Origin and population structure of major prawn and shrimp species in Bangladesh

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Dissertation submitted in partial fulfillment of a Philosophiae Doctor degree in Biology

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Abstract

The Indo-Pacific region possesses the world’s largest biodiversity, with a unique ecosystem. Origins and population structures of six commercially and ecologically important prawn and shrimps in Bangladesh are the subject of this thesis. The species are five penaeid shrimps: *Fenneropenaeus indicus, Penaeus monodon, Penaeus semisulcatus, Metapenaeus monoceros, Parapenaeopsis sculptilis*, and the palaemonid prawn *Macrobrachium rosenbergii*. The species were studied at different depth, using mitochondrial DNA markers, microsatellites, single nucleotide polymorphisms (SNPs) and short DNA sequences from restriction sites sampled randomly from the genome. Analysis of the genetic variation allows in addition to an assessment of the phylogenies, the diversity and the demographic history of the species. Mitochondrial DNA based phylogeny of the genus *Fenneropenaeus* and *Metapenaeus* showed polyphyletic relationships within the genus and revealed three distinct lineages which indicate cryptic species in *F. indicus*, one in Bangladesh and India, the second in India, Sri Lanka and Australia, and the third in south-eastern Africa, Iran and Oman. A further support for cryptic species were obtained within *P. monodon*. The phylogeographic analyses revealed a match between the genetic divergence and the known biogeographic barriers in the western Indian Ocean, between the Bay of Bengal and the south-eastern Africa, and across the Sunda-Shelf and the Isthmus of Kra. Mitogenomic variation revealed population structure in *P. semisulcatus* from Sri Lanka and in *M. rosenbergii* from Bangladesh. Microsatellites and SNPs revealed clear genetic patterns in *P. monodon* sampled along the coast in Bangladesh from west to southeast. Similarly, at least three distinct populations were observed in *M. rosenbergii*, sampled from four watersheds in Bangladesh, based on SNPs and alleles obtained from double digest restriction-site associated DNA sequencing (ddRADseq). The high genetic variation of the Bangladesh prawn and shrimps, but shallow mitochondrial genealogy, were in accordance with expectation of sudden expansion model for population changes, dating back to 74 kyr ago in *P. sculptilis* to 466 kyr in *M. monoceros*. The expansion of the Bangladesh *F. indicus* population started more recently (~78 kyr ago) than the population in Sri Lanka (~120 kyr ago). The phylogeographic lineages identified in this study should be considered as evolutionary significant units (ESUs) or conservation units and should be considered in the management of these valuable species,
both in aquaculture and fisheries in order to maintain the large diversity within
the species in the Indo-Pacific region. The populations detected in Bangladesh
*P. monodon* and *M. rosenbergii* should be considered as separate management
units. The use of gravid and ovigerous females of wild *P. monodon* and *M.
rosenbergii* in hatcheries should consider the population structures for the
quality postlarva production. The sequences from this study have been
deposited in GenBank and can be used to trace an unknown catch and control
illegal fishing of shrimps from different lineages.
Útdráttur

Indlandshaf og Kýrrahaf búa yfir einstöku vistkerfi og mesta liffræðilega fjölbreytileika jarðar. Uppruni og stofngerð sex rækjutegunda sem eru mikilvægar jafnt efnahagslega sem og fyrir vistkerfi Bangladesh er viðfangsefni þessarar ritgerðar. Tegundirnar eru fimm þursarækjur: *Fenneropenaeus indicus*, *Penaeus monodon*, *Penaeus semisulcatus*, *Metapenaeus monoceros*, *Parapeneaeopsis scutillis*, og strandrækjan *Macrobrachium rosenbergii* sem lifir að mestu í ferskvatni. Tegundirnar voru rannsakaðar með því að greina erfðabreytileika þeirra á misítarlegan hátt, með raðgreiningu hvatbera DNA, greiningu örtungla, afgerðagreiningu á stönum breytilegum sætum viðsvegar úr erfðamengjum (e. SNP) og með raðgreiningum á stuttum röðum viðsvegar úr erfðamengi einnar tegundarinnar (e. ddRadSeq). Niðurstöður greininga á erfðabreytileikanum gáfu einnig tækifæri til að rannsaka fokkunarfræði tegundanna, breytileika þeirra og sögulegar stofnastærðarbreytingar. Fokkunartré *Fenneropenaeus* og *Metapenaeus* ættkvíslanna sýndu fjölstofna tengsl í þeirra. Duldar tegundir greindust innan *F. indicus* og í *P. monodon* með athugun á hvatberaerfðaefni tegundanna. Greining á upprunalaðufræði tegundanna sýndi samsvörun milli erfðafjöldilegra aðgreiningar og þekktra liffræðilegra svæða, milli Bengalflóa og austurstranda Afriku, og síthvorum megin við Malakkaskaga. Aðgreining milli stofna greindist í erfðamengi *P. monodon* meðfram strönd Bangladesh frá vestri til suðausturs og einnig meðal stofna *M. rosenbergii*, frá vatnasvæðum fjögurra á dannan Bangladesh. Erfðabreytileiki rækkjanna var mikill, þrátt fyrir grunn ættartré sem bendir til stórra stofna sem hafa vaxið ört á síðustu 466 þúsund árum. Stofnþvoxtur *F. indicus* hefur byrjað síðar í Bangladesh (~78 000 ár) en í Sri Lanka (~120 000 ár). Hinar landfræðilega aðskildu hvatberagerðir sem greindust í þessari rannsókn flokkað sem þróunarfræðilega marktækra einingar sem taka ætti tillit til við stjórnnun á þessum mikilvægu tegundum, þæði í eldi og í veiðistjórnun, til að vernda þennan mikla breytileika innan tegundanna. Stofnir sem greindust innan Bangladesh *P. monodon* og *M. rosenbergii* ætti að flokka sem sérstaka veiðistofna. Niðurstöður úr þessari rannsókn má einnig nýta til að rekja uppruna rækja af óljósum uppruna, m.a. frá ólöglegum veiðum.
Dedication

To my parents
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The world is facing a severe biodiversity crisis, threatening supply of essential goods and services for human existence (Barber et al. 2014). Biodiversity deals with the variety of life on Earth, ranging from genes to ecosystems, and the ecological and evolutionary processes for sustainability (AMNH 2016). With the mechanical industrialization and development during the last century, human influences have led to rapid changes in the world ecosystems, and possibly to unrecoverable loss of the biodiversity (AMNH 2016). In a recent assessment of the International Union for Conservation of Nature (IUCN) of 76,000 species throughout the world, 22,000 were found to be at risk of extinction, 75% of genetic diversity of agricultural crops is considered to be extinct, 75% of the world’s fisheries fully or over exploited, and up to 70% of the world’s known species are thought to be in risk of extinction if the global temperature rises by more than 3.5 °C (Global Issues 2014; IUCN 2015). Production practices, based on e.g. chemical fertilizers, pesticides, herbicides etc. for agricultural production, are known to harm the environment and the ecosystem, and may result in further loss of biodiversity (MEA 2005; FAO 2011), including the genetic diversity and structure of the species (Olsson et al. 2007). Genetic diversity provide essential information for the sustainable management and conservation of wild populations by revealing the scale and patterns of connectivity and relatedness (Ward 2000; Koljonen 2001; Laikre et al. 2005; Olsson et al. 2007). In addition, genetic variation is of crucial importance for the adaptation of species to a changing environment. Quantifying genetic diversity and population structure is essential for designing and implementing a suitable programme for the sustainable management and conservation of prawns and shrimps (Mandal et al. 2012).

The Indo-Pacific region possesses the world’s highest marine biodiversity in terms of species richness and unique ecosystems (Crandall & Riginos 2014; Keyse et al. 2014). This diverse biogeographic region is insufficiently studied and unevenly sampled to unveil genetic diversity (Crandall & Riginos 2014; Keyse et al. 2014). The Bangladesh region, with its extensive mangrove forests and large flow of freshwater, may harbour a rich and unexplored diversity of the aquatic fauna. Bangladesh waters are enriched with 251-267 species of freshwater fish, 402-475 of marine water fishes, 60-62 species of shrimp and prawn, 71 amphibians, 181 reptiles, 15 crabs, 6 lobsters, 362 molluscs, 66 corals, 3 sponges and 12 species of mammals (Quader 2010; Department of Fisheries Bangladesh 2013; Hossain 2014; IUCN Bangladesh 2014; Department of Fisheries Bangladesh 2015). Twenty three
percent of the vertebrates, 57% of reptiles and 36% of mammals found in Bangladesh have been facing different level of threats of extinction (IUCN Bangladesh 2014). Thirty indigenous riverine fishes have already become extinct and over 100 fishes are at risk of extinction (Hossain 2014). The diversity of shrimp in Bangladesh is threatened by overexploitation (e.g. postlarvae collection, the use of banned fishing gears), coastal and marine pollution, the impact of coastal aquaculture, natural disasters (e.g. cyclones, sea level rise) (Quader 2010; Department of Fisheries Bangladesh 2013). Thus, it is important to look closer into its prawn and shrimp diversity to unveil the underlying patterns of taxonomy, phylogeography, population differentiation and demographic history for proper management and conservation of the species in the region.

1.1 Prawn and shrimp in Bangladesh

Diverse waters in Bangladesh are the habitat of wide varieties of crustaceans (Quader 2010; Hossain 2014; IUCN Bangladesh 2014). Among the crustaceans, shellfish (mainly shrimps and prawns) are the dominant species, and have considerable commercial and nutritional values (Department of Fisheries Bangladesh 2013; Department of Fisheries Bangladesh 2015). Prawns and shrimps of Bangladesh are of great importance in foreign exchange earnings and livelihood development of millions of coastal people through direct engagement in the shrimp industry (Department of Fisheries Bangladesh 2013). The total production of prawn and shrimp was about 224 KMT, of which over 57% was contributed by aquaculture, and the country earned over 528 million USD by exporting about 48 KMT prawn and shrimp products in 2013-2014 fiscal year (Department of Fisheries Bangladesh 2015). The country’s saline water is enriched with 36 species of shrimps, including 24 penaeid shrimps and the freshwater with 24 prawns, including 18 palaemonid prawns (Department of Fisheries Bangladesh 2013; Department of Fisheries Bangladesh 2015). *Fenneropenaeus indicus* (H. Milne-Edwards 1837), *Penaeus monodon* (Fabricius 1798), *Penaeus semisulcatus* (De Haan 1844), *Metapenaeus monoceros* (Fabricius 1798) and *Parapeneaepsis sculptilis* (Heller 1862) are important penaeid shrimps (Kamal & Khan 2009; Quader 2010). *Macrobrachium rosenbergii* (De Man 1879) is highly valued palaemonid prawn for commercial aquaculture and capture fishery (Kamal & Khan 2009; Quader 2010).

1.2 Phylogeny of penaeid shrimps

Phylogenetics is an important tool in systematics and taxonomy, examining the evolutionary relationships among organisms (Nei 1996; Sinclair 2005), and to define species boundaries (Purvis et al. 2005). Phylogenetic reconstruction is
useful to study biodiversity, habitats and genetic patterns from the higher
taxonomic rank to the species level (Sinclair 2005). Traditionally, phylogenetic
studies were done using morphological and physiological characteristics (Lio &
Goldman 1998), now-a-days, DNA sequences are commonly used in phylogenetic studies (e.g. Lio & Goldman 1998; Sinclair 2005).

Numerous studies have addressed the phylogenetic relationships and evolutionary
histories of the penaeid shrimps. The genus *Penaeus* sensu lato (Fabricus 1798) is
a giant and a commercially important group of marine shrimps or prawns (Lavery
et al. 2004; Flegel 2007) with six subgenera based on morphological variation
(Pérez Farfante 1969; Tirmizi 1971; Burukovsky 1972). Pérez Farfante and
Kensley (1997) ranked the six subgenera as genuses (i.e. *Fenneropenaeus, Penaeus, Farfantepenaeus, Litopenaeus, Melicertus, Marsupenaeus*), based on
anatomical differences. However, several studies, based on variation in
mitochondrial and nuclear genes, refuted the recent taxonomic revision of
*Penaeus s.l.* proposed by Pérez Farfante and Kensley (1997) and even the
formation of previous six subgenera (Lavery et al. 2004; Flegel 2007; Ma et al.
2011). The taxonomic revision of *Penaeus s.l.* was considered to be controversial
as its monophyly was supported (Flegel 2007; Ma et al. 2011). Thus,
phylogenetics of penaeid shrimps has been a debated issue and several species are
known for a large molecular variation. The penaeid shrimp binomials in the thesis
are used according to Pérez Farfante and Kensley (1997).

### 1.3 Phylogeography of prawn and shrimp

Phylogeography is an important tool to study the historical processes associated with
geographical distributions of individuals (Lowe et al. 2006; Paule et al. 2012). It can
play an important role in defining evolutionary significant units (ESUs) or
conservation units characterized by reciprocally monophyletic mitochondrial DNA
lineages (Moritz 1994) and in understanding how the range of species changed due
to historical biogeographical processes (Paule et al. 2012) e.g. after the last glacial
period of Ice Age. It can provide a valuable platform for studying selection and
adaptation of organisms which is enriched by an understanding of the biology and
ecology of the studied organisms (Bowen et al. 2014).

A number of studies have applied molecular markers to infer genetic patterns in
prawns and shrimps in the Indo-West Pacific region and have revealed distinct
lineages associated with known biogeographic barriers i.e. the Sunda-Shelf
(Baldwin et al. 1998; Duda & Palumbi 1999; Benzie et al. 2002; You et al.
2008; Zhou et al. 2009; Walther et al. 2011; Waqairatu et al. 2012) and the
Isthmus of Kra (Hurwood et al. 2014; De Bruyn et al. 2005). The genus *Penaeus*
is considered to have originated in the center of the Indo-West Pacific region and dispersed eastward and westward forming two groups (i.e. western Pacific and Indian Oceans), which spread during the Tertiary and Pleistocene periods (Baldwin et al. 1998; Waqairatu et al. 2012). Such diversification has also been observed within *P. monodon* where populations east and west of the Sunda-Shelf are characterized by distinct mtDNA lineages (Benzie et al. 2002). Based on mtDNA, *P. monodon* populations in the Indo-Pacific region are broadly structured as three groups, i.e. in South-East Africa, South and South-East Asia and the Pacific (Benzie et al. 2002; You et al. 2008; Waqairatu et al. 2012). The South and South-East Asia populations are further divided into two groups, west and east of the Sunda-shelf (Benzie et al. 2002; Kumar et al. 2007; You et al. 2008; Mandal et al. 2012). The populations in the South-East Africa group are unique and genetically differentiated from the other main groups, which are partially admixed (Kumar et al. 2007; You et al. 2008; Mandal et al. 2012). Due to the large divergence between South-East Asia and South-East Africa haplotypes, the South-East Asia populations are considered to have migrated towards the Indo-West Pacific region (Benzie et al. 2002). Other studies, based on the mitochondrial control region (CR), group the haplotypes into three lineages (A, B and C), where A is mostly found in the Pacific Ocean region, B from the juncture of the Indo-West Pacific Oceans and C from India and Africa (Zhou et al. 2009; Walther et al. 2011). Limited or no information are available about phylogeography of *P. semisulcatus*, *M. monoceros* and *P. sculptilis*.

Distinct phylogeographic lineages have also been observed in Palaemonid prawn *M. rosenbergii* (De Bruyn et al. 2005; Hurwood et al. 2014). Evolutionary relationships among haplotypes of *M. rosenbergii* sampled from the Indo-West Pacific region grouped them into three distinct lineages, i.e. western (from Gujarat, north-western India to the Kraburi river, south-western Thailand, including the individuals from two locations of Bangladesh) lineage consistence with the biogeographic barrier the Isthmus of Kra, central (from locations around the Sunda-Shelf, including Kerala, India) lineage and eastern (includes samples from Bengawan and Sandakan rivers, Indonesia, including Kerala, India) lineage (De Bruyn et al. 2005; Hurwood et al. 2014).

The phylogeographic patterns in the Indo-West Pacific region were largely influenced by historical sea-level fluctuations associated with the known biogeographic barriers: the Sunda-shelf and the Isthmus of Kra, which connected the Malay Peninsula, Sumatra, Java and Borneo with extensive land bridges at 50 m depth contour below present level around 11 kyrs ago (Figure 1-1; Voris 2000).
1.4 Genetic variation and population structure in prawn and shrimp

Population genetic variation, the differences in DNA or genes within a population, is essential for evolution and provides the raw material for natural selection. Genetic diversity acts as a key ecosystem function and thus, maintenance of genetic diversity is considered internationally as a common goal in the management of wild populations (Ward 2000; Koljonen 2001; Laikre et al. 2005; Olsson et al. 2007). Population structure deals generally with the patterns in neutral genetic variation, resulting from the past or present departure from panmixia of a population (Guillot & Orlando 2015). Population structure is a very important part of evolutionary genetics as it allows populations to diversify (Futuyma 1998). Each population can be considered as separate management unit that is important to manage with respect to its population dynamics and to monitor e.g. due to the influences of human activities (Palsbøll et al. 2007), such as fisheries, and thus, to ensure effective and sustainable management (Olsson et al. 2007). Unlike the evolutionary significant unit, which presents reciprocally monophyletic groups and have diverged over
sufficient period of time, management unit reflect recent divergence of populations which can be characterized solely by differences in frequencies of different genetic variants (Moritz 1994).

Several studies have investigated population differentiation in prawn and shrimps in the Indo-West Pacific region based on genetic variation. A mitogenomic study of *F. indicus* sampled from the coastal belt of Sri Lanka revealed a high variation in COI gene of *F. indicus*, indicating the presence of different populations along the coast (De Croos & Pálsson 2010). Polymorphic microsatellite markers have revealed population differentiation in *P. monodon*, and tagging nonindigenous farmed shrimps (Pan et al. 2004). Based on ten microsatellite loci, significant genetic structure was identified among eight *P. monodon* populations from the Bay of Bengal, south of Bangladesh, with the highest genetic diversity in the Andaman Islands (Mandal et al. 2012). High levels of polymorphism, based on variation in 30 microsatellites, was also reported from Malaysian waters with highly significant deficiencies of heterozygotes possibly due to admixture of populations or breeding units (Aziz et al. 2011). In a recent review, Keyse et al. (2014) compiled data of genetic variation in 116 species of six phyla, including 11 species of Arthropods from the tropical Indo-Pacific region but no samples were included from Bangladesh. *M. rosenbergii* sampled from South-West Bangladesh (the Pashur and the Paiera rivers) and South-East Bangladesh (the Naf river), based on seven microsatellites, revealed a panmictic population with a heterozygote deficiency, possibly due to high level of inbreeding (Khan et al. 2014). Due to its commercial importance for both aquaculture and capture fishery, *P. monodon* and *M. rosenbergii* need to be studied using more powerful markers to detect population structure or management units for a sustainable harvest of the species.

### 1.5 Demography

Demography deals with the size, distribution and composition of a population, and the underlying causes of changes therein (Hauser & Duncan 1959). The effective population size (*N_e*) is essential to evaluate the rate of inbreeding and loss of genetic variation in wild populations (Frankham 1995). The genetic estimation of *N_e* and the related adult census size (*N*) are useful for the conservation and management of endangered or exploited species (Palstra & Fraser 2012), where the census size *N* is often about $10^3$ to $10^6$ times of *N_e* for highly fecund species i.e. fish, oysters and shrimp (Frankham et al. 2010). The demographic history of Asian species is known to have been affected by climatic fluctuations, and several Pleistocene refugia for different species have been described in this region (Liao et al. 2010, Stewart et al. 2010, Tsang et al. 2012).
1.6 Study species

Considering the size of wild catch, commercial value and importance for aquaculture, management and conservation, five penaeid shrimps i.e. *F. indicus*, *P. monodon*, *P. semisulcatus*, *M. monoceros* and *P. sculptilis* and one palaemonid prawn *M. rosenbergii* were selected for analyses of genetic variation. Information of genetic patterns could contribute to a sustainable management of these species within Bangladesh.

The Indian white shrimp (locally known as Chaka) *F. indicus* (Figure 1-2a) is distributed throughout the Indo-West Pacific region, covering East and South-East Africa to South China, New Guinea and Northern Australia (Holthuis 1980; De Grave 2012). *Fenneropenaeus indicus* is one of the dominant commercial penaeid species of the world (Mehanna et al. 2012; Bindu et al. 2013; Sarada 2006) and has significant importance in offshore fishing and aquaculture in Bangladesh (Holthuis 1980).

The giant tiger shrimp (locally known as Bagda) *P. monodon* (Figure 1-2b) makes an important contribution to aquaculture production and marine capture in the Indo-West Pacific region (Motoh 1985; Kumar et al. 2007; You et al. 2008; Azad et al. 2009; Kamal & Khan, 2009; Quader 2010; Mandal et al. 2012; Waqairatu et al. 2012; Vaseeharan et al. 2013; Debnath et al. 2015). It is the most targeted penaeid shrimp for both capture and aquaculture in Bangladesh, due to its high economic value (Quader 2010; Department of Fisheries Bangladesh 2014).

The green tiger prawn (locally known as Flower Tiger) *P. semisulcatus* (Figure 1-2c) is a commercially important penaeid shrimp distributed throughout the Indo-West Pacific region from South-East Africa to Japan (Holthuis 1980; Grey et al. 1983). The species plays an important role in aquaculture-breeding programmes (Seidman & Issar 1988; Türkmen 2007), polyculture with rice-fish farming and offshore fishing in the Ganges delta in Bangladesh, eastern India (Holthuis 1980; Rao et al. 1993; Quader 2010) and Sri Lanka wild catches (De Croos & Palsson 2013).

The speckled shrimp (locally known as Harina) *M. monoceros* (Figure 1-2d) is an important penaeid shrimp distributed in the coastal and marine waters of the Indian Ocean ranging from the Bay of Bengal in the east to South-East Africa, including the Red Sea and the Mediterranean Sea (FAO 1970; Fransen 2011). It is one of the commercially important marine shrimps for both Gher farming (rearing of natural postlarva in coastal paddy fields) and wild capture, contributing about 56% of the total wild catch in Bangladesh (Quader 2010).

The rainbow shrimp (locally known as Bagtara) *P. sculptilis* (Figure 1-2e) is a penaeid shrimp distributed throughout the Indo-West Pacific region, including
north-eastern Australia, northern Bay of Bengal, west coast of India and south-eastern Africa (De Grave 2015; ITIS 2016). It is an important marine shrimp for wild capture in Bangladesh (Department of Fisheries Bangladesh 2013).

Figure 1-2  The six species (a-f) studied in this thesis

The giant freshwater prawn (locally known as Galda) *M. rosenbergii* (Figure 1-2f) is found naturally in rivers and adjacent freshwater reservoirs (i.e. lakes, swamps, canals) in South and South-East Asia, from western Pakistan to western Java of Indonesia (FAO 2002; De Bruyn 2005; Hurwood et al. 2014). It has been introduced into 40 countries due to its importance both for fishing and aquaculture (Iketani et al. 2011). Aquaculture of *M. rosenbergii* in Bangladesh has been growing for two decades due to its export potential (Ahmed et al.
2008; Wahab et al. 2012), and has a high potential to expand its culture in a large number of ponds in Bangladesh (Alam & Alam 2014).

1.7 **Aims of the study**

The main aim of the study was to investigate the genetic patterns of several major prawn and shrimp species from Bangladesh for better management. The following research questions were addressed in five separate papers (I-V):

1. What are the main phylogeographic patterns of the species and what are the origin of the populations in Bangladesh? Does variation in mtDNA suggest different evolutionary lineages or even cryptic species found in different regions within the Indo-Pacific Ocean? This was the subject of papers I-III which analysed variation in *F. indicus, P. monodon* and *P. semisulcatus*. In addition, the results from analyses of *M. monoceros* and *P. sculptilis* are presented in this thesis.

2. Are there genetically differentiated populations or management units within Bangladesh waters? In papers IV-V nuclear markers for *P. monodon* and *M. rosenbergii* were utilized, in addition, mitochondrial DNA variation in all species was analyzed in all papers.

3. Does the genetic variation reflect large population sizes or recent expansion? This was the subject of papers I-III and V which analysed historical demography of *F. indicus, P. monodon, P. semisulcatus* and *M. rosenbergii*. The results from analyses of *M. monoceros* and *P. sculptilis* are also presented in this thesis.
2 Material and methods

2.1 Study area

Wild origin of 400 specimens of five penaeid shrimps: *F. indicus*- 157, *P. monodon*- 100, *P. semisulcatus*- 48, *M. Monoceros*- 55 and *P. sculptilis*- 40 ind., were collected from four locations along the Bangladesh coastline i.e. Sundarban mangrove forest (A, 104 ind.), the coast of Barguna (B, 55 ind.), Middle ground of the Bay of Bengal (C, 143 ind.) and the coast of Teknaf (D, 98 ind.) during the period of December 2012 to September 2013 (Figure 2-1). Wild origin 83 *M. rosenbergii* were collected from four rivers in Bangladesh i.e. the Bishkhali river (BR, 20 ind.), the Meghna river (MR, 20 ind.), the Karnaphuli river (KR, 22 ind.) and postlarvae (PL) from the Naf river (NR, 21 ind.) during the same period (Figure 2-1). Mitochondrial DNA sequences of the studied species throughout their ranges of distribution from South-West Africa to the West Pacific Ocean, were retrieved from GenBank and used in the analyses along with the newly collected Bangladesh specimens.

2.2 Markers used

In recent years, molecular methods, have been used for studying genetic variation and population patterns and its underlying evolutionary processes, and facilitated sustainable management of wild populations (Lowe et al. 2006; Olsson et al. 2007). Both mitochondrial and nuclear markers have been used to identify species and to infer population structures in shrimps (Vaseeharan et al. 2013).

2.3 Mitochondrial DNA

Mitochondrial DNA is a circular molecule and inherited maternally with limited recombination in some species. MtDNA is useful as a molecular marker (e.g. Vaseeharan et al. 2013, Bajpai & Tewari 2010) due to its high mutation rate (Liu & Cordes, 2004) and has been used to study genetic variation of crustaceans and their phylogeny upto the species level (e.g. Chu et al. 2003). MtDNA has been a principal tool for phylogeographic studies due to the accessibility of its sequence information (Paule et al. 2012). MtDNA was used in this study to reevaluate the phylogeny of penaeid shrimps, to reassess the phylogeography in the Indo-West Pacific region and to reveal historical demography, genetic diversity and population differentiation.
Figure 2-1  Sampling locations in Bangladesh. Letters A-D present four sampling sites with colour pies representing the proportion of specimens from five penaeid shrimps and letters: BR- Bishkhali river, MR- Meghna river, KR- Karnaphuli river and NR- Naf river show the four sampling locations for only Palaemonid prawn Macrobrachium rosenbergii.

Figure 2-2  Selected genes of Mitochondrial DNA used in this study
Five regions of mtDNA (Figure 2-2) were amplified and sequenced from five penaeid shrimp species, including *F. indicus, P. monodon, P. semisulcatus, M. monoceros* and *P. sculptilis* and one palaeonid prawn *M. rosenbergii* sampled from Bangladesh: two adjacent regions of the cytochrome oxidase subunit 1 (the barcode region CO1b and a downstream region CO1d), control region (CR) short fragment and long fragment, 16S rRNA, combined 16S rRNA and tRNA^val^ genes with the primer pairs: LCO-1490 and HCO-2198 (Folmer et al. 1994), COIF (Palumbi & Benzie 1991) and TL2N (Quan et al. 2001), 12S and IR (Chu et al. 2003), PmCON-2F (Wilson et al., 2000) and IR (Chu et al. 2003), 16STf (MacDonald et al. 2005) and 16Sbr (Palumbi et al. 1991) and 16ScruC4 and 16ScruC3 primers (Pascoal et al. 2008), respectively. Gene sequences of mtDNA varied with the species, depending on the sequences available for the particular species in GenBank.

2.4 Microsatellites and SNPs in *Penaeus monodon*

Microsatellites have been a preferred nuclear DNA marker to infer individual genetic diversity and population structure (Vaseeharan et al. 2013). More recently, single nucleotide polymorphisms (SNPs) have been accessible in various species due to genomic studies and have been used e.g. to diagnose diseases in aquaculture (Vaseeharan et al. 2013).

Ten polymorphic microsatellites (Pan et al. 2004) and 14 unlinked SNPs (six unannotated, seven in C-type lectin genes and one in HLA; Baranski et al. 2014) were genotyped in the giant tiger shrimp *P. monodon*, sampled from four locations along the Bangladesh coastline, to identify the population structure or putative management units, and to assess potential selection in immune-related genes due to microbial pathogens. Microsatellite allele sizes at each locus were determined from electropherogram images using the GeneMapper software (version 4.1, Applied Biosystems), after having run the PCR-products on Applied Biosystems 3500xL Genetic Analyser. SNP genotypes were determined from allelic discrimination plots (Figure 2-3) of qPCR analysis for each of the SNP loci, amplified on 7500 real time PCR system, Applied Biosystems), applying the KASP method, following the manufacturer’s instructions (LGC Limited; www.lgcgenomics.com).

2.5 SNPs in *Macrobrachium rosenbergii*, obtained from ddRADseq

Double digest restriction-site associated DNA sequencing (ddRADseq) offers major advantages for population genomics by screening single nucleotide polymorphisms (SNPs) throughout the genome and has been proven as an
effective tool to delineate population structure in organisms (Hohenlohe et al. 2010; Peterson et al. 2012; Benestan et al. 2015).

**Figure 2-3** Allelic discrimination plot of SNP genotypes produced from qPCR. The axis presents the strength of the signal for each allele. Green and blue dots present individuals which are homozygous for allele 1 and allele 2, heterozygotes are in purple and blank or unamplified are in red.

**Figure 2-4** Double digest RAD sequencing (ddRADseq). The method uses a two enzymes double digest, modified from Peterson et al. (2012). X in black and grey refer to the restriction sites, Colours: Red= Fragments selected for library preparation, Green= Individual 1, Yellow=Individual 2; Letters: ’a’ and ’b’ represent the fragments excluded.

Four hundred and thirty six SNPs and 481 alleles obtained from ddRADseq were used to unveil the genetic diversity and population structure in the giant freshwater prawn *M. rosenbergii* sampled from four watersheds in Bangladesh. The double digest RADseq (Figure 2-4) library was prepared following
protocols modified from Peterson et al. (2012) and Elshire et al. (2011). The library was run on an Illumina MiSeq2000 for 300 cycles (2x150 paired-end) using v2 chemistry. Raw FASTQ files from the MiSeq runs were demultiplexed into reads unique for each individual using the process_radtags command in STACKS v.1.09 (Catchen et al. 2013). Variant detection and genotyping was performed in PyRAD v.3.0 (Eaton 2014). The output files for STRUCTURE, presenting a single SNP from each locus with the least missing data across loci, and alleles were used for all analyses.

### 2.6 Data analysis

#### 2.6.1 Phylogeny and phylogeography

Phylogenetic analysis based on mitochondrial markers was performed for the genus *Fenneropenaeus* and *Metapenaeus*. Phylogeography of the targeted species in the Indo-West Pacific region (included *F. indicus*, *P. monodon*, *P. semisulcatus*, *M. monoceros*, *P. sculptilis* and *M. rosenbergii*) was studied through construction of phylogenetic tress using BEAST v1.7.5 (Drummond and Rambaut 2007) and PhyML-3.1 (Gouy et al. 2010), multi-dimensional scale plots (Venables & Ripley 2002), minimum spanning tree (Sedgewick 1990) and unrooted cladograms using a median-joining algorithm (Bandelt et al. 1999). Two criteria were considered to evaluate cryptic diversity: first, the ratio of species-screening-threshold ($R_{SST}$ > 10; Witt et al. 2006; Hebert et al. 2004) calculated from inter and intra-species distances, and the second, the species delimitation threshold (0.16 subst./site; Lefébure et al. 2006) calculated from patristic distances.

#### 2.6.2 Demographic history and population expansion

Population demographic changes and deviation from neutrality were estimated by analysing the mismatch distribution, using sum of square deviation (SSD) and the raggedness index (Harpending 1994), and with Tajima’s D (e.g. Tajima 1993) and Fu’s Fs (Fu 1997) using ARLEQUIN v3.5 (Excoffier & Lischer 2011). The demographic changes were further analysed with the Bayesian Skyline Plot (BSP) using BEAST v1.7.5 (Drummond et al. 2007). The time since expansion was based on the median of the mismatch distribution ($\tau$) and the mutation rate, $\mu$ as $t = \tau/(2\mu L)$, where L is the length of the sequence. Posterior probability of the effective population size ($N_e$) was estimated with the BSP analysis.
2.6.3 Genetic diversity and population differentiation

Genetic diversity based on mitochondrial DNA, including gene diversity \((h)\), nucleotide diversity \((\pi)\) and its partition among sampling sites with analysis of molecular variance (AMOVA) applying both the conventional F-statistics from haplotype frequencies and the distance method \((\Phi)\), was calculated using ARLEQUIN v3.5 (Excoffier & Lischer 2011). Haplotype richness was calculated using the HIERFSTAT package (Goudet 2005) in R (R Core Team 2015). Evolutionary relationships were investigated with an unrooted cladogram, using a median-joining algorithm (Bandelt et al. 1999), implemented in NETWORK v 5.0.0.0 (www.fluxus-engineering.com).

Basic statistics based on microsatellites, SNPs and alleles (allelic richness, observed heterozygosity, expected heterozygosity and \(F_{IS}\)) were summarized with the HIERFSTAT (Goudet 2005) and PEGAS (Paradis et al. 2016) packages in R (R Core Team 2015). The F-statistics were calculated and tested, using HIERFSTAT in R and, using exact tests in GENEPOP (Rousset 2008) which was also used to evaluate the genotypic disequilibrium. Identification of candidate loci under natural selection was done using BayeScan (Foll 2012). The multivariate ordination of the different genotypes was investigated using Discriminant Analysis of Principal Components (DAPC) with the ADEGENET package (Jombart et al. 2015) in R (R Core Team 2015). As the \(F_{ST}\) is dependent on heterozygosity within populations (Meirmans & Hedrick 2011), two unbiased methods of population comparisons, \(G''_{ST}\) (Meirmans & Hedrick 2011) and \(D_{ST}\) (Jost 2008) were calculated based on the microsatellite and SNP variation in \(P. monodon\) with the MMOD package (Winter et al. 2015) in R (R Core Team 2015). Wilcoxon test was used to compare the heterozygosities and the \(F_{ST}\) between the SNPs in \(P. monodon\) from the C-type lectin genes and the \(HLA3\) and the unannotated SNPs.
3 Results

3.1 Phylogeny of penaeid shrimps

Figure 3-1  Bayesian inference tree for the genus Fenneropenaeus. A 421 bp fragment of CO1d for 15 sequences were utilized under ‘HKY + G’ model following a mutation rate of 1% per million year. Numbers at the nodes represent divergence in million years and shadings represent Bayesian posterior probabilities (PP, %). The tree is rooted with Penaeus monodon with a divergence time of ~21.8 Myr (PP>90). Note: the time estimates are two times of that reported in Paper I, which have been estimated using divergence rate rather than mutation rate.

Mitochondrial DNA based phylogeny of *F. indicus* from different populations revealed a polyphyletic relationships within the species (Paper I). Two distinct clusters, based on CO1d (Figure 3-1), diverged from each other for 15.6 (CI: 10.8 - 23.4) Mya. Cluster I includes *F. indicus* of the western Indian Ocean and a specimen from Thailand with a recent common ancestor (0.4 Mya, CI: 0.2 - 0.8), grouping nominally (PP < 70) together with *Fenneropenaeus chinensis* (Osbeck, 1765). Cluster II includes *F. indicus* from the eastern Indian Ocean except for the Thailand
sample, i.e. from Bangladesh, Australia, India and Sri Lanka, grouping together with *Fenneropenaeus merguiensis* (De Man 1888), *Fenneropenaeus penicillatus* (Alcock 1905) and *Fenneropenaeus silasi* (Muthu & Motoh 1979). Bangladesh *F. indicus* diverged earliest from the other populations of *F. indicus* and species within cluster II (PP > 90). The four *F. indicus* specimens from India were similar either to Bangladesh or Sri Lanka, but single specimen from both Australia and Thailand showed closest similarity with Sri Lanka and western Indian Ocean populations, respectively. A phylogenetic tree based on combined data set of COI and 16S rRNA showed a greater overall support than obtained for either marker analyzed separately (Paper I). Net genetic distances between populations for COIb, COId and 16S rRNA genes of *F. indicus* from Bangladesh, Sri Lanka and for COId from western Indian Ocean were significant (P < 0.05) (Figure 3-2).

![Figure 3-2](image)

*Figure 3-2* Genetic variation within and between populations of *Fenneropenaeus indicus*. Net genetic distances based on COI barcode, COId and 16S rRNA of mtDNA regions. B: within Bangladesh, S: within Sri Lanka, BS: between Bangladesh and Sri Lanka, I: within cluster I (Western Indian Ocean and Thailand, see figure 3-1), BI: between Bangladesh and cluster I, SI: between Sri Lanka and cluster I.

Phylogeny of the genus *Metapenaeus* for CR revealed that *M. monoceros* samples from different populations were polyphyletic with respect to other *Metapenaeus* spp. (Figure 3-3). Two distinct clusters (I and II) for CR were observed, which diverged from each other for 7.4 (CI: 4.84 – 10.52) Myr: cluster I included *M. monoceros* of
the eastern Indian Ocean from Bangladesh, grouping together with *Metapenaeus ensis* from China (PP > 90), and cluster II included *M. monoceros* from the western Indian Ocean from Kenya (Figure 3-3). Phylogeny for 16S rRNA revealed that *M. monoceros*, *M. ensis* and *M. affinis* samples from different populations were also polyphyletic (tree not shown). Two distinct clusters (I and II) were observed in the phylogeny based on 16S rRNA, which had diverged from each other for 5.6 (CI: 3.42 – 8.28) Myr: I) with *M. monoceros* of the eastern Indian Ocean from Bangladesh, grouping together with *M. ensis* and *M. affinis* from China, and II) with *M. monoceros* from the western Indian Ocean from Tanzania grouping together with *M. ensis*, *M. affinis* and *M. joyneri* from China (tree not shown).

![Bayesian inference tree for the genus Metapenaeus.](image)

*Figure 3-3 Bayesian inference tree for the genus Metapenaeus. A 388 bp fragment of CR for 86 sequences were utilized under ‘GTR + G’ model following a mutation rate of 1.72% per Myr. Numbers at the nodes represent divergence in million years and shadings represent Bayesian posterior probabilities (PP, %). The tree is rooted with Penaeus monodon (GenBank accession number: KT006166) with a divergence time of ~110.26 (CI: 63.92 – 172.68) Mya (PP ≥ 90). Numbers following names of countries represent the number of haplotypes.*

### 3.2 Phylogeography in the Indo-West Pacific

Mitochondrial DNA based phylogeographic analyses revealed a match between the genetic divergence associated with known biogeographic barriers: in the western Indian Ocean between the Bay of Bengal and south-eastern Africa, the Sunda-Shelf and the Isthmus of Kra, which resulted two to four phylogeographic lineages or even cryptic species among the studied species: the first in the western Indian Ocean with an additional lineage of *F. indicus* in India and Sri Lanka, the second in the Bay of Bengal region, the third in South-East Asia and the forth in the western Pacific ocean.

Phylogeography of *F. indicus* revealed two main lineages: the first in the eastern (includes Bangladesh, Sri Lanka, India and West Australia) and the second in
the western Indian Ocean regions (includes South-Eastern Africa, Oman and Iran) with an individual from Thailand, which diverged well before the onset of Ice age 5.4 Myr ago. A further split is observed within the eastern group which has occurred during the Ice Age. The values of the $R_{SST}$ (the ratio of average distances among and within groups) and the substitutions per site exceeded the Crustacean species thresholds ($R_{SST} > 10$; substitutions per site = 0.16) suggested by Hebert et al. (2004), Witt et al. (2006) and Lefébure et al. (2006) in most cases defined for CO1 gene, despite the substitution rate for the CO1d region being apparently lower than the CO1b region.

![Figure 3-4](image)

**Figure 3-4** Bayesian inference tree for *Penaeus monodon*. A 496 bp fragment of CR (lineage C) for 311 sequences (without Indonesia) were utilized under ‘GTR + G + I’ model following a mutation rate of 1.72% per Myr. Numbers at the nodes represent divergence in million years and shadings represent Bayesian posterior probabilities (PP, %). The tree is rooted with *F. chinensis* (GenBank accession number: DQ518969) with a divergence time of ~92.4 (CI: 64.6 – 130.0) Mya (PP ≥ 90). Numbers following names of countries represent the number of haplotypes/sample size. Note: the clustering within clades I to III is insignificant except for a single node with PP ≥ 90, and different haplotypes from the same country have been grouped into a single branch. The time estimates are two times of that reported in Paper II which have been estimated using divergence rate rather than mutation rate.

Phylogeography of *P. monodon* showed that the most recent common ancestor of three monophyletic CR lineages (A, B and C) dated back to 13.2 (CI: 9.6 - 17.4) Mya, and 8.2 (CI: 5.6 - 10.8) Mya for lineages A and B. Individuals in lineage C
were found in all specimens in west of the Sunda-Shelf from Madagascar to Bangladesh, except for two specimens from Sri Lanka, and C haplotypes were in lower frequency in south-east Asia, from 50% in Thailand to 12 % in China. Lineages A and B were solely found in populations from the south-east Asia and the Pacific Ocean regions. The Bayesian inference analyses of lineage C for CR showed a complex pattern in the Indo-West Pacific region, with five distinct clusters (PP ≥ 90; Figure 3-4). Pair-wise genetic distances (\( \Phi_{ST} \)) for CR showed clear differentiation between populations of all lineages and also within lineage C (Figure 3-5). The Madagascar and Kenya *P. monodon* populations, which cluster within the C lineage, are significantly different from other populations (\( \Phi_{ST} \) ranges from 0.44 to 0.82, \( p < 0.01 \)). The Bangladesh population is most similar to the populations of India and Sri Lanka (\( \Phi_{ST} = 0.08 - 0.11 \), omitting the small sample from India West) but significantly different from east and south India (Figure 3-5). The differentiation of the Bangladesh population from Sri Lanka, Thailand (West and East), Indonesia and China were insignificant when lineages A and B are omitted (\( \Phi_{ST} \) ranges from 0 to 0.03, \( p > 0.05 \)). The distances between populations based on the 16S rRNA gene support the split based on CR.

Figure 3-5  Multidimensional scale plot for *Penaeus monodon*. Pairwise genetic distances (\( \Phi_{ST} \)) between 14 *P. monodon* populations, based on variation in 714 sequences of all mtDNA CR lineages in the Indo-West Pacific region, were used. Letters: MG= Madagascar, KY= Kenya, IW= India West, IS= India South, IE= India East, SL= Sri Lanka, BD= Bangladesh, TW= Thailand West, TE= Thailand East, ID= Indonesia, VN= Vietnam, CH= China, PH= Philippines, AU= Australia.
Phylogeography of *P. semisulcatus* showed clear patterns in the Indo-West Pacific region with a split across the Sunda-Shelf. The Indian Ocean samples (Bangladesh, Sri Lanka, India, Malaysia and Iran) clustered together in a West Sunda-Shelf group but the samples from China and Philippines clustered in an East Sunda-Shelf group. Individuals from Iran showed admixture of two lineages and high nucleotide diversity, one shared with Bay of Bengal and possibly western Indian Ocean populations and one distinct. Pair-wise genetic distances ($\Phi_{ST}$) for all markers (included CO1b, CO1d, CR and 16S) showed clear differentiation between all populations except for the samples from Malaysia and India (Figure 3-6). The $R_{ST}$ for CO1b (12.9) and substitution rate per site for CR (ranged from 0.68 to 0.83) between Indian Ocean samples and West Pacific Ocean samples from Philippines and China, respectively exceeds the crustacean species threshold developed for CO1. The corresponding values based on other mitochondrial markers were smaller than the crustacean species threshold developed for CO1.

Two main lineages were observed in *M. monoceros*: one in the eastern Indian Ocean, including individuals from Bangladesh, and the second in the western Indian Ocean, including individuals from Kenya and Tanzania. The net distances between

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**Figure 3-6**  *Multidimensional scale plot for Penaeus semisulcatus. Ordination is based on genetic distances ($\Phi_{st}$) between six *P. semisulcatus* populations in the Indo-West Pacific region, based on all markers (includes COI barcode, CO1d, CR and 16S rRNA), were used. Letters: BD= Bangladesh, SL= Sri Lanka, MA= Malaysia, IN= India, IR=Iran, CH= China.*
Results

**Figure 3-7**  Intra-population and inter-populations genetic variation of *Metapenaeus monoceros*. Net genetic distances based on CR and 16S rRNA of mtDNA regions. BD = within Bangladesh, KY = within Kenya, TN = within Tanzania, BDvKY = between Bangladesh and Kenya, BDvTN = between Bangladesh and Tanzania.

**Figure 3-8**  Bayesian inference tree for *Macrobrachium rosenbergii*. A 577 bp fragment of CO1b for 127 sequences were utilized under ‘GTR + I + G’ model following a mutation rate of 1% per million year. Numbers at the nodes represent divergence in million years and shadings represent Bayesian posterior probabilities (PP, %). The tree is rooted with *Macrobrachium nipponense* (GenBank accession number: NC_015073) with a divergence time of ~27.4 (CI: 18.56 – 37.84) Mya (PP ≥ 90). Eastern clade (including haplotypes from Australia) is not shown in the figure which diverged from both central and western clades by ~16.04 (CI: 11.08 – 21.68) Mya (PP ≥ 90). Numbers following the names of countries represent the number of haplotypes.
Bangladesh with Kenya based on CR and between Bangladesh and Tanzania based on 16S rRNA were significant with the $R_{SST}$ of 1.34 and 36.63, respectively, and the later exceeded the crustacean species threshold ($R_{SST} > 10$) developed for CO1 (Figure 3-7). The substitutions per site between Bangladesh with Kenya based on CR and Tanzania based on 16S rRNA ranged from 0.45 to 0.55 and from 0.057 to 0.061, respectively, and the former exceeded the Crustacean species thresholds for CO1 (substitutions per site = 0.16).

Two main lineages in *M. rosenbergii* were also observed in both sides of the Sunda-Shelf, one in the western Pacific Ocean (includes Australia, Papua New Guinea and Indonesia) and the second in the western Indian Ocean, which diverged well before the onset of the Ice Age around 16.04 (CI: 11.08 – 21.68) Mya (Figures 3-8 and 3-9). The western Indian Ocean lineage was further split into two clusters: western (includes Bangladesh, India, Sri Lanka and Thailand) and central (includes Indonesia, Malaysia, Vietnam and Thailand) which diverged around 1.66 (CI: 0.94 - 2.28) Mya (Figure 3-9). Individuals from China and Brazil resolved onto both clusters.

![Minimum spanning tree for Macrobrachium rosenbergii](image)

*Figure 3-9* Minimum spanning tree for Macrobrachium rosenbergii. Pairwise genetic distances ($\Phi_{ST}$) for mitochondrial CO1b among 10 *M. rosenbergii* populations in the Indo-West Pacific region. Letters: BD= Bangladesh, IN=India, SL= Sri Lanka, TH= Thailand, MA= Malaysia, VN= Vietnam, CH=China, AU= Australia, BR= Brazil.
3.3 Genetic diversity and population differentiation

Bangladesh prawn and shrimps are characterized by high genetic diversity for both mitochondrial and nuclear markers. Mitogenomic variation revealed high gene diversity with a shallow genealogies. The highest genetic diversity for mitochondrial CO1b was observed in *M. monoceros* followed by *M. rosenbergii*, *P. monodon*, *F. indicus*, *P. semisulcatus* and *P. sculptilis* (Table 3-1). CR showed similar genetic diversity in *P. monodon*, *P. semisulcatus* and *M. monoceros* but much higher than that for CO1b where haplotype diversity reached to maximum value of one (Table 3-1).

The overall observed heterozygosity (*H*<sub>O</sub>) and expected heterozygosity (*H*<sub>E</sub>) for *P. monodon* populations, based on 10 microsatellites and 14 SNPs, ranged from 0.557 in MG to 0.596 in BC and from 0.617 in SB to 0.640 in BC, respectively, with an overall inbreeding coefficient *F*<sub>IS</sub> = 0.07 ± 0.13 (Paper IV). The overall genetic diversities, based on 436 SNPs, were almost similar among four *M. rosenbergii* populations (H ranged from 0.074 in KR to 0.085 in MR), with an overall *F*<sub>IS</sub> = 0.689, and based on 481 alleles, were also similar among the four populations (H ranged from 0.315 in NR to 0.339 in MR), with an overall nucleotide diversity of 0.0094 (Paper V).

The median-joining haplotype networks for mitochondrial CO1b reflects the genetic diversity and form starlike networks in all shrimp species throughout Bangladesh (Figure 3-10). The proportion of variance among populations (AMOVA), based on mitogenomic variation, did not reveal any significant differentiation within *M. monoceros*, *P. monodon*, *F. indicus*, *P. semisulcatus* and *P. sculptilis* sampled from Bangladesh (*Φ*<sub>ST</sub> and *F*<sub>ST</sub> were insignificant with P > 0.05), except for *M. rosenbergii* with a significant differentiation between BR with KR and NR from Bangladesh, and between the *P. semisulcatus* populations from Sri Lanka (*Φ*<sub>ST</sub> and *F*<sub>ST</sub> were significant with P < 0.05).

Differences between the four samples of *P. monodon* along the Bangladesh coastline, based on all loci (nine microsatellites and 14 SNPs), were significant, except for the comparisons of SB with BC and MG, and thus, revealed three distinct populations: one in SB-BC, the second in SB-MG and the third in SM near Myanmar. Significant *F*<sub>ST</sub> for all loci ranged from 0.004 to 0.011, *G"*<sub>ST</sub> from 0.042 to 0.093 and *D*<sub>ST</sub> from 0.022 to 0.053. The DAPC analysis with prior information also support the three distinct *P. monodon* populations in Bangladesh (Figure 3-11).
Table 3-1  Genetic diversity of major prawn and shrimps in Bangladesh based on mtDNA. \( N \) = No. of individuals, \( L \) = Length of sequence, \( N_h \) = No. of haplotypes, \( H_R \) = Haplotype richness, \( h \) = haplotype diversity, \( \pi \) = Nucleotide diversity, \( S \) = No. of segregating sites, \( N_e \) = Current effective population size, \( t \) = Time since population expansion estimated from mismatch \( \tau \), with the confidence interval (CI) of 5% and 95%. The effective population size of Fenneropenaeus indicus and Penaeus monodon are two times of that reported in Paper I and II, which have been estimated using divergence rate rather than mutation rate.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>N</th>
<th>L (bp)</th>
<th>( N_h )</th>
<th>( H_R )</th>
<th>( h )</th>
<th>( \pi )</th>
<th>S</th>
<th>( N_e \times 10^6 ) ind.</th>
<th>t kyers ago</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Macrobrachium rosenbergii</em></td>
<td>83</td>
<td>579</td>
<td>27</td>
<td>15.26</td>
<td>0.84</td>
<td>0.0027</td>
<td>24</td>
<td>10.56 (CI: 1.08 - 43.06)</td>
<td>142 (CI: 98 - 182)</td>
</tr>
<tr>
<td></td>
<td><em>Fenneropenaeus indicus</em></td>
<td>157</td>
<td>594</td>
<td>48</td>
<td>14.92</td>
<td>0.72</td>
<td>0.0020</td>
<td>43</td>
<td>37.56 (CI: 5.00 - 154.18)</td>
<td>100 (CI: 78 - 132)</td>
</tr>
<tr>
<td>CO1b</td>
<td><em>Penaeus monodon</em></td>
<td>46</td>
<td>584</td>
<td>20</td>
<td>16.77</td>
<td>0.73</td>
<td>0.0030</td>
<td>21</td>
<td>12.46 (CI: 1.10 - 52.40)</td>
<td>234 (CI: 42 - 454)</td>
</tr>
<tr>
<td></td>
<td><em>Penaeus semisulcatus</em></td>
<td>37</td>
<td>583</td>
<td>11</td>
<td>11.00</td>
<td>0.69</td>
<td>0.0018</td>
<td>12</td>
<td>8.24 (CI: 0.38 - 38.92)</td>
<td>94 (CI: 48 - 140)</td>
</tr>
<tr>
<td></td>
<td><em>Metapenaeus monoceros</em></td>
<td>55</td>
<td>586</td>
<td>24</td>
<td>17.32</td>
<td>0.85</td>
<td>0.0063</td>
<td>29</td>
<td>23.72 (CI: 1.96 - 108.62)</td>
<td>466 (CI: 186 - 752)</td>
</tr>
<tr>
<td></td>
<td><em>Parapeneopsis sculptilis</em></td>
<td>40</td>
<td>589</td>
<td>12</td>
<td>11.32</td>
<td>0.58</td>
<td>0.0014</td>
<td>11</td>
<td>6.98 (CI: 0.48 - 31.20)</td>
<td>74 (CI: 40 - 116)</td>
</tr>
<tr>
<td>CR</td>
<td><em>Penaeus monodon</em></td>
<td>86</td>
<td>517</td>
<td>83</td>
<td>23.72</td>
<td>1.00</td>
<td>0.0242</td>
<td>124</td>
<td>205.6 (CI: 45.48 - 823.08)</td>
<td>286 (CI: 176 - 802)</td>
</tr>
<tr>
<td></td>
<td><em>Penaeus semisulcatus</em></td>
<td>37</td>
<td>487</td>
<td>36</td>
<td>23.59</td>
<td>1.00</td>
<td>0.0319</td>
<td>84</td>
<td>56.40 (CI: 8.94 - 233.24)</td>
<td>694 (CI: 472 - 1320)</td>
</tr>
<tr>
<td></td>
<td><em>Metapenaeus monoceros</em></td>
<td>24</td>
<td>495</td>
<td>21</td>
<td>21.00</td>
<td>0.98</td>
<td>0.0268</td>
<td>51</td>
<td>24.94 (CI: 2.74 - 110.44)</td>
<td>1302 (CI: 652 - 1620)</td>
</tr>
</tbody>
</table>
Figure 3-10  Median-joining haplotype networks of penaeid shrimps. Letters a-e represent five species in Figure 1-2. Number of individuals and haplotypes correspond to the sample size and haplotypes for mitochondrial CO1b gene in Table 1. Four locations (i.e. A, B, C and D) along the Bangladesh coastline correspond to Figure 2-1.
Discriminant analysis of principal components (DAPC) of Bangladesh Penaeus monodon. a. with prior information and b. proportion of individuals from four locations in “a” (SB, BC, MG and SM, see Figure 2-1). Shadings (from black to white) represent the four locations, SB, BC, MG and SM, respectively.

Discriminant analysis of principal components (DAPC) of Bangladesh Macrobrachium rosenbergii. a. with prior information and b. proportion of individuals from four locations in “a” (BR, KR, MR and NR, see Figure 2-1). Shadings (from black to white) represent the four locations, BR, KR, MR and NR, respectively.

The differentiations between *M. rosenbergii* populations from the four watersheds in Bangladesh, were significant with $F_{ST}$ for haploid SNPs ranged
from 0.007 to 0.031 except for the comparisons between BR and MR, and \( \phi_{ST} \) for haploid alleles ranged from 0.030 to 0.079 and, thus, identified three distinct populations: one in BR-MR, the second in KR and the third at NR in Bangladesh-Myanmar. The DAPC analysis with prior information of the sampling sites support the three genetically distinct populations in *M. rosenbergii* in Bangladesh (Figure 3-12).

3.4 Historical demography

Historical demographic analyses revealed large current effective population sizes for prawn and shrimps in Bangladesh, which have all undergone a gradual increase for the last 74 - 466 kyr, following a bottleneck or a selective sweep. The mismatch analyses of all Bangladesh prawn and shrimps, based on mitochondrial CO1b, followed the sudden expansion model both for the SSD and the raggedness index (P > 0.05), and the expansion started with *M. monoceros* around 466 (CI: 186 - 752) kyr ago followed by *P. monodon*, *M. rosenbergii*, *F. indicus*, *P. semisulcatus* and more recently with *P. sculptilis* about 74 (CI: 40 - 116) kyr ago (Table 3-1). The expansion was further supported by Tajima’s D and Fu's Fs which were both negative and significant (P < 0.05), suggesting an expansion from a bottleneck or recovery from a selective sweep. The time to recent expansion varied among the markers, as the earliest expansion for CR started around 652 - 1620 kyr ago in *M. monoceros* followed by *P. semisulcatus* and *P. monodon* (Table 3-1). The BSP analysis revealed higher current effective population sizes (\( N_e \)) for CR than that for CO1b of the studied species, reflecting high variation between the markers. The highest effective population size for CR was observed in *P. monodon* (\( N_e = 205.6 \times 10^6 \); CI: 45.5 \times 10^6 – 823.1 \times 10^6 ind.) followed by *P. semisulcatus* and *M. monoceros*, but for CO1b the highest was in *F. indicus* (\( N_e = 37.56 \times 10^6 \); CI: 5.0 \times 10^6 – 154.18 \times 10^6 ind.) followed by *M. monoceros*, *P. monodon*, *M. rosenbergii*, *P. semisulcatus* and *P. sculptilis* (Table 3-1). The expansion of the Bangladesh *F. indicus* population started more recently than that in Sri Lanka (t = ~ 120 kyr ago) with a current \( N_e \) of 68 \times 10^6 (CI= 11.86 \times 10^6 – 242.86 \times 10^6) individuals, but the Bangladesh *P. semisulcatus* has a higher current effective population than that in Sri Lanka (\( N_e = 4.08 \times 10^6 \); CI: 0.18 \times 10^6 – 19.04 \times 10^6). The current effective population size (\( N_e \)) was highly correlated with sample size for both CO1 (\( r = 0.696 \)) and CR (\( r = 0.999 \)).
4 Discussion

This thesis contributes to two main areas of interest by identifying evolutionary significant units and management units, which are of importance in sustainable management of these species but add also to our understanding of their biology and history. The findings add furthermore to numerous studies on the phylogeography of the West Indo-Pacific region and the diversity of the studied prawn and shrimps, showing there are different mtDNA lineages or evolutionary significant units (ESUs) in different regions of the Indo-Pacific Oceans which can be linked to the east and west biogeographic zones of the Sunda-Shelf and the western Indian Ocean Province. The evidence for these phylogeographic splits varies somewhat among the species studied. A more extensive analysis of genetic markers revealed clear population differentiation or management units (MUs) in Bangladesh waters within two species.

4.1 Phylogeny and phylogeography

Phylogenetic of penaeid shrimps is still a debated issue, and the data presented here contributes significant new knowledge to our current understanding. The phylogenies of the genera Fenneropenaeus and Metapenaeus showed large discrepancies between the molecular taxonomy and the morphological and anatomical classifications. Both Fenneropenaeus and Metapenaeus geographic distributions used in this study showed polyphyletic relationships within the genera despite different geographical and taxonomic coverage. Inclusion of Bangladeshi and Sri Lankan samples in the phylogeny indicated that the Sri Lanka F. indicus is a sister taxon to Australian F. merguiensis and the divergence of Bangladesh F. indicus occurred earlier than all remaining Fenneropenaeus spp. except for F. chinensis. The phylogenetic relationships within Fenneropenaeus revealed three cryptic species (or even a species complex) within F. indicus: the first in Bangladesh and India, the second in Sri Lanka and India and the third in the western Indian Ocean (including Thailand). The Thailand sample, clustering within the western group and highly divergent from the eastern Indian Ocean, represents a geographical discrepancy. This could possibly be explained by historical currents, colonization routes or recent anthropogenic introductions from other regions either purposely for aquaculture or accidentally through transfer of ballast water. The phylogeny of the genus Metapenaeus revealed two distinct clusters: cluster I includes M. monoceros of the
eastern Indian Ocean from Bangladesh, and cluster II includes *M. monoceros* from the western Indian Ocean from Kenya and Tanzania. Individuals of *M. ensis* and *M. affinis* from China, based on 16S rRNA gene, resolve onto both clusters. Both the R_{SST} and substitution per site between the individuals of *M. monoceros* from Tanzania and Bangladesh exceed the crustacean species delimiting threshold proposed and suggest two cryptic species. The distinct lineages of *P. monodon* could also be considered as cryptic species: the first in the western Pacific ocean (lineage A), the second in the South-East Asia around the Sunda-Shelf (lineage B), the third in the western Sunda-Shelf including the Bay of Bengal region (lineage C1) and the forth in south-eastern Africa (lineage C2). Several studies, based on both mitochondrial and nuclear genes, also concluded the recent penaeid phylogeny as a controversial and debated issue (Lavery et al. 2004; Flegel 2007; Ma et al. 2011).

The overall phylogeographic patterns in the studied prawn and shrimps, based on mitochondrial DNA markers, closely follows the geographic structure in the West Indo-Pacific region, where neighbouring populations are generally more similar to each other than those sampled from further away. The Bangladesh *P. monodon* population shows the greatest similarities with the neighbouring populations in India and Sri Lanka but differs clearly from all other samples. Such a pattern was also observed in *M. rosenbergii* and *P. semisulcatus* populations from Bangladesh, which showed closest similarity with the Indian, Sri Lankan and western Thailand populations. In general, two to four phylogeographic lineages or ESUs, associated with different known biogeographic barriers, were observed for the studied species: I) the western Pacific Ocean, west of the Sunda-Shelf (includes *P. monodon, P. semisulcatus, M. rosenbergii*), II) the South-East Asia, around the Sunda-Shelf but south-east of the Isthmus of Kra (includes *P. monodon, M. rosenbergii*), III) the Bay of Bengal region, west of the Sunda-Shelf and the Isthmus of Kra (includes *F. indicus, P. monodon, P. semisulcatus, M. monoceros, M. rosenbergii* and IV) the south-eastern Africa (includes *F. indicus, P. monodon, M. monoceros*, possible *P. Semisulcatus*), coming from a different biogeographical province, the Western Indian Province (Bowen et al., 2012). An additional lineage of *F. indicus* was observed between the Bay of Bengal and the western Indian Ocean, in India and Sri Lanka.

Biogeographic events related to the Sunda-Shelf are known to have affected diversification of marine organisms in the Indo-West Pacific region (Gopurenko et al. 1999; Tsoi et al. 2007), and have been proposed to explain the splits (among South Pacific, eastern Australia and south-east Asia) and a split (between eastern Indian Ocean and western Pacific) within *P. monodon* as the two oceans were completely separated at certain times during the Tertiary and Pleistocene periods (Waqairatu et al. 2012). The oldest divergence within *P. monodon* (estimated as
the most recent common ancestor for all lineages), based on the mutation rate for the CR, predates the main temperature fluctuations and sea level changes, which started around 5.6 Mya or at the onset of the Pleistocene, when the Indian and Pacific Oceans were completely separated with extensive land bridges (Voris 2000; Benzie et al. 2002). An admixture of all lineages may have occurred around the Sunda-Shelf in Indonesia, Thailand, Vietnam and China, when the Indian and Pacific Oceans connected through the Sunda-Shelf or even after the last glacial period (Bird et al. 2005). The admixture appears to have been directional, as lineage C of *P. monodon* from the Bay of Bengal area, rather than from the distinct African populations as proposed by Benzie et al. (2002), may have migrated eastwards through the region, but only a small number of lineage A individuals have been found west of the region, in western Thailand and Sri Lanka. Baldwin et al. (1998) concluded that the *Penaeus* genus originated in the center of the Indo-West Pacific region and migrated eastwards and westwards forming two groups, in the eastern Pacific and the Indian Ocean. Such divergence has been reported for other Indo-West Pacific species, e.g. in mud crab (*Scylla serrata*; Forskal 1775) (Gopurenko et al. 1999), kuruma shrimp (*Penaeus japonicus*; Bates 1888) (Tsoi et al. 2007). The Sunda-Shelf may not be the only geographic barrier in the Indian Ocean. The large split between the African samples and the samples from India, Sri Lanka and Bangladesh might have occurred due to large geographical distances, and due to different surface and subsurface equatorial ocean currents in western Indian Ocean (Pidwirny 2006). The distinct phylogeographic lineages (eastern, central and western) of *M. rosenbergii* are also associated with the Sunda-Shelf and Isthmus of Kra (De Bruyn et al. 2005; Hurwood et al. 2014). A strong concordance between intraspecific and biogeographic boundaries have also been reported for several other marine species (Ewers-Saucedo et al. 2016; Altman et al. 2013; Dawson 2001; Wares 2002; Pelc et al. 2009; Haye et al. 2014).

### 4.2 Genetic diversity and population structure

Bangladesh prawn and shrimps are characterized by high gene diversity with a shallow genealogy. Mitochondrial CR in *P. monodon*, *P. semisulcatus* and *M. monoceros* reveals high degree of variation where almost all individuals carry a unique haplotype. High genetic diversities have also been reported in *P. monodon* populations from the Indo-West Pacific region (You et al. 2008; Zhou et al. 2009; Waqairatu et al. 2012; Mkare et al. 2014). Mitochondrial CO1 barcode gene also reveals high gene diversity, but not as high as for CR, with the highest diversity in *M. monoceros* followed by *P. monodon*, *F. indicus*, *M. rosenbergii*, *P. sculptilis* and *P. semisulcatus*, which also reflects the size of
wild catch from Bangladesh waters (Quader 2010; Department of Fisheries Bangladesh 2013). Variation in microsatellites and SNPs also revealed high genetic diversity (i.e. observed and expected heterozygosities) in Bangladesh *P. monodon* and *M. rosenbergii*, with a slight deficiency of heterozygotes in *P. monodon*. Genetic variation estimated using microsatellites was high, and characterized by a large number of private alleles. Similar genetic variability was reported in *P. monodon* sampled from India (Mandal et al. 2012), Malaysia (Aziz et al. 2011) and the Indo-West Pacific region (You et al. 2008). In general, most marine decapods, including penaeids, have shown high microsatellite diversity (Benzie et al. 2000), reflecting large population sizes. Variation at the SNP sites within *M. rosenbergii* populations did not deviate from the expectation of neutral evolution and did not show any deviation from random mating within populations.

The distribution of the mtDNA variation along the Bangladesh coast does not provide any evidence of population structure. A single population in Bangladesh based on mtDNA variation was observed in *F. indicus*, characterized by a large number of closely related haplotypes that form a previously undescribed divergent lineage in the Indo-Pacific region. Single panmictic population was also observed in *P. monodon*, *P. semisulcatus*, *M. monoceros* and *P. sculptilis* sampled along the coast, except for Sri Lanka *P. semisulcatus* and Bangladesh *M. rosenbergii*. Mitochondrial markers were unsuccessful to detect population structure in most cases, possibly due to lack of statistical power. The shallow genetic networks and the corresponding low nucleotide diversity in Bangladesh prawn and shrimps may have resulted from population recovery after reduction in population size (bottleneck) due to climate change, overfishing, range expansion after colonization (e.g. Hewitt 1996) or recovery from reduction in effective population size due to natural selection.

*Penaeus monodon* sampled from four locations along the Bangladesh coastline of the Bay of Bengal, based on microsatellites and SNP variation, revealed three distinct populations: one in the east in the mangrove forest of the Sundarban-Barguna coast (south Bangladesh), the second at the Sundarban-Middle ground in the Bay of Bengal and the third at St. Martin’s island in south eastern Bangladesh. The population samples from both Middle ground and Barguna coast had a certain level of similarities with Sundarban samples, indicating ongoing gene flow among these three locations, which may have resulted via the large mangrove forest, Sundarban, which is a major nursery ground for shrimps (Hoq 2007; Ghosh et al. 2015). Previous studies revealed genetic structure in *P. monodon* sampled from the Bay of Bengal (Mandal et al. 2012), Malaysia (Aziz et al. 2011), the Gulf of Thailand (Tassanakajon et al. 1998) and Australia (Li et
al. 2007). *Macrobrachium rosenbergii* sampled from four major rivers in Bangladesh, based SNP and allele variation obtained from ddRAD sequencing, revealed three distinct populations: first, in the Naf river in between Bangladesh and Myanmar, the second in the Bishkhali and Meghna rivers and the third in the Karnaphuli river. These findings differ from a previous genetic diversity study on *M. rosenbergii* sampled from the Pashur and the Païra rivers, South-West Bangladesh and the Naf river, South-East Bangladesh performed by Khan et al. (2014), based on seven microsatellites, where no differentiation was observed between the sampling sites. The population sampled in the Meghna river showed highest similarity with the samples from the Karnaphuli river, indicating connectivity and ongoing gene flow between these two river estuaries either due to natural through possible migrations of larva, juveniles and adults or human-mediated mixing.

### 4.3 Demography

Bangladesh prawn and shrimps have a large current effective population size ($N_e$), reflecting a large real population ($N$) which is often about 1000 times of $N_e$ (Frankham et al. 2010). The estimation of $N_e$ and the subsequent census population should though be taken with caution as $N_e$ is highly correlated with the sample size and varies with species (Frankham 1995 and this study). Mitochondrial CR and CO1b present different estimation of $N_e$, but the 95% confidence intervals overlap the estimated $N_e$ for both markers. Bangladesh prawn and shrimps follow the prediction of a sudden expansion model, which started during the late Pleistocene (74 - 234 kyr ago) or even earlier as in *M. monoceros* (466 kyr ago), with an expansion from a bottleneck or recovery from a selective sweep. The expansion from a bottleneck event is also supported by the negative and significant Tajima’s D and Fu’s Fs, and the BSP analysis. The Bangladesh *F. indicus* and *P. semisulcatus* populations started expansion more recently than the Sri Lanka population. Hewitt (2004) reported that the effects of Ice Ages on organisms varied with latitude and topography. As Bangladesh is located at a higher latitude than Sri Lanka, and in the vicinity to the Himalaya Mountains, its genetic diversity may have been more affected by climatic and glacial fluctuations. Bangladesh may have experienced a series of abrupt oscillations during the cold periods of the last Ice Ages between 40 and 80 kyr ago (Schmidt & Hertzberg 2011). This time estimate may though be an overestimate due to a possible admixture of distinct mtDNA clusters, as in Grant et al. (2012) who demonstrated also how declines in population size may eradicate historical information; thus the interpretation of estimates of demographic changes should be taken with caution.
To conclude, this study provides an increased knowledge on the phylogenetics and phylogeography of penaeid shrimps in the Indo-West Pacific region, including insights about the origin, genetic variation, population structure and historical demography of major prawn and shrimps in Bangladesh. Mitochondrial DNA based phylogeny revealed three cryptic species in *F. indicus*. Phylogeography revealed an additional split in the western Indian Ocean between the Bay of Bengal and the south-eastern Africa, in addition to, the known biogeographic barriers i.e. the Sunda-Shelf and the Isthmus of Kra, with an additional lineage in *F. indicus* from India and Sri Lanka. Both mitochondrial and nuclear markers revealed high genetic diversity in the studied prawn and shrimps from Bangladesh, despite the shallow mitochondrial genealogy, reflecting a large population and no indication of depletion in the stock. Mitochondrial markers were unsuccessful to detect population structure in the studied prawn and shrimps, except for *P. semisulcatus* from Sri Lanka and the some extent of differentiation in *M. rosenbergii* between the Bishkhali river with the Naf and the Karnaphuli rivers. Variation in microsatellites and immune-related SNPs identified three distinct populations in *P. monodon* sampled from four locations along the Bangladesh coastline. Sundarban is identified as an important nursery ground used by the *P. monodon* postlarva from two neighbouring populations, Barguna coast and Middle ground of the Bay of Bengal. Immune-related genes did not show any evidence of natural selection in the wild *P. monodon*, indicating no sign of viral infections. Variation in SNPs and alleles obtained from ddRADseq also detected three distinct populations in *M. rosenbergii* sampled from four watersheds in Bangladesh.

The phylogeographic lineages identified in this study should be considered as evolutionary significant units (ESUs) or conservation units, which need regional cooperation for conservation and management. The use of individuals stocked in aquaculture should take into account the phylogeographic patterns in order to maintain the large diversity within the species, which could harbour diverse adaptations in different regions, and can be useful for different breeding programs and for coping with environmental change such as climate change. The information about genetic diversity can be used to monitor and sustain the harvest of prawn and shrimps in the region. The populations detected in Bangladesh *P. monodon* and *M. rosenbergii* should be considered as separate management units (MUs) with respect to guidance of fisheries of these different units. The sequences from this study have been deposited in GenBank and can be used to trace an unknown catch and control illegal fishing of shrimps by the international fishing trawlers. Further studies for the revision of the taxonomic
status of the penaid shrimps, including more extensive geographic sampling and other genetic markers, are required to resolve the controversies in the penaeid shrimps phylogeny. Like as *P. monodon* and *M. rosenbergii*, other prawn and shrimps from Bangladesh should be studied using nuclear markers (i.e. SNPs) to identify putative management units. Genetic variation should also be assessed in aquaculture populations to detect possible selective effects due to pathogenic infections and selective breeding.
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