographic history and deduce whether a population has undergone population expansion (Rogers and Harpending 1992). Goodness of fit between the observed and expected distribution was tested with 1 000 permutation replicates under a sudden expansion model (Rogers 1995). To further examine the population history, neutrality tests of Tajima's D (Tajima 1989) and Fu's F statistics (Fu 1997) were carried out in the same software. These statistics are widely used to detect changes in population size (Mousset et al. 2004) once they estimate the deviation from neutrality, which is based on the expectation of a constant population size at mutation-drift equilibrium (Lee and Boulding 2007). We calculated the time of expansion (t) using the equation $\tau = 2ut$, where u = mutation

rate/nucleotide/year*sequence length. We used a mutation rate of 2.81% per million years estimated for *Cerithidea* snails using K2P distances in Miura et al. (2010).

Morphometric Analysis

The morphology of the shell was characterized in two dimensions, using geometric morphometric methods, for 1799 specimens from 32 sites. These methods yield detailed information about variation in the shape of objects while retaining a visual representation of them throughout the analysis (Mitteroecker and Gunz 2009). Landmarks, which are coordinates of points upon which geometric morphometric methods are based (Bookstein 1991), were recorded from high-resolution digital images taken with a Nikon D70 digital camera with a 55-mm micro lens and using consistent capture conditions for all specimens. The 15 homologous points chosen (Figure 2) were digitized on the shells using software TPSDIG 2.16 (Rohlf 2010a). The criteria used to choose the landmarks were their relative ease in identification across samples and the ability of the suite of landmarks to capture the general shape of the shell.

Gastropods are known to have allometric growth so it was necessary to evaluate the effect of size on shape variation, using the software TPSREGR 1.37 (Rohlf, 2009). This was achieved by regressing each shape variable (relative warps) against a measure of body size and estimating the residual shape variation, which was then used for further morphometric analyses. Landmarks of each specimen were optimally aligned using a Generalized Procrustes Analysis (Rohlf and Slice, 1990), in which the configurations of the specimens are superimposed via translation, scaling and rotation, using the minimal bending energy method (Bookstein, 1997; Mitteroecker and Gunz, 2009). Centroid size, which is the square-root of the sum of the squared distances between each landmark and the centroid of the landmark configuration (Sneath, 1967) and is used as scaling factor during the superimposition process (Bookstein 1991; Dryden and Mardia 1998), was subsequently used as a measure of size for each specimen. The term “shape” used in this study is thus defined as a geometric representation of an object after removing all non-shape variation, due to measurement associated errors (Claverie et al. 2011).

From the aligned specimens, shape variables (including uniform and non-uniform components) were generated by performing a relative warp analysis, (Bookstein, 1991), which is the analogue of a principal components analysis (Rohlf and Bookestein, 2003; Zelditch et al. 2004). This analysis reduces the dimensionality of multivariate data by transforming a set of many correlated variables into a small number of significant uncorrelated variables (Claverie et al. 2011) called relative warps, therefore condensing shape information. These new sets of

shape variables are then used for statistical comparisons of shape variation within and among groups. The present study also used the thin-plate spline approach, using the software TPSRELW 1.49 (Rohlf, 2010b), which allowed the visualization of shape change as deformation grids.

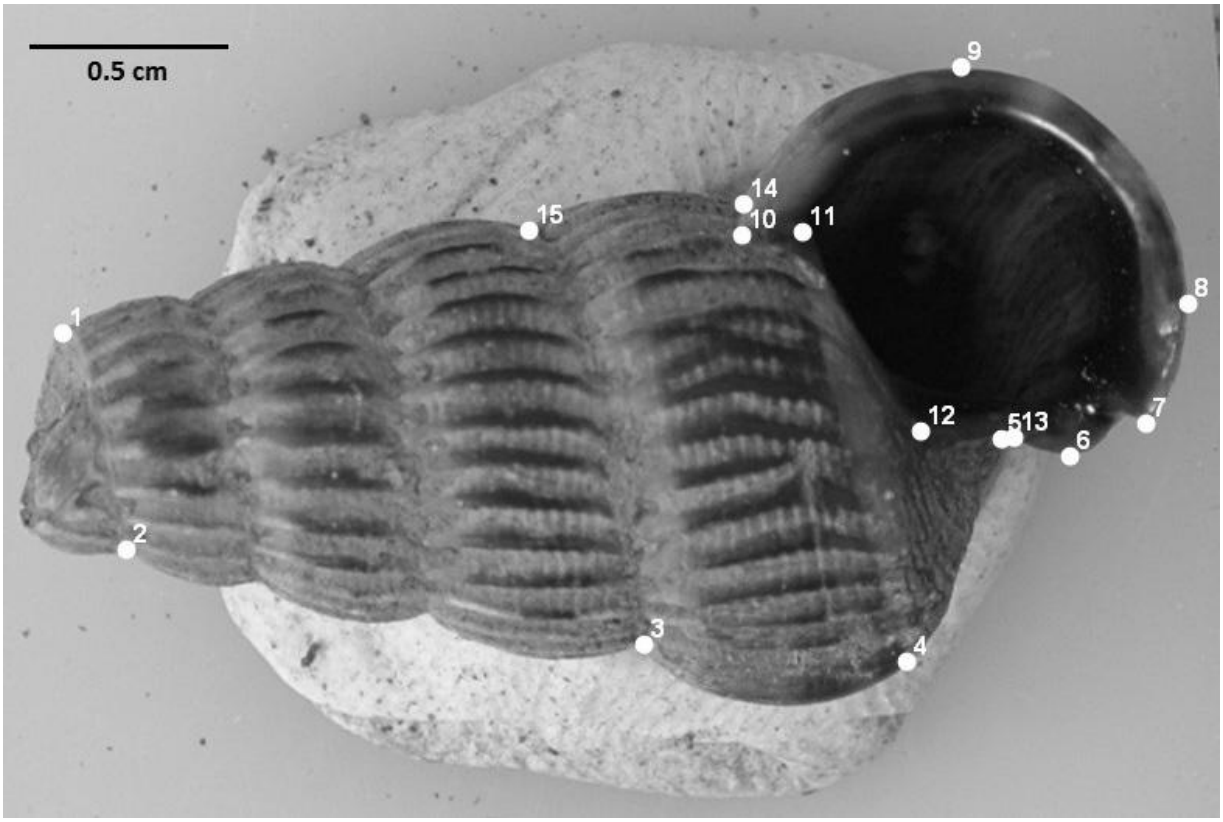

Figure 2 – *Cerithidea decollata*. Position of the 15 landmarks on the shell.

The software STATISTICA 9.0 (StatSoft Inc) was used to investigate shell shape variation significance. To test the significance of the morphometric differentiation patterns generated by the relative warp analysis, a nested multivariate analysis of variance (MANOVA) was performed on the relative warp scores. In this way we determined the degree of variation between regions, study areas within regions and populations within areas. To test which groups differed within each hierarchic level, we performed post-hoc Tukey HSD tests for unequal N, which perform pairwise comparisons. A stepwise discriminant analysis was also performed on the shape variables. Classification methods were then used to evaluate the power of the functions in discriminating groups.

In order to assess a possible differentiation of shape due to clinal variation, a correlation between the first three relative warps (which condense the majority of shape variation) and latitude was tested.

Results

Genetic Analysis

The sequencing of 420 bp from 172 specimens revealed a total of 60 distinct haplotypes (H1 to H60; Table 1) defined by 49 polymorphic sites, of which 26 were parsimony informative. Out of the 50 point mutations, 41 were synonymous substitutions and nine were replacement changes. The sequences' overall AT content was 61.7%, similar to those found in other gastropods (Marko and Vermeij 1999, Holznagel and Lydeard 2000, Kirkendale and Meyer 2004). Forty-nine (81.67%) haplotypes were unique accounting for 28.49% of the overall specimens. The most common haplotype (H1) was present in 60 of the 172 individuals (34.88%). H1 was found in every sampling locality from Kenya to south Mozambique (except for Shirazi and Saco), but it did not appear in the populations of the Republic of South Africa. The second (H10) and third (H6) most common haplotypes accounted for 16.27% and 6.40% of the total individuals, respectively. H10 was found across the entire geographical range of this study, appearing in three of the four regions defined (but not in every population) – exception for Mozambique centre, which was represented by a single point location, Rio Savane). Haplotype 6 was private to Mozambique. Almost all haplotypes (seven out of 10) found in Mozambique central region were private. Within a given location, the highest haplotype richness was found in Rio Savane (Mozambique centre), with 10 haplotypes, and the lowest haplotype richness was found in Shirazi (Kenya) and Pemba (Mozambique north) samples with only 1 haplotype. For the rest of the samples the number of haplotypes ranged from 2 to 5. The distribution of haplotypes per sampling locality is summarized in Table 1.

Table 1 – Number of haplotypes, haplotype and nucleotide diversity for *C. decollata* populations using a 420bp fragment of the COI mtDNA gene. For the complete name and location of the sampling sites see figure 1. K/TzN: Kenya/Tanzania north; TzS/MozN: Tanzania south/Mozambique north; MozC: Mozambique centre; MozS/RSA: Mozambique south/Republic of South Africa.

	Sampling sites																												
	K/TzN						TzS/MozN														MozC	MozS/RSA							
	Mi	G	Sh	K	MK	RD	Mn	RE	Ol	MP	U	Lu	IK	T	O	P	Me	NV	N	CG	RS	CS	MF	S	PR	KB	SL	HB	Mlz	Nr of specimens
H1	1	1		1	1	2	3	2	4	6	5	7	2	4	1	6	3	1	3	3	1	1	1		1					60
H2														1															1
H3																					1				1	2		2	1	7
H4																											2			2
H5																											2			2
H6															1				1		3	3	2		1					11
H7																								1						1
H8																								1	1					2
H9						1																								1
H10		1			1	1	1		1			1	2		1		1	3	1	2		2	2	2		5	1			28
H11																												1		1
H12																													1	1
H13																										1				1
H14																					1									1
H15																					1									1
H16									1																					1
H17																								1						1
H18																												1		1
H19			1																											1
H20																										1				1
H21																		2												2
H22																										1				1
H23				1																										1
H24	2	2																												1
H25				1																										4
H26				1																										1
																														1

Table 1 – Continued.

	Sampling sites																												
	K/TzN						TzS/MozN														MozC	MozS/RSA								
	Mi	G	Sh	K	MK	RD	Mn	RE	Ol	MP	U	Lu	IK	T	O	P	Me	NV	N	CG	RS	CS	MF	S	PR	KB	SL	HB	Mlz	Nr of specimens
H27																										1				1
H28																													1	1
H29																					1									1
H30																													1	1
H31																			1											1
H32				1																										1
H33											1																			1
H34						1																								1
H35															1															1
H36													1																	1
H37					2			1																						3
H38						1																								1
H39										1																				1
H40														1																1
H41														1																1
H42																						1								1
H43																		1												1
H44																					1									1
H45																							1							1
H46																						2								2
H47												1																		1
H48							1																							1
H49					1																									1
H50													1																	1
H51								1																						1
H52							1																							1

Table 1 – Continued.

	Sampling sites																													
	K/TzN						TzS/MozN														MozC	MozS/RSA								
	Mi	**G**	**Sh**	**K**	**MK**	**RD**	**Mn**	**RE**	**Ol**	**MP**	**U**	**Lu**	**IK**	**T**	**O**	**P**	**Me**	**NV**	**N**	**CG**	**RS**	**CS**	**MF**	**S**	**PR**	**KB**	**SL**	**HB**	**Mlz**	**Nr of specimens**
H53																													1	1
H54																					1									1
H55																					1									1
H56																	1													1
H57																					1									1
H58																	1													1
H59																				1										1
H60																							1							1
Haplotype diversity	0.6667	0.8333	0.0000	1.0000	0.9000	0.9333	0.8000	0.8333	0.6000	0.2857	0.3333	0.4167	0.8667	0.7143	1.0000	0.0000	0.8000	0.8095	0.8000	0.7333	0.9545	0.8611	0.9048	0.9000	1.0000	0.8333	0.8000	0.8333	1.0000	0.8480
Nucleotide diversity	0.0032	0.0079	0.0000	0.0081	0.0081	0.0111	0.0048	0.0024	0.0048	0.0048	0.0007	0.0026	0.0060	0.0034	0.0119	0.0000	0.0048	0.0098	0.0081	0.0059	0.0120	0.0108	0.0099	0.0124	0.0119	0.0087	0.0057	0.0087	0.0143	0.0091
Nr haplotypes	2	3	1	5	4	5	4	3	3	2	2	3	4	4	4	1	4	4	4	3	10	5	5	4	4	6	3	3	5	60
Nr private haplotypes	0	0	1	4	1	3	2	1	1	1	1	1	2	3	1	0	2	2	1	1	7	2	2	2	0	4	2	2	4	53
Nr specimens	3	4	1	5	5	6	6	4	6	7	6	9	6	7	4	6	6	7	6	6	12	9	7	5	4	11	5	4	5	172

Considering the total study area, the eastern coast of Africa, the overall values of haplotype diversity and nucleotide diversity for the entire mtDNA data set were 0.8478 (± 0.023) and 0.00912 (± 0.00052), respectively, meaning that, on average individuals differed by much less than 1% per base pair. The levels of within population genetic diversity were quite variable among localities, ranging in haplotype diversity (*h*) from 0.000 to 1.000 and in nucleotide diversity (π) from 0.00000 to 0.01429 (Table 1). The regions with the highest overall level of *h* were Mozambique centre (*h* = 0.955 ± 0.057), Mozambique south/Republic of South Africa (*h* = 0.916 ± 0.026) and Kenya/Tanzia north (*h* = 0.909 ± 0.039), and the lowest level was found in Tanzania south/Mozambique north (*h* = 0.643 ± 0.056). Considering nucleotide diversity, the highest overall value of π was found in Mozambique centre (π = 0.01205 ± 0.00138) and Mozambique south/Republic of South Africa (π = 0.01105 ± 0.00091) and the lowest value was found in Tanzania south/north Mozambique (π = 0.00504 ± 0.00069).

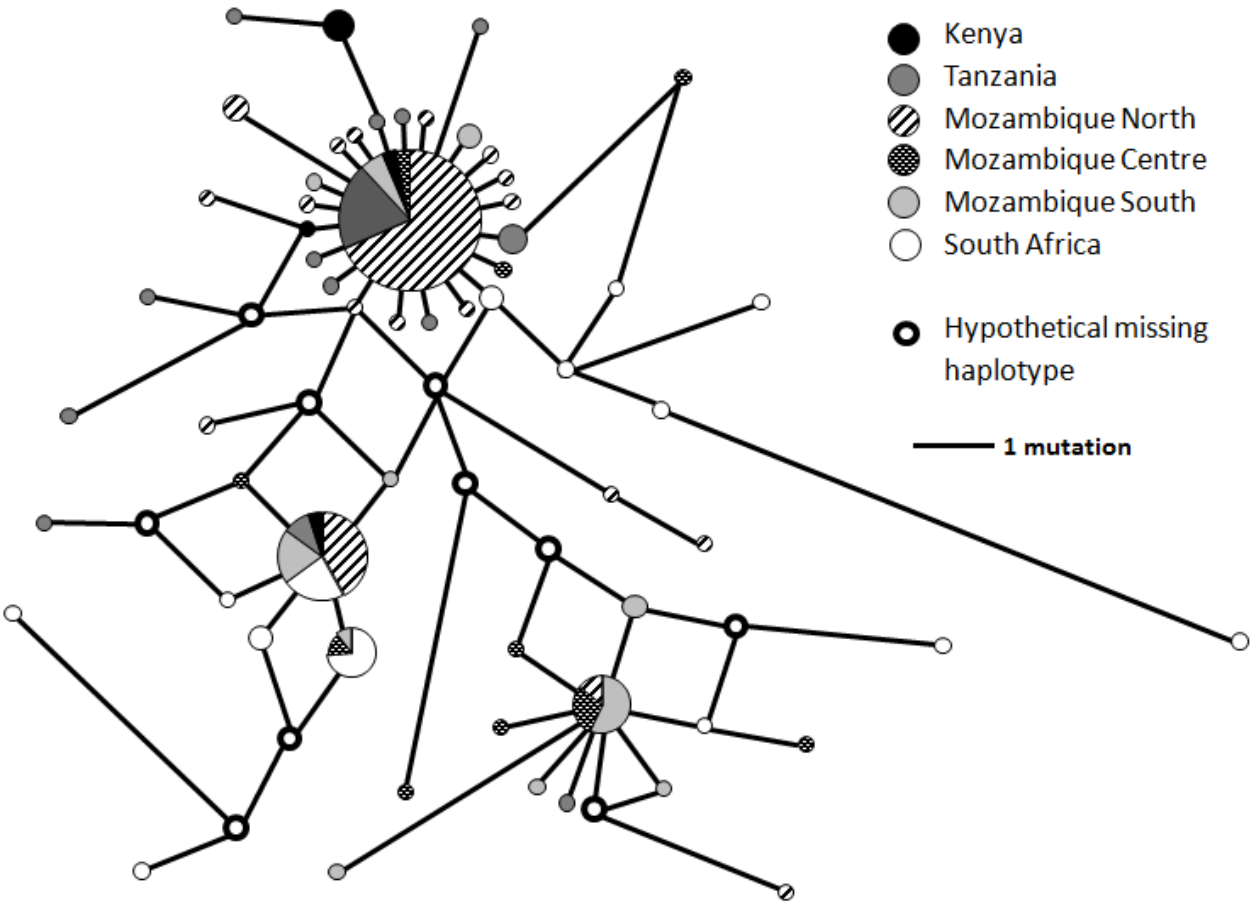

Figure 3 – Median-joining network constructed with NETWORK 4.6.0.0 for *C. decollata* haplotypes. Circles represent haplotypes and circle size represents haplotype frequency. The length of the lines connecting haplotypes is proportional to the number of mutational steps. The open circles represent hypothetical missing haplotypes that were not found in the samples but are necessary to connect haplotypes.

The mtDNA haplotype network for *C. decollata* (Figure 3) was not fully resolved, presenting various ambiguities (loops). The three most common haplotypes (H1, H10 and H6), presented central positions within the network connected by a few mutational steps. H1 was in the center of a clear star-like topology. Several exclusive alleles were interspread in the network and connected by a few mutational steps, without a clear assignment to a cluster.

The evolutionary model that best fits the mitochondrial COI gene data set was the three-parameter model (TPM), including two unequal frequencies (+2uf), proportion of invariable sites (I) and gamma distribution (G) (TPM2uf+I+G; empirical base frequencies: A = 0.2903, C = 0.1755, G = 0.1731, T = 0.3611; substitution rates: A-C = 0.2345, A-G = 19.1650, A-T = 0.2345, C-G = 1.0000, C-T = 19.1650 and G-T = 1.0000; I = 0.6530 and gamma shape distribution = 1.8660). The NJ and MP methods revealed identical tree topologies and low levels of bootstrap for all nodes (not shown). The phylogenetic analysis was in accordance with the network results, as they failed to recover distinct haplotype evolutionary lineages.

Pairwise Φ_{ST} comparisons carried out for each pair of sample sites ranged from -0.443 to 0.905 and allowed us to identify significantly different groups of localities. Pairwise Φ_{ST} values were higher and most of them were significant when comparing samples from the northern (area A to F) and southern regions (area H to L) of the latitudinal gradient. It is also important to note that the population of Rio Savane (Mozambique centre, area G, separates between the northern and southern regions) showed significant p-values for most of the pairwise comparisons (see Appendix, Figure 1). The full COI data set revealed a positive and significant correlation between genetic divergence and geographic distance, as indicated by the results of the Mantel test (Figure 4, Z = 671.480, r = 0.392, P < 0.001), fitting an isolation by distance model.

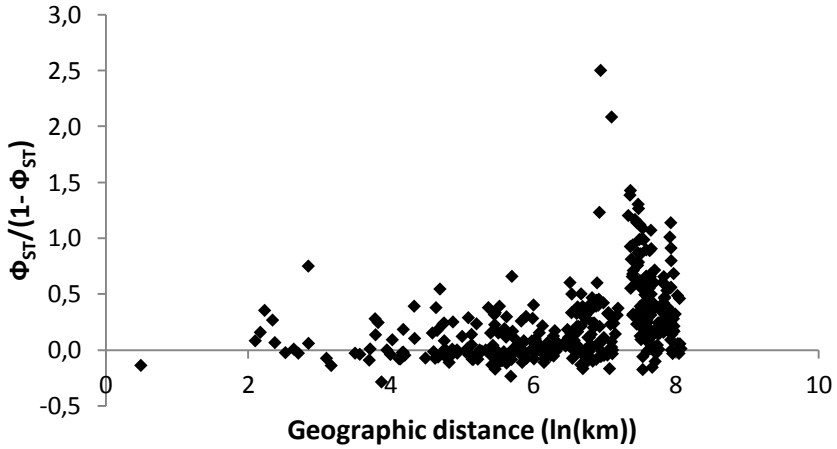

Figure 4 – Isolation by distance in *C. decollata* samples. The genetic divergence estimates ($\Phi_{ST}/(1-\Phi_{ST})$) are plotted versus geographic distance for each pair of populations.

The SAMOVA algorithm (Dupanloup et al. 2002) was used to investigate the hypothesis of finding population groups using both genetic information and geographic location of the populations sampled. A specific search for three differentiated population clusters (K=3, to check for consistence with the structure suggested by pairwise Φ_{ST} comparisons and our *a priori* expectations: northern-central-southern subdivision) revealed one group consisting of northern samples (from Mikindani to Cabaceira Grande, area A to F), a second group consisting of the central population Rio Savane (area G) and the populations from south Mozambique (Costa do Sol, Mangal do Farol, Saco and Ponta Rasa, areas H and I) and a third cluster composed by the populations of the Republic of South Africa (Kosi bay, Santa Lucia, Honeymoon bend and Mlalazi, area J to L). This analysis was further developed and performed several times by increasing the user-defined number of clusters, K (from K=2 to K=6). The cluster formed by the northern localities did not collapse with the increase in the number of groups, while the two other clusters were broken progressively. In most cases, samples were in fact grouped not breaking the defined sampling areas (A to L, Figure 1).

When considering the hierarchical partioning of mtDNA variation accomplished by AMOVA, for AMOVA I, in which individuals from the same locality were treated as individual populations, the large majority of the genetic variance was due to differences within populations (78.76%) and only 21.24% to differences among populations. Nevertheless a significant Φ_{ST} value was achieved (Φ_{ST} = 0.21245, P < 0.001), indicating significant overall genetic differentiation among populations (Table 2). In accordance to our *a priori* expectation and to pairwise Φ_{ST} values achieved, a three group genetic structure was tested (AMOVA II, Table 2): (1) from Mikindani (area A) to Cabaceira Grande (area F) – northern region, (2) Rio Savane (area G) – central region and (3) from Costa do Sol (area H) to Mlalazi (area L) – southern region (see Appendix, Figure 1). The genetic differentiation among groups represented 25.00% of the total variance observed. The greatest source of variation, 69.37%, was due to genetic differences within populations and only a smaller fraction of the total variance, 5.63%, was due to differentiation among populations within groups (Table 2). However, not only significant genetic differentiation was found among populations (Φ_{ST} = 0.30632, P < 0.01) and among groups (Φ_{CT} = 0.25004, P < 0.001), but also significant genetic differentiation was found among populations within groups (Φ_{SC} = 0.07505, 0.01 < P < 0.05).

When performing AMOVAs considering the genetic structures defined by SAMOVA for the various user-defined number of clusters (from K=2 to K=6), the results (not shown) actually indicate a tendency of Φ_{CT} to increase and Φ_{SC} to decrease with increasing K, reaching a plateau at K=4 (AMOVA III; Table 2). The genetic structure tested in AMOVA III consisted of four groups: (1) from Mikindani to Cabaceira Grande (area A to F), (2) from Rio Savane to Ponta

Rasa (area G to I), (3) from Kosi bay to Honeymoon bend (area J and K), and (4) Mlalazi (area L). For this population genetic structure, the partition of total variance reached its highest value for genetic differentiation among groups (32.41%) and its lowest value for genetic differentiation among populations within groups (near 0.00%), being the genetic differentiation among populations within groups nonsignificant (P>0.05). The K=4 structure differs from K=3 (described above) in respect to the southern populations, being the cluster with the South African populations in the K=3 structure broken in two in the K=4 structure: one containing Kosi bay, Santa Lucia and Honeymoon bend and the other cluster formed by a single population, Mlalazi.

Table 2 – Analysis of molecular variance (AMOVA). AMOVA I: overall genetic differentiation among samples; AMOVA II: three groups (north-centre-south) according to Φ_{ST} pairwise comparisons and *a priori* expectation of spatial population differentiation (based on Silva et al. 2010a); AMOVA III: four groups obtained by SAMOVA (1 - from area A to F; 2 – from area G to I; 3 – area J and K; 4 – area L).

Source of variation	*d.f.*	Sum of squares	Variance components	% variation	P	Fixation indices
AMOVA I						
Among populations	30	113.25	0.40983	21.24		
Within populations	141	214.215	1.51926	78.76	0.00000	Φ_{ST} =0.21245
Total	171	327.465	1.92909			
AMOVA II						
Among groups	2	52.423	0.54762	25.00	0.00000	Φ_{CT} = 0.25004
Among populations within groups	28	60.827	0.12327	5.63	0.00391	Φ_{SC} =0.07505
Within populations	141	214.215	1.51926	69.37	0.00000	Φ_{ST} =0.30632
Total	171	327.465	2.19015			
AMOVA III						
Among groups	3	72.554	0.72572	32.41	0.00000	Φ_{CT} = 0.32412
Among populations within groups	24	33.137	-0.02680	-1.20	0.53366	Φ_{SC} = -0.01771
Within populations	144	221.774	1.54010	68.78	0.00000	Φ_{ST} = 0.31216
Total	171	327.465	2.23902			

For the mismatch analysis and neutrality tests (Table 3), populations were grouped in northern (1), central (2) and two southern regions (3 and 4), according to the 4-group structure generated by SAMOVA that maximized the variance among groups. Both P_{SSD} and P_{Ragged}

showed that the observed mismatch distribution patterns did not significantly differ from the expected distribution under a sudden expansion model (except P_{SSD} value for group 2). Tajima´s D and Fu's Fs statistics for group 1 were negative with significant p-values, and therefore the hypothesis of sudden population expansion could not be rejected, indicating that this expansion was recent, being consistent with a scenario of dispersal and demographic expansion. The calculated time of expansion for group 1 was 227 155 years. For groups 2, 3 and 4, the results of the several tests are not all consistent with the hypothesis of sudden demographic expansion.

Table 3 – MtDNA haplotypes' mismatch distribution analysis for *C. decollata*. Tajima's D and Fu's Fs tests and p-values are also presented. θ_0 and θ_1: pre and post expansion population size; τ: time in number of generations elapsed since the episode of sudden expansion; SSD: sum of squared deviations; Ragged.: raggedness index; P_{SSD} and P_{Ragged}: probability that expected mismatch distributions have significantly larger frequencies than observed mismatch distributions; * significant values at $P < 0.05$.

	Parameters			**Goodness-of-fit tests**				**Tajima´s D test**		**Fu's Fs test**	
	θ_0	θ_1	τ	SSD	P_{SSD}	Ragged.	P_{Ragged}	D	P	Fs	*p*
Group1	0.002	2.799	5.355	0.012	0.716	0.041	0.795	-2.033	0.004*	-26.212	0.000*
Group2	0.000	14.707	6.398	0.035	0.030*	0.054	0.089	-0.525	0.306	-6.146	0.018*
Group3	0.002	5.510	5.156	0.015	0.659	0.048	0.640	-0.249	0.447	-2.554	0.094
Group4	0.002	99999.000	6.904	0.031	0.736	0.100	0.737	0.301	0.673	-1.060	0.143

Morphometric Analysis

The shape analysis approach revealed the existence of morphological variations in the shells among populations. Shape components analysis (including uniform and non-uniform components of shape) performed on all the specimens showed a decreasing amount of variation explained by the shape variables, with the first relative warp (RW1) accounting for 19% of the explained variance, the second relative warp (RW2) explaining 14% of the variance and the third relative warp (RW3) explaining 12% of the variance. The first axis revealed shape differences on the length and flattening of the shell, whereas the second axis revealed differences at the shell aperture level, mainly in the distances between landmarks 7, 8, 9 and 10, characterizing a progressive narrowing/opening of the aperture. In Figure 5 the thin plane spline grids allow the visualization and interpretation of the deviation values (both positive and negative) in geometric terms, for RW1 and RW2. The results graphically support the visual differences in shell morphology that can be observed.

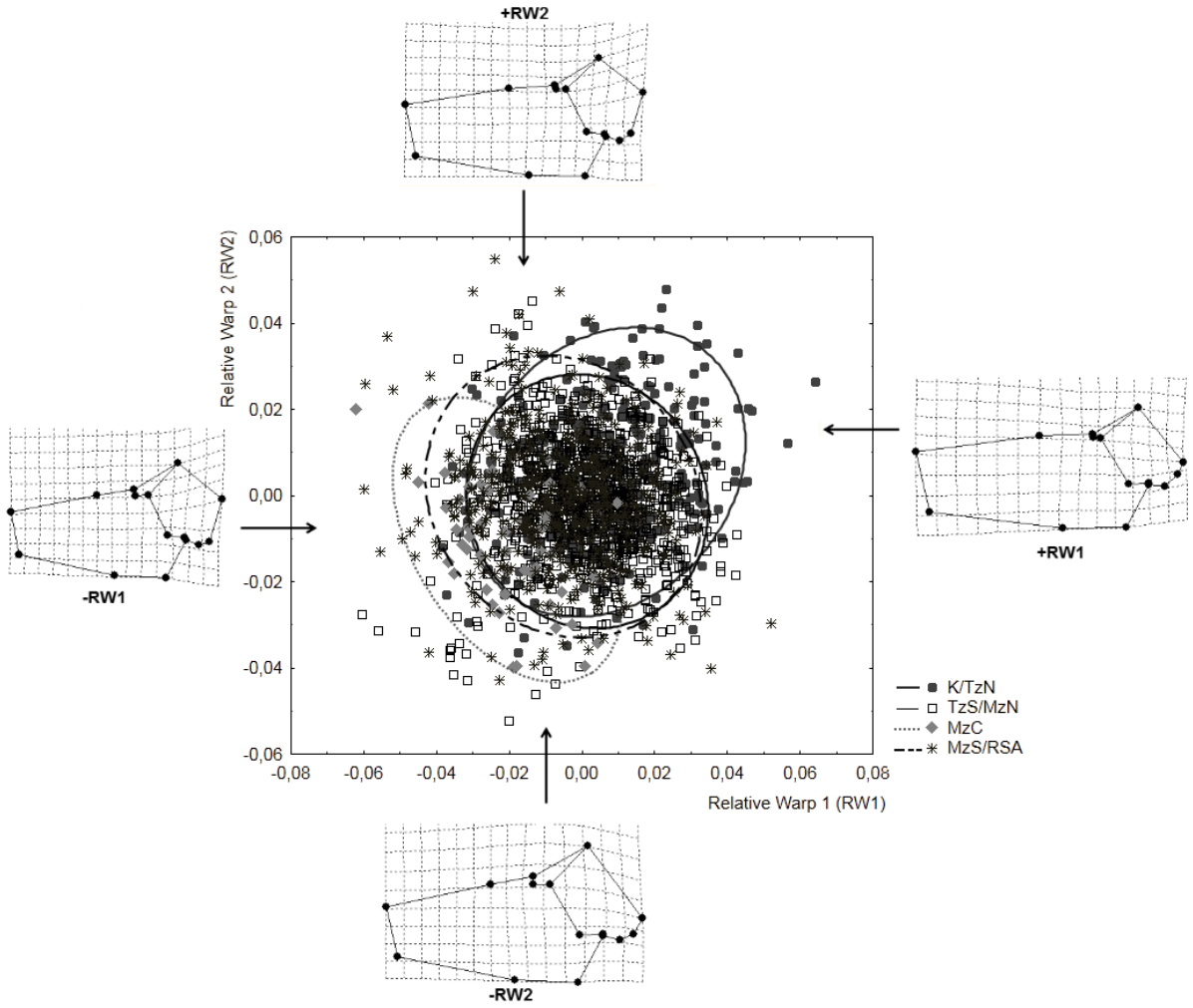

Figure 5 – Spline grids showing shape deformation along relative warps 1 and 2 of the shell of *C. decollata*. The graph illustrates the specimens' distances to the mean configuration of the shell (graph origin 0.0). Each point in the scatterplot represents a unique shape and the axes represent vectors of shape change. The ellipses correspond to a probability of 90% of the points being distributed within their limits. K/TzN: Kenya/Tanzania North; TzS/MzN: Tanzania South/Mozambique North (these two groups represent the northern region); MzC: Mozambique Centre (central region); MzS/RSA: Mozambique South/Republic of South Africa (southern region).

The advantage of RW1 is that it summarizes the main differences in shape (Carvajal-Rodriguez et al., 2006) and can be used as a formal statistical variable (Silva et al. 2010b). Results from the nested MANOVA showed that there were no significant differences among regions and among areas within the regions, revealing an overlap of shape across the latitudinal gradient. When testing for differences among populations (Figure 6) however, results showed significant differences among them (Wilks' $\lambda = 0.055$; $F = 10.676$; $P < 0.0001$), which indicates that differences in shell shape are mainly local. The post hoc Tukey HSD tests

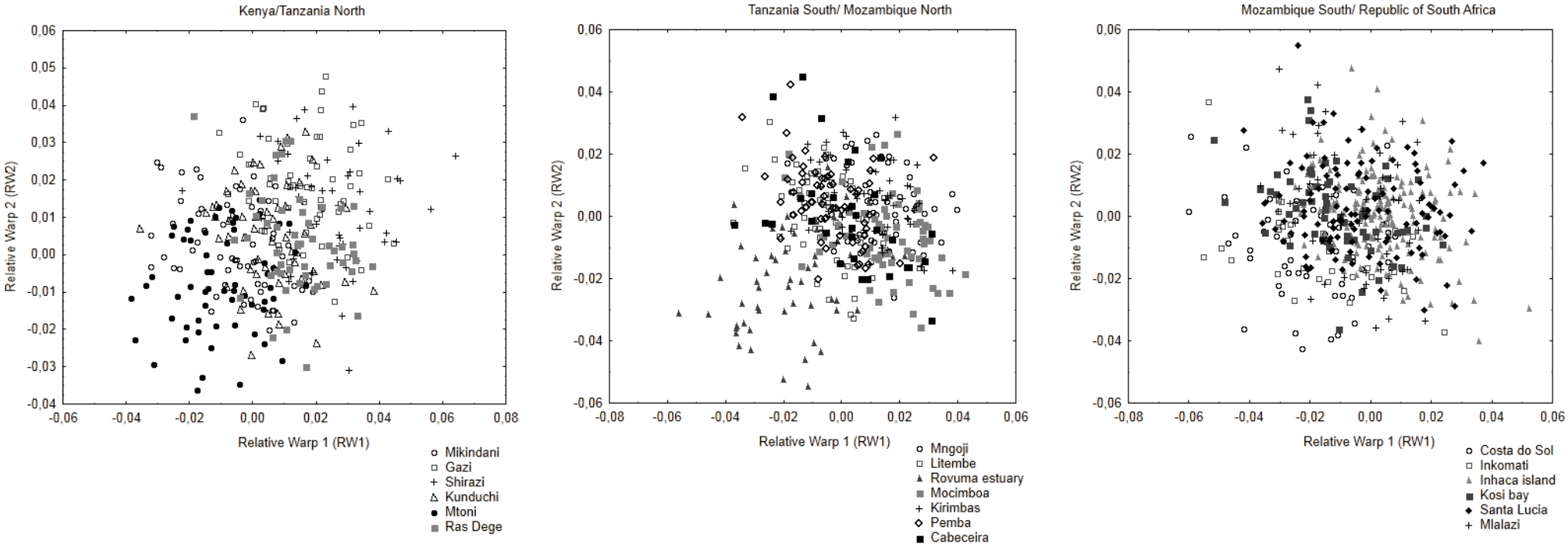

Figure 6 – Scatterplots of individual scores from the relative warp analysis (RW1xRW2), comparing populations within the main geographical regions of the latitudinal gradient in study (Mozambique centre not included once it is represented by only one population – Rio Savane).

performed on the populations to test which were different confirmed this possibility, showing that within all areas, almost all of the populations were significantly different from each other.

The degree of separation between regions, areas and populations was quantified using a step-wise discriminant analysis applied to the shape variables. Significant differences were observed for all cases for the discriminant function: regions (canonical correlation = 0.571; P < 0.0001), study areas (canonical correlation = 0.625; P < 0.0001) and populations (canonical correlation = 0.795; P < 0.0001) and the most efficient variable for discrimination was RW1. A cross validation statistical analysis also verified the efficiency of the discrimination.

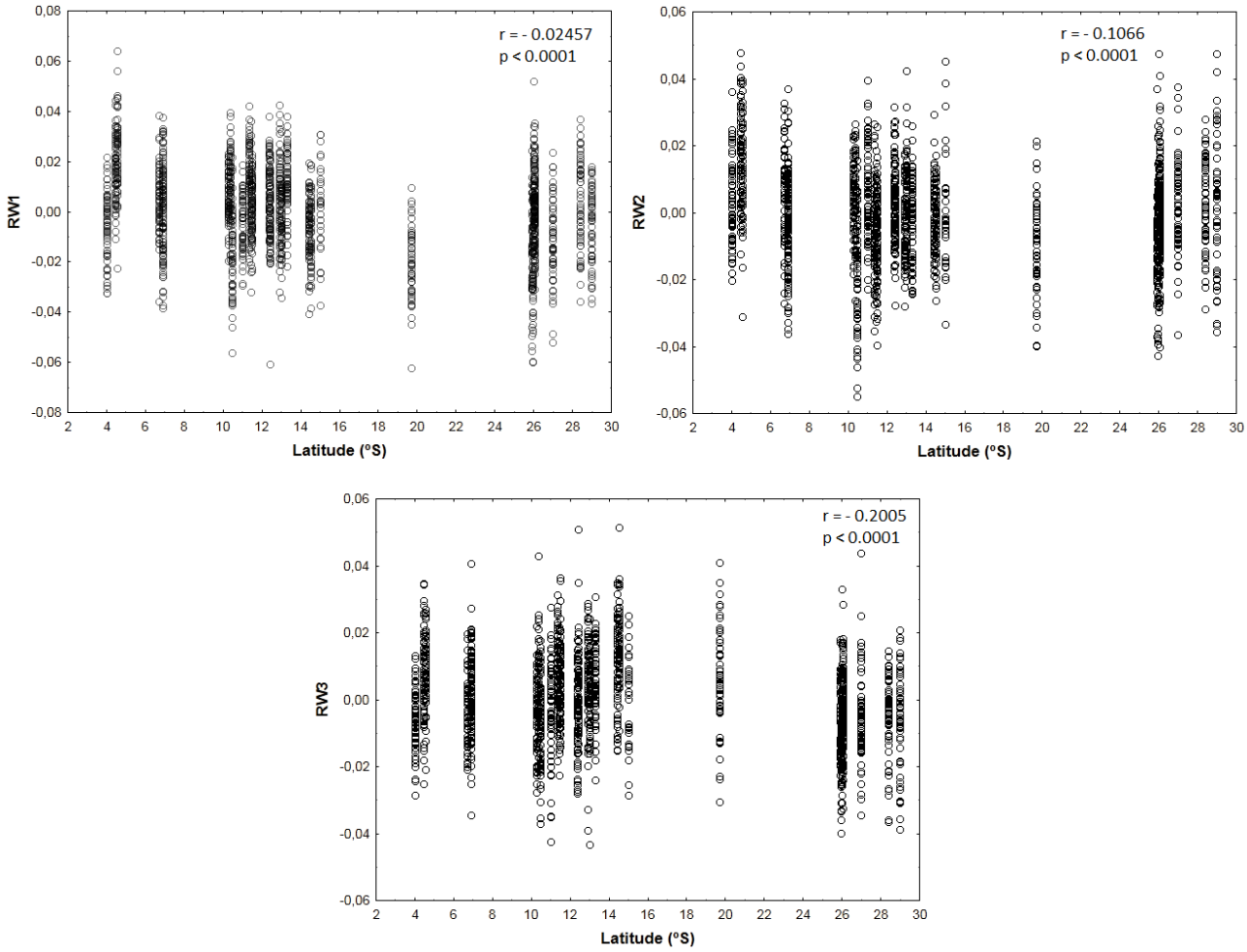

Figure 7 – *C. decollata*: correlation made between the first 3 relative warps (RW) and the latitude.

Considering the gradient in study, correct classification of specimens according to shape similarities/differences was 44.7% for Kenya/Tanzania north, 77.4% for Tanzania south/Mozambique North, 90.2% for Mozambique Centre, and finally 67.4% for Mozambique south/Republic of South Africa. The overall rate of specimens correctly classified into regions was 58.3%. When analyzing each area and each locality separately, 47.4% and 58.4% of the

overall specimens, respectively, were correctly classified into groups. Considering localities, correct classification ranged from 41% (Pemba, Cabaceira Grande) to 84% (Rio Savane). The majority of misclassified individuals were assigned to geographically adjacent localities or areas, although there are cases of misclassification that show no obvious pattern of assignment.

The correlation made between the first three relative warps and the latitude showed a negative and significant correlation for all variables tested, as shown in Figure 7.

Discussion

The assessment of population differentiation along the eastern African coast for the mangrove gastropod *C. decollata* through the combined analysis of mtDNA and shell shape variation revealed different patterns of divergence for genetic and morphological traits. Considering the genetic differentiation pattern, the major source of variation was found to be at within-localities level. However, differences at a macrogeographical scale were found to be significant and correlated with geographic distance, suggesting the possibility of the existence of discrete groups (northern-central-southern) along the coast with diminuished gene flow among them, which is concordant with the results obtained for the crab *Perisesarma guttatum* in the same geographical area (Silva et al. 2010a). The results further suggest that the northern region seems to be more homogenous, while central and southern groups present some genetic divergences within them. This was evidenced by the SAMOVA in which, by increasing the number of groups to which populations were assigned, the northern group remained cohese while the central and southern groups were progressively bronken. Considering the morphological differentiation pattern, results suggest that the major source of variation occurs within areas at among-localities level, where significant differences were obtained, but not at a macro spatial scale, showing an overlap of shape across the major regions of the latitudinal gradient, which is opposed to the genetic significant differences obtained at this geographical scale. Summarizing the general patterns obtained: (1) Genetic pattern: significant differentiation among regions (macrospatial scale – suggesting a 4-group structure defined by the SAMOVA), significant genetic differentiation within localities (microspatial scale) along the coast, but non significant differentiation among localities within groups (mesospatial scale); (2) Morphological pattern: non significant differentiation among regions (macrospatial scale), non significant differentiation within localities (microspatial scale) but significant differentiation among localities within areas (mesospatial scale).

Considering nucleotide diversity values, π, similar ranges of values were found (0.0000-0.0143) when compared with other *Cerithidea* species (Kojima et al. 2006: *C. cingulata*: 0.0004-0.0119, *C. djajariensis*: 0.0000-0.0088, *C. largillierii*: 0.0059-0.0068, *C. rhizophorarum*: 0.0007-0.0054). Furthermore, results show that COI gene is polymorphic in this species, confirmed by the high genetic diversity within populations. In the specific case of Tanzania south/Mozambique north, where lower levels of *h* and π diversities were found, these may be due to a population bottleneck followed by demographic expansion, which is corroborated by the mismatch analysis. In any case, the analysis of mitochondrial genes in gastropod species with pelagic and nonpelagic larvae has revealed higher levels of nucleotide diversity in species with pelagic larvae. Due to the presence of many rare haplotypes within our sampling localities, our results suggest that female effective population size is very large (Lewontin 1974). This is consistent with the hypothesis that pelagic lineages have higher effective population sizes (*Ne*), which is correlated with higher levels of gene flow among populations (Lee and Boulding 2009). In fact, the sweepstakes-like reproductive success of the planktotrophic species could allow a few related females to populate hundreds of kilometres of coastline (Lee and Boulding 2009). In accordance, the population genetics theory states that planktotrophic species have large *N*e and so the effects of genetic drift might be greatly diminished, in the way that rare mitochondrial haplotypes/alleles will be more likely to persist in the population (Slatkin 1985). Our results further indicate that since no apparent haplotype saturation level is attained with increasing sample size, it is possible to speculate that many unidentified haplotypes still exist in the populations sampled, as was also observed by Cassone and Boulding (2006) and Silva et al. (2010a).

Our analysis of DNA sequences suggests that *C. decollata* displays a moderate and significant population structure along the 3200 km of the eastern African coast. This was reflected by 1) a reasonable and significant overall value of Φ_{ST} (0.21245), 2) significant pairwise Φ_{ST} values among populations from the northern vs central vs southern regions of the latitudinal gradient and 3) the existence of private haplotypes (i.e., haplotypes that are geographically restricted) in most populations. These results are consistent with a number of other intertidal and nearshore species with planktonic larvae that despite the high potential for dispersal and gene flow, display a weak to moderate but significant population structure (*Batillaria zonalis*, Kojima et al. 2005; *Carcinus maenas*, Roman and Palumbi 2004, Pascoal et al. 2009; *Conus ebraeus*, Duda and Lessios 2009). Similarly to our results, high levels of genetic variation within populations have been reported for several gastropod species such as *Littorina scutulata* along the west coast of North America (Kyle and Boulding 2000), *Nerita atramentosa*

in southeastern Australia (Waters et al. 2005) and *Littoraria scabra* and *Littoraria glabrata* in eastern Africa (Silva et al. submitted).

The different genetic differentiation patterns attained for different geographical scales in this study seems to indicate that the presence and duration of the planktotrophic larval phase are key determinants of the magnitude of spatial population structure in marine invertebrate species, as observed by Lee and Boulding (2009). Another possibility to explain the genetic differences observed along the gradient is the zonation of plant communities in intertidal habitats to which *C. decollata* is associated. Mangrove zonation quite often occurs in a mosaic style that varies with the complex of physical, chemical, and biological interactions occurring in a particular area (Neukermans et al. 2007, Rey and Rutledge 2009). In this way, *C. decollata* should be distributed in clumps near *Avicennia marina* tree patches, which serve as shelter during the high tide to escape from predators. Such correlation between mangrove vegetation and fauna distribution as been suggested for several intertidal species, in particular for grapsid crabs (Dahdouh-Guebas et al. 2002). This type of distribution would lead to a network of spatial genetic variation explaining the significant overall subdivision observed in the study area.

We should note that the mild genetic differentiation obtained at a macrogeographical scale (4-group structure obtained by SAMOVA) might have been underestimated once fixation indices derived from the AMOVA are not completely accurate when used for the analysis of haplotypic data and, hence, should be regarded as indicators rather than absolute values (Schneider et al. 2000). This is important because samples from large populations, as in the case of *C. decollata,* along the sampled geographical gradient, tend to underestimate population differentiation, causing the results to be conservative (Waples 1998).

The attained structure pattern is also consistent with increasing evidence that a variety of physical oceanographic factors, including temperature gradients, wind patterns, ocean mesoscale currents and eddies can restrict larval dispersal (e.g. Reeb and Avise 1990; McCartney et al. 2000). Once it was predicted a high dispersal ability for *C. decollata*, due to its planktonic larval stage of 2-3 weeks, these results indicate the possibility of isolation by distance as consequence of physical barriers with the existence of a correlation between coastal hydrology and population connectivity. In particular, the coastal divergence of the south equatorial current in North Mozambique and the eddy system and countercurrents near the coast along the Mozambique Channel, are the most likely physical mechanisms that may act as barriers reducing dispersal. These phenomena may, therefore, contribute to the retention of larvae and restrict offshore dispersal as suggested by Paula et al. (2001), which

would prevent gene flow, explaining the observed differences among groups obtained in the AMOVAs. Also, Larson and Julian (1999) maintain that oceanographic currents and entrainment of larvae lead to genetic patchiness, which may counteract the effect of fecundity and dispersal time.

In IBD analysis, genetic differentiation was found to be correlated with coastline distance among populations, suggesting that dispersal occurs across limited distances and through successive events (i.e. following a step-by-step process along the coastline), fitting an isolation-by-distance model. This is concordant with other studies in which isolation by distance has been suggested for rocky and shallow intertidal organisms in south Mozambique and South Africa to date, irrespective of their pelagical larval duration (Evans et al. 2004, Teske et al. 2007). In addition, theory states that stable IBD slopes (with increasing geographic distance) are generated and maintained by a balance between genetic drift and migration. As a consequence, there is an inverse relationship between the IBD slope and the average dispersal distance of a species (Rousset 1997; Raybould et al. 2002). A review of IBD slopes for several marine invertebrates inferred that planktonically developing gastropods dispersed from 20 to 140 km on average (Kinlan and Gaines 2003). So, at this stage, our results suggest that a physical break can be in the origin of the population structure. Nevertheless, it seems that within each group defined in the 4-group genetic structure, the larval export strategy (see Paula et al. 2004) might apply, once the degree of differentiation among populations within groups was really low and non-significant (AMOVA III). A hypothesis for explaining the overall significant genetic subdivision (AMOVA I) might be the pre and post settlement natural selection events: the larval pool is possibly not always mixed homogenously, explaining genetic heterogeneity among populations (Silva et al. 2009). In addition, the differential mortality of larval recruits that can cause genetic differentiation (see Koehn et al. 1980, Kordos and Burton 1993), can also lead to a failure in the reproduction of migrant individuals within the new population to which they are dispersed (Silva et al. 2010c). Researchers have, in fact, reported the possible influence of selection in population structuring of marine species (Nielsen et al. 2004, Banks et al. 2007), and selective factors may account for spatial population differentiation. However, as we have employed supposedly neutral markers, such possibility cannot be tested with the present dataset (Machado-Schiaffino et al. 2010).

The network topology, the mismatch distribution and Tajima's D and Fu's Fs tests for *C. decollata* for the northern region are indicative of a population that has recently expanded in size from one or a small number of founders following a population bottleneck (Slatkin and Hudson 1991), showing an excess of recent mutations. Thus, the limited degree of genetic

differentiation among populations within this area may also be a reflection of recent expansion. The calculated time of expansion, 227 155 years, coincides with the beginning of a warm high sea level period in the Holsteinian interglacial period. Similar patterns appear to be shared by many other western and central Pacific marine *taxa* (Duda and Lessios 2009). For the central and two southern groups, the results of the tests mentioned above were not all concordant with the hypothesis of sudden expansion, which seems to indicate the existence of several colonization episodes (i.e., from a founder event or from mutations that accumulated *in situ* over time) resulting in the admixture of different lineages. In line with these findings, Benzie et al. (2002) and Kochzius and Nuryanto (2008) suggest that the fall in sea level during the Pleistocene ice ages is likely to have removed shelf habitat in southern Africa. In fact, the mangrove habitat is highly sensitive to sea-level change, being greatly reduced in global area during low sea-level stands (Woodroffe and Grindrod 1991, Sun et al. 2000). Consequently, the distributions of marine species must have been profoundly disrupted along the broad continental shelves in the central Indo-West-Pacific, when these were exposed during Pleistocene sea-level fluctuations (Voris 2000). These habitats would then have been reinvaded by the surviving populations after the occurrence of a major sea level rise, at the end of the glacial periods (Forbes et al. 1999), enabling recolonization and growth of the reduced populations. This scenario also seems to be applicable for many marine organisms from other disparate geographic regions (Gopurenko and Hughes 2002, Couceiro et al. 2007, Espinosa et al. 2010).

Contrary to the genetic results, differences in morphological characters were not so obvious when analyzing the thin-plate deformations and the nested MANOVA showed morphological similarity along the gradient at a macrogeographical scale, due to overlap of morphotypes. Nevertheless, we observed the existence of significant among-locality (within areas) divergence of shape. The main morphometric differences concerned the shell length and flattening and the size of the aperture. The greatest deviations to the mean configuration were found in the central region (represented by Rio Savane) vs the northern (from area A to F) and southern regions (from area H to L), when displaced in a morphological space. Interestingly, morphotypes of different regions seem to be alike, and the northern region shows more variability in terms of shell shape. Therefore, our data does not show any particular geographical assignment of shapes among the regions defined, which was confirmed by the low levels of correct classification obtained by classification methods, although we could find a significant correlation between shapes and latitude. These results show a divergence between differentiation patterns for genetic and morphological traits. This may

indicate that similar ecological pressures are acting along the coast, leading to the similar development of morphological characters. However, when acting in the opposite direction, site-specific ecological pressures may cause *C. decollata* to respond plastically, causing changes in the expression of a particular morphological trait, which may explain the significant diferences observed among sampling sites. A similar phenomenon was also observed by Silva et al. (2010a) for a crab species, where the same environmental factors that possibly justified the lack of morphological differentiation across several hundreds of kilometers of coast were, in fact, the same factors contributing to local shape variation, when acting in opposite directions. These ecological pressures can have diverse origins, such as foraging, defence, habitat availability, mating, food acquisition and even human caused disturbances (Smith 2004, Brian et al. 2006). It is hard at this point to determine whether the observed pattern of morphological variation is a result of genetic traits or the action of plastic mechanisms in response to habitat pressures (Beaumont & Wei 1991, de Aranzamendi et al. 2008, Hoffman et al. 2010). This is due to the fact that usually, the markers used to study population genetics are generally selectively neutral universal markers and so they cannot be directly associated with the expression of morphological characters or either tested for selection by environmental factors. Nonetheless, it seems evidences are accumulating for different hypotheses: 1) plasticity in gastropods (Trussel 1996), 2) weak relationship between phenotypic characteristics and genetic similarity (Brian et al. 2006), and 3) morphometric pattern coincides with genetic pattern at macrogeographical scales (Silva et al. 2010b). The results achieved in the current study, in accordance with Brian et al. (2006), suggest that genetic and phenotypic characters are in some way independent. Understanding the cues inducing these changes and their immediate adaptive value is being improved (Schlichting and Pigliucci 1998), but there is still much left to comprehend about how this phenomenon contributes to broader spatial (and temporal) patterns of variation.

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CHAPTER 3

FINAL REMARKS

Evolution requires genetic variation. Understanding the mechanisms that generate genetic variation is one of the basic persuits of evolutionary studies. The present work improved the knowledge about mechanisms that produce geographic variation in the western Indian Ocean populations of *Cerithidea decollata*. This study demonstrated that *C. decollata*, a species thought to have a 2-3 week time of planktonic life, presents a mild genetic structuring across the eastern African coast. There is no question about the importance of the dispersal ability of a species as it can influence not only species' geographical range, but also population genetic structure and evolutionary trajectory. Nonetheless it seems that oceanographic factors may play an important part in maintaining or restraining gene flow between populations. In *C. decollata*, the combination of a high dispersal potential with the environmental features, in particular Mozambique Channel's eddy system and the Equatorial convergent current, along the coast, seem to have lead to a mild genetic divergence, suggesting the existence of four groups (northern, central and two southern groups) with low gene flow among them. Genetic analyses of marine population structure often find slight geographic differentiation in species with high dispersal potential but interpreting this genetic signal has been difficult, once it can be a reflection of recent expansion, levels of gene flow or even sampling error. Genetic patterns of isolation by distance have the potential for estimating measures for larval dispersal distance but many more studies of widespread species will be needed before the factors that contribute to modern-day patterns can be elucidated.

The pattern of morphological variation showed an overlap of shape across the eastern coast of Africa although differences at local levels were found; suggesting that similar morphogical changes of identical magnitude might be produced in a plastic way. This suggests that morphological variation may be somewhat independent of genetic traits. However this possibility needs to be further investigated and so, caution is needed when interpreting patterns of shape differentiation. It is not possible to conclude that morphological differences reflect genetic differences or the action of selection without an experimental verification.

The use of morphological and genetic data of *C. decollata* allowed an interpretation of variability patterns searching for the source of a possible inter and intrapopulation variation, and also the recognition of the existence of discrete groups of individuals along coastal areas, to a better understanding of the ecology and biogeography of the species. The combined application of different approaches should help resolve outstanding questions about the effective population sizes of marine populations, how these change through time, and the

movements between marine populations that shape their evolutionary history and sustain their dynamics today.

Analysis of the genetic structures of marine organisms not only provides information about historical variations in oceanic environments but also provides fundamental data for estimations of the effects of future environmental changes on marine ecosystems. Measuring genetic variation and interpreting these data in a phylogeographic and population genetics context also enables us to understand the evolutionary context of species, and to improve the development of management strategies. As an example, the design of marine reserve systems requires an understanding of connectivity between protected and adjacent areas and whether reserve networks can exchange recruits. To do this, the assessment of connectivity, biodiversity within and among populations of a vast number of marine species is essential. This will allow the identification of areas, which should be a subject of protection for safeguarding the marine ecosystems. Ultimately, it is important not to forget that human welfare depends on the stability of ecosystems.

As a note for the future, the recent phenomenon of global warming is expected to help warm-water species such as *C. decollata* extend their distribution. Thus, it is necessary to monitor changes in the geographical distribution and genetic structure of such species over a long period.

All things considered, it is important to continue researching the different ecological, genetic and evolutionary contexts that play an important role promoting adaptive radiation and speciation in marine species.

APPENDIX

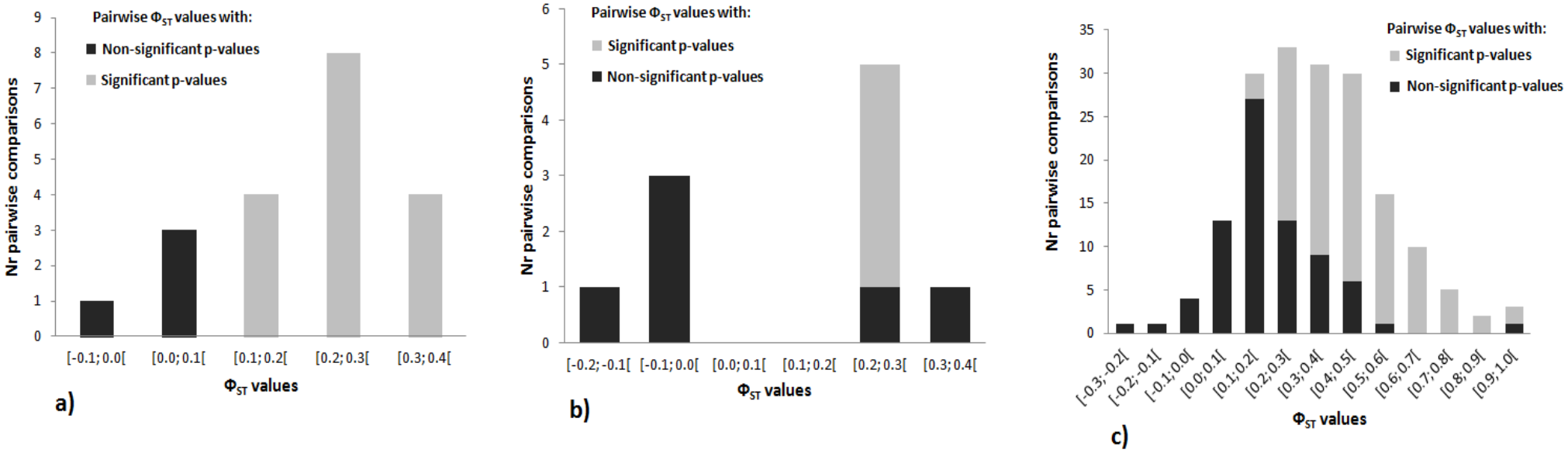

Figure 1 – Pairwise Φ_{ST} comparisons among populations from different groups: a) northern (from Mikindani to Cabaceira Grande) and central (Rio Savane) groups; b) southern (from Costa do Sol to Mlalazi) and central groups; c) northern and southern groups. This subdivision in northern-central-southern groups is similar to the one suggested by Silva et al. (2010a) for *Perisesarma guttatum* in the same geographical area and it represents our *a priori* expectation of a possible genetic structure, to be tested by an analysis of molecular variance (AMOVA II). Pairwise Φ_{ST} comparisons among populations within a group are not shown here.