Genetic population structure of penaeid prawns *Penaeus monodon* Fabricius 1798, *Fenneropenaeus indicus* H. Milne Edwards 1837 and *Metapenaeus monoceros* Fabricius 1798 in the Malindi–Ungwana Bay, Kenya

By

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Abstract

Comparative analyses of genetic diversity, population structure and evolutionary relationships among co–distributed species can provide useful insights into fisheries management. In this study, mitochondrial DNA control region (mtCR) sequences were used to investigate genetic population structure and recruitment patterns of three co–occurring shallow water penaeid prawn species; *Penaeus monodon*, *Fenneropenaeus indicus* and *Metapenaeus monoceros.* These taxa dominate artisanal and commercial prawn catches in the Malindi–Ungwana Bay in Kenya, where juvenile prawns inhabit estuarine habitats, and adults occur further offshore, on mudbanks in the bay. A total of 296 [i.e. (*P. monodon*; n = 129), (*F. indicus*; n = 96), (*M. monoceros*; n = 71)] specimens were sampled from five sites; two estuarine nursery areas (juveniles), a nearshore mid–station (adults), and two offshore areas (adults). The sites were chosen to represent the bulk of the Kenyan fishery activities, and to include juvenile and adult cohorts that are presumably connected to each other through larval dispersal processes and migrations. Juveniles were obtained during 2010 from local fishermen, and adult prawns during 2011 using a commercial prawn trawler. Analysis of the mtCR sequences indicated high haplotype diversity (*P. monodon*; $h = 0.9996 \pm 0.0010$; *F. indicus*; $h = 0.9998 \pm 0.0015$; *M. monoceros*; $h = 0.9815 \pm 0.0110$) for all three species. Genetic differentiation results for each species using AMOVA indicated no significant population differentiation (*P. monodon*; $\Phi_{ST} = 0.000$, $= p > 0.05$; *F. indicus*; $\Phi_{ST} = 0.000$, $= p > 0.05$; *M. monoceros*; $\Phi_{ST} = 0.0164$, $= p > 0.05$) and pairwise Φ_{ST} statistics among sampling sites indicated the complete absence of spatial differentiation of female genes for all three species. In addition, the mtDNA data of *P. monodon* (i.e. $n = 103$) was augmented by using six polymorphic nuclear microsatellite loci. The pattern of panmixia was supported by the

microsatellite analyses of *P. monodon* where AMOVA (i.e. R_{ST} = 0.00113, = p > 0.05), pairwise R_{ST} statistics (i.e. R_{ST} = 0.0000–0.0223, = p > 0.05) and STRUCTURE all confirmed the complete absence of genetic differentiation, among all sampled localities. Based on the absence of genetic population structure, each of the three species can be regarded as a single management unit throughout the Malindi–Ungwana Bay area. Spatial management strategies for prawn fisheries in the bay should therefore rely on factors other than genetic metapopulations, such as seasonal prawn recruitment and distribution patterns, ecosystem functioning and socio–economic implications to fishing communities and commercial trawl fishing companies.

Opsomming

Vergelykende analise van genetiese diversiteit, bevolkings stuktuur en evolutionêre verwantskappe tussen spesies wat ‘n verspreidingsgebied deel kan nuttige insigte lewer oor vissery bestuur. In hierdie studie was die mitokondriale DNS kontrole area (mtCR) volgordebepalings gebruik om die bevolkings genetiese stuktuur en werwingspatrone van drie mede-verspreide vlak water penaeid garnaal spesies; *Penaeus monodon, Fenneropenaeus indicus* and *Metapenaeus monoceros* te ondersoek. Hierdie taksa domineer die ambagtelike en kommersiële vangste in die Malindi-Ungwanabaai in Kenya waar, onvolwasse garnale in riviermondings voorkom en volwassenes in dieper waters op modderbanke in die baai voorkom. ‘n Totaal van 296 [(*P. monodon*; n = 129), (*F. indicus*; n = 96), (*M. monoceros*; n = 71)] monsters was geneem vanaf vyf lokaliteite; twee in riviermondings (onvolwassenes), ‘n naby-kus mid stasie (volwasse) en twee diep water (volwasse) areas. Hierdie lokaliteite was gekies om die oorgrote meerderheid van Kenya se vissery aktiwiteite, asook die onvolwasses en volwassene kohorte te verteenwoordig wat vermoedelik geneties verbind is aan mekaar deur larwale verspreidingsprosesse en migrasies. Onvolwasse diere was verkry in 2010 vanaf plaaslike vissermanne en volwasse diere was in 2011 gekollekteer deur gebruik te maak van ‘n kommersiële garnaal vissersboot. Analise van die mtCR volgorde bepaling het gewys dat daar ‘n hoë haplotipiese diversiteit (*P. monodon*; h = 0.9996 ± 0.0010; *F. indicus*; h = 0.9998 ± 0.0015; *M. monoceros*; h = 0.9815 ± 0.0110) vir al drie spesies bestaan. Genetiese differensiasie resultate vir elke spesie, bepaal deur ‘n AMOVA toets, dui op geen beduidende bevolking differensiasie nie (*P. monodon*; Φ_{ST} = 0.000, = p > 0.05; *F. indicus*; Φ_{ST} = 0.000, = p > 0.05; *M. monoceros*; Φ_{ST} = 0.0164, = p > 0.05) en paarsgewyse Φ_{ST} statistiek tussen die lokaliteite waar

monsters geneem was, dui op geen ruimtelike differensiasie van die vroulike gene in al drie spesies nie. Hierbenewens is die mtDNS datastel van *P. monodon* (i.e. n = 103) uitgebrei deur ses polimorfiese kern mikrosatelliete in te sluit. Die patroon van mtCR panmixia was ondersteun deur die mikro-satelliet analise van *P. monodon* waar die AMOVA (i.e. R_{ST} = 0.00113, = p > 0.05), paarsgewyse R_{ST} statistiek (i.e. R_{ST} = 0.0000-0.0223, = p > 0.05) en STRUCTURE bevestig het dat daar totale afwesigheid is van genetiese differensiasie tussen alle vergelyk-te lokaliteite. Gebaseer op die afwesigheid van genetiese bevolking-struktuur kan elk van die drie spesies beskou word as ‘n enkele bestuurseenheid deur die Malindi-Ungwanabaai area. Die bestuurstrategieë vir garnaal vissery aktiwiteite in die baai moet dus steun op ander faktore as genetiese meta-bevolking. Belangrike faktore om in ag te neem is seisoenale garnaal werwing en verspreidings patrone, ekosisteem funksionering en sosio-ekonomiese implikasies van vissers gemeenskappe en kommersiële visserymaatskappye.

Dedication

I dedicate this thesis first to my mother Salome Dzidza Mkare and my loving wife Janet Ningala Kalama for their love, care, support and prayers that have kept me going.

Second, I dedicate this thesis to you my first child and daughter Faith Dzidza Kalama. Faith, you were born when your father was spending sleepless nights writing this thesis. Thank you for your calmness.

Third, I dedicate this thesis to my late Dad Mr. Mkare Ndiro (1952–2012) who was not fortunate to see the glorious moments of his son.

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Table of Contents

List of Tables

List of Figures

List of plates

CHAPTER 1

General introduction

1.1 Zoogeography and systematics of penaeid prawns

The terms prawns or shrimps are synonyms and are used interchangeably in different parts of the world. Penaeid prawns (Crustacea, Decapoda, Penaeidae) have a global distribution and occur in four major marine basins including the Indo–West Pacific, eastern Pacific, the western and the eastern Atlantic (Holthuis 1980; Dall *et al*. 1990). The Indo–Pacific region has a larger continental shelf area and more suitable habitat when compared to the Atlantic, and this has given rise to higher marine species diversity (i.e. about five times more species) and increased abundance (Dall *et al*. 1990; Briggs 1999).

The four marine basins are also regarded as bioregions (Spalding *et al*. 2007) and some features can be put forward separating some of the regions. For instance, the eastern Atlantic is separated from the western Atlantic by deep oceanic waters. Similarly, the western Pacific is separated by deep oceanic waters from the eastern Pacific, and isolated islands and cold water masses add further diversity. There is no documented barrier separating the Indian and west Pacific Oceans, and this region is regarded as a single and complex bioregion, the Indo–West Pacific (Briggs 1999). Contemporary barriers (including land masses, temperature gradients, ocean currents and ocean deeps) can all restrict the dispersal of pelagic larvae or eggs by ocean currents, or migrations by fish or benthic organisms such as penaeid prawns, giving rise to structured populations and eventually to speciation. Similarly, vicariance events (formation of physical barriers which disconnect previously continuous regions) such as seaway closures (e.g.

closure of the Tethys seaway between the Atlantic and Indo-Pacific via the Mediterranean during the Oligocene/Miocene [Hrbek and Meyer, 2003] or of the Isthmus of Panama [Keigwin, 1978; Coates *et al.* 1992]) in the marine environment can limit dispersal and result in the formation of genetic differentiations among taxa (Teske *et al*. 2007).

The taxonomic revision of the family Penaeidae Rafinesque–Schmaltz 1815 recognises 17 extant genera (Table 1), with at least 200 extant species across the four basins (Dall *et al*. 1990; Chan *et al*. 2008). Many of the genera inhabit shallow waters with the exception of *Metapenaeopsis*, *Parapenaeus* and *Penaeopsis* which are distributed in deeper oceanic waters (Dall *et al*. 1990). The Indo–West Pacific (IWP) is characterized by 11 of the 17 genera (Table 1). Moreover, nine of the 11 genera constitute about 24 species which occur in the South West Indian Ocean (SWIO) (Table 1; Dall *et al*. 1990). Endemism in the SWIO is however extremely low and most of these taxa have wide distributions (Dall *et al*. 1990).

The latest taxonomic revision of the family Penaeidae (Pérez Farfante and Kensley 1997) suggests a total of 26 genera (Table 1); in the revision *Trachypenaeus* was divided into five genera and *Penaeus* into six. Several phylogenetic analyses using mitochondrial fragments (e.g. Baldwin *et al*. 1998; Maggioni *et al*. 2001; Lavery *et al*. 2004; Quan *et al*. 2004; Voloch *et al*. 2005; Chan *et al*. 2008), nuclear genes (Ma *et al*. 2009) and a combination of mitochondrial and nuclear genes (e.g. Ma *et al*. 2011) have questioned the validity of the classification by Pérez Farfante and Kensley (1997). Based on available phylogenetic evidence, it is clear that taxonomic revision, especially for the traditional genus *Penaeus* (currently including *Penaeus*,

Fenneropenaeus, *Litopenaeus*, *Farfantepenaeus*, *Melicertus* and *Marsupenaeus*) is needed (Dall 2007; Flegel 2007; 2008; Chan *et al*. 2008; Ma *et al*. 2011).

Table 1. The taxonomy of the family Penaeidae showing the old and the latest taxonomic revisions. The genera occurring in the IWP and SWIO regions are listed. The penaeidae species inhabiting the SWIO waters are listed

Old genera (Dall *et al.* 1990)	**Added genera (Pérez Farfante and Kensley 1997)**	**IWP genera**	**SWIO genera**	**SWIO species**
Atypopenaeus	*Farfantepenaeus*	*Atypopenaeus*	*Macropetasma*	*Macropetasma africanus* Balss 1913
Macropetasma	*Fenneropenaeus*	*Macropetasma*	*Metapenaeopsis*	*Metapenaeopsis hilarula* De Man 1911
Metapenaeopsis	*Litopenaeus*	*Metapenaeopsis*	*Metapenaeus*	*Metapenaeopsis mogiensis* M. J. Rathbun 1902
Metapenaeus	*Marsupenaeus*	*Metapenaeus*	*Parapenaeopsis*	*Metapenaeopsis scotti* Champion 1973
Parapenaeopsis	*Melicertus*	*Parapenaeopsis*	*Parapenaeus*	*Metapenaeopsis provocatoria* Racek and Dall 1965
Parapenaeus	*Megokris*	*Parapenaeus*	*Penaeopsis*	*Metapenaeopsis quiquedentata* De Man 1907
Penaeopsis	*Miyadiella*	*Penaeopsis*	*Trachypenaeopsis*	*Metapenaeus monoceros* Fabricius 1798
Trachypenaeopsis	*Trachysalambria*	*Trachypenaeopsis*	*Trachypenaeus*	*Metapenaeus stebbingi* Nobili 1904
Trachypenaeus	*Rimapenaeus*	*Trachypenaeus*	*Penaeus*	*Parapenaeopsis acclivirostris* Alcock 1905
Penaeus		*Penaeus*		*Parapenaeus fissoides* Crosnier 1985
Heteropenaeus		*Heteropenaeus*		*Parapenaeus investigatoris* Alcock and Anderson 1899
Protrachypene				*Parapenaeus longipes* Alcock 1905
Xiphopenaeus				*Parapenaeus sextuberculatus* Kubo 1949
Artemesia				*Penaeopsis balssi* Ivanov and Hassan 1976
Tanypenaeus				*Trachypenaeopsis richtersii* Miers 1884
Funchalia				*Trachypenaeus curvirostris* Stimpson 1860
Pelagopenaeus				*Trachypenaeus sedili* Hall 1961
				Penaeus monodon Fabricius 1798
				Penaeus canaliculatus Olivier 1811
				Penaeus indicus H. Milne Edwards 1837
				Penaeus japonicus Bate 1888
				Penaeus latisulcatus Kishinouye 1896
				Penaeus marginatus Randall 1840
				Penaeus semisulcatus De Haan 1844

1.2 Reproduction and life history characteristics of penaeid prawns

Penaeid prawns can roughly be divided into two groups based on the morphology of the thelycum: genera with a closed thelycum (e.g. *Penaeus*, *Fenneropenaeus*, *Marsupenaeus*, *Melicertus*, *Farfantepenaeus* and *Metapenaeus*); and those with an open thelycum (e.g.

Litopenaeus). The term thelycum (woman–like) in penaeids refers to a structure usually found in female prawns and its function is to receive and store male spermatophores during mating. An open thelycum has ridges and protuberances to allow for the attachment of spermatophores, whereas a closed thelycum has lateral plates which lead into a seminal receptacle where spermatophores can be inserted (Primavera 1979; 1985; Yano *et al*. 1988).

Reproduction in penaeids is through copulation and their mating behaviour begins with an approach, crawling, chasing and finally insertion/attachment of the male spermatophore into the female thelycum (Alfaro-Montoya 2010). Mating behaviour differs between the two groups: species with a closed thelycum mate when the gonads of moulted females are still immature, whereas those with an open thelycum mate after ovarian maturation, when females are at an intermolt stage. Irrespective the shape of the thelycum, fertilization is always external.

The life cycles of all extant species of the family Penaeidae involve eggs, planktonic larvae (with naupliar, protozoeal, mysis, postlarvae stages), followed by juvenile and adult stages. Four types of life cycles are recognized and these depend on habitat preferences among postlarvae, juveniles and adults and the nature of the eggs (either demersal or pelagic; Dall *et al*. 1990). Type I represents a life cycle that is exclusively estuarine. The postlarve of type I species migrate to upstream waters that are characterised by lower salinities where they feed and grow before recruiting back to estuarine waters of higher salinities where they join adult populations. Type II species require both estuarine and offshore marine waters to complete their life cycle; the post–larvae of this group prefer estuaries or estuarine–like environments, whereas the juveniles and sub–adults emigrate from estuaries to offshore adult breeding grounds (Forbes and Demetriades 2005). Species with a Type III life cycle are highly restricted to sheltered inshore

waters, preferably those with higher salinities, whereas Type IV species complete their entire life cycle in offshore waters (Dall *et al*. 1990).

Most *Penaeus* and *Metapenaeus* species have a Type II life cycle, in which larval, juvenile and adult migrations between estuaries and offshore areas play a major role. Larvae and postlarvae can migrate both passively (along prevailing currents) and actively (vertical movements), but they probably don't swim against prevailing currents. Larvae are thus transported by oceanic currents while juveniles and adults migrate by drifting in prevailing currents, swimming against them, or by benthic migrations (Dall *et al.* 1990; Criales *et al*. 2005; Vance and Pendrey 2008).

1.3 Study species

The three species selected for this study were *P. monodon*, *F. indicus* and *M. monoceros* (Plates1–3). *Penaeus mondon* and *F. indicus* are restricted to the Indo–West Pacific whereas *M. monoceros* has a wider distribution spanning the Indo–West Pacific and the Eastern Atlantic bioregions (Dall *et al*. 1990). At the local scale, the three species co–occur along the Kenyan shallow water continental shelf, but they are more abundant in the Malindi–Ungwana Bay (MUB) (Wakwabi and Jaccarini 1993; Mwaluma 2002). Identification of the three species can easily be achieved by using the FAO species catalogue (Holthuis 1980). They differ in general body colour patterns, rostrum shape and structure (presence/absence of rostral teeth on the ventral and dorsal sides) and thelycum (even though they are all closed thelycum species) for females or petasma for males (e.g. Plates1–3) throughout their range of distribution. Some

morphological differences have been suggested between Western Indian Ocean and Western Pacific populations of *P. monodon* (You *et al.* 2008).

Plate 1. Dorsal view of *Penaeus monodon* photographed during the commercial prawn trawling expedition in the Malindi–Ungwana Bay in 2011.

Plate 2. Dorsal view of *Fenneropenaeus indicus* photographed during the commercial prawn trawling expedition in the Malindi–Ungwana Bay in 2011.

Plate 3. Lateral view of *Metapenaeus monoceros* photographed during the commercial prawn trawling expedition in the Malindi–Ungwana Bay in 2011.

Adult females of *P. monodon*, *F. indicus*, and *M. monoceros* spawn in offshore waters where eggs hatch into planktonic larvae that progress through a series of developmental stages over a period of typically 14–21 days (Dall *et al*. 1990; Niamaimandi *et al*. 2007). Postlarvae enter coastal and estuarine nursery areas to feed and grow to juvenile stages; these then recruit to offshore waters to join adult populations (Dall *et al*. 1990). Whereas *M. monoceros* is a habitat generalist (inhabits muddy and sandy substrates, seagrass meadows and mangrove creeks), *P.*

monodon and *F. indicus* co–occur in more limited habitat types, such as sandy and muddy substrates, and mangrove creeks (Sheridan and Hays 2003; Macia 2004). All three species have high fecundity, and may produce several broods throughout their lifetime (Nandakumar 2001; Jayawardane *et al*. 2002; Mgaya and Teikwa 2003).

1.4 Economic importance and management of Kenyan prawn fisheries

Prawns are the economic mainstay of fisheries along the Kenyan coast, and artisanal and commercial fisheries are focussed on the MUB area. Five co–occurring species are harvested: *F. indicus*, 55–70% of landings; *M. monoceros*, 10–15%; *P. monodon*, < 10%; *P. semisulcatus*, < 10%; and *M. japonicus*, < 5% (Fulanda *et al.* 2011). The prawn fisheries contribute to the local economy through job creation, food security and as a source of foreign earnings (Kenya Fisheries Department 2006a; van der Elst *et al.* 2009). For instance, during 2006, 10,726 artisanal fishers were active, and > 250,000 Kenyan people depended on marine organisms for food security and commerce (Kenya Fisheries Department 2006b). It is therefore important that the prawn resources of MUB are managed sustainably.

The MUB is traditionally divided into three main fishing zones: an artisanal prawn fishing zone (also called a trawling exclusion zone) between zero and five nautical miles (nm) from the coast; a commercial trawling zone between five and 12 nm; and an Exclusive Economic Zone (EEZ) between 12 and 200 nm (Cap 378; Kenya Gazette 1999; 2000; 2001). However, a recently gazetted prawn fishery management plan designated the area between zero

and three nm from the shore to artisanal fishing, while permitting commercial fishing further offshore than three nm (Kenya Gazette 2011).

Artisanal and commercial trawl fisheries have been managed by the Kenyan Department of Fisheries since 1963 and 1970, respectively (FAO 1971), and have been faced with conflicts among resource users. Conflicts stemmed from poorly defined prawn fishing zones, gear damage, use of environmentally damaging fishing gears, and reduced prawn catches and revenues (Fig. 1) (McClanahan *et al.* 2005; Munga *et al.* 2012). The commercial trawl fishery was suspended between 2006 and June 2011, whereafter trawling was continued. The motivation for the closure was to recover prawn catch rates, and to allow for scientific investigation to assist in fisheries management decisions. The present genetic population analysis of the three dominant prawn species in the MUB fisheries should be seen in this light.

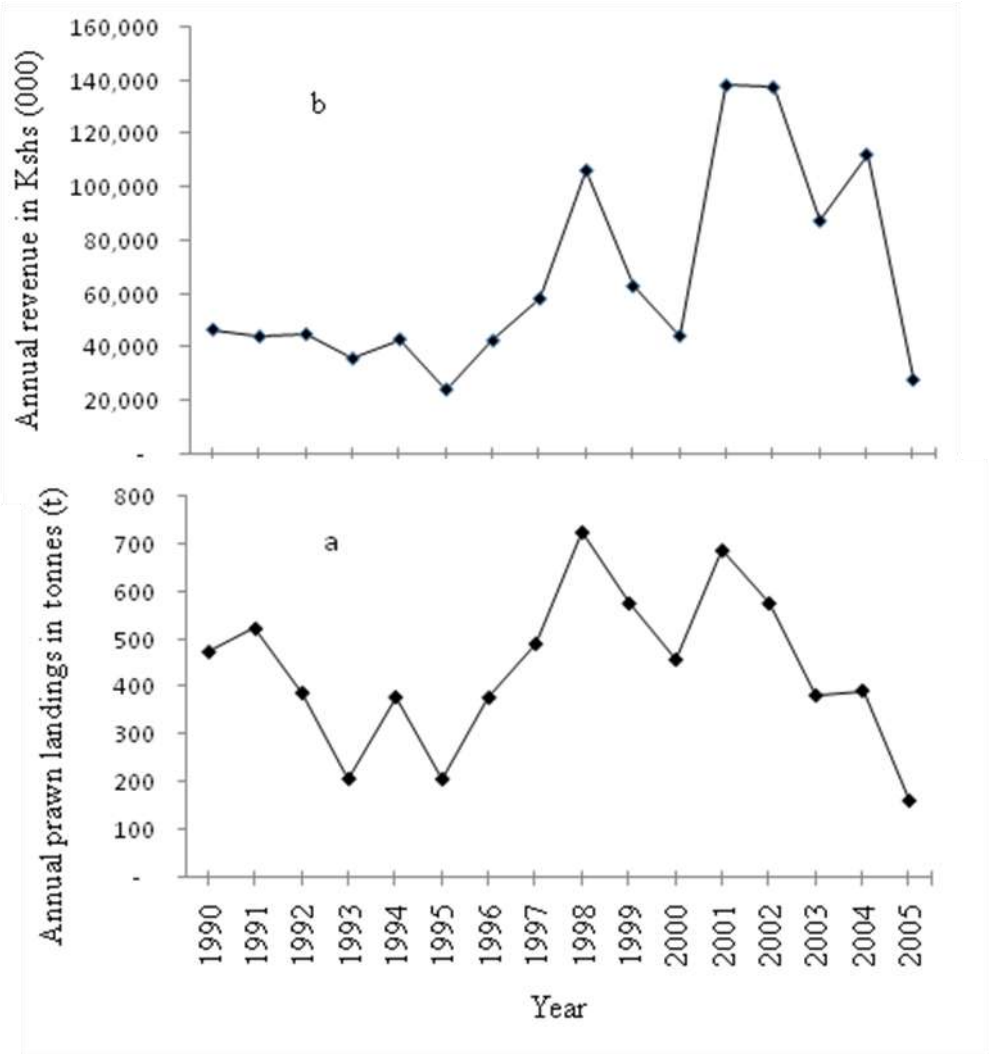

Fig. 1. Total annual prawn landings (a) and revenues (b) obtained from artisanal and commercial trawl fisheries in the Malindi–Ungwana Bay between 1990 and 2005, just prior to the closure of the commercial trawl fishery. Data were obtained from the Kenya Fisheries Department.

1.5 The study area

The MUB starts from Malindi in the south and extends to Ras–Shaka in the north, and lies between latitudes 2°30´–3°30´S and longitudes 40°00´– 41°00′E (Fig. 2). The bay is characterised by a shallow continental shelf that ranges from 15 to 60 km offshore (Kitheka

2002). The Sabaki and Tana rivers are the largest in Kenya and discharge their waters into the MUB; both rivers provide estuarine environments at their outflows (Kitheka 2002; 2005), and also deposit sand and mud sediments in the bay, thus maintaining favourable prawn habitats.

The MUB region is influenced by the South East (SE) monsoon winds between April and October and the North East (NE) monsoon winds between November and March (McClanahan 1988). Ocean currents that influence the MUB (Fig. 2) are the northerly flowing East Africa Coastal Current (EACC) and the southerly flowing Somali current (SC). The Somali current reverses its flow direction between April and October to align itself with the SE monsoonal wind direction (McClanahan 1988). The area where the SC and EACC converge marks the beginning of an offshore South Equatorial Counter Current (SECC). These oceanographic features of the MUB presumably facilitate prawn larval dispersal and mixing, with implications for recruitment patterns and genetic population structure. Nevertheless, it remains unclear whether prawn populations in the MUB comprise of genetically panmictic populations, or whether distinct metapopulations exist for specific estuarine / offshore assemblages.

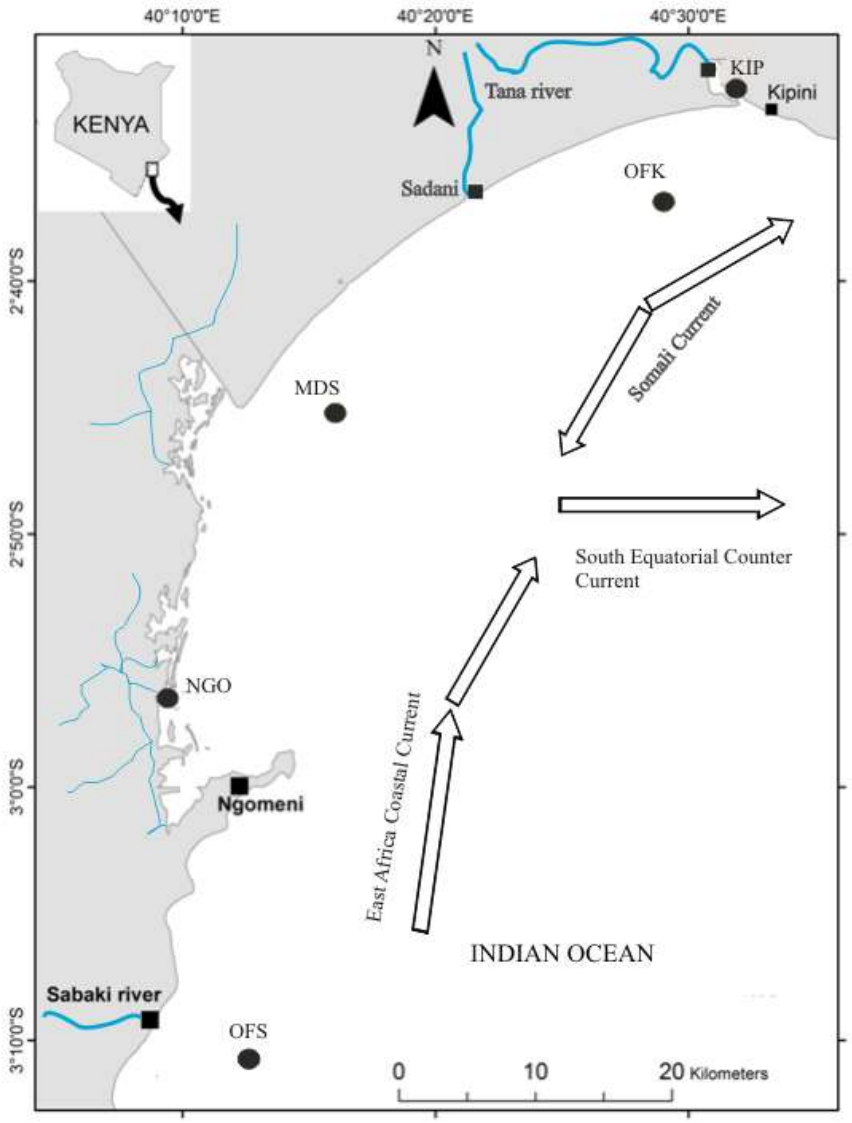

Fig. 2. Map of the Malindi–Ungwana Bay region, showing the Tana and Sabaki Rivers, as well as a schematic representation of the Somali Current, East Africa Coastal Current, and the South Equatorial Counter Current. Sampling stations for prawns (black filled circles) were at Ngomeni (NGO), Kipini (KIP), mid station (MDS), and offshore of Sabaki (OFS) and Kipini (OFK).

1.6 Recruitment patterns, genetic diversity and population structure in the marine environment

Marine species with a planktonic larval dispersal phase (e.g. the three study species) can be considered to be demographically open because larvae may originate from local or geographically distant sources (Caley *et al.* 1996; Lowe and Allendorf 2010). This means that local nursery grounds can receive larvae from many sources, which recruit to local adult populations. This pattern in larval dispersal, settlement and recruitment has implications for genetic population structure and fisheries dynamics. A comparative genetic analysis of genealogical relationships among larvae and adult genes (e.g. haplotypes) is appropriate to assess the geographic origin of larvae and/or juveniles (e.g. Bunn and Hughes 1997; Malhi *et al.* 2002; Silva–Rocha *et al*. 2012).

Genetic variation is an important element with regard to the ability of species to adapt and evolve and this measure is also used by conservation officials to form management opinions (Schwartz *et al*. 2007; Reynolds *et al*. 2012). The neutral population genetic theory suggests that genetic variation within species at mutation–random drift equilibrium is correlated with effective population size. However, other factors that could affect genetic diversity include; differences in evolutionary rate between genes and species, life histories, distribution, abundances, bottlenecks, overfishing exploitation and founder effects (Amos and Harwood 1998; Bromham 2009; Leffler *et al*. 2012).

The spatial pattern in which genetic variation is organised within and among animal populations is referred to as genetic population structure, and in the marine environment it has been reviewed extensively by Laikre *et al.* (2005) and Waples and Gaggiotti (2006). Three

general patterns are recognized; no differentiation (homogeneous populations), continuous genetic change (isolation by distance; Wright 1943) and complete differentiation (island model; Wright 1931).

In the absence of genetic differentiation, genetic variation within a species may be homogeneous over large geographic ranges, and could extend to its entire distribution. In this case, geneflow or effective migrations between geographic areas would be extensive and individuals within a species might migrate and randomly mate without constraints (Laikre *et al*. 2005). Sometimes, species belonging to this pattern could have a single geographic spawning area where mature adults gather to reproduce before dispersing to adult feeding grounds.

In a continuous change in genetic structure scenario (i.e. isolation by distance), random mating and geneflow is extensive between individuals that are geographically close to each other. However, individuals that occur at extreme opposite ends of a species distribution tend to have a limited chance of mating, thus leading to an increase in the change in allele frequencies as geographic distances become greater.

In the complete genetic differentiation scenario (island model), organisms are organised into systems (local populations) where gene flow among them is limited by factors such as physical barriers (e.g. Williams and Benzie 1998; von der Heyden *et al*. 2011), oceanographic barriers (e.g. Gilg and Hilbish 2003) or reproductive isolation (e.g. Dai *et al*. 2000). Given enough time, populations separated from their most recent common ancestor (MRCA) without gene exchange may result in allopatric speciation.

Shank *et al.* (2003) and Shanks (2009) suggested that animal species with an extended pelagic larval duration (PLD) would be more dispersive showing homogeneous genetic

population patterns (e.g. Crandall *et al*. 2010), even over very long distances (Groeneveld *et al*. 2012), while brooding or live–bearing taxa and those with short PLD could exhibit genetic discontinuities over small geographic ranges (e.g. Hellberg 1996; Baird *et al*. 2012; Ledoux *et al*. 2012). However, the link between PLD and population structure is not straightforward (Sponer and Roy 2002; Becker *et al*. 2007; Weersing and Toonen 2009; Faurby and Barber 2012), and life history characteristics alone are thus unlikely to explain genetic patterns among marine crustaceans (but see McMillen–Jackson and Bert 2003; Sivasundar and Palumbi 2010).

Despite life history, sudden intraspecific genetic disjunctions can also occur in areas where interchange between individuals is constrained by factors associated with physical barriers and other environmental factors (e.g. sharp salinity gradients, deep waters and circular currents/eddies etc.) (Gilg and Hilbish 2003; von der Heyden *et al*. 2011). In the MUB, no barrier has yet been documented that may cause genetic differentiation among prawn populations, however it is interesting to report that a large–scale genetic population analysis of *P. monodon* indicated significant genetic differentiation of the Kenyan population when compared to western Madagascar (You *et al*. 2008). In addition, studies on *P. monodon* populations in the SWIO have shown a genetic break between Tanzania and western Madagascar (Duda and Palumbi 1999), and no genetic structure among populations in South Africa, Mozambique and Madagascar (Benzie *et al*. 2002). A previous genetic analysis of *F. indicus* indicated a general lack of genetic differentiation among populations from South Africa, Tanzania and Oman, using Cytochrome c oxidase sub–unit 1 gene (COI) sequences (Querci 2003). Conversely, Random Amplification of Polymorphic DNA (RAPD) analysis indicated genetic differentiation among

the above three geographic units (Querci 2003). This latter result emphasised that using different genetic markers can provide contrasting insights, based on the level of polymorphism.

Presently, there are two main classes of DNA markers frequently used in phylogeographic investigations; those derived from mitochondrial DNA (e.g. mitochondrial control region fragment) and those obtained from nuclear DNA (e.g. nuclear microsatellite; reviewed in Sunnucks 2000; Selkoe and Toonen 2006; White *et al.* 2008; Fromentin *et al*. 2009; Galtier *et al*. 2009). The selection of genetic markers to be used in population genetics studies depends on individual marker characteristics (e.g. sample requirement, reproducibility, mode of inheritance and scoring, level of polymorphism) and the nature of the research questions being addressed (Sunnucks 2000; Karl *et al*. 2012).

Recently, the presence of paralogous sequences (either due to Numts [nuclear insertions of mitochondrial sequences], duplication of the control region, or heteroplasmy) have been suggested for *P. monodon* (Walther *et al.* 2011). Paralogous sequences may not have similar evolutionary rates when compared with mtDNA genes, thus accidental inclusion of these sequences into analysis may introduce erroneous interpretations. Thus it was important to ensure that paralogous sequences were not present in the DNA of *P. monodon* analysed in the present study.

1.7 Aims of the study

The aims of this research were to investigate genetic population structure of *P. monodon*, *F. indicus* and *M. monoceros* in the Malindi–Ungwana Bay fishing area in Kenya using mitochondrial DNA control region sequences and nuclear microsatellite loci.

1.7.1 Research Questions

The following research questions were posed:

1) Do juvenile and adult *P. monodon, F. indicus* and *M. monoceros* on offshore banks in the MUB originate from the local estuarine and/or nursery areas of Ngomeni and Kipini, or do they come from distant larval sources?

The null hypothesis suggesting an offshore recruitment that originates from the two local nursery (local recruitment) areas of Ngomeni and Kipini was tested. The alternative hypothesis, that adults in the bay originate from distant sources, supposed that long–lived pelagic larvae might have drifted from afar in oceanic currents. To test the above hypotheses, a genetic analysis of sequences (haplotypes) obtained from juveniles that occur in local estuaries and adults from offshore banks was undertaken.

2) Do the prawn populations of MUB belong to a single mixed population, or is there genetic structure in the bay congruent with separate nursery grounds?

The null hypothesis which suggests a lack of genetic differentiation was tested. The alternative hypothesis suggesting genetic differentiation in the bay supposed that physical/environmental factors (current systems) and life histories (habitat preferences) might have acted to separate populations. In the event of genetic differentiation between estuaries or among sampling sites; the alongshore ocean currents occurring in the bay (detailed in section 1.5) is not responsible for mixing/homogenising populations; perhaps due the less migratory nature of the benthic

juveniles. In addition, in the event of genetic differentiation for the habitat specific species (*P. monodon* and *F. indicus*) but not for the habitat generalist species (*M. monoceros*); differences in life histories (habitat preferences) should then be invoked to explain for such observations. To test the above hypotheses, analyses of the mitochondrial DNA control region sequences were undertaken. In addition, microsatellite data for *P. monodon* were included to validate sequences results.

CHAPTER 2

Materials and Methods

2.1 Collection of samples and DNA extraction

Sampling localities were chosen to represent the MUB prawn populations that support both artisanal and commercial trawl fisheries, and to include juvenile and adult cohorts that are presumably connected to each other through larval dispersal processes and migrations. A total of five sites were sampled; Ngomeni (NGO), Kipini (KIP), mid station (MDS), offshore of Sabaki (OFS) and offshore of Kipini (OFK) (Fig. 2). Ngomeni sampling station is situated far from the mouth of the Sabaki river whereas Kipini station is located within the river mouth (Fig. 2). Prawns sampled for this study included juveniles and adults of *P. monodon*, *F. indicus* and *M. monoceros*, and between seven to 28 specimens per species were collected from each of the five localities (Fig. 2 & Table 3). Juveniles were obtained during 2010 from local fishers in NGO and KIP (Fig. 2). Adults were obtained from MDS, OFS and OFK (Fig. 2) during 2011 using a commercial prawn trawler under survey SWIOFP2011C201a. Total genomic DNA was extracted from ethanol (96%) preserved muscle tissues using the Wizard® SV Genomic DNA Extraction Kit (Promega, Madison, WI, USA) following the manufacturers instructions and stored at –20°C prior to further analysis.

Table 2. Details of geographic localities within Malindi–Ungwana bay from where genetic prawn samples were obtained. The start and end positions of offshore trawl transects are included

			Geographic Coordinates (Latitudes and Longitudes)			
Locality	Abbreviation	Transects	Start_lat. (S)	End_lat. (S)	Start_Long. (E)	End_Long. (E)
Kipini	KIP	—	02° 31' 688"	—	040° 31' 388"	—
Ngomeni	NGO	—	02°59' 994"	—	040°10' 588"	—
Mid station	MDS	1—2	02°44' 708"	02°42' 862"	040°13'456"	40°14' 882"
"	"	1—3	02°39' 802"	02°41' 709"	040°16' 571"	40°14' 989"
Offshore of Kipini	OFK	1—5	02°34' 591"	02°35' 513"	040°25' 25"	40°22' 862"
"	"	1—6	02°33' 577"	02°34' 138"	040°29' 208"	40°26' 644"
Offshore of Sabaki	OFS	1—1	03°11' 078"	03°10' 914"	040°08' 502"	40°08' 601"
"	"	3—3	03°11" 488"	03°90' 747"	040°10' 943"	40°12' 154"

2.2 PCR amplification and sequencing of mtDNA control region (mtCR) fragment

PCR amplification of the mtDNA control region of *P. monodon* was performed using the species specific primer pair PmCON–2F and PmCON–2IR published by You *et al*. (2008). The thermal PCR profile for *P. monodon* in this study was adopted from You *et al*. (2008) without modifications. The PCR amplification of *F. indicus* and *M. monoceros* control region fragment used the penaeid prawns universal primers. The forward primer DLA was published by Chu *et*

al. (2003) and the reverse primer DLB by McMillen–Jackson and Bert (2003). The thermal profile for the latter two species was one cycle of 3 minutes at 95˚C; 35 cycles each 50 seconds at 95˚C; 60 seconds at 48˚C, 90 seconds at 72˚C, and one cycle of 5 minutes at 72˚C. PCR products were gel purified and the reverse strand was sequenced using the BigDye terminator chemistry (Applied Biosystems) and analysed on an ABI 3100 automated sequencer.

In addition, a subset of *P. monodon* DNA samples sequenced previously using the species specific primers, were re–amplified and sequenced using the universal primers (i.e. PCR amplification and sequencing of the control region for each of the selected DNA samples was conducted on separate reaction tubes for each of the two primer pairs. This was to ensure two control region sequences were generated from a single DNA sample). This was conducted specifically to confirm whether sequences generated using the You *et al.* (2008) primers would amplify the authentic control region, or instead the paralogous genes as was recently reported by Walther *et al.* (2011).

2.3 Mitochondrial DNA data analysis

SEQUENCHER v.4.8 (Gene Codes, Corp., Ann Arbor, Michigan) was used to edit all sequences, which were then aligned using Clustal W (Thompson *et al*. 1994) as implemented in MEGA v.5 (Tamura *et al*. 2011). The correctness of specimens sampled as belonging to each of the three species was confirmed by blasting each of the mtCR sequences using GenBank (http://blast.ncbi.nlm.nih.gov). When sequences blasted to nothing (i.e. sequence unavailable on database for that species), the sequences were aligned and a phylogenetic analysis (Neighbour Joining trees) using MEGA v.5 (Tamura *et al*. 2011) was used in order to see how such sequence(s) clustered with others. When more than one clade was observed, whole prawn

specimens were compared morphologically before proceeding with further analyses. Data for each species was treated separately throughout the analysis. DNASP v.5.0 (Librado and Rozas 2009) was used to prepare input files for subsequent analyses. Genetic diversity summary statistics were calculated for juveniles (NGO & KIP) and adult (OFK, MDS & OFS) sampling localities. Moreover, sampling localities (including both juveniles & adults) were also combined to obtain overall species specific diversity statistics. Sampling localities were also treated as natural groupings in order to conduct genetic differentiation analyses.

Genetic diversity estimates [i.e. number of polymorphic sites (*s*), number of haplotypes (*k*), haplotype diversity (*h*) and nucleotide diversity (π)] were obtained from ARLEQUIN v.3.11 (Excoffier *et al*. 2005). The predefined groupings (i.e. localities) were tested for genetic differentiation using ARLEQUIN v.3.11 (Excoffier *et al*. 2005) under the null assumption of no differentiation. First, pairwise Φ_{ST} statistics among sampling localities (which takes into account haplotype frequencies and genetic distances information) were calculated and significance was obtained using 10,000 random permutations. Second, analysis of Molecular Variance (AMOVA) was performed and the significance level of the population fixation index Φ_{ST} was obtained through a nonparametric permutation procedure (Excoffier *et al*. 1992) with 10,000 permutations. Within species evolutionary divergence between sequences (haplotypes) was estimated using the uncorrected *p*–distance model (uncorrected site changes between haplotypes) using MEGA v.5 (Tamura *et al*. 2011). We determined evolutionary relationships among juvenile and adult haplotypes in each of the three datasets (species wise) using a statistical parsimony network (Templeton *et al*. 1992) using TCS v.1.21 (Clement *et al*. 2000), and enforcing a 95% connection limit. The above networks (showing evolutionary relationships

among juvenile and adult haplotypes) were used to give indications of the recruitment pattern for each of the three species.

2.4 PCR amplification and genotyping of *Penaeus monodon*

Microsatellite analyses were performed on *P. monodon* and six out of 10 polymorphic di–nucleotide microsatellite loci developed for this species were successfully amplified (Brooker *et al*. 2000; Pan *et al*. 2004). Microsatellite loci were grouped into three panels (groups) for multiplex PCR amplifications. This grouping relied on fluorescent dyes of the forward primer (e.g. FAM, VIC, NED or PET), published allelic size ranges and annealing temperatures. Panel 1 included loci PM09 (accession number AF068826), PM25 (AF068827), PM27 (AF068828) and PM2345 (AY500860); Panel 2 consisted of loci PM138 (AY500853), PM3854 (AY500863) and PM1713 (AY500858) and Panel 3 consisted of PM580 (AY500856), PM3945 (AY500864) and PM4018 (AY500865).

Multiplex PCR amplification was carried out in a 10µl reaction final volume containing 1µl of (5–50ng) template DNA, 6µl of Qiagen multiplex PCR master mix, 2µl of ddPCR H_2O and 1µl of primer mix (0.2µM final concentration). The annealing temperature (Ta°C) was 57.3^{o}C for panel 1 and 3, and 58.0^{o}C for panel 2. The thermal profile followed that of Pan *et al*. (2004). The internal size standard Genescan™ 500Liz (Applied Biosystems) was added to the amplified PCR products and run in an ABI PRISM 3730 genetic analyzer (Applied Biosystems). Microsatellite alleles were obtained using GeneMapper™ software version 3.7 (Applied Biosystems) using the size standard GS500(–250)LIZ. Allele scoring was done automatically from established bins and edited manually. Individuals that had ambiguous peaks were re–

amplified using positive and negative controls (i.e. one sample for each case) and rescored. About 20% of individuals were randomly chosen for re–amplification and genotyping so as to determine consistency of results. The scored alleles were all verified by an independent researcher before proceeding to further analysis.

2.5 Microsatellite data analysis

Genetic diversity summary statistics were estimated for each sampling locality and also for the combined localities. The programme GENAIEX v.6.41 (Peakall and Smouse 2006) was used to perform quick exploratory analyses as well as to prepare input files for other software.

Genotypic linkage disequilibrium (LD) between pairs of loci was determined as implemented in FSTAT v.2.9.3 (Goudet 2002). We used sequential Bonferroni correction (Rice 1989) to adjust *p* values for multiple tests when a significant level was observed. Deviations from Hardy–Weinberg equilibrium (HWE) were determined using GENEPOP v.4.1 (Rousset 2008), where the Wright's (1951) inbreeding coefficient (F_{IS}) with heterozygosity deficit as the alternate hypothesis was used. Genotyping errors which normally take the form of null alleles (alleles that fail to be detected through PCR amplification), stuttering and large allele dropouts were investigated using MICROCHECKER v.2.2.3 (Van Oosterhout *et al.* 2004). When null alleles were suspected, their frequencies were estimated using the Oosterhout and sequential Bonferroni method (Rice 1989).

Genetic diversity summary statistics [i.e. number of alleles (NA), observed heterozygosity (*Ho*) and expected heterozygosity (*He*) were obtained using the MICROSATELLITE TOOLKIT (Park 2001). Allelic richness (AR) which is not affected by

sample sizes was obtained as implemented in FSTAT v.2.9.3 (Goudet 2002). The rarefaction method (Petit *et al*. 1998) was used to obtain AR.

Population differentiation was examined using ARLEQUIN v.3.1 (Excoffier *et al*. 2005) where pairwise R_{ST} values were used to test the null hypothesis of panmixia. Significance levels were obtained using the exact test of population differentiation which is robust even when sample sizes are small and also when alleles with low frequencies are included (Raymond and Rousset 1995). The same program was also used to perform an analysis of Molecular Variance (AMOVA; Excoffier *et al*. 1992). To determine the number of homogenous genetic clusters (*K*), the program STRUCTURE v.2.3 (Pritchard *et al*. 2000) was used. The Admixture model (Pritchard *et al*. 2000) in combination with the correlated allele frequencies model (Falush *et al*. 2003) was used. A burnin length of 1,000,000 and 10,000 Markov chain Monte Carlo (MCMC) samples and sequential independent runs were performed with values of *K* ranging from one to five. However, because the structure program does not automatically give the correct number of possible *K* present in the dataset (Kalinowski 2011), we identified the correct *K* through the ad hoc guidelines suggested by the STRUCTURE manual v. 2.3 and the statistic (delta *K*) as suggested by Evanno *et al*. (2005).

CHAPTER 3

Results

3.1 Mitochondrial DNA data

Penaeus monodon

Electropherograms that were obtained from the two pairs of primers [PmCON–2F/PmCON–2IR (You *et al*. 2008) and DLA/ DLB (Chu *et al*. 2003; McMillen–Jackson and Bert 2003)] did not show any signs of double reads (evidence for co–amplification of pseudo genes and/or paralogous genes; Walther *et al.* 2011) and sequences for the same individuals were identical. Alignment of sequences to the mtCR fragment of You *et al*. (2008) produced a 570 base pair region of perfect matching for 129 specimens. A total of 126 haplotypes (*k*) were obtained (including three shared and 123 unique haplotypes) which were defined by a total of 120 polymorphic nucleotide sites (*s*) (Table 3). All haplotypes were deposited in GenBank (accession numbers). There were 120 transitions and 32 transversions present in the aligned data set. Nucleotide frequencies estimated from the entire data set was A–T rich—i.e. A = 39.55%, T = 39.46%, C = 11.61% and G = 9.38%. Haplotype diversity was generally high coupled to lower nucleotide diversity in each of the five localities; this pattern was consistent in the overall dataset (Table 3). The within species uncorrected sequence divergences between haplotypes (±SE) ranged from 0.2% ± 0.2% to 3.3% ± 0.7% (mean: 1.49% ± 0.18%).

Pairwise Φ_{ST} values among sampling localities were not significant (Table 4; Φ_{ST} ~0, p > 0.05) and AMOVA supported the complete absence of genetic differentiation among localities

(i.e. Table 5; Φ_{ST} = 0, p > 0.05). The distribution and evolutionary relationships among juvenile and adult sequences (haplotypes) are illustrated in the TCS haplotype networks (Figs. 4 & 5). Haplotypes were not distributed according to a geographic pattern (Fig. 4), and in many occasions, haplotypes originating from a similar sampling locality were randomly connected among haplotypes originating from other sampling sites (Fig. 4). In this species also, the gene sequence of a few juvenile individuals were similar to those of adult specimens (shared haplotypes: Fig. 5).

Fenneropenaeus indicus

The DNA from a total of 96 specimens of *F. indicus* was PCR amplified and sequenced. Alignment of those sequences produced a region of 791 base pairs. Ninety–five haplotypes (including one shared and 94 unique haplotypes) were obtained and deposited in GenBank (accession numbers). Haplotype nucleotide frequencies estimated from the overall population was A = 37.96%, T = 42.68%, C = 9.84% and G = 9.52%. High haplotype and lower nucleotide diversity values were observed in each of the five localities and this pattern was concordant with the combined dataset (Table 3). The within species uncorrected sequence divergences between haplotypes (±SE) ranged from 0.1% ± 0.1% to 7.1% ± 0.9% (mean = 1.48% ± 0.2%). Pairwise Φ_{ST} values for *F. indicus* among localities were not significant (Table 4; Φ_{ST} ~0, p > 0.05) and AMOVA supported the complete absence of genetic differentiation (i.e. Table 5; Φ_{ST} = 0, p > 0.05). The distribution and pattern of evolutionary relationships among juvenile and adult haplotypes of *F. indicus* indicated by the TCS networks were similar to the observations made

for *P. monodon* above. However, three haplotypes of *F. indicus* were not connected to the main network (Figs. 4 & 5).

Metapenaeus monoceros

A total of 88 DNA samples from individuals prawns identified in the field as *M. monoceros* were PCR amplified and sequenced. However, only 71 sequences (Clade A; Fig. 6) were confirmed as belonging to *M. monoceros* and were thus available for analysis. The 71 sequences of authentic *M. monoceros* produced 774 base pairs for analysis, and a total of 61 haplotypes (including two shared and 59 unique haplotypes) were obtained and deposited in GenBank (accession numbers). The nucleotide frequencies estimated from the entire data set was A = 40.67%, T = 43.33%, C = 7.97% and G = 8.03%. High haplotype and lower nucleotide diversity values were observed in each locality and from the combined dataset (Table 3). The within species uncorrected sequence divergence (±SE) ranged from 0.1% ± 0.1% to 4.7% ± 0.7% (mean = 1.1% ± 0.18%). Significant pairwise Φ_{ST} value between NGO and OFK populations was observed (Table 4) (i.e. Φ_{ST} = 0.08809, $p < 0.002$), although the overall Φ_{ST} value for AMOVA did not support differentiation (i.e. Table 5; Φ_{ST} = 0.01638, $p > 0.05$). The TCS network for this species (Figs. 4 & 5) were comparable to those of *P. monodon* and *F. indicus* above, except that more juvenile and adult *M. monoceros* shared sequences than in the other two species (Fig. 5).

Table 3. Genetic diversity summary statistics of *P. monodon*, *F. indicus* and *M. monoceros* from five sampling localities in the MUB. Sample size (n), number of haplotypes (*k*), polymorphic sites (s), haplotype diversity (*h*) and nucleotide diversity (π) are shown. Abbreviations for sampling locations correspond to those in Table 2

Genetic diversity indices						
Spp.	Station	*n*	*s*	*k*	*h*	π
P. monodon	KIP	28	65	28	1.0000 ± 0.0095	0.0139 ± 0.0070
	NGO	24	55	24	1.0000 ± 0.0120	0.0134 ± 0.0070
	MDS	28	70	28	1.0000 ± 0.0095	0.0163 ± 0.0090
	OFK	27	64	26	0.9972 ± 0.0111	0.0150 ± 0.0080
	OFS	22	67	22	1.0000 ± 0.0137	0.0153 ± 0.0080
	Total	129	120	126	0.9996 ± 0.0010	0.0147 ± 0.0076
F. indicus	KIP	25	92	25	1.0000 ± 0.0113	0.0161 ± 0.0083
	NGO	24	69	24	1.0000 ± 0.0120	0.0153 ± 0.0080
	MDS	15	53	15	1.0000 ± 0.0243	0.0149 ± 0.0080
	OFK	17	42	17	1.0000 ± 0.0202	0.0123 ± 0.0066
	OFS	15	51	15	1.0000 ± 0.0243	0.0143 ± 0.0077

	Total	96	159	95	0.9998 ± 0.0015	0.0147 ± 0.0020
	KIP	22	30	20	0.9870 ± 0.0201	0.0094 ± 0.0051
	NGO	15	37	15	1.0000 ± 0.0243	0.0111 ± 0.0061
M. monoceros	MDS	7	16	6	0.9524 ± 0.0955	0.0090 ± 0.0055
	OFK	10	50	8	0.9333 ± 0.0773	0.0165 ± 0.0092
	OFS	17	32	16	0.9926 ± 0.0230	0.0099 ± 0.0055
	Total	71	91	61	0.9815 ± 0.0110	0.0109 ± 0.0057

Table 4. Pairwise Φ_{ST} values for *P. monodon*, *F. indicus* and *M. monoceros* obtained from the mtCR. Pairwise R_{ST} values for *P. monodon* from the microsatellite data is given at the end of this table. Abbreviations for sampling locations correspond to those in Table 2. Significant tests are indicated by bold values ($p < 0.05$)

Species	Locality	KIP	NGO	MDS	OFK	OFS
P. monodon	NGO	0.00000	—			
	MDS	0.00000	0.00000	—		
	OFK	0.00000	0.00000	0.00000	—	
	OFS	0.00000	0.00000	0.00000	0.00367	—
F. indicus	NGO	0.00313	—			
	MDS	0.00000	0.00000	—		
	OFK	0.00000	0.00000	0.00000	—	
	OFS	0.00000	0.00000	0.00000	0.00000	—

M. monoceros	NGO	0.02396	—			
	MDS	0.00000	0.02548	—		
	OFK	0.04597	**0.08809**	0.00000	—	
	OFS	0.00000	0.00000	0.0000	0.0362	—
P.monodon(microsat)	NGO	0.00292	—			
	MDS	0.00000	0.00441	—		
	OFK	0.00047	0.02225	0.00807	—	
	OFS	0.00719	0.01094	0.00743	0.01487	—

Table 5. Results of the Analysis of Molecular Variance (AMOVA) of *P. monodon*, *F. indicus* and *M. monoceros* obtained from the comparisons among the five sampling localities using mtCR sequences. AMOVA obtained from the microsatellite data for *P. monodon* is given at the bottom of this table

Species	Source of variation	Degree of freedom	Sum of squares	Variance contribution	Percentage of variation	Φ_{ST}	*p*
P. monodon	Among localities	4	13.6030	0.0000	0.0000	0.0000	(p = 0.9960)
	Within localities	124	523.8780	4.1928	100.0000		
	Total	128	537.4810	4.1928			
F. indicus	Among localities	4	20.5260	0.0000	0.0000	0.0000	(p = 0.8804)
	Within localities	91	531.4740	5.8030	100.0000		

	Total	95	552.000	5.8030			
M. monoceros	Among localities	4	20.584	0.06978	1.6384	0.01638	(p = 0.10861)
	Within localities	66	276.487	4.18919	98.3616		
	Total	70	297.070	4.25897			
Species	Source of variation	Degree of freedom	Sum of squares	Variance contribution	Percentage of variation	R_{ST}	p
P.monodon(microsat)	Among localities	4	13.926	0.00305	0.1131	0.00113	(p = 1.00000)
	Within localities	98	328.875	0.66337	2.4610		
	Within individuals	103	209	2.02913	75.2770		
	Total	205	551.801	2.69555			

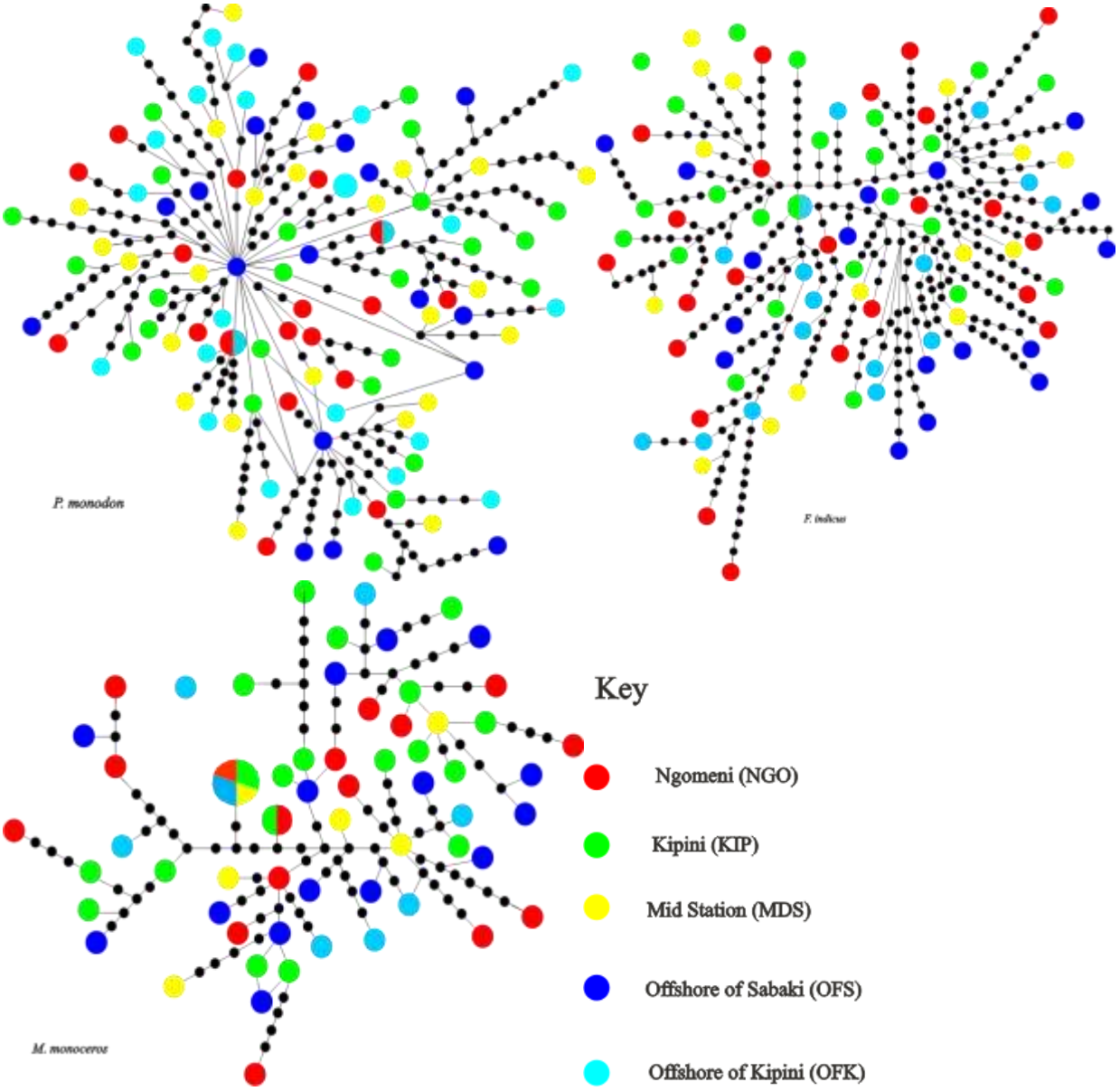

Fig. 3. Statistical parsimony network for *P. monodon*, *F. indicus* and *M. monoceros*. Haplotypes are proportionally represented by coloured circles. Colour represents geographical localities from where haplotypes were sampled. Intermediate haplotypes (i.e. black circles) represent unsampled or extinct haplotypes. A black line connecting haplotypes represents one mutational step.

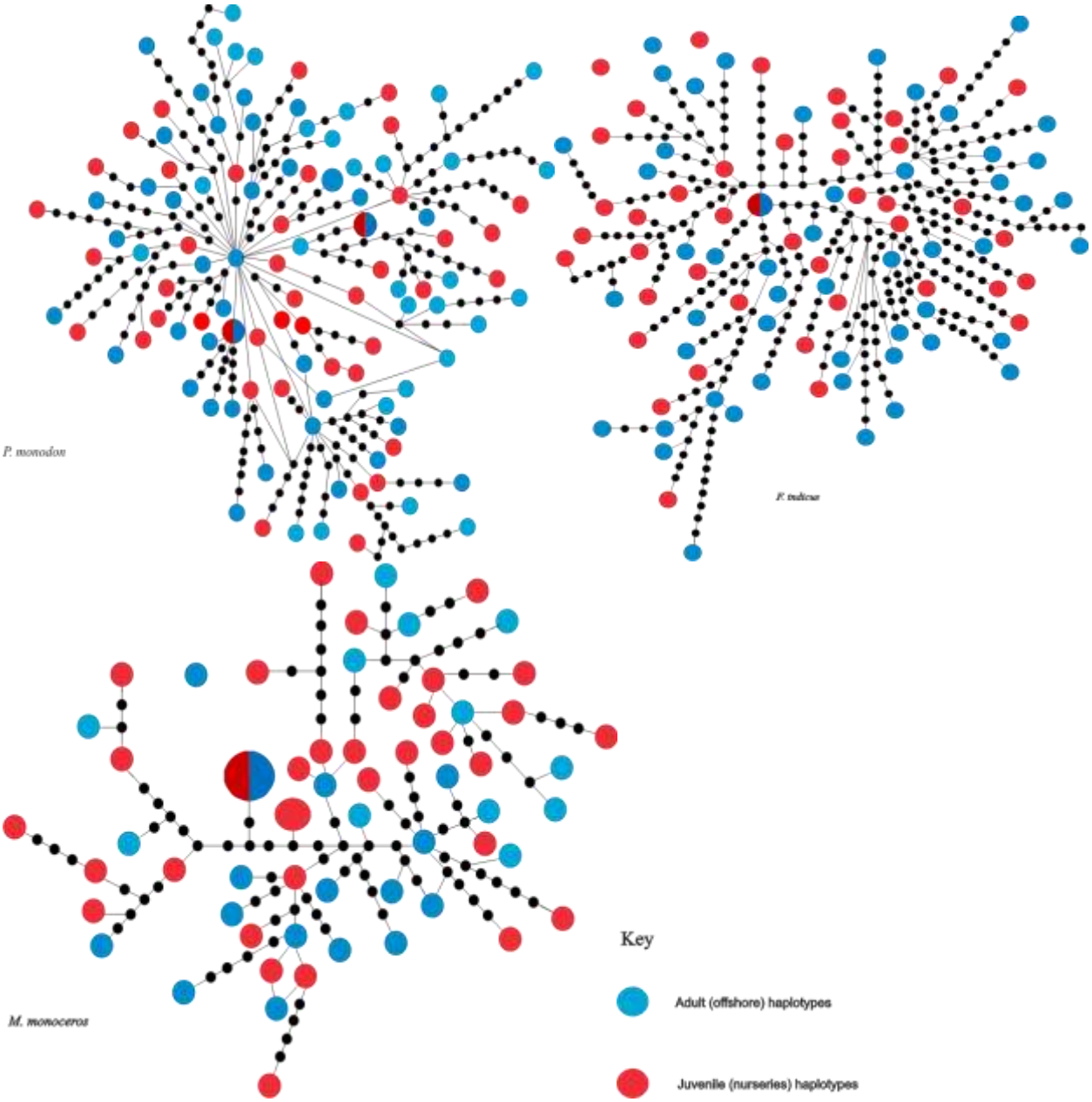

Fig. 4. Statistical parsimony network for *P. monodon*, *F. indicus* and *M. monoceros* showing evolutionary relationships of juvenile and adult haplotypes. Haplotypes are proportionally represented by coloured circles. Colour represents maturity stages (juvenile/adults). Intermediate

haplotypes (i.e. black circles) represent unsampled or extinct haplotypes. A black line connecting haplotypes represents one mutational step.

3.2 Microsatellite data

Penaeus monodon

Optimization of the three multiplex PCR reactions allowed for successful amplification of six polymorphic loci. All six microsatellite loci in our study indicated significant deviations from Hardy–Weinberg Equilibrium (HWE) when samples were combined (Table 6). Nonetheless, there were 24 out of 30 cases where locality–locus significant HWE deviations were observed (Table 6). The significant deviations from HWE were all indicated by positive and significant inbreeding coefficient F_{IS} relative to heterozygote deficiency. MICROCHECKER analysis suggested the presence of null alleles in each of the six loci and their estimated frequencies were 0.1119 for PM25, 0.0489 for PM27, 0.1438 for PM580, 0.103 for PM3854, 0.1652 for PM3945 and 0.1761 for PM4018. We did not detect any two loci that had significant genotypic linkage disequilibrium, thus each locus represents a unique evolutionary pathway. All loci except PM4018 were highly polymorphic as indicated by high values of allelic richness (AR) and expected heterozygosity (summarised in Table 6).

Pairwise R_{ST} values among localities were not statistically significant [Table 4; (R_{ST} range = 0.000–0.0222, $p > 0.05$)]. AMOVA results indicated an absence of genetic differentiation

(Table 5). Structure analysis in combination with the more formal algorithms (Evanno *et al*. 2005), suggested the presence of a single genetic population (*K* = 1; Fig. 5).

Table 6. Genetic characteristics of six nuclear microsatellite loci for *P. monodon* samples obtained from five sampling localities. Abbreviations for sampling locations correspond to those in Table 2. *NA* = number of alleles, *AR* = allelic richness, *Ho* = observed heterozygosity, *He* = unbiased expected heterozygosity, *F*IS inbreeding coefficient (Bold *F*IS indicate significant departure from HWE)

Locus		Sampling localities					
		KIP (n=21)	NGO (n=20)	MDS (n=22)	OFK (n=20)	OFS (n=20)	Total (N=103)
PM25	*NA*	17	14	14	16	17	20
	AR	16.710	14.000	13.622	16.000	17.000	15.119
	Ho	0.857	0.650	0.636	0.700	0.750	0.718
	He	0.942	0.894	0.919	0.923	0.932	0.929
	*F*IS	**0.092**	**0.278**	**0.312**	**0.246**	**0.199**	**0.225**
PM27	*NA*	18	19	15	20	19	24
	AR	17.660	19.000	14.786	20.000	19.000	17.245
	Ho	0.714	0.850	0.864	0.850	0.950	0.845
	He	0.942	0.937	0.938	0.954	0.94	0.938
	*F*IS	**0.246**	0.095	0.081	0.111	-0.011	**0.106**
PM580	*NA*	16	17	15	15	15	29
	AR	15.617	17.000	14.617	15.000	15.000	16.222
	Ho	0.667	0.700	0.636	0.700	0.600	0.660
	He	0.916	0.933	0.932	0.922	0.906	0.926
	*F*IS	**0.277**	**0.255**	**0.323**	**0.245**	**0.344**	**0.289**
PM3854	*NA*	24	18	24	22	12	34
	AR	23.373	18.000	22.617	22.000	12.000	20.262
	Ho	0.762	0.800	0.727	0.750	0.750	0.757

	He	0.966	0.942	0.961	0.958	0.910	0.957
	FIS	**0.216**	**0.154**	**0.247**	**0.221**	**0.180**	**0.206**
PM3945	*NA*	18	16	14	19	17	32
	AR	17.613	16.000	13.797	19.000	17.000	18.645
	HO	0.476	0.650	0.546	0.700	0.800	0.631
	He	0.934	0.946	0.923	0.953	0.949	0.946
	FIS	**0.496**	**0.319**	**0.415**	**0.270**	**0.160**	**0.335**
PM4018	*NA*	7	4	6	6	6	10
	AR	6.95	4.000	5.727	6.000	6.000	5.983
	HO	0.381	0.650	0.318	0.350	0.550	0.447
	He	0.743	0.676	0.651	0.641	0.676	0.687
	FIS	**0.494**	0.039	**0.517**	**0.460**	0.190	**0.348**
AR		24.833	14.667	14.667	16.333	14.333	24.833
HO /locality		0.643	0.717	0.621	0.675	0.733	0.676
He /locality		0.907	0.888	0.887	0.892	0.886	0.897
FIS		**0.297**	**0.197**	**0.305**	**0.248**	**0.176**	**0.246**

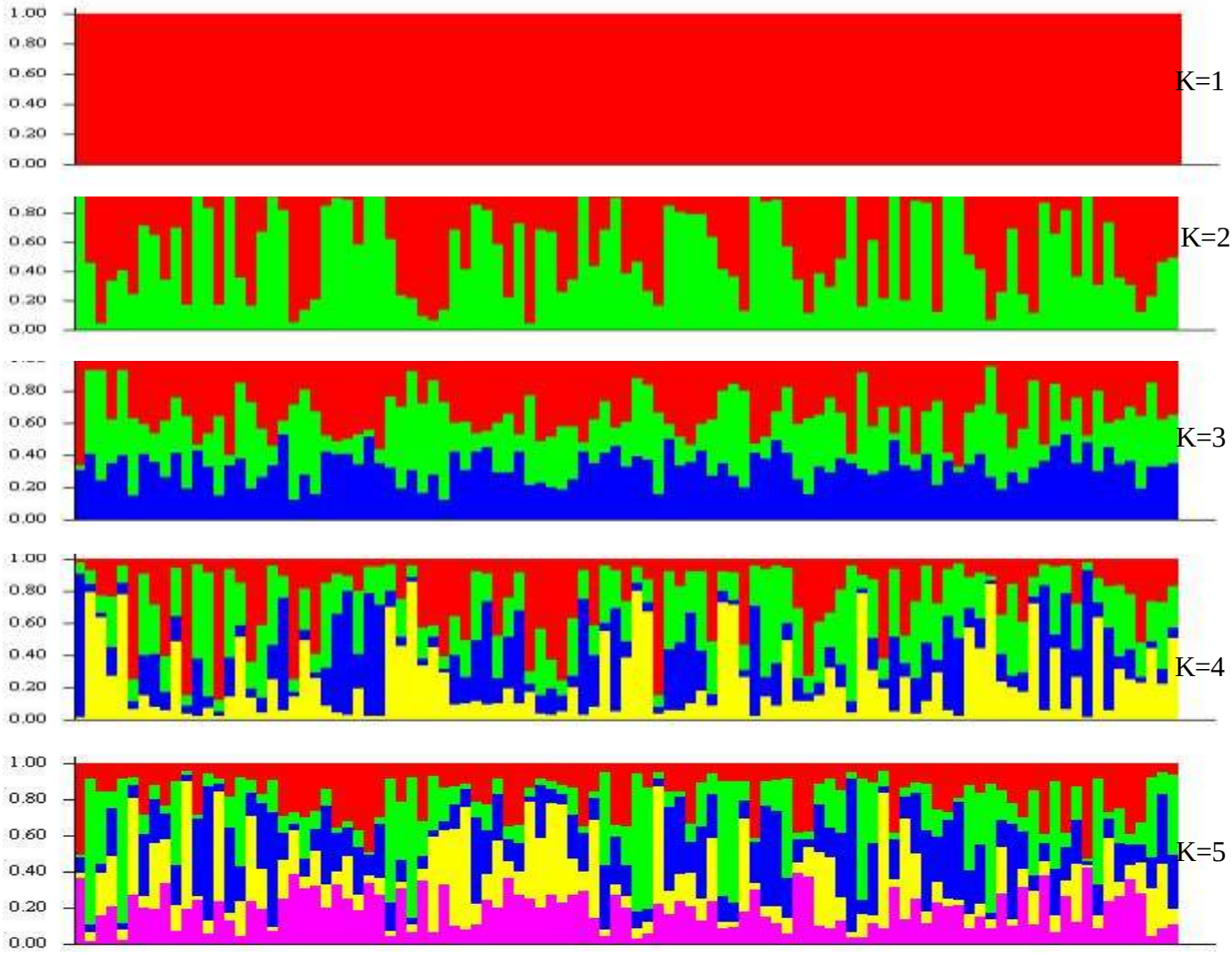

Fig. 5. Results from the structure analysis (performed using six microsatellite loci) showing genetic population clusters ranging from *K* = 1 to *K* = 5. Each colour represents a single inferred genetic cluster irrespective of the geographic origin of samples. Each individual is represented by a vertical bar. The numbers and proportions of colours (ranging from 0 to 1) contained in each individual indicates the extent of genetic admixture of that individual.

CHAPTER 4

Discussion

4.1 Genetic diversity of *P. monodon*, *F. indicus* and *M. monoceros* in the Malindi–Ungwana Bay, Kenya

Genetic diversity at the mtCR at each of the five localities was generally high for all three species analysed (i.e. *P. monodon*: *h* = 0.9972–1.0000; *F. indicus*: *h* = 1.0000; *M. monoceros*: *h* = 0.9333–1.0000). A high genetic diversity estimate per species was also observed when haplotypes from the five sampling localities were combined. High genetic diversity of penaeids in the MUB is congruent with previous results for *P. monodon* from IWP (*h* = 0.969–1.000; You *et al*. 2008), *Farfantepenaeus duorarum* from the southeastern United States (*h* = 1.000; McMillen–Jackson and Bert 2004) and *Fenneropenaeus chinensis* from northern China seas (*h* = 0.9500–0.9900; Kong *et al*. 2010). The microsatellite analysis confirmed the high genetic diversity of *P. monodon*, based on heterozygosity levels (*He* = 0.886–0.907) and allelic richness (*AR* = 14.333–24.833).

The high heterozygosity is comparable to *P. monodon* from IWP (*He* = 0.82–0.91; Waqairatu *et al*. 2012), but these values are much higher than those detected in the open thelycum *Litopenaeus vannamei* from the eastern Pacific (*He* = 0.241–0.388; Valles–Jimenez *et al.* 2005). The discrepancy might be due to a homozygote excess in the present data set, compared to a homozygote deficit in the data set used for *L. vannamei* by Valles–Jimenez *et al.* (2005). Also, the life histories of the two species differ in that the *P. monodon* life cycle involves

both marine and estuarine waters, making them highly migratory species. To the contrary, *L. vannamei* inhabits predominantly estuarine waters, thus limiting its dispersal range.

The high genetic diversity of the three species reflects the large effective population sizes of these taxa (Ovenden *et al.* 2007; Leffler *et al.* 2012), high rates of mitochondrial evolution (Palumbi and Benzie 1991; Baldwin *et al.* 1998; McMillen–Jackson and Bert 2003) and at the nuclear microsatellite DNA (Chakraborty *et al.* 1997). Moreover, the neutral theory of molecular evolution (Kimura and Crow 1964; Kimura 1983) suggests that species with large effective population sizes tend to reach mutation–random drift equilibrium, thus such species retain high levels of genetic diversity. This is consistent with present observations. There was no genetic evidence of inbreeding or overfishing, both of which may be associated with a smaller effective population size (reviewed in Charlesworth and Wright 2001; Allendorf *et al.* 2008; Leffler *et al.* 2012). Lower effective population sizes may lead to a faster rate of loss of variation (alleles) due to genetic random drift (Charlesworth 2009), which was not evident in this study.

Penaeus monodon, *F. indicus* and *M. monoceros* in the MUB exhibit some differences in habitat preference, and abundance and distribution patterns (see sections 1.2 and 1.4), but likely share similar mechanisms of dispersal and recruitment between the local estuaries and offshore habitats. Presumably, therefore, comparable levels of genetic diversities can be explained by a combination of similar life–history patterns, rates of molecular evolution and effective population sizes. Nevertheless, *M. monoceros* indicated the lowest haplotype diversity when compared to the other two species, despite its more generalist habitat preferences and higher abundance than *P. monodon.* A possible explanation to the above genetic diversity patterns could be an imprint due to a difference in the timing of historical demographic events. For

instance, *M. monoceros* might have experienced a recent population bottleneck, or alternatively, the effective population size of *M. monoceros* might be lower than the other two species. Ramos-Onsins *et al.* (2004) and Piganeau and Eyre–Walker (2009) showed that effective population sizes could vary even among closely related species. Another cause might be due to differences in mutation rates among the three taxa. Kumar *et al*. (2012) found that the rate of mitochondrial evolution is not uniform among penaeid species.

Exceptionally high numbers of unique haplotypes (as shown in the present study, especially for *P. monodon* and *F. indicus*) appear to be relatively common among penaeid prawns, and were previously found at the mtCR fragment of *P. monodon* (You *et al.* 2008; Waqairatu *et al*. 2012), *F. duorarum* (McMillen–Jackson and Bert 2004) and *F. chinensis* (Kong *et al*. 2010). This characteristic of penaeid prawns suggest that the rate at which neutral mutations are being incorporated into the mtCR fragment is very rapid, and given the possession of large effective population sizes usually associated with these taxa, a large number of haplotypes can thus be maintained. It is noteworthy that a high number of haplotypes can generally be expected in the highly evolving markers, but not from every gene in those species. This is because the intra–species evolutionary rate among mitochondrial genes is not similar. For instance, the mitochondrial Cytochrome c Oxidase Subunit 1 (CO1) gene of *F. indicus* did not show as many unique haplotypes (Querci 2003; De Croos and Palsson 2010).

The significant deviation from Hardy–Weinberg Equilibrium (HWE) indicated by heterozygote deficiency in this study is also not unique, and has been shown elsewhere for *P. monodon* (Brooker *et al.* 2000; Pan *et al*. 2004; You *et al*. 2008; Waqaitaru *et al.* 2012), and in other marine invertebrates (Raymond *et al*. 1997; Huang *et al*. 2000; Addison and Hart 2005)

and fish (Hoarau *et al*. 2002; Morin *et al*. 2009). As mentioned earlier, factors that can cause a microsatellite locus to deviate from HWE due to heterozygote deficits include stuttering, large allele dropouts, null alleles, selection, inbreeding and Wahlund effect (reviewed in Selkoe and Toonen 2006). In our study, however, heterozygote deficit (i.e. positive significant F_{IS} or excess in homozygotes) could be caused either by null alleles or Wahlund effect. This is because stuttering, which is most often detected in microsatellite loci that contain di–nucleotide repeat motifs, was rare or completely eliminated from our study because individuals that produced ambiguous peaks were re–amplified and scored more than once, using controls. In addition, alleles were consistently scored in established bins and edited manually prior to verification by an independent researcher, thus eliminating the inclusion of wrongly scored alleles in our analysis.

Inbreeding, which is expected to affect all neutral loci, is also unlikely to explain our data since all samples originated from the wild, and the mtDNA results indicate an extreme high level of haplotypic diversity (i.e. indirectly suggesting the absence of inbreeding). Selection is also unlikely to explain the present findings, because the microsatellite loci analysed (with di–nucleotide repeat units) occurred in the non–coding region of the nuclear genome (Selkoe and Toonen 2006). Heterozygosity at neutral loci can be extensively reduced through genetic hitch hiking when loci are linked to selected regions of the genome (Maynard–Smith and Haigh 1974; Kim and Stephan 2000) – no signature for this was detected because no two loci were linked.

The presence of null alleles cannot be completely excluded from our study because they have previously been reported in many invertebrates species (Pan *et al.* 2004; Gruenthal and

Burton 2008; You *et al*. 2008), and were also suggested by MICROCHECKER in the present analysis. However, if the presence of null alleles was indeed driving "non equilibrium", it would be unique to this study. For instance, null alleles arise if the conserved microsatellite flanking region from where priming was expected to occur inadvertently mutates, the PCR condition is not fully optimised to allow amplification of all alleles present in a locus. In our case it is unlikely that null alleles caused the heterozygote deficit, since the deficit occurred across all six loci and at 24 out of 30 locus/locality cases.

The Wahlund effect (the occurrence of individuals in a single sampling unit that originated from genetically distinct populations, but were brought together erroneously during sampling) may be a plausible reason for present deviations from HWE expectation, however there was no genetic differentiation among the five geographical localities from where samples were obtained (see Ni *et al.* 2011; You *et al.* 2008). Alternatively, the Wahlund effect may have been caused by inadvertent sampling of individuals occurring in similar localities, but belong to different age groups (cohorts) thus forming a temporal Wahlund effect (Selkoe and Toonen 2006). This phenomenon can occur in the complete absence of genetic differentiation and has been observed in larval cod *Gadus morhua* (Ruzzante *et al*. 1996) and flat fish *Pleuronectes platessa* (Hoarau *et al.* 2002). Penaeid prawns are highly fecund, iteroparous and characterised by several broods per year (Rao 1968; Jayawardane *et al*. 2002). These life history characteristics coupled with ocean currents might facilitate dispersal among localities and the occurrence of different cohorts in the same localities, thus leading to temporal Wahlund effect. It is noteworthy that the Wahlund effect is expected to affect all microsatellite loci (reviewed in Selkoe and Toonen 2006) which is the case we observed in our study.

4.2 Dispersal and recruitment patterns in the Malindi–Ungwana Bay

A complete lack of genetic differentiation in the MUB, as shown for the three penaeids in the present study, was also observed in parrot fish *Scarus ghobban* (Visram *et al*. 2010), and two mangrove crab species; *Neosarmatium meinerti* (Ragionieri *et al*. 2010) and *Perisesarma guttatum* (Silva *et al*. 2010). These six species all rely on larval dispersal processes driven by water movements, and the lack of genetic structure therefore suggests the absence of clear barriers to gene flow in the MUB region of Kenyan marine waters. It therefore appears that pelagic larvae of several taxa, including fish and crustaceans, are mixed throughout the MUB region, where water movements are facilitated by river outflow, tidal exchange, and monsoonal winds coupled with ocean currents (see Chapter 1). Furthermore, it appears that differences in distribution ranges and habitat preferences in the MUB among the three penaeids (Chapter 1), and among several other taxa, do not influence the genetic differentiation patterns at the local scale that was studied here.

The absence of genetic differentiation observed for the three prawn species in MUB, coupled to the distribution pattern of juveniles and adult haplotypes (see Fig. 3 & 4) strongly suggest local recruitment patterns. Nevertheless, the MUB populations may also be receiving larvae from more distant sources along the East African coast, such as Tanzania, Mozambique or South Africa.

The alongshore currents and absence of clear physical barriers to geneflow along the East African coast may facilitate long distance dispersal of pelagic larvae, and therefore some prawns in the MUB might have originated from distant sources. This is supported by the genetic panmixia of *F. indicus* along the East African coast (Querci 2003), as well as the genetically

diverged haplotypes (i.e. unconnected haplotypes; Fig 3 & 4) in the present study. Even though the occurrence of unconnected haplotypes to the main network may signal under–sampling (inadvertent failure to sample intermediate haplotypes; Chen *et al.* 2010) or sympatric speciation (Barluenga *et al.* 2006), it is more likely an indication of some recruitment from distant sources. This hypothesis is supported by previous studies on marine crustaceans that have shown extensive regional geneflow along the East African coast, but disjunct from populations surrounding island states such as Madagascar, Seychelles and Mauritius (Duda and Palumbi 1999; You *et al.* 2008; Ragionieri *et al.* 2010).

4.3 Management of prawn fisheries in the MUB region

4.3.1 Local management recommendations

Each of the three prawn species studied exhibited single genetically panmictic populations in the MUB region. Consequently, the traditional and recent demarcations of the MUB to form artisanal (< 3nm) and commercial (3< nm) fisheries is not supported by molecular data, and both fisheries target the same stocks with a single gene pool per species. The present study therefore shows that indicators other than genetically separate stocks need to be developed to support fisheries management strategies, and particularly the division of fishing grounds among artisanal and commercial fishing sectors. These may more appropriately be based on factors such as prawn distribution and abundance patterns, recruitment trends, and socio–economic criteria.

The present study showed that nearly all prawn recruits into the MUB originate from the nearby estuaries. It is therefore important that estuaries be recognized as nurseries that support artisanal and commercial prawn fisheries, and that they are conserved and managed to maintain prawn nursery habitats. The destruction of nursery habitats through discharge of untreated wastes or chemicals, clearance of mangrove habitats for human settlements or commerce (e.g. ports and harbour constructions), and upstream building activities such as dams, that may reduce river discharge and therefore alter juvenile recruitment to offshore habitats, need to be identified and mitigated. Failure to do so may eventually collapse prawn fisheries in MUB, as was illustrated for the shallow-water prawn fishery on the Tugela Banks off eastern South Africa (Turpie and Lamberth 2010). The latter fishery also depended on *P. monodon, F. indicus* and *M. monoceros*.

A high genetic diversity of prawns in the MUB implies that they are more likely to be able to adapt to human and/or climatic induced pressures. However, management of these fisheries need to conserve and protect this diversity from overfishing exploitation, because these prawn stocks support numerous anthropogenic needs in the region.

4.3.2 Regional management issues

The three penaeid species addressed in the present study are the mainstay of the artisanal and commercial trawl fisheries in the MUB region of Kenya, and are also caught as primary target species in similar fisheries in several other countries of the SWIO (Tanzania, Mozambique, South Africa, Madagascar) (van der Elst *et al.* 2009). To date, prawn fisheries in the SWIO are managed individually by countries in which they occur, and consequently a variety of management policies and methods has developed in the region (see FAO 2006).

It is likely that the genetic panmixia observed in prawn populations in the MUB extends further along the East African coast, because of the effects of alongshore current regimes on drifting larvae. Indeed, previous studies on *P. monodon* have indicated that East African coastal populations differ genetically from those occurring along the west coast of Madagascar (Duda and Palumbi 1999; You *et al.* 2008). Furthermore, studies on mangrove crabs *Neosarmatium meinerti* and *Perisesarma guttatum* have also indicated a lack of genetic differentiation along the East African coast, but differentiated crabs from these areas from those at SWIO island states (Ragionieri *et al.* 2010; Silver *et al.* 2010). The present study can therefore be extended to include populations from Tanzania, Mozambique, South Africa and Madagascar, to obtain genetic population structure of the dominant shallow-water penaeid prawns of the SWIO region. Such a study would contribute to the development of regional management strategies for shallow-water prawn fisheries in the SWIO region.

CHAPTER 5

General conclusions

This study presents the first finer scale comparative population genetic analyses of closed thelycum species *P. monodon*, *F. indicus* and *M. monoceros* within the range of their natural distribution. Further, the study increases the number of genetic population examinations in the Kenyan coastal waters conducted for prawn species (i.e. *P. monodon* from one [You *et al*. 2008] to two), but presents the first genetic analyses of *F. indicus* and *M. monoceros,* not only in the Kenyan waters but also throughout their range of distributions, when using mtDNA control region sequences data.

The use of two DNA markers with different evolutionary histories (i.e. mitochondrial and nuclear microsatellite DNA) is usually a more robust approach in genetic studies (see section 1.6 and Karl *et al*. 2012). The lack of spatial genetic differentiation among the three species of prawns in the MUB compares well with results from studies on other taxa (fish, crabs), all of which found single genetic populations. Contributing factors are likely to include dispersive planktonic larvae, monsoonal winds, ocean tides and currents, and the complete absence of physical or environmental barriers to dispersal in the MUB.

Recruitment into the bay is dominated by larvae/juveniles that come from local/nearby estuarine nursery areas. However, recruitment from distant origins (especially along the mainland of the east African coast) cannot completed be precluded from this study (discussed in section 4.2).

The present study needs to be expanded regionally, to include samples from other SWIO countries (i.e. Tanzania, Mozambique, South Africa and Madagascar), to investigate the extent to which ocean current systems in the SWIO contribute to larval dispersal patterns, and whether isolation by distance theory can be applied to prawn populations along the East African coastline. From a fisheries management perspective, a regional extension of this study will likely inform on whether prawn stocks are shared among neighbouring countries, or regionally, thus opening the door for regional fisheries management strategies.

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Appendix 1

Table 7. Raw genotypic data obtained from six polymorphic nuclear microsatellite loci for *P. monodon*

Loci	**PM25**		**PM27**		**PM580**		**PM3854**		**PM3945**		**PM4018**	
Sample	A	B	A	B	A	B	A	B	A	B	A	B
KIP02	209	221	145	145	318	326	210	220	312	312	254	254
KIP03	223	231	131	157	328	328	236	236	310	310	258	258
KIP04	219	227	147	163	316	324	232	250	298	298	248	260
KIP05	227	227	135	135	324	330	228	256	288	332	258	258
KIP06	203	223	131	149	328	328	222	236	298	298	258	258
KIP07	223	231	149	171	314	330	234	234	256	308	248	258
KIP09	205	233	133	145	284	326	236	250	306	306	248	260
KIP10	207	217	149	159	314	330	218	234	302	302	260	260
KIP12	219	231	161	161	330	330	224	254	270	302	258	258
KIP13	211	217	151	151	288	320	226	246	304	304	260	260
KIP14	205	213	147	147	320	330	214	234	260	302	248	260
KIP15	215	215	133	165	298	322	208	248	260	316	258	258
KIP17	219	231	137	151	300	318	222	244	264	318	260	260
KIP18	205	213	137	147	320	320	224	224	308	308	258	262
KIP19	209	229	145	165	296	332	236	268	254	310	258	258
KIP20	219	227	131	169	320	328	232	244	306	318	260	260
KIP21	219	219	141	149	324	324	240	248	298	308	250	258
KIP22	209	223	143	151	308	308	216	272	294	308	260	260
KIP25	201	221	149	149	318	328	220	230	280	280	262	264
KIP27	217	233	139	151	320	326	228	228	302	302	260	260
KIP28	225	231	147	157	330	330	242	242	308	308	250	262
NGO01	223	233	135	143	318	324	226	242	306	306	250	260

NGO02	223	223	153	161	332	332	216	224	286	312	248	258
NGO03	213	223	139	151	326	336	230	236	270	270	250	258
NGO04	221	221	149	161	292	326	234	240	310	310	258	260
NGO05	203	203	145	149	304	332	256	256	302	302	258	258
NGO08	209	209	133	147	284	312	224	232	260	312	248	258
NGO09	215	221	133	157	320	326	218	256	316	316	258	260
NGO10	225	225	137	159	334	334	220	238	294	316	258	260
NGO12	201	227	149	165	316	334	236	244	304	310	258	258
NGO13	201	223	147	155	324	324	242	242	272	290	258	260
NGO15	217	227	149	149	318	324	222	236	298	304	248	248
NGO16	223	223	147	147	330	330	210	244	258	272	248	248
NGO19	219	227	147	147	328	328	234	234	298	316	248	260
NGO20	223	223	153	157	314	324	226	234	286	308	248	258
NGO21	209	223	127	151	320	320	234	246	260	302	260	260
NGO24	199	219	147	157	304	326	216	234	260	288	258	260
NGO25	207	221	131	159	296	320	256	276	298	298	248	258
NGO26	209	225	149	159	294	326	224	236	302	302	258	258
NGO27	203	223	141	169	320	326	230	240	290	308	248	258
NGO29	219	225	139	161	316	326	242	242	298	312	258	258
MDS01	205	217	135	165	326	336	210	210	298	312	260	260
MDS02	213	219	147	157	330	330	216	228	298	298	258	258
MDS03	213	229	141	171	314	322	212	238	308	308	260	260
MDS04	205	219	145	145	308	332	240	240	256	302	258	258
MDS05	219	219	145	153	326	332	224	230	260	308	258	258
MDS06	209	219	141	149	314	330	226	250	266	318	258	258
MDS07	221	221	141	149	320	320	206	228	312	312	260	260
MDS08	207	213	137	147	326	326	234	256	298	298	260	260
MDS09	207	223	131	175	312	328	218	236	290	290	248	260
MDS11	203	227	151	151	312	330	220	238	260	306	258	258
MDS12	209	209	147	165	332	332	232	242	322	322	258	260
MDS13	223	223	149	149	328	328	246	262	306	306	256	258

MDS14	209	223	131	157	332	332	226	244	298	310	260	260
MDS15	217	225	137	147	320	332	234	240	308	308	248	258
MDS16	211	223	139	151	294	336	228	240	260	318	248	258
MDS17	225	225	153	171	312	318	208	240	302	302	258	258
MDS18	229	229	149	171	296	326	210	210	298	298	260	260
MDS19	209	217	139	145	320	328	224	252	310	318	260	260
MDS20	205	225	137	141	308	308	214	236	262	290	258	258
MDS21	209	209	135	149	312	322	226	226	262	304	250	258
MDS25	201	223	157	161	324	324	236	236	262	304	248	266
MDS27	219	219	149	171	316	324	216	216	262	298	260	260
OFK01	203	221	151	151	318	330	212	258	294	316	258	258
OFK02	213	223	151	151	306	324	222	236	274	322	260	260
OFK03	203	225	151	157	302	328	204	250	302	318	260	260
OFK04	217	217	131	157	316	322	236	250	302	318	248	258
OFK05	209	233	143	161	330	330	240	256	258	258	248	260
OFK06	205	235	141	147	330	330	224	224	294	306	258	258
OFK07	217	231	127	157	324	330	222	254	260	260	260	260
OFK09	219	219	135	169	318	334	214	236	298	298	258	258
OFK10	217	231	139	159	320	330	224	238	298	314	250	256
OFK11	217	227	143	171	284	310	222	282	278	312	258	260
OFK12	217	225	135	147	328	328	224	224	304	304	246	260
OFK13	217	225	149	159	324	332	244	270	262	312	258	258
OFK14	219	219	135	157	332	332	234	246	260	260	260	260
OFK16	215	215	133	157	326	326	222	240	310	322	250	260
OFK19	211	219	135	141	320	330	232	232	288	288	260	260
OFK23	209	217	145	145	286	320	214	214	270	294	260	260
OFK25	203	203	127	169	334	334	238	262	262	306	250	260
OFK26	225	231	153	163	316	330	222	242	294	298	260	260
OFK27	227	227	149	173	310	326	220	220	260	294	258	258
OFK28	209	229	145	155	306	334	220	248	312	324	260	260

OFS01	217	229	147	165	332	332	222	230	304	316	256	258
OFS02	225	225	139	149	312	324	218	232	306	314	258	260
OFS03	205	225	147	163	318	328	226	226	304	304	248	248
OFS05	205	225	145	153	324	324	226	250	308	316	260	260
OFS06	209	223	147	153	290	326	218	232	254	232	258	258
OFS07	227	235	147	159	330	330	226	230	266	312	258	258
OFS08	217	217	147	163	282	328	226	232	288	306	258	260
OFS09	225	225	145	155	326	326	210	250	262	310	258	258
OFS10	217	239	151	157	328	328	228	250	268	298	248	258
OFS12	203	211	131	151	326	334	228	242	260	312	258	260
OFS13	217	229	141	147	328	334	216	228	260	306	250	260
OFS15	203	221	151	151	326	326	210	224	316	316	258	258
OFS16	213	223	149	153	328	328	210	230	304	314	258	260
OFS17	203	225	151	157	304	336	234	234	288	310	258	258
OFS18	209	209	129	157	282	330	232	242	314	314	258	260
OFS19	209	233	143	161	316	330	228	228	306	306	258	258
OFS20	205	235	143	147	318	332	232	232	254	308	248	260
OFS21	217	231	127	157	328	328	224	232	282	298	250	262
OFS22	219	219	135	169	330	344	218	230	282	310	260	260
OFS23	207	227	139	149	310	332	226	226	290	308	256	258

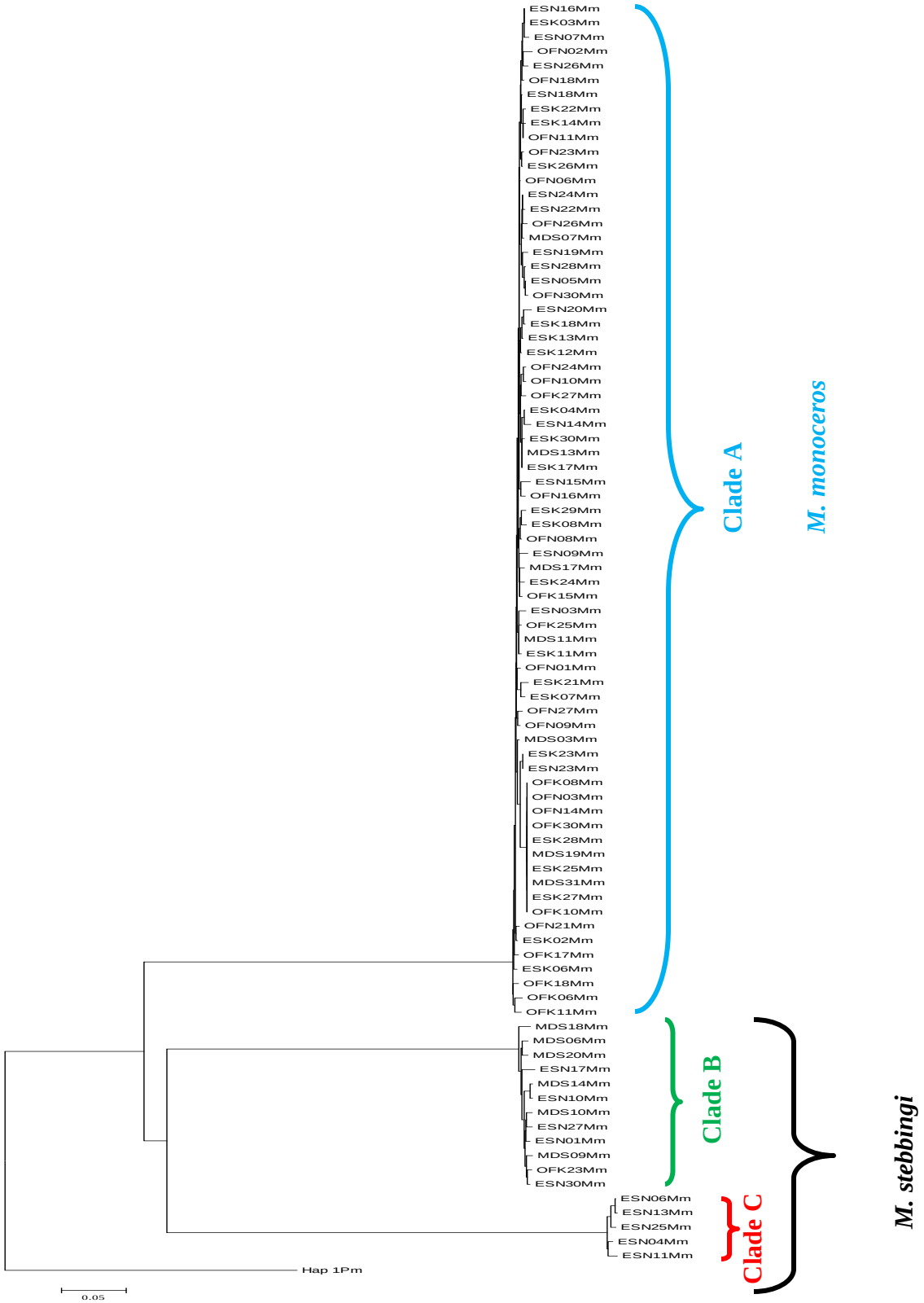

Fig. 6. Neighbour joining tree reconstructed from the mtCR sequences, showing *M. monoceros* and *M. stebbingi* clades, originating from the MUB. The three clades were confirmed using morphological examinations of sub–adults or adults specimens.