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Phylogeography of Weaver Ant, Oecophylla smaragdina, in Bangladesh (Hymenoptera: Formicidae)

モハメッド マムヌール ラーマン

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Phylogeography of weaver ant, *Oecophylla smaragdina,* in Bangladesh (Hymenoptera: Formicidae)

Md. Mamunur Rahman

2018

Phylogeography of weaver ant, *Oecophylla smaragdina,* in Bangladesh (Hymenoptera: Formicidae)

by

Md. Mamunur Rahman

A dissertation submitted to the Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Japan

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ABSTRACT

The weaver ant species, *Oecophylla smaragdina,* is widely distributed from India through Southeast Asia to northern Australia including many tropical Western Pacific islands. The ant is arboreal observed in natural forests, fruit orchards and homestead woodlands, and is one of the important biological control agents. A recent phylogenetic study of *O. smaragdina* showed that the species is divided into 7 groups based on mtDNA and that the Bangladesh populations belong to SE Asian mainland clade despite of its geographical proximity to India. However, the samples analyzed from Bangladesh were limited and distribution pattern of Bangladeshi populations was not clearly presented.

The present study aims to reveal the phylogeographic aspect of *O. smaragdina* in Bangladesh including phylogenetic analysis, haplotype network analysis and divergence time estimation as well as hybridization detection based on extensive materials. The sampling was executed according to zonation of 5 areas which are demarcated by 3 main rivers during the years 2013 to 2017. Ninety five colonies from 87 localities of 47 Districts were collected. The molecular analyses were carried out using 2 mitochondrial loci: Cytochrome b oxidase subunit 2 (Cytb) consisting of 507 bp and Cytochrome c oxidase subunit I (COI) consisting of 639 bp. The possibility of occurring hybridization of *O. smaragdina* in the zone of contact was examined by using LW *Rh* of nuclear DNA and microsatellite markers.

The phylogenetic analysis of 84 colonies revealed that 47 are the Indian clade (Indian type) and 37 are the SE Asian mainland clade (SE Asian type). The distribution of mtDNA types showed that the western parts of Bangladesh is predominantly occupied by the Indian type, whereas, the eastern part was dominated by the SE Asian type, and the central parts is the mixture of both types. The haplotype network analysis indicated that a total of 25 haplotypes

were identified in Bangladesh based on COI genes, comprises 13 and 12 of Indian and SE Asian types, respectively. The divergence time analysis in Bangladesh populations, resulted that Indian type diverged ca. 2.2 Ma and SE Asian type diverged ca. 0.20 Ma corresponding to early to late Pleistocene. Two microsatellite markers, MS 6.45 and MS 8.24 possibly detected identical allele between Indian and SE Asian types of *Oecophylla* population in Bangladesh. Nuclear gene sequences from LW *Rh* region showed inconsistency of Indian and SE Asian types from two colonies of the overlapped zone. Discordance between mitochondrial and nuclear DNA genes suggests the possibility of hybridization in the zone of contact.

The results of the study suggest a scenario of dispersal: 1) before Last Glacial Maximum (LGM), the haplotypes observed in Bangladesh would be already established; 2) during the LGM, the Bengal Delta supposed to be vacant for *Oecophylla smaragdina* distribution because the northern limit of the species would be located down to the south; 3) after the LGM, the isolated populations in southern refuge areas of India and SE Asia extended to south, and in Bengal Delta the Indian type populations came from west and the SE Asian type populations from east; 4) when the Indian type populations and the SE Asian type populations contacted in the Bengal Delta, each of the types already contained several haplotypes of mtDNA, and reproductive isolation is not established.

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

General Introduction

1.1 Background of the study

Phylogeography is comparatively a new discipline that deals with the spatial arrangement of genetic lineages, among and within the closely related species (Avise, 2009). The study of phylogeography combined with classical population analyss deals with historical, phylogenetic components of the spatial distributions of gene lineages, distinguishes between vicariant allopatric divergence, long-distance dispersal or expansion events and investigates the effects of recurrent gene flow in shaping geographical molecular variation (Hewitt, 2004). Mitochondrial DNA (mtDNA) has been widely used in phylogenetic studies, because it evolves rapidly and provides an abundance of genotypic characters, either by restriction fragment analysis or amplification by PCR and subsequent nucleotide sequencing (Randi et al. 2001). The mitochondrial Cytochrome b (Cytb) gene and Cytochrome oxidase subunit I (COI) gene were used in many phylogeographic studies, since this region seems to has a proper substitution rate for analyzing intra species phylogeny of Hymenoptera and some insect-specific oligonucleotide primers are already designed for this gene (Patwardhan et al. 2014). Mitochondrial DNA, in conjunction with morphological data and nuclear-gene (e.g., allozyme) studies, can be a powerful tool for detecting hybridization (Dowling et al. 2015; Dowling and DeMaris, 1993). In addition, comparisons of nDNA and mtDNA suggest that interspecific hybridization has been a persistent feature in the history of this group.

Oecophylla (Hymenoptera, Formicidae) is the weaver ant and is a broadly distributed genus. Weaver ants constitute the genus *Oecophylla* (Formicidae, Formicinae) which consists of only three extant species, *O. longinoda*, *O. smaragdina* and *O. kolhapurensis*. *Oecophylla. longinoda* (Latrelille, 1802) is distributed in tropical and subtropical Africa and *O. smaragdina* (Fabricius, 1775) in southeastern Asia and Australia including many tropical western pacific island (Bolton, 1995). The recently identified species *O. kolhapurensis* is distributed in western India (Kurane et al. 2015). *Oecophylla* originated in the early Paleogene of the Palaearctic realm, radiating strongly during the climatic changes of the Eocene–Oligocene transition (Dlussky et al. 2008). *Oecophylla smaragdina* and *O. longinoda* were estimated to be diverged at 13.3-11.3 Ma, while the diversification within the groups of *O. smaragdina* was estimated between 7.8 to 3.6 Ma, from the middle Pliocene to early Pleistocene (Azuma et al. 2006). Since *O. smaragdina* is an arboreal species, inseminated queens lifted by the wind than terrestrial ants. Rafting has been considered effective for between-island dispersal of several species of insects (Thornton, 1996), and weaver ants construct light and waterproof nests of leaves which appear to be a preadaptation for rafting dispersal.

Azuma et al. (2002) first analyzed populations of *O. smaragdina* using molecular data and samples of *O. smaragdina* from Bangladesh. Including additional populations of *O. smaragdina* from India, Southeast Asia and Australia, Azuma et al. (2006) proposed an outline of the phylogeography of *O. smaragdina* and categorized the sampled populations into 7 major clades: group 1 from India; group 2 from Southeast Asian mainland including the Indochinese and Malayan Peninsula as well as the Greater Sunda Islands; group 3 from the Philippines; group 4 from Flores; group 5 from Sulawesi; group 6 from Halmahera; group 7 from Australia and New Guinea. Hereafter, I call their group 1 as the Indian type and group

2 as Southeast Asian type. Asaka (2010) extended the survey of *O. smaragdina* to South Asia, and collected several samples from India and Sri Lanka. Her phylogenetic analysis showed that all analyzed samples belong to Indian type with low levels of sequence divergence (Fig. 1.1).

Based on those data, Bangladesh is considered a major transition zone between Indian and Southeast Asian populations as Bangladesh populations of *O. smaragdina* is identified as SE Asian types. This is the unique case of population boundaries without any distinguished geographical borders (e.g., deep sea or high mountains) although the seven groups of *O. smaragdina* based on haplotype grouping by Azuma et al. (2006) are geographically bordered by the sea, except the group 1 and group 2 (Fig. 1.2). The major rivers are considered as the geographical border between India and Bangladesh, and as all Indian isolates are found to be Indian type (Asaka, 2010; Azuma et al. 2006), Bangladesh is a major focusing area to find out the border between Indian and SE Asian types. As in Bangladesh, 3 main rivers Ganges, Jamuna and Meghna originated during Pleistocene and crisscrossed throughout the mostly flat territories of the country, these rivers might have some influence of separating the Indian type and the SE Asian type in Bangladesh.

1.2 Problem statement

Azuma et al. (2006) characterized the mitochondrial sequence identity of the Bangladesh populations as the Southeast Asian clade in spite of geographical proximity of Bangladesh to India. So, there might be some barriers of Indian type to reach into Bangladesh, although no such evidence was found. Moreover, Bangladesh is a plane land area with the 3 big rivers

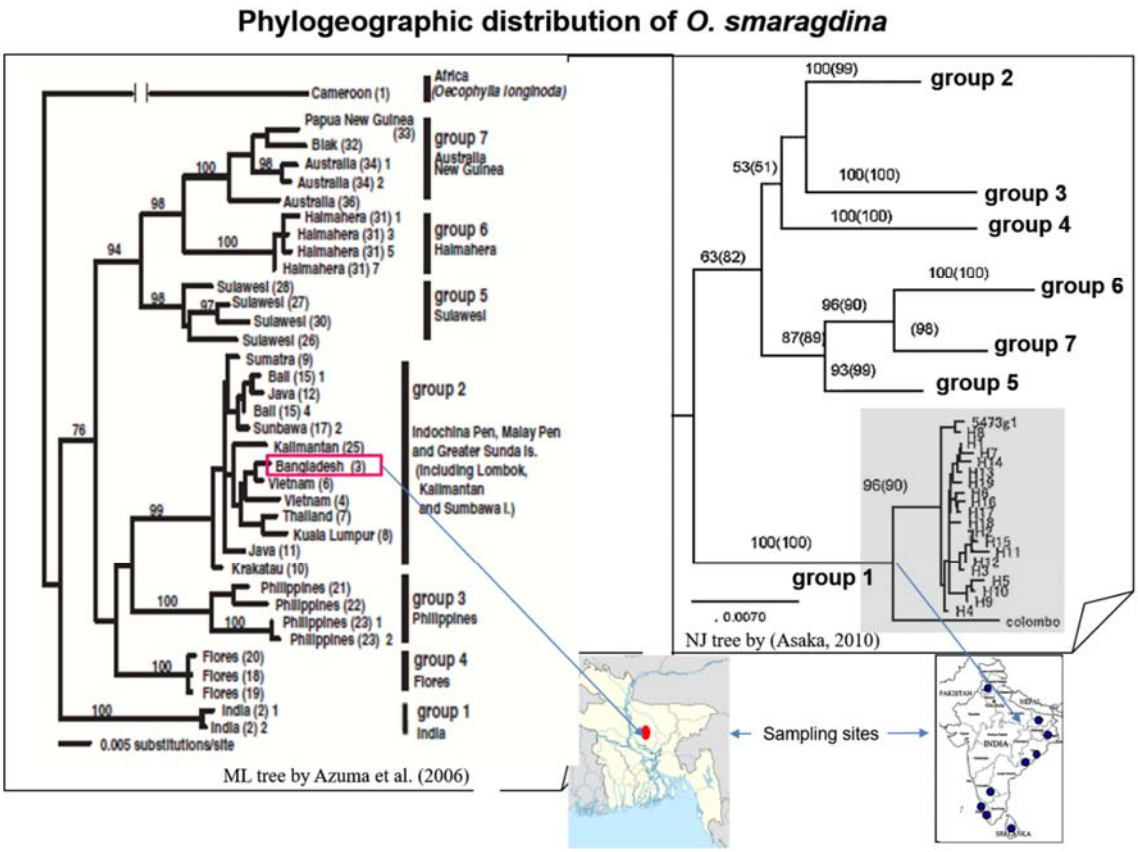


Fig.1.1 Summary of the phylogeographic study of *O. smaragdina* by Azuma et al. (2006) and Asaka (2010)

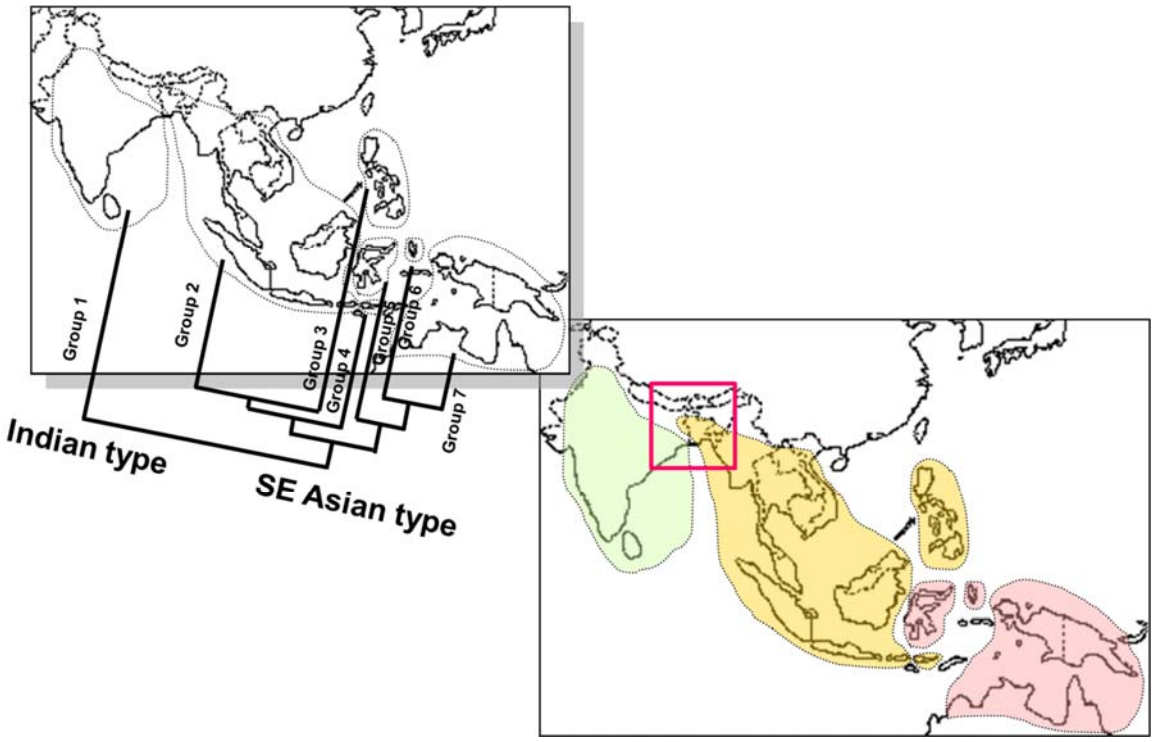


Fig.1.2 Phylogeography of population groups of weaver ant, *O. smaragdina* with reference to geography modified from Azuma et al. (2006).

separated the Bangladesh geography from India. The river might be the barrier but no such a study was carried out before. On the contrary, rafting method of dispersal along with the nuptial flight is very common in *O. smaragdina*. So, barrier effect along with the geological history needs to be considered with a strong evidence which is also lacking. Azuma (2006) analyzed the Bangladesh sample from just one locality i.e., Nurbag. However, for getting the actual phylogeographic history, it is necessary to analyze the samples from different parts of the country more comprehensively. Data on the hybridization scenario of Indian and SE Asian types of *O. smaragdina* in this subcontinent was also lacking. Therefore, with this study, can have a glimpse of the phylogenetic study as well as the hybrid scenario of *O. smaragdina* in Bangladesh.

For checking those 3 rivers, Ganges, Jamuna and Meghna as borders, *O. smaragdina* was collected from 5 broadly categorized area based on three main rivers for phylogeographic analysis. From the analysis if it is found that all populations belong to Indian type, then it can be said that the previous results of Azuma et al. (2006), who showed that the *Oecophylla* population from area 3 was SE Asian type, will be exceptional.

For analyzing the existing border, if the collected sample from area 1 and area 2 is found SE Asian type, then it can be stated that the river Ganga will be the possible border for separating the two populations. If area 1 and area 2 denotes Indian Populations but area 3, area 4 and area 5 present the SE Asian type of population, then it can be said that, the river Jamuna is the border. If, area 1, area 2 and area 3 found Indian type but area 4 and area 5 as SE Asian type, then there is the possibility of Meghna river as existing border. If all populations from the 5 area found SE Asian type, then it will be clear that the river Ganga is the border as this river separating this two countries. If all population from the 5 area are found to be Indian type, then it can be said that the border is not existing in Bangladesh. It

may be the Arakan mountains that separate Bangladesh from Myanmar and also the two populations of *Oecophylla* as well.

1.3 Objectives of the study

The present study aims to show an evolutionary aspect of the distribution of *O. smaragdina*. The first purpose is to test whether all the *O. smaragdina* populations in Bangladesh belong to SE Asian type or not. If not, the question arises where the boundary of SE Asian type and Indian type is. In other word, the second purpose of the study is to depict the distribution pattern of types at mtDNA level. This could be clarifying the limitation of the SE Asian type distribution in westward. Additionally, the hybridization of SE Asian and Indian types will be verified. Finally the study attempts to propose the probable cause of the revealed distribution pattern of *O. smaragdina* in Bangladesh.

1.4 Outline of the dissertation

This doctoral dissertation combines 6 chapters. In **Chapter 1**, the background, justification, hypothesis and objectives of the research are presented. **Chapter 2** included some previous studies and their finding regarding the Phylogeography of *O. smaragdina*. **Chapter 3** focuses on the general methodology of conducting the several experiment for phylogeographic study. **Chapter 4** sheds light on the phylogenetic study, distribution pattern and haplotype network analysis of *O. smaragdina* in Bangladesh. **Chapter 5** describes the divergence time and the hybridization scenario of the two types of *Oecophylla smaragdina* in Bangladesh. **Chapter 6** represents general discussion and the comprehensive conclusion of the study. **The last parts** outline the acknowledgement, references and appendix.

CHAPTER 2

REVIEW OF THE PREVIOUS STUDY

CHAPTER 2

Review of the previous study

2.1 Phylogeographic study

The study of phylogeography combined with classical population genetics deals with historical, phylogenetic components of the spatial distributions of gene lineages, distinguish between vicariant allopatric divergence, long-distance dispersal or expansion events and investigate the effects of recurrent gene flow in shaping geographical molecular variation (Hewitt, 2004).

In molecular phylogeny, the relationships among, usually extant, organisms along with the modified total DNA content due to evolution are examined by comparing homologous DNA or protein sequence. Phylogeography offers the tools to shed light on the evolutionary relationship among different taxa with reference to the structure of population genealogies by demographic history. Moreover, it can also make inferences about temporal changes in the physical and biotic environment of a population using present day genetic data (Beheregaray, 2008).

2.1.1 Mitochondrial DNA analysis

Mitochondria, the powerhouse of a cell plays a crucial role in respiration, genetic illness, aging and self-destruction of a cell (Faure and Casanova, 2006). The genetic material in mitochondria, the mitochondrial DNA (mtDNA) contains genes involved in production of enzymes for oxidative phosphorylation and protein synthesis. Mitochondrial DNA (mtDNA)

has been widely used in phylogenetic studies, because it evolves rapidly and provides an abundance of genotypic characters, either by restriction fragment analysis or amplification by PCR and subsequent nucleotide sequencing (Randi et al. 2001). Mitochondrial genome sequence and structure provide evolutionary and comparative genomics information as well as information on molecular evolution, and patterns of gene flow, phylogenetics and population genetics resources. Like other animals, insect mitochondrial genome is a double stranded molecule consists of 37 genes encoding the large and small subunit ribosomal RNAs, 22 transfer RNAs (trnI, trnQ, trnM, trnW, trnC, trnY, trnL1, trnK, trnD, trnG, trnA, trnR, trnN, trnS1, trnE, trnF, trnH, trnT, trnP, trnS2, trnL2, trnV) necessary to translate the protein-coding genes and 13 protein coding genes that are all components of the oxidative phosphorylation process (Faure et al. 2011).

In phylogenetic study, mitochondrial DNA has many advantages. They possess strict maternal transmission with high mutation rate due to limited repair system (5-10 times that of nuclear DNA) (Mauro et al. 2006) and conserved simple structure. These unique properties allow the development of universal primers and easy recovery from small or degraded biological sample due to its high copy number in most cells with a different evolution rate in different regions of mitochondrial DNA. This fact facilitates the monitoring of its transmission along the evolutive lines starting in the early evolution. In the case that one individual is not available for a direct comparison with a biological sample, any sample which comes from the maternal genitor can be a good and usable one (Mauro et al. 2006).

The mitochondrial cytochrome b (Cytb) gene and cytochrome oxidase subunit I (COI) gene were used in many phylogeographic studies, since this region seems to have a proper substitution rate for analyzing within-species phylogeny of Hymenoptera and some insect-specific oligonucleotide primers are already designed for this gene (Patwardhan et al. 2014).

The enzyme cytochrome c oxidase I is a very well known protein of electron transport chain and is found in both bacteria and mitochondria. The COI and COII genes code for two of seven polypeptide subunits in the cytochrome c oxidase complex. The COI gene consists of approximately 894 bp. COI and/or COII sequences have been applied to phylogenetic problems at a wide range of hierarchical levels in insects, from closely related species to genera and subfamilies, families, and even orders. The COI gene is slowly evolving compared to other protein coding mitochondrial genes and is widely used for estimating molecular phylogenies and is a good performer in recovering an expected tree (Rawlings et al. 2001). Sequencing both the genes represents one of the largest sequence data sets generated for phylogenetic study of any group and also fulfils the putative phylogenetic accuracy. COI has recently been suggested as a potential 'barcode' for insect identification in general.

Cytochrome-b gene (~1,143 bp) is reported as the most useful marker in recovering phylogenetic relationships among closely related taxa but it can lose resolution at deeper nodes (Randi et al. 2001). Although the cytochrome-b gene has proven useful in recovering phylogenetically useful information at a variety of taxonomic levels, strength of its utility can be lineage-dependent and declines with evolutionary depth. Study of the haplotype network based on mitochondrial DNA nucleotide sequences and estimation of time of divergence were also very important for accurate tracing of the phylogeographic study of weaver ant population.

2.1.2 Nuclear DNA for detecting hybridization

Genetic variation within maternal lineages as reflected by mitochondrial DNA (mtDNA) is commonly used to trace ancient population migrations, demographic history, phylogeography and phylogeny. Since in vertebrates mtDNA population genetic analyses is confined to tracing the migration patterns of maternal lineages, a more complete picture of

population genetic structure requires analysis of nuclear DNA (nDNA)-encoded markers inherited from both parents. Moreover, being maternally inherited, the mtDNA population diversity reflects maternally directed natal site fidelity, whereas genome-wide bi-parentally inherited nDNA markers assist in quantifying levels of gene flow among subpopulations for both sexes (Bar Yaacov et al. 2012). Ancient hybridization would be most evident in comparisons among phylogenies based on independent character sets, such as mtDNA, allozymes, specific nuclear-gene trees, or morphology (Dowling and DeMaris, 1993). Biogeographical evidence would often support these inferences, for example, changes in distributions associated with Pleistocene glaciation (Dowling and DeMaris, 1993). However, the more likely outcome would be that the bifurcation between the hybridizing species would simply appear more recent than it actually was, because the hybridization would most likely involve sister taxa. Several points need to be considered with regard to hybridization. Hybridization is a problem in phylogenetic analysis only if it goes undetected. If hybridization is apparent, its effects can be taken into account and its occurrence incorporated into the historical narrative of the group as it should be. After all, the purpose of the phylogenetic analysis is to determine the evolutionary history of the group, which may have involved reticulate as well as cladistic events. For detecting the hybridization phenomenon, distribution of mitochondrial haplotypes must be compared with those of nuclear genes as only mt DNA cannot detect (Dowling and DeMaris, 1993). However, the nuclear genes need not be explicitly known Mitochondrial DNA, in conjunction with morphological and nuclear-gene (e.g., allozyme) studies, can be a powerful tool for detecting hybridization (Dowling et al. 2015; Dowling and DeMaris, 1993). More generally, hybridization results in intermediacy of traits determined by nuclear genes and increased variance because the genetic determinants assort independently. The nDNA phylogeny, along with preliminary evidence from amplified fragment length polymorphism data corroborates hypothesis of multiple invasions, providing

additional details that elucidate the patterns and processes of speciation in Hawaiian Lauapla. In addition, comparisons of nDNA and mtDNA suggest that interspecific hybridization has been a persistent feature in the history of this group.

2.1.3 Microsatellite study for determining heterozygosity

Microsatellites, also called short tandem repeats (STRs) or simple sequence repeats (SSRs), are sequential repeats of 1 to 6 base pair motifs that have been used as genetic markers. Microsatellites have been used to measure population differentiation and hybridization (Butler et al. 2014), to investigate ploidy levels and to reconstruct parentage and pedigrees in wild and domestic populations (Steiner et al. 2011). Microsatellites are comparatively cheap to genotype and can be used with low concentrations of DNA. Furthermore, they typically have more alleles per locus than single nucleotide polymorphisms (SNPs) and thus provide more information per locus (Kronauer et al. 2011). In this study, we have conducted microsatellite analysis for determining the heterozygosity and finding the identical alleles. Azuma et al. (2004) characterized the 13 microsatellite loci for the *O. smaragdina,* which was found to be useful to analyze the genetic structure of *Oecophylla* species at both the colony and population levels.

2.1.4 Phylogenetic tree for inferring phylogeography

Phylogenetic tree is used for representing the phylogeographic relationship of groups of organism. The tree is a simple object consisting of two elements: nodes and branches. Branch is a line that connects two nodes. Bootstrap analyses are performed to test the support for branches of a phylogenetic tree. Mainly four primary methods are used for constructing phylogenetic tree. They are distance method (of which Neighbor-Joining is currently the favored implementation), maximum parsimony (MP), maximum likelihood (ML) and

Bayesian (BAY) method. Haplotype network is also used to see the correlation pattern raised in phylogenetic tree. This network represents the relationships among the different haploid genotypes observed in the dataset (ie. identical sequences are pooled into a single terminal). They are usually drawn unrooted, which is quite sensible for within-species data, where the root location is often unknown. Bayesian phylogenetic methods were introduced in the 1990s and have since revolutionized the way we analyse genomic sequence data (Ronquist and Huelsenbeck, 2003). Examples of such analyses include phylogeographic analysis of virus spread in humans (Wilfert et al. 2016), inference of phylogeographic history and migration between species, analysis of species diversification rates (Hoorn et al. 2010), divergence time estimation and inference of phylogenetic relationships among species or populations. The popularity of Bayesian methods seems to be due to two factors: (1) the development of powerful models of data analysis; and (2) the availability of user-friendly computer programs to apply the models. In phylogenetics, the tree topology and the substitution model together specify the statistical model for the data. Different tree topologies thus correspond to different models, while the branch lengths or divergence times as well as the substitution parameters (such as the transition/transversion rate ratio) are parameters in the model. The data are usually a molecular sequence alignment or an alignment of morphological characters (or a combination of both). An appealing property of Bayesian inference is that it makes direct probabilistic statements about the model or unknown parameter.

2.1.5 Phylogeographic feature in the study at East Asia

Recently, a great number of phylogeographic studies have been pubslished on insects, plants amphibians, birds, fishes, and several mammals. The result of those study mainly focused on the importance of Pleistocene climate changes and biogeographic barrier such as mountains, rivers, seas and deserts for the diversification, radiation and isolation of the genetic lineage within the species (Riddle et al. 2000). Besides the phylogeographic study (Asaka, 2010; Azuma et al. 2006, 2002) on *O. smaragdina*, several studies on Asian elephants denoted some phenomenon. Vidya et al. (2009) reported endangered Asian elephant (*Elephas maximus*), which revealed two highly divergent mitochondrial DNA (mtDNA) lineages, an elucidation of which is central to understanding the species's evolution. They identified several clades based on the divergence history of Asian elephants and suggested a contraction–expansion scenario during severe climatic oscillations of the Quaternary, with range expansions from different refugia during warmer interglacial leading to the varying geographical overlaps of the two mtDNA clades. Brandon-jones (1996) reported similar trends of declining the *Oecophylla* populations and other mammals after Pleistocene glaciation. Their study also discussed the possibility of variation of the *Oecophylla* types between Indian and Bangladesh. He described the divergence pattern between the Indian and Bangladeshi types might be due to a combination of submergence of lowlands or islands, deforestation caused by cold and drought in inland Asia, and other environ-mental changes.

2.2 Taxonomy, distribution and divergence history of *Oecophylla*

2.2.1 Taxonomy of *O. smaragdina*

The genus *Oecophylla* was established by F. Smith in 1860 including two species, *Formica smaragdina* Fabricius and *Formica virescens* Fabricius. Subsequently Bingham (1903) designated *Oecophylla virescens* as type species. The genus is distinct in having triangular mandibles with long apical tooth, palp formula of 5 maxillary and 4 labial, elongate petiole with a low node as well as aggressive behavior and arboreal nesting habit. The latest phylogenetic study (Blaimer et al. 2015) showed that *Oecophylla* is the sister group of *Gesomyrmex*, and that the two genera were diverged in 70 to 75 Ma. Presently, 3 names of species are recognized as valid.

(1) *Oecophylla smaragdina* (Fabricius, 1775)

Formica smaragdina Fabricius, 1775: 828. Type locality: India

Combination in *Oecophylla*: Smith 1860: 102

Formica virescens Fabricius, 1775: 392. Type locality: Australia

Combination in *Oecophylla virescens*: Smith, 1860: 102.

As subspecies of *Oecophylla smaragdina*: Emery, 1887: 242; Forel, 1915: 95; Wheeler, 1922: 228, Emery, 1926: 52.

Junior synonym of *Oecophylla smaragdina*: Mayr, 1872: 143, Taylor & Brown, 1985: 127.

Formica viridis Kirby, 1819: 478. Type locality: Australia

Junior synonym of *Oecophylla virescens*: Roger, 1863:10; Dalla Torre, 1893: 177; Emery, 1925: 52; of *Formica smaragdina*: Smith, 1857: 53; of *Oecophylla smaragdina*: Taylor & Brown, 1985: 127.

Formica macra Guérin-Méneville, 1831: pl. 8, fig.1. Type locality: Indonesia

Junior synonym of *Oechopylla virescens*: Smith 1858:29; of *Oecophylla smaragdina*: Roger, 1863:10; Dalla Torre, 1893: 176; Arnold, 1922: 609.

Formica zonata Guérin-Méneville, 1838: 295. Type locality: "Port Praslin", Australia.

Junior synonym of *Oechopylla smaragdina*: Roger, 1863:10; Dalla Torre, 1893: 176.

(2) *Oecophylla longinoda* (Latreille, 1802)

Formica longinoda Latreille, 1802: 184. Type locality: Senegal

Combination in *Oecophylla*: Mayr, 1863: 439.

Junior synonym of *Oecophylla virescens*: Smith, 1858: 29.

Subspecies of *Oecophylla smaragdina*: Emery, 1892: 564; Forel, 1907: 15; Santschi, 1914: 128; Santschi, 1919: 345; Emery, 1925: 52; Prins, 1965: 77.

Status as species: Dalla Torre, 1893: 176; Emery, 1921: 102; Wheeler, 1922: 227; Santschi, 1928: 211; Bolton, 1995: 298.

Oecophylla brevinodis André, 1890: 313. Type locality: Sierra Leone.

Subspecies of *Oecophylla longinoda*: Dalla Torre, 1893: 176; of *Oecophylla smaragdina*: Stitz, 1916: 396.

Junior synonym of *Oecophylla longinoda*: Wheeler, 1922: 945.

(3) *Oecophylla kolhapurensis* Kurane, Bhoje & Sathe, 2015

Oecophylla kolhapurensis Kurane, Bhoje & Sathe, 2015: 39. Type locality: Kolhapur, Maharashtra, India.

The Asian weaver ant has been represented by a single species, *Oecophylla smaragdina*, in spite of variations of body color and size. The African weaver ant, *Oecophylla longinoda*, is

similar to *O. smaragdina*. Indeed, it was treated as subspecies of *O. smaragdina* by some authors at one time.

According to Wheeler (1922), *O. smaragdina* is distinguished from *O. longinoda* by the shape of the petiole as follows:

1) In *O. smaragdina*, the petiole is very slender; its stigmata is very prominent in dorsal view; its ventral surface is nearly straight or very feebly convex in profile
2) In *O. longinoda*, the petiole is stouter and higher; its stigmata is not prominent in dorsal view; its ventral surface is strongly convex in profile.

The third species, *O. kolhapurensis*, was recently described from India, but the diagnose showed by Kurane et al. (2015) were obscure and the status as species is doubtful. Indeed, an opinion in "AntWiki*" mentioned that the species is "undoubtedly a junior synonym of common and widespread *Oecophylla smaragdina*." Although, the statement does not have value as a taxonomic treatment, the species is not taken in consideration here.

The species of the genus exhibits a high degree of variation in worker body color. For example, the workers are light to dark brown in Southeast Asia, but in Australia they are known as ''green tree ants'' due to the intense green color of the abdomen. Thus there has been a long history of descriptions of infraspecific forms.

Presently 12 valid subspecies names has been recognized:

Subspecies of *Oecophylla smaragdina*

nominal plus

Oecophylla smaragdina fuscoides Karawaiev, 1933. Type Locality: Indonesia (Java).

Oecophylla smaragdina gracilior Forel, 1911. Type Locality: Indonesia (Java).

Oecophylla smaragdina gracillima Emery, 1893. Type Locality: Indonesia (Batjan I.).

Oecophylla smaragdina selebensis Emery, 1893. Type Locality: Indonesia (Sulawesi).

Oecophylla smaragdina subnitida Emery, 1892. Type Locality: New Guinea.

Subspecies of *Oecopylla longinoda*

nominal plus

Oecophylla longinoda annectens Wheeler, 1922. Type Locality: Democratic Republic of Congo.

Oecophylla longinoda claridens Santschi, 1928. Type Locality: Ivory Coast.

Oecophylla longinoda fusca Emery, 1899. Type Locality: Cameroun.

Oecophylla longinoda rubriceps, Wheeler, 1922. Type Locality: Democratic Republic of Congo.

Oecophylla longinoda rufescens, Santschi, 1928. Type Locality: Congo.

Oecophylla longinoda taeniata, Santschi, 1928. Type Locality: Democratic Republic of Congo.

Oecophylla longinoda textor, Wheeler, 1922. Type Locality: Democratic Republic of Congo.

In the case of *Oecophylla*, the infraspecific names were described in 1890s to 1930s. The concept of subspecies at that time is quite different from modern usage (Brown, 1955). Wheeler (1922) compiled these taxa to the date and showed the distinction in key as below.

1. Petiole very slender, its stigmata seen from above very prominent, its ventral surface nearly straight or very feebly convex in profile (*smaragdina*) - - - - - - - - - - - - - - 2
- Petiole stouter and higher, its stigmata seen from above not prominent, its ventral surface strongly convex in profile (*longinoda*) - 7
2. Body ferruginous or testaceous - 3
- Gaster and sometimes the head pea-green, head more rouinded and less truncated

behind; size smaller, petiole somewhat shorter (Queensland, New Guinea, the Islands Aru and Key) - - - - - - - - - - - subspecies *virescens* (Fab.)

3. Integument opaque or subopaque - 4
- Integument more or less distinctly shining -- 5
4. Color ferruginotis (India, Ceylon, Cochin China, Indonesia) - *smaragdina* (typical)
- Smaller and more testaceous, mesonotum and petiole alittle narrower (Java) - - - - - - - - - - - - - -- var. *gracilior* Forel
5. Large forms, integument slightly shining (Papua, Philippines, Melanesia) - - - - - - subspecies *subnitida* Emery
- Smaller forms, integument more shining - 6
6. Body very shining and slender, color testaceous, head rather elongate - - - - - - - - - - var. *gracillima* Emery
- Less shining and less slender, head shorter (Celebes) - - - - - - - var. s*elebensis* Emery
7. Ferruginous or testaceous throughout - 8
- Brown or black - 9
8. Color ferruginous (West Africa) - *longinoda* (typical)
- Color paler, more testaceous, petiole shorter, head slightly broader, apical tooth of mandibles shorter (Zanzibar) - var. *textor* (Santschi)
9. At least the thorax and mandibles black - 10
- Body rather uniformly brown (Belgian Congo) - - - - - - - - - - - - - - - - - var. *annectens* Wheeler
10. Head dull red, gaster often brownish (Belgian Congo) - - - - - - - - - - - var. *rubriceps* (Forel)
- Head and gaster black or dark brown (Belgian Congo, Nigeria, Liberia, Cameroon, Spanish Guinea) - var. *fusca* (Emery)

Since then, 4 names were added by Santschi (1928) and Karawaiew (1933) in *Oecophylla smaragdina*. In term of zoological nomenclature these are valid because no one synonymized then. But it is seldom to refer subspecies categories.

Subspecies refers to geographical populations that have some morphological features distinguishing from other populations. Before the biological species concept was established by Mayr (1942), the terms concerning infraspecies (subspecies, varieties, forma, etc.) were confused. Although ICZN describes the rule of trinomen where only the subspecies is a rank below species, Wilson and Brown (1953) argued that subspecies is not a “real taxa”, because of the absence of criteria and thus arbitrariness (see also Mallet, 2007). In ant taxonomy, since both Wilson and Brown were researchers of ants, the application of subspecies were not popular until today.

If the population is defined by genetic basis and it links with morphological characters, the naming of population or subspecies name might be important to evolutionary work. Phylogeography treats with populations, but in the case of *Oecophylla smaragdina*, the relationship between the named subspecies and populations treated in phylogeographic studies remains ambiguous. The classification of subspecies is not consistent with grouping based on mtDNA (Azuma et al. 2002, 2008).

2.2.2 Biology of *O. smaragdina*

Oecophylla colonies are arboreal, large and polydomous in nature. Workers show the polymorphic characters with diversified organizing behavior in the colony. They are aggressive in nature and well known for their predatory characters. They are able to protect a variety of terrestrial plants against insect pests. Weaver ants formed their nest in the tree canopy by their unique nest building behavior. Workers construct pendulous bag-like nests from cluster of green leaves which are bound together with silk produced by their mature larvae. (Chapuisat and Keller, 2002). Their prominent leaf nests glued ("woven") together with silk from the larvae, can readily be seen on many trees in open and closed forests, and their large colony sizes, often over 500,000 workers, (Lokkers, 1990), is considered as one of the background of dominating behavior.

Oecophylla smaragdina colony starts the life cycle with a mated queen and laying a batch of about 35 eggs within 5 - 10 days after dealation / shedding her wings (Lokkers, 1990). The development of brood depends on temperature however the optimum temperature is 30°C. Emerging of larvae occurred about day eight. Pupae follow after day 17 and the first adult worker appeared after 28 days (Lokkers, 1990). Those colonies surviving the founding stage will develop into colonies consisting of at least half a million individuals occupying several

good sized trees (Hölldobler and Wilson, 1990). When the queen dies the workers activate their ovaries and produce a last set of male brood before the colony shrinks as worker numbers reduce over the following months (Lokkers, 1990). The colony can live about 8 years.

Schlüns et al. (2009) reported that *O. smaragdina* promises to be a very interesting study organism because its genetic colony structure deviates from the archetypical case and varies among populations. This is a rare case where colony relatedness is reduced by both polygyny and polyandry at levels specific to each population. *Oecophylla smaragdina* involves the manner in which nest mates of multiple castes (including larvae) cooperate to construct arboreal silk nests. For crossing gaps, workers form living chains and bring leaves together. The rest of the workers by the help of mandibles hold final instar larvae for using the silk produced by the larvae at the binding position of the leaves, to fasten together leaves to form the nest walls (Hölldobler and Wilson, 1977). This is considered as one of the key factors of achieving such a big colony and dominating behavior in the ecosystem (Schlüns et al. 2009).

2.2.3 Distribution of *O. smaragdina* and the factors affecting such distribution

Oecophylla smaragdina is distributed from Australia to tropical and subtropical Asia, India and many tropical islands. *Oecophylla longinoda* is distributed mainly in tropical and subtropical Africa (Lokkers, 1986; Wheeler, 1922). Among all ant species, *O. smaragdina* is the most widespread over ocean islands. Behavioral characters of this species along with the environmental effect played the major roles of such distribution. The dispersal of *O. smaragdina* without human intervention indicates that the species has the ability to disperse across wide water barriers. In *O. smaragdina*, the mating swarming has not been observed and

described, but only assumed when fertilized queens are found in large quantities in the vegetation. Lokkers, (1990) stated that sexuals 'take part in aerial swarm mating in north Queensland, but he had 'never witnessed the release of queens from a nest. Therefore, he suggested that swarming probably occurs under the cover of darkness. With discriminant analyses, Lokkers, (1986) observed the distribution pattern and suggested that both rainfall and temperature have the marked effect for the distribution of *Oecophylla*, as low temperatures directly inhibit larval development, and both rainfall and temperature levels limit the distribution of the forest-woodland vegetation required by this arboreal ant. Thus, the climatic condition also has the marked impact on the swarming behavior often leading to change in distribution pattern. Wetterer, (2017) and Peel et al. (2007) reported that the climatic condition of this continent has marked influence of some diverged distribution of *Oecophylla* and also explained the delimiting weather factors for *Oecophylla* distribution. According to them, the climatic conditions was much variable in this part might cause the behavioral changes in swarming and mating of weaver ant. Wetterer, (2017) based on the history of distribution and climatic condition with a Köppen-Geiger climate map resulted that the vast majority of *O. smaragdina* having tropical climates with tropical rainforest, monsoon climate. In arid climatic condition, fewer number of *O. smaragdina* distribution were observed. However, most of them comes from semi arid areas and majority from India or Australis. The moderate range of distributions were recorded in the sub tropical climate with dry winter and rainy summer.

2.2.4 Divergence time of *O. smaragdina*

Paleontological Information

1) Fossil species

In spite of only 2 extant species, *Oecophylla* has many described fossil species. Although some were described based on forewings only from imprints, 16 valid species are recognized (Bolton, 2018). The species list of fossils, as shown below, is broadly based on the data from "Antcat.org", a database of ant taxonomy and from "Fossilworks", a web-based portal to the Paleobiolgy Database.

(1) *Oecophylla atavina* Cockerell

Oecophylla atavina Cockerell, 1915: 485, pl.64, fig. 7 (m) Bembridge Marls, UK (Eocene) [Age range: 37.2 to 33.9 Ma].

Oecophylla perdita Cockerell 1915: 485, pl. 64, figs. 5, 6 (q.m.)

Junior synonym of *O. atavina*: Dlussky and Perfilieva 2014: 424.

(2) *Oecophylla bartoniana* Cockerell

Oecophylla bartoniana Cockerell, 1920: 277, pl. 16, fig. 4 (wing) Bagshot Beds, Bournemouth, UK (Eocene) [Age range: 48.6 to 40.4 Ma]

(3) *Oecophylla brischkei* Mayr

Oecophylla brischkei Mayr, 1868: 31, pl. 1, figs. 12, 13 (w) Baltic amber, Poland, (Eocene). [Age range: 37.2 to 33.9 Ma]

[also Wheeler, 1915]

(4) *Oecophylla crassinoda* Wheeler

Oecophylla crassinoda Wheeler, 1922: 27. Baltic amber (Eocene)

Replacement name for *Oecophylla brevinodis* Wheeler, 1915: 116

[Junior primary homonym of *Oecophylla brevinodis* André, 1890: 313.]

(5) *Oecophylla eckfeldiana* Dlussky, Wappler and Wedmann

Oecophylla eckfeldiana Dlussky, Wappler and Wedmann, 2008: 619, fig. 4 (w.q.). Eckfeild Maar, Germany (Eocene) [Age range: 48.6 to 40.4 Ma]

(6) *Oecophylla grandimandibula* Riou

Oecophylla grandimandibula Riou, 1999: 130 (alate, sex not indicated) Ardèche, France (Miocene) [Age range: 8.7 to 5.3 Ma]

(7) *Oecophylla kraussei* (Dlussky and Rasnitsyn)

Camponotites kraussei Dlussky and Rasnitsyn, 1999:74, fig 2 (q.) U.S.A. (Early Eocene). [Age range: 55.8 to 48.6 Ma]

Combination in *Oecophylla*: Perfilieva,Dubovikoff and Dlussky, 2017: 399.

See also Dlussky & Rasnitsyn, 2003: 418; Dlussky, Karl, Brauckmann, Gröning and Reich, 2011:452; Archibald, S. B., Rasnitsyn, A. P., Brothers, D. J.; Mathewes, R. W. 2018: fig. 12G

(8) *Oecophylla leakeyi* Wilson and Taylor

Oecophylla leakeyi Wilson and Taylor, 1964: 93, fig. 2, pl. 2 (w.l.). Mfwangano Island, Kenya (Miocene) [Age range: 20.43 to 15.97 Ma]

(9) *Oecophylla longiceps* Dlussky, Wappler and Wedmann

Oecophylla longiceps Dlussky, Wappler and Wedmann, 2008: 617, figs. 2, 3 6A (q.m.) Hessen, Germany (Eocene). [Age range: 48.6 to 40.4 Ma]

(10) *Oecophylla macroptera* (Dlussky)

Camponotites macroptera Dlussky, 1981: 76, fig. 53 (q.). RUSSIA (Middle Miocene).

Combination in *Oecophylla*: Perfilieva,Dubovikoff and Dlussky, 2017: 399.

(11) *Oecophylla megarche* Cockerell

Oecophylla megarche Cockerell, 1915: 486, pl.65, fig. 1-3 (q.) Bembridge Marls, UK (Eocene). [Age range: 37.2 to 33.9 Ma] [See also Dlussky and Perfilieva, 2014:426]

(12) *Oecophylla obesa* (Heer)

Formica obesa Heer, 1849: 108, pl. 8, fig. 1 (q.) Radoboj, Croatia (Miocene) [Age range: 37.2 to 11.608 Ma]

Combination in *Oecophylla*: Mayr, 1867b: 50; Dlussky & Putyatina, 2014: 252, figs. 5A-F, 18A-B, 18E (q.m.).

Attopsis longipes Heer, 1867: 29, pl. 2, fig. 15 (m.?) Croatia (Miocene).

See also Förster, 1891: 442.

Junior synonym of *Oecophylla obesa*: Dlussky & Putyatina, 2014: 252.

Formica pinguis Heer, 1849: 110, pl. 8, figs. 3, 4 (q.) Croatia (Miocene).

Combination in *Camponotus*: Mayr, 1867b: 51. Junior synonym of *Oecophylla obesa*: Dlussky & Putyatina, 2014: 252.

Attopsis anthracina Heer, 1849: 156, pl. 12, fig. 12 (m.) Croatia.

Combination in *Cataulacus*: Mayr, 1867b: 58.

Senior synonym of *Cataulacus nigra*: Mayr, 1867b: 58.

See also: Bolton, 1974a: 87.

Junior synonym of *Oecophylla obesa*: Dlussky & Putyatina, 2014: 252.

Attopsis nigra Heer, 1849: 157, pl. 12, fig. 13 (q.) Croatia.

Combination in *Cataulacus*: Mayr, 1867b: 58.

Junior synonym of *Cataulacus anthracinus*: Mayr, 1867b: 58.

(13) *Oecophylla praeclara* Förster

Oecophylla praeclara Förster, 1891: 432, pl. 13, fig. 6 (q.) Brunstatt, France (Oligocene). [Age range: 33.9 to 28.4 Ma]

(14) *Oecophylla sicula* Emery, 1891

Oecophylla sicula Emery, 1891b: 156, pl. 3, figs. 36, 37 (w.) Sicilian amber, Italy (Tertiary). [Age range: 11.608 to 5.332 Ma] [also Brown & Wilson, 1978]

(15) *Oecophylla superba* Théobald, 1937

Oecophylla superba Théobald, 1937a: 212, pl. 4, fig. 17; pl. 15, fig. 1 (q.) Kleinkembs, Germany (Eocene/Oligocene Boundary). [Age range: 33.9 to 28.4 Ma]

(16) *Oecophylla taurica* Perfilieva, Dubovikoff & Dlussky, 2017

Oecophylla taurica Perfilieva, Dubovikoff & Dlussky, 2017: 399, fig. 7, pl. 7.6 (q.) Malyi Kamyshlak, Crimean Peninsula, Russia (Middle Miocene).

In addition,Viehmeyer (1913) reported *O. smaragdina selebensis* found at Celebes copal (Quaternary of Indonesia). Zhang (1989: 297) transfered *Rabidia xiejiaheensis* Hong from Miocene under genus *Oecophylla*. Later the species was treated under *Camponotites* by Dlussky et al. (2008: 616). Bolton (2018) considered both *Oecophylla xiejiaheensis* and *Rabidia xiejiaheensis* as unavailable and uncategorized.

There was a confusion in the genus *Camponotites*. Dlussky established monotypic extinct genus *Camponotites* in 1981, based on the *Camponotites macropterus* from Russia, which is a forewing impression fossil. But this name is a junior homonym of *Camponotites* Steibach, 1967. The taxon was treated as incertae sedis in Formicidae by Hölldobler & Wilson (1990: 18) and Dlussky & Rasnitsyn (2002: 418), or in Formicinae by Bolton (1994: 50), Bolton (1995b: 83) and Bolton (2003: 112). Dlussky et al. (2011) treated *Camponotites* Dlussky as junior homonym and junior synonym of *Campnotites* Steinbach. The latest taxonomic treatment of the taxon is that of Perfilieva et al. (2017) in text where the genus *Camponotites* is a junior synonym of *Oechophylla* by implication as type-species of Camponotites Dlussky transferred to *Oecophylla*, but not not fully accepted (Bolton, 2018)

2) Geochronology and distribution of fossil species.

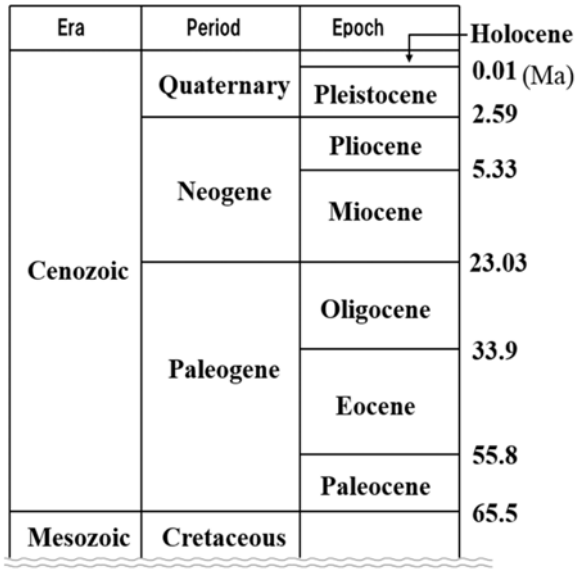


Fig. 2.1 Geochronologic classification of the fossil species

The fossil species are predominantly found from middle Eocene to Miocene in Europe (Fig. 2.1). Apart from *O. kraussei* from Klondike Mountain Formation, Washington State, USA in Early Eocine, the early fossils of the genus were described based on the materials from Europe. Two species are described from the Middle Eocene, *O. longiceps* and *O. eckfeldiana* both from Germany. In the Upper Eocene, six species are described: *O. brischkei*, *O. crassinoda* from Baltic amber; *O. praeclara* from Germany, *O. bartoniana, O. atavina, and O. megarche* from UK. In the Oligocene, two species are described; *O. superba* from Kleinkembs, France and *O. sicula* from Sicilian amber. In the Miocene, *O. obesa* from Croatia, *O. leakeyi* from Kenya and *O. taurica* from Russia.

The age of the *Oecophylla* is back to 48.6 Ma and the distribution is summarized as follows (Fossilwork):

Quaternary: Indonesia (Viehmeyer, 1912)

Miocene: Croatia (Uger, 1841, Heer, 1849, 1865), France (Weidner and Riou, 1986), Germany (Heer, 1847, 1849), Italy (Brown & Carpenter, 1978), and Kenya (Wilson & Taylor, 1964)

Oligocene: France (Förster, 1891) and Germany (Theobald, 1937)

Eocene: the Czech Republic (Deichmüller, 1881), Germany (Dlussky et al, 2009), Poland (Holl, 1829, Menge, 1854), Russia (Mayr, 1868, Cockerell, 1909, Enderlein, 1910, Ulmer, 1912), and UK (Cockerell, 1915)

Azuma et al. (2006) estimated the divergence time of *Oecophylla smaragdina*. Based on that estimation, *O. smaragdina* and *O. longinoda* diverged 13.3- 11.3 Ma, in the late Miocene. Diversification within the seven groups have been estimated between 7.8 and 3.6 Ma. Most likely *Oecophylla* originated in the early Paleogene in the Palaearctic realm, radiating strongly during the climatic changes of the Eocene–Oligocene transition (Dlussky et al. 2008). According to the fossil records, *Oecophylla* might have originated in the early Paleogene (ca. 60 Ma) in the Palaearctic region, and dispersed during the climatic changes of the Eocene–Oligocene transition at ca. 43 Ma (Dlussky et al. 2008). Recently, Blaimer et al. (2015) estimated the divergence time of the genus *Oecophylla* based on the fossil records and ultra conserved elements (UCEs). They estimated that *Oecophylla* crown group evolved during Oligocene at ca. < 30 Ma and stem-group evolved during early Eocene at ca.50 Ma. The divergence time of *Oecophylla* are shown here based on (Azuma et al. 2006; Dlussky et al. 2008) resulted that diversification within the groups of *O. smaragdina* occurred between 4.7 Ma to 0.7 Ma ago.

2.3 Geology of Bangladesh and its importance for phylogeographic study of weaver ant

Bangladesh is a riverine country with three main rivers Ganga, Jamuna and Meghna crisscrossed throughout the mostly flat territories of the country. Bangladesh is surrounded in the north, west and south by India, by Myanmar in the Southeast and by Bay of Bengal in the south. The river Ganges (Known all over the Bangladesh as Padma) is a trans-boundary river acting as a barrier of India and Bangladesh. The river rises in the western Himalayas in the Indian state of Uttarakhand, and flows south and east through the Gangetic Plain of North India into Bangladesh, where it empties into the Bay of Bengal. Bangladesh's geology began 350 million years ago when the Pangean supercontinent broke apart (Mannan, 2002). The Bengal Basin began 127 million years ago when the Indian Plate rifted away from Antartica at 18 cm per year for 20 million years.

This rapid velocity stopped 55 million years ago and was followed by a period in which little or no spreading took place west of the Ninety East Ridge for 20 million years. The Indian subcontinent lies atop the Indian tectonic plate, a minor plate within the Indo-Australian Plate (Gibbons et al. 2012). Its defining geological processes began a north eastwards drift lasting fifty million years across the then unformed Indian Ocean. The subcontinent's subsequent collision with the Eurasian Plate and subduction under it, gave rise to the Himalayas, the planet's highest mountains (Ali, 2005; Gibbons et al. 2012). In the former seabed immediately south of the emerging Himalayas, plate movement created a vast trough, which, having gradually been filled with sediment borne by the Indus and its tributaries and the Ganges and its tributaries, now forms the Indo-Gangetic Plain (Uddin and Lundberg, 1999). The second largest river Jamuna is derived from the Himalayas as Brahmaputra river. The river drains the

Himalaya east of the Indo-Nepal border, southern-central portion of the Tibetan plateau above the Ganges basin and adjoint with Ganga, near chandpur and fall into Bay of Bengal. The river mainly formed during the period Holocene (Pickering et al. 2014). The Meghna is a distributary of the Brahmaputra. The Meghna is formed inside Bangladesh by the joining of the Surma and Kushiyara rivers originating from the hilly regions of eastern India. Down to Chandpur, Meghna is hydrographically referred to as the Upper Meghna. This rivers forms the Bengal basin at around 5-10 milliion years ago (Uddin and Lundberg, 1999). So, all the main rivers originating from different period of time, separating the countries with some definite geological areas. The southeast border of Bangladesh is separated by the mountain Arakans from Myanmar. Arakan Mountains formed by the compression as Indian plate collided with the Eurasian plate approximately along the boundary between India and Myanmar at around 71 ma ago. This mountain are submerged in Bay of Bengal for sufficiently long stretch and emerges against in the form of Andaman and Nicobar Island and the highest peak was found about 3055 m (Aitchison et al. 2008).

Formation of the Bengal basin in led to development of Bangladesh is corresponded to the divergence of *O. smaragdina.* Bengal delta being prograding in nature since its inception covers entire Bengal basin. The prograding nature of the delta has been intervened with sea transgression number of times in its geologic history. With, a low-lying delta complex associated with the Ganges, Brahmaputra-Jamuna, Meghna rivers form one of the largest depositional systems in the World (Goodbred and Kuehl, 2000). The major rivers, viz., the Ganges, the Brahmaputra-Jamuna, the Meghna and their tributaries and distributaries have been acting as the primary transporting media of the sediments in the delta building and subsequently emerged as the Bengal delta. In view of the contribution in the delta building by the rivers Ganges, Brahmaputra-Jamuna, Meghna and their tributaries and distributaries, the

delta may be termed as Ganges Brahmaputra- Meghna (GBM) delta. Similarly inbred tectonism of the Indian plate has been playing a significant role in the development of the GBM delta (Chowdhury, 2012). The vital tectonic element, viz., Himalayas in the north and Indo-Burma range in the east, have been acting virtually as in exhausting sources of sediments. The river Ganges and Brahmaputra drain the NW and NE portion of the Himalayas to the Bay of Bengal over the delta. Subduction tectonics of the Indo-Burma convergent system and differential subsidence and up liftment largely controlled the basin architecture. Mannan (2002) studied on the Surma basin in Bangladesh, and according to him, most of the country is covered by thick layers of sediments. The country occupies most of the Bengal basin eroded from the eastern Himalayas and the Indo–Burman Ranges, and carried by major river systems similar to the present-day Ganges and Brahmaputra. The basin is bordered by the Indian plate to the west; the Himalaya to the north; the Shillong plateau, India, to the northeast; the Indo–Burman Ranges to the east; and the Bay of Bengal to the south. Sedimentation in the basin has been almost continuous for 65.5 million years. The first clearly orogenic detritus is in the lowermost 23 million year old strata. The Surma Basin is a sub-basin of the Bengal Basin, which was formed about 127 million years ago when the Indian plate rifted away from Antarctica.#The onshore part of the Bengal Basin is the site of the world's largest formed by rivers (Ganges, Brahmaputra/Jamuna, Padma, Meghna) that drain a large portion of the Himalayas. Formation of the basin and its subsequent development might have some impact for the development of vegetation and other ecological niche as suitable host for *Oecophylla.* As the Bengal basin formation has a long history influenced by the origination and flow channel of the riverine border, so this could be the key issue for further phylogenetic study. In all the cases of such basin formation, climate change has the marked impact specially in the last glacial maximum (LGM). During LGM, most of the river system in Bangladesh was largely inactive in terms of sediment deposition but after glaciation the magnitude of sediment discharge increased

substantially which facilitate the formation of different basins and also facilitate the formation of vegetation of the concrete locality (Goodbred, 2003).

2.4 Phylogenetic study of *O. smaragdina* in Asian continents

Weaver ant has an evolutionary importance which is corresponding to geography and geological history of the earth. Azuma et al. (2002) first analyzed populations of *O. smaragdina* including samples from Bangladesh. She also added comprehensive samples of *O. smaragdina* from India, Southeast Asia and Australia using molecular data, Azuma et al. (2006) proposed an outline of the phylogeography of *O. smaragdina* and categorized the sampled populations into 7 major clades: group 1 from India; group 2 from Southeast Asian mainland including the Indochinese and Malayan Peninsulas, as well as the Greater Sunda Islands; group 3 from the Philippines; group 4 from Flores; group 5 from

Sulawesi; group 6 from Halmahera; group 7 from Australia and New Guinea. Asaka, (2010) extended the survey of *O. smaragdina* to South Asia and collected several samples from India and Sri Lanka. Her phylogenetic analysis showed that all analyzed samples belong to Indian clade with low levels of sequence divergence. Azuma et al. (2006) characterized the mitochondrial sequence identity of the Bangladesh populations as Southeast Asian clade in spite of the geographical proximity of Bangladesh to India.

CHAPTER 3

GENERAL METHODOLOGY

CHAPTER 3

General Methodology

3.1 Sampling of *Oecophylla smaragdina* in Bangladesh

The setting of the study sites

Based on three main rivers, Ganges, Jamuna, and Meghna, the study area in Bangladesh was broadly categorized into five areas under 8 division. The detailed information of that 5 broad areas is presented in the following figure (Fig. 3.1).

Area 01 included the northwestern part of Bangladesh. This area include 16 districts under 2 divisions. The climate of this area is comparatively dry with very hot summer and cold winter to other areas. This area is surrounded by the river Ganges and Jamuna. The study area lies between 24°07′ N to 25°13′ N latitude and 88°00′ E to 89°10′ E longitude. The average temperature ranges from 35°C to 25°C in the dry season and 9°C to 15°C in the winter season (Yasmin et al. 2013). This northwestern part of Bangladesh has been evolved during Pleistocene Terraces due to tectonic upliftment and /or exists as an erosional geomorphic feature (Rashid et al. 2015).

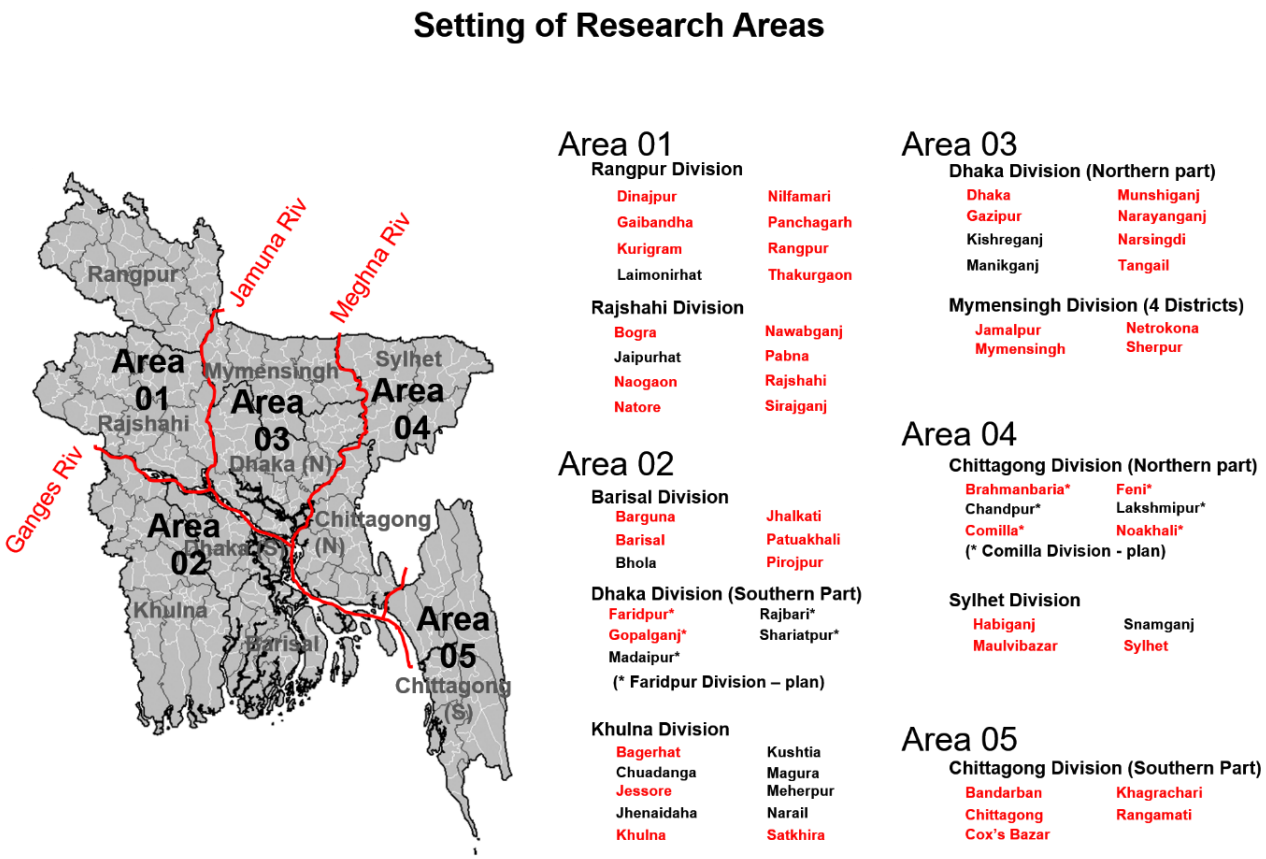
Area 02 was located atthe western districts of the country consisting Khulna, Barisal and western part of Dhaka divisions. The important feature of this area is the existence of Mangrove forest, “The Sundarbans”. So, this area passess some unique vegetation. The Sundarban forest lies in the vast delta on the Bay of Bengal formed by the super confluence of the Ganges, Hooghly, Padma, Brahmaputra and Meghna rivers across the southern

Bangladesh. The soil is saline in nature with the availability of less fresh water. The lower boundary of that area 02 is flooded by the Bay of Bengal. The major river channels are derived from this bay of Bengal along with the flow of Ganges.

Area 03 was located in the central and northern part of the country with the northern part of Dhaka division and Mymensingh division. The major characteristics of this area is the availability of Shal forest and it is mainly covered by the plain land. This area is the characterized of Pleistocene upliftment resulted Bhawal and Madhupur tract (Rashid et al. 2006). Some hilly areas including tectonically uplifted blocks created during Pleistocene are also the major characteristics in this area. This area is developed on the Ganges-Brahmaputra delta on late quaternary stratiphication (Goodbred and Kuehl, 2000).

Area 04 was located the northern part of Chittagong division and Sylhet. Northern Chittagong is a plane landed area with plenty of fresh water sources and many rivers crisscross acrossed while Sylhet division is mountainous with enormous number of tea garden with natural water fall. The major geological feature of that area is the Bengal basin. The Surma Basin is a sub-basin of the Bengal Basin situated in the northeastern part of Bangladesh. The basin is bounded on the north by the Shillong plateau, east and southeast by the Chittagong-Tripura fold belt of the Indo-Burman ranges, and west by The Indian Shield platform. This area is mainly created during Holocene (Mannan, 2002).

Area 05 includes the southern Chittagong division. This area is divided from others region of Bangladesh by the border of Meghna rivers. It is a mountainous area and the longest sea beach, Coxsbazar, is situated in this area. The area is mainly composed of Hill Tracts (CHT) in the south-eastern part of Bangladesh encompassing three hill districts: Rangamati, Khagrachari and Bandarban. It shares borders with Myanmar on the south and southeast, India on the north and northeast, and the Chittagong district of Bangladesh on the west. CHT is



The red letters show the District names where we sampled during March 2013 to December 2017.

Fig. 3.1 Sampling sites of 5 broad areas based on three main rivers in Bangladesh. The red line across the map showing the river flow that separated 5 broad areas in Bangladesh. In the text, red color indicating the district name from where sampling were done in each area.

located between 21°40′ degrees and 23°47′ degrees north latitude and 91°40′ degrees and 92°42′ degrees east longitude. It is a unique territory with marked socio-economical and cultural differences from the rest of Bangladesh.

Localities of *O. smaragdina* sampling

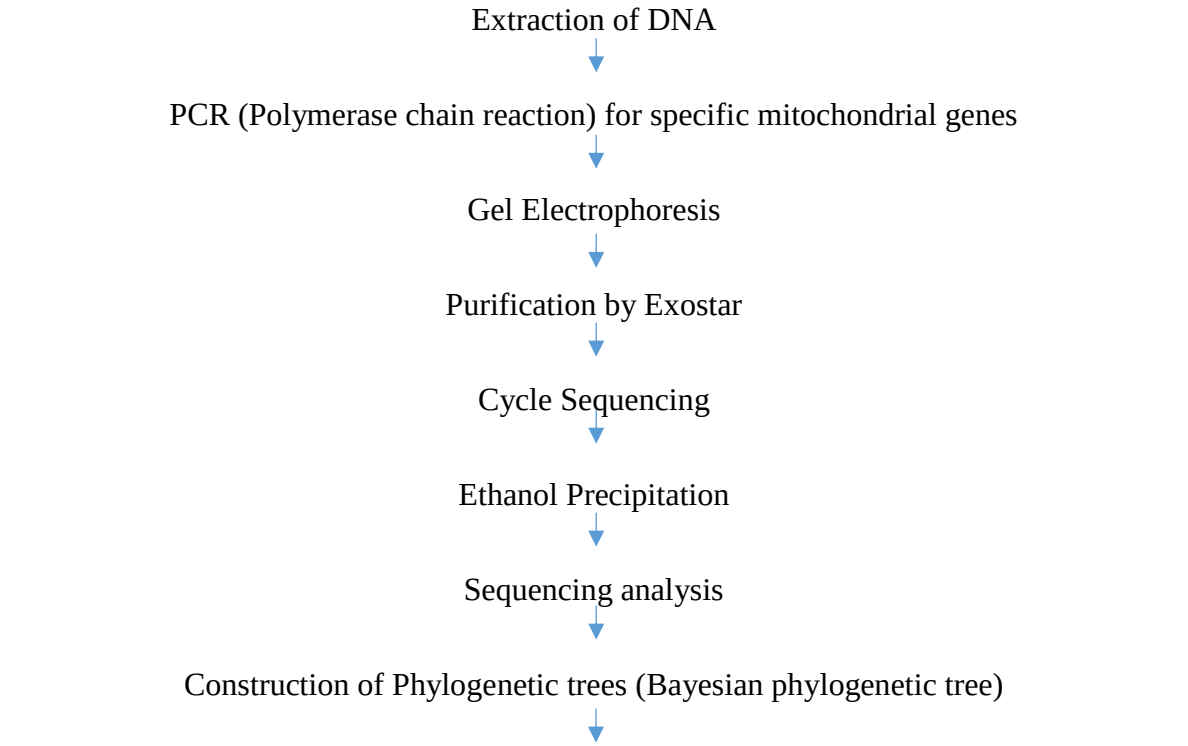
A total of 95 *O. smaragdina* colonies had been collected from 87 localities of 47 districts belonging to 8 divisions of Bangladesh during September 2013 to December 2017. The locality position and detailed information are presented in Fig. 2.2 and Table 2.1.

Sample preparation

The collected samples from Bangladesh were analyzed for sequencing in the laboratory Institute of Tropical Agriculture of Kyushu University, Japan. The collected colonies of *Oecophylla smaragdina* were preserved in 99% ethanol prior to DNA extraction.

3.2 Molecular studies

The sequential procedure for extraction of DNA to final sequencing is shown in the following diagram.



The inference is drawn by haplotype network analysis, divergence time estimation, Nuclear DNA (LWRh) analysis, and microsatellite study; The details will be in each chapter.

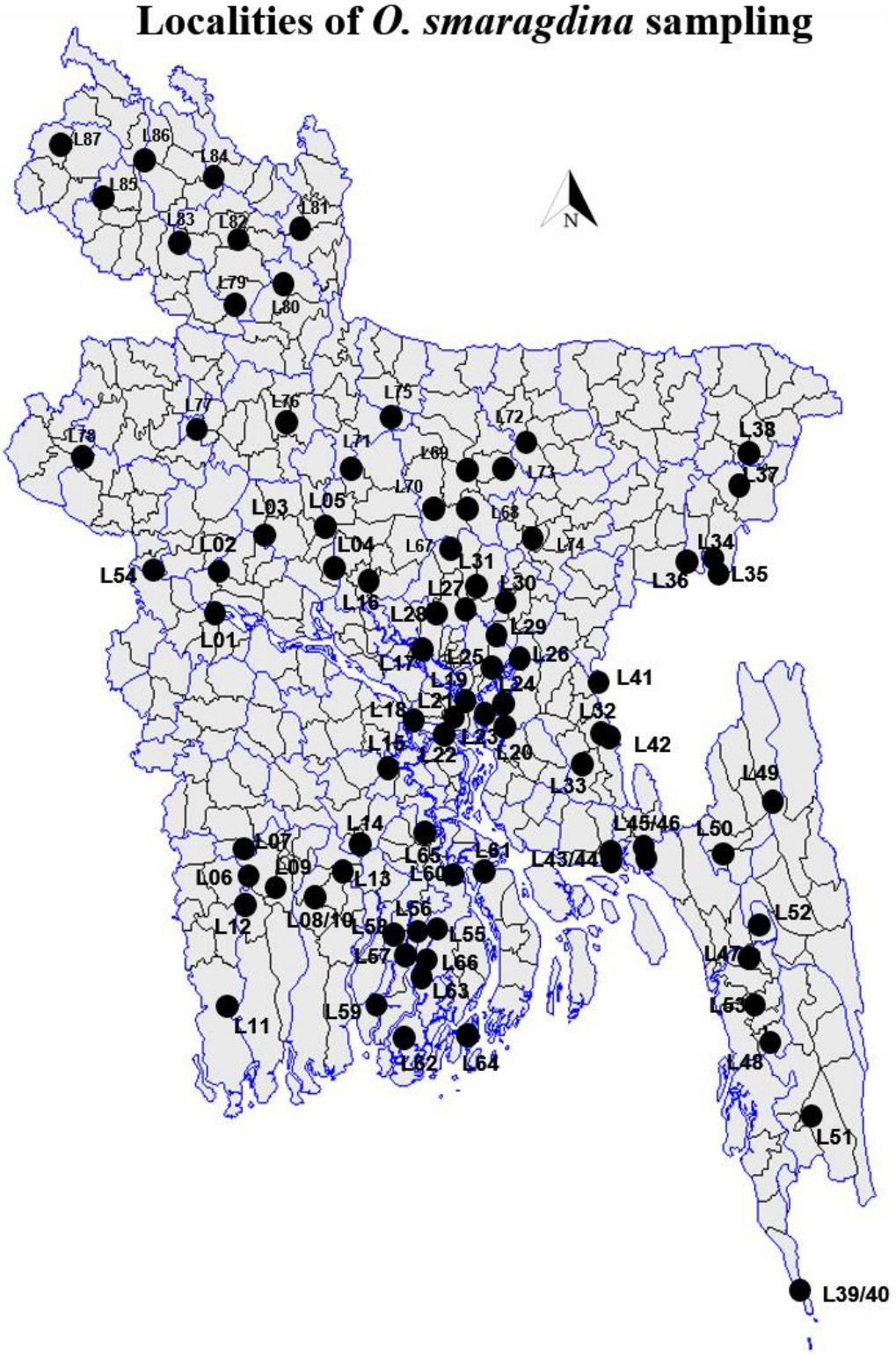


Fig. 3.2 Sampling sites of *Oecophylla smaragdina* in Bangladesh.

Table 3.1 Detailed locality information of *O. smaragdina* sampling sites in Bangladesh

Legends	Locality No.	Locality Name	No. of colonies	District	Division	Area Code
AB	L01	Ishwardi	1	Pabna	Rajshahi	A01
ABC	L02	Bonpara	1	Natore	Rajshahi	A01
AB	L03	Tarash	1	Sirajganj	Rajshahi	A01
AB	L04	Chauhali	1	Sirajganj	Rajshahi	A01
ABC	L05	w side of Jamuna Bridge	1	Sirajganj	Rajshahi	A01
ABD	L06	Panjia	1	Jessore	Khulna	A02
AB	L07	Monirampur	1	Jessore	Khulna	A02
ABC	L08	Khulna Univ. Campus	2	Khulna	Khulna	A02
AB	L09	Chuknagar	1	Khulna	Khulna	A02
ABC	L10	Batiaghata	1	Khulna	Khulna	A02
AB	L11	Atulia	1	Satkhira	Khulna	A02
AB	L12	Modonpur	1	Satkhira	Khulna	A02
ACE	L13	Mollahat Bazar	1	Bagerhat	Khulna	A02
AB	L14	Pachuria	1	Gapalgonj	Dhaka	A02
AB	L15	Bhanga	1	Faridpur	Dhaka	A02
AB	L16	Elenga	1	Tangail	Dhaka	A03
AB	L17	Kumrail	1	Dhaka	Dhaka	A03
AB	L18	Thanamore	1	Dhaka	Dhaka	A03
AB	L19	Ruhitpur	1	Dhaka	Dhaka	A03
A	L20	Baluakandi	1	Munshiganj	Dhaka	A03
ABCD	L21	Nimtali	1	Munshiganj	Dhaka	A03
AC	L22	Bejgaon	1	Munshiganj	Dhaka	A03
A	L23	Nababganj Bazar	1	Narayanganj	Dhaka	A03
A	L24	Shiddirganj	1	Narayanganj	Dhaka	A03
A	L25	Vulta	1	Narayanganji	Dhaka	A03
ABCD	L26	Panchdona	1	Norsingdi	Dhaka	A03
ABE	L27	Bhawal National Park	2	Gazipur	Dhaka	A03
ABC	L28	Nurbag	1	Gazipur	Dhaka	A03
ABC	L29	Charpara	1	Gazipur	Dhaka	A03
	L30	Zamirarchala	1	Gazipur	Dhaka	A03
A	L31	Rajbari	1	Gazipur	Dhaka	A03
ABD	L32	Nimshar	1	Comilla	Chittagong	A05
C	L33	Madhoyia	1	Comilla	Chittagong	A05

Continued

ABCE	L34	Tea Resort Center	3	Moulovibazar	Sylhet	A04
AB	L35	Lauachra National Park	1	Moulovibazar	Sylhet	A04
ABC	L36	Bahubal	1	Habiganj	Sylhet	A04
ABCD	L37	Tarau	1	Sylhet	Sylhet	A04
ABC	L38	Doradarpur	1	Sylhet	Sylhet	A04
AB	L39	Marishbunia	1	Cox's Bazar	Chittagong	A05
	L40	Noakhali para	1	Cox's Bazar	Chittagong	A05
AB	L41	Mondabag	1	Bramhanbaria	Chittagong	A04
A	L42	Jogotpur	1	Comilla	Chittagong	A04
AB	L43	Sebarhat	1	Noakhali	Chittagong	A04
A	L44	Senbag Upozilla Hospital	1	Noakhali	Chittagong	A04
ABC	L45	Mohipal	1	Feni	Chittagong	A04
ABDE	L46	Mohipal Primary School	1	Feni	Chittagong	A04
A	L47	Raujan Bazar	1	Chittagong	Chittagong	A05
ABC	L48	Satkania	1	Chittagong	Chittagong	A05
ABC	L49	Dighinala HRC	1	Khagrachari	Chittagong	A05
ABE	L50	Matiranga Dhibi	1	Khagrachari	Chittagong	A05
AB	L51	Ruma Karai	1	Bandarban	Chittagong	A05
ABCD	L52	Kawkhali Bazar	1	Rangamati	Chittagong	A05
A	L53	Patia	1	Chittagong	Chittagong	A05
ABD	L54	Thanapara Sadah	1	Rajshahi	Rajshahi	A01
AB	L55	Nalchiti prim. sch. field	1	Jhalokati	Barisal	A02
AB	L56	BRAC more	1	Jhalokati	Barisal	A02
ABCD	L57	Baghribazar	1	Jhalokati	Barisal	A02
ABCD	L58	Kawkhali Upz P Chottor	1	Pirojpur	Barisal	A02
ABC	L59	Shakharikathi	1	Pirojpur	Barisal	A02
A	L60	Rupatoli	1	Barisal	Barisal	A02
ABC	L61	Patarhat	1	Barisal	Barisal	A02
A	L62	Barguna Sadar bus stand	1	Barguna	Barisal	A02
AD	L63	PSTU	1	Patuakhali	Barisal	A02
AB	L64	Panpatti	1	Patuakhali	Barisal	A02

Continued

ABD	L65	Agailjhara Uni P office	1	Barisal	Barisal	A02
ABED	L66	Mohespur	1	Barisal	Barisal	A02
ABCE	L67	Bhaluka Bazar	1	Mymensingh	Mymensingh	A03
ABD	L68	Trishal Primary School	1	Mymensingh	Mymensingh	A03
ABC	L69	BAU Campus	1	Mymensingh	Mymensingh	A03
ABC	L69	BAU, Sesh Matha More	1	Mymensingh	Mymensingh	A03
AB	L70	Nandail	1	Mymensingh	Mymensingh	A03
ABCD	L71	Sarisha Bari High School	1	Jamalpulr	Mymensingh	A03
AC	L71	Bazar Pukur	1	Jamalpur	Mymensingh	A03
ABC	L72	Sadar Hospital	1	Netrokona	Mymensingh	A03
ABC	L72	Sadar Primary School	1	Netrokona	Mymensingh	A03
AB	L73	Gauripur Upz Complex	1	Mymensingh	Mymensingh	A03
AB	L74	Higher par	2	Kishorgonj	Mymensingh	A03
AB	L75	Sadar Thana more	1	Sherpur	Mymensingh	A03
ABC	L76	Dhunat Upz. Chattor	1	Bogra	Rajshahi	A01
ABC	L77	Municipality Orchard	1	Naogaon	Rajshahi	A01
ABC	L78	Nijampur	1	Chapainawab ganj	Rajshahi	A01
ABC	L79	Hakimpur Nursery	1	Dinajpur	Rangpur	A01
ABC	L80	Shibpur	1	Gaibandha	Rangpur	A01
ABC	L81	Gharialdanga	1	Kurigram	Rangpur	A01
ABC	L82	Rasulpur School ground	1	Rangpur	Rangpur	A01
ABC	L83	Saidpur airport surrounding	1	Nilfamari	Rangpur	A01
ABC	L84	Barobala	1	Rangpur	Rangpur	A01
ABC	L85	Pirganj fire station orchard	1	Thakurgaon	Rangpur	A01
ABC	L86	Debiganj bus stand	1	Panchagar	Rangpur	A01
ABC	L87	Atwari sadar thana more	1	Panchagar	Rangpur	A01

A=used in phylogenetic analysis, B= used in Haplotype network study, C= in Nuclear DNA study, D= in Divergence time estimation, E= in Microsatellite study

Extraction of DNA

Genomic DNA was extracted from the fore, middle and hind legs of specimens that were preserved in 99% ethanol by using *QIAGEN DNeasy Blood and Tissue kit* (Qiagen, Maryland, USA) following manufacturer's instruction. Samples were vortexed by adding 180 µl Buffer ATL and 20 µl proteinase K. Sample were incubated at 55°C for 48 hours. DNA extraction was completed by adding two wash buffer AW1 and wash buffer AW2 and Buffer AE and lysis buffer AL and elusion buffer AE, as per manufacturer instruction. All centrifugation steps were completed at room temperature. The colony mates of the specimens used for DNA analysis were preserved in the laboratory of Institute of Tropical Agriculture, Kyushu University after DNA extraction.

PCR (Polymerase chain reaction)

Amplification of mitochondrial DNA was done by polymerase chain reaction (PCR) using *TaKaRa Ex Taq* PCR kit, according to the manufacturer's instructions. The kit contains 10X *Ex Taq* Buffer (20mM Mg^{2+} plus) and dNTP mixture (2.5mM each). The storage buffer contain 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40 and 50% glycerol. dNTP mixtures contains TAPS, KCl, $MgCl_2$, DTT, dATP, dGTP, dCTP with activated salmon sperm DNA. Reaction mixtures for PCR for 50 µl, *TaKaRa Ex Taq* (0.25 µl), 10XExTaq Buffer (5 µl), dNTP mixture (4 µl), a pair of oligonucleotide primers (0.2-1.0 µM; Table 2) and sterilized distilled water (up to 50 µl) were run in Biometra Genomics and Proteomics analyzer with the following instructional status. The thermal cycling parameters for *Cytb* and *COI* basically followed the protocols established by (Crozier and Crozier, 1993) and (Sameshima et al., 1999), including 95 °C for 5 min for initial denaturation, 35 cycles of dissociation (92 °C, 1 min), annealing (50 °C for *Cytb* and 54 °C for *COI*, 1 min), and extension (70 °C, 2 min). The primers used for amplification are identical to primers

reported by Crozier et al. (1995), Lunt et al. (1996), Azuma et al. (2002), and Azuma et al. (2006) (Table 3.2). For LW *Rh*, the PCR parameters were the same as for mitochondrial genes; thermal cycling parameters were nearly the same, except the annealing temperature was 60°C for this region. The sequences and positions of mitochondrial DNA of the primers for PCR and sequencing are shown in Table 3.3 and Table 3.4.

Gel electrophoresis

After PCR, gel electrophoresis was performed to check the result. The gel was prepared with boiling 100 ml 1xTAE buffer, 1.0g Agarose and 10µl Gel Red at 800 watts for 1.30 minutes. Then the PCR product having mixed with 6X coding buffer was put into the gel of gel electrophoresis plate for 20 minutes to compare the results of the PCR products with the standard product that containing 100 bp DNA ladder.

Exostar

For Enzymatic PCR and sequencing clean-up, Illustra and ExoProStar were followed according to the instruction of the manufacturer GE Healtcare. Exonuclease (1 reaction μl^{-1}, Solution in 20mM Tris-HCl (pH 7.5), 0.1mM EDTA, 1mM DTT, 50% (v/v) glycerol) and Alkaline phosphate (1 reaction μl^{-1}, Solution in 20Mm HEPES-NaOH (pH 7.4), 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 0.1% (v/v) Triton X-100, 50% (v/v) glycerol were added to the PCR product and incubate 37^0C for 15 minutes and 80^0C for 15 minutes to inactivate the enzymes.

Cycle sequencing

For cycle sequencing, ABI PRISM Big Dye Terminator v3.1 Cycle sequencing kits from Applied Biosystems were used in an automated sequencer. The operational status that

Table 3.2 Primers for amplifying and sequencing mitochondrial Cytb and COI and nuclear genes. The positions of primers for mitochondrial genes follow the complete sequence of mitochondrial DNA of *Apis mellifera* (Crozier and Crozier, 1993)

Region	Name	Direction	Sequence (5'-3')[a]	Position
Cytb	Cb1[d]	Forward	TATGTACTACCATGAGGACAAATATC (1)	11400-11425
	tRs[c]	Reverse	TATTTCTTTATTATGTTTTCAAAAC (1)	12250-12226
	Cb1.5F[b]	Forward	GAGATTTATATAAAATTCCT	11596-11616
	Cb3[b]	Forward	CCAATTCATATTCAACC	11777-11794
	Cb4R[d]	Reverse	CTCATATTTTTATAATTAGAAATGAT	12100-12126
COI	COI 1-3[d]	Forward	ATAATTTTTTTTATAGTTATACC (2)	1981-2002
	COI 2-4[d]	Reverse	TCCTAAAAAATGTTGAGGAAA (2)	3063-3083
	COI 66OR[b]	Reverse	GCTGAAGTAAAATAAGCTCGTG	2688-2710
	COI 2-1[b]	Forward	CTTTATCAACATTTATTTTGATTTTT (2)	2481-2499
LW Rh	LW RhF [d]	Forward	AATTGCTATTAYGARACNTGGGT (3)	NA
	LW RhR [d]	Reverse	ATATGGAGTCCANGCCATRAACCA (3)	NA
	LR 798 F [d]	Forward	GCH GCY CAY GAG AAG AAY ATG CG (4)	NA
	LR 1047 R [d]	Reverse	GG ATT RTA YAC RGC RTT GGC TTT BGC(4)	NA
	LR 482 FCR [d]	Forward	ATA TGG ACG ATG ACR ATG ATC GC(4)	NA
	LR 855 R [d]	Reverse	GA TCG YAR VGA AGC RAC GTT CAT(4)	NA

[a] (1) (Crozier et al. 1995), (2) (Lunt et al. 1996) [b] Used only for sequence; (Cameron et al. 2001) (Cameron and Williams, 2003),(3) (Mardulyn and Cameron, 1999), (4) (Blaimer, 2012)

[c] Used for PCR; [d] Used for both PCR and sequence

Table 3.3 The operational status of PCR and Cycle sequencing

PCR for Cytb					
Steps	Times (X)	^{0}C	M:S	Go to	Loops
1		95.0	05:00		
2		92.0	01:00		
3	35	50.0	01:00		
4		70.0	02:00	2	34
5		70.0	01:00		
6		4.0	Pause		

PCR for CO1					
Steps	Times (X)	^{0}C	M:S	Go to	Loops
1		95.0	05:00		
2	35	92.0	01:00		
3		54.0	01:00		
4		70.0	02:00	2	34
5		70.0	01:00		
6		4.0	Pause		

Exoster		
Steps	^{0}C	M:S
1	37.0	15:00
2	80.0	15:00
3	4.0	Pause

Cycle sequence					
Steps	Times (X)	^{0}C	M:S	Go to	Loops
1		94.0	00:30		
2	25	96.0	00:30		
3		50.0	04:50	2	24
4		4.0	99.59		

performed is presented in Table 2.4. Cycle sequencing and ethanol precipitation were done for final sequencing analysis by ABI 3100 Avant DNA Sequencer (Applied Biosystems).

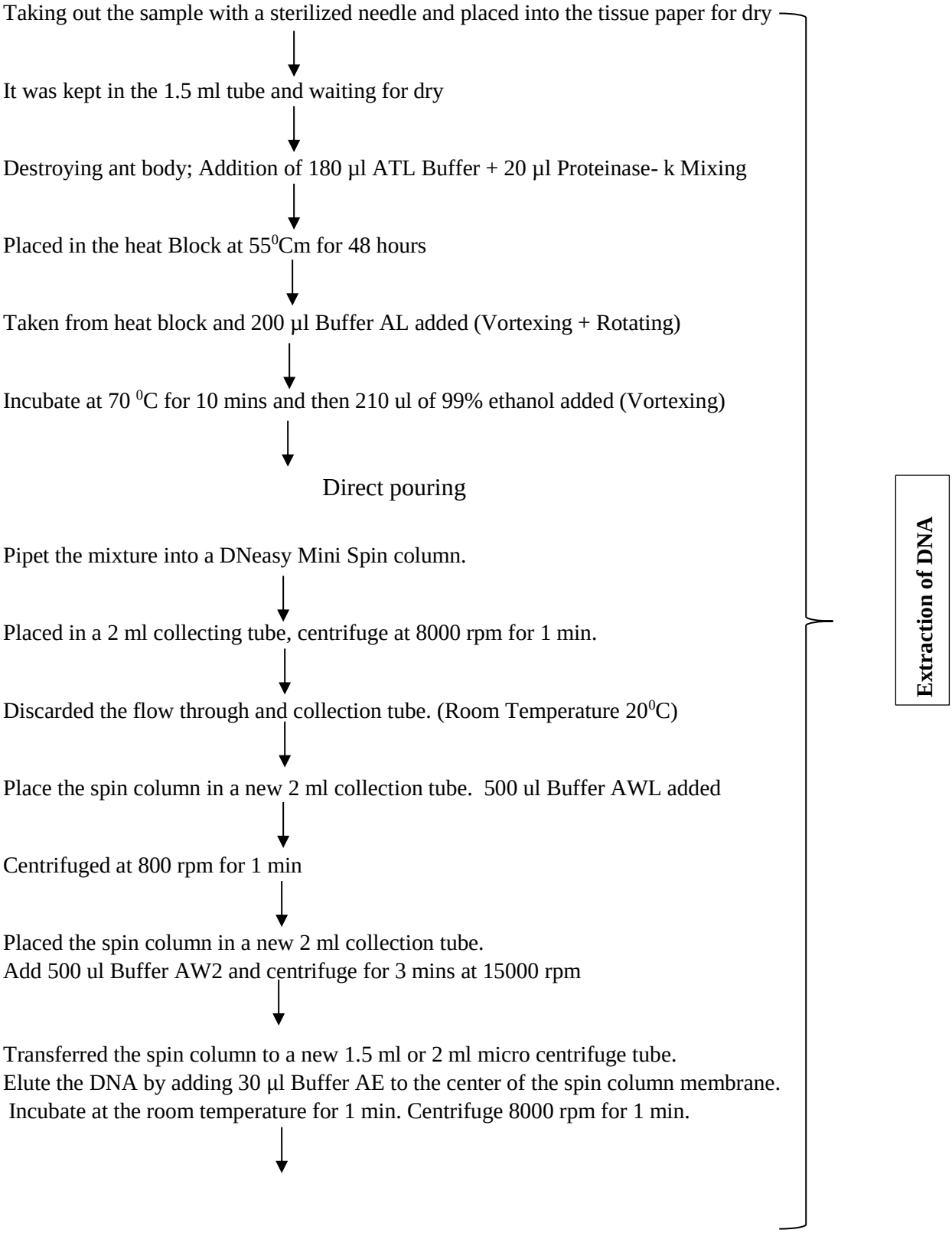
Ethanol precipitation

After completing the cycle sequencing, the products were added with sodium acetate and 99% ethanol. Then flash together and centrifuge at 15000 rpm for 10 minutes, removed all the solutions and then added 70% ethanol. Recentrifuged again at 15000 rpm for 5 minutes and then total ethanol was excluded. Then the product was kept for 90^0C for 2 minutes to remove moisture.

Sequencing

For melting DNA of the ethanol precipitated product, Hidiformamid was added and heat shock at 94°C for 2 minutes was applied. Then it was placed in the final sequencer analyzer to get the final sequence by using ABI 3100 Avant DNA Sequencer (Applied Biosystems).

3.3 Detailed flow chart of the experiments



Combination of MilliQ + 10x E7Q Buffer + DNTP Buffer + Primar (forward and reverse) + Ex Taq. All in one tube (0.6 ml tube) was done

↓

9 µl taken from the mixed solution of 0.6 ml tube and put it into 0.2 µl micro tube according to the number of samples. Extracted DNA solution of 1 ul was added by Pipetting and then flashed in refrigerated centrifuge machine at 4^0C.

↓

Placed in the PCR machine with set programing

PCR

After PCR:

Parafilm was taken and dropped 6x coding Buffer of 1.5 µl and PCR product 4 µl

↓

4 µl Drop of 100 bp DNA ladder to the additional one of the Parafilm.

↓

Poured into stained gel keeping on the plate and started Gel Electrophoresis:
For the preparation of gel, 100 ml 1XTAE Buffer + 1 g Agarose + 10 µl ethyl bromide (staining) was added Boiling to the oven at 800 watts for 1:30 min.

Gel Electrophoreses

↓

Taken the PCR product after the successful result of gel electrophoresis

↓

Add 1 µl alkaline phosphate and 1 µl exonuclease

↓

Placed in the PCR machine for summer cycle for 30 mins

Exostar

↓

For cycle sequencing 1 µl exostar product of each sample are taken in 0.2 ml tube

↓

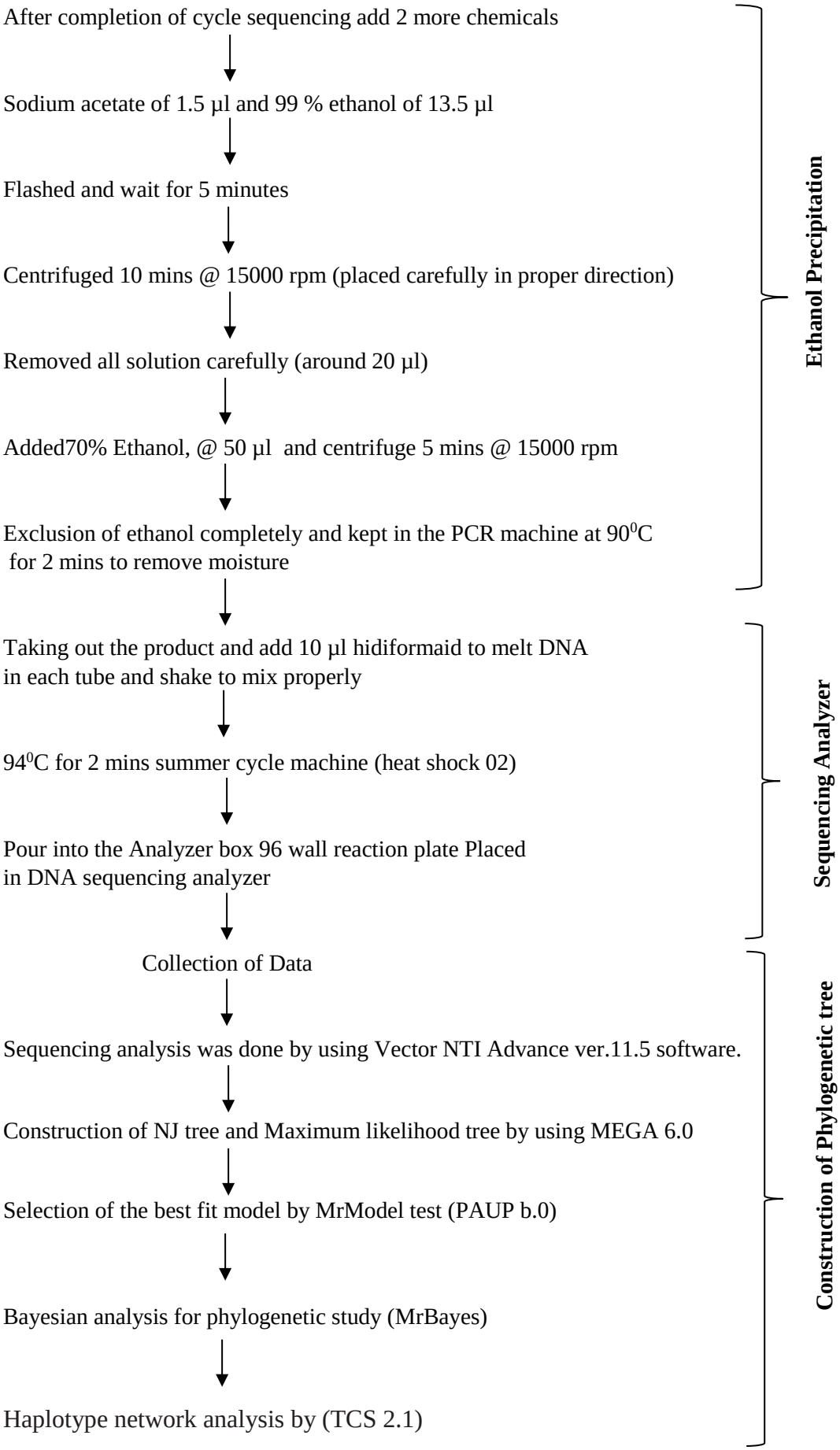
MilliQ, dilution buffer, big dye buffer, and primer are mixed in an aforesaid concentration

↓

3.9 µl mixture was added with each 1µl of Exostar product and placed for cycle sequencing

↓

Cycle sequencing


After completion of cycle sequencing add 2 more chemicals
Sodium acetate of 1.5 µl and 99 % ethanol of 13.5 µl
Flashed and wait for 5 minutes
Centrifuged 10 mins @ 15000 rpm (placed carefully in proper direction)
Removed all solution carefully (around 20 µl)
Added70% Ethanol, @ 50 µl and centrifuge 5 mins @ 15000 rpm
Exclusion of ethanol completely and kept in the PCR machine at 90^{0}C for 2 mins to remove moisture
Taking out the product and add 10 µl hidiformaid to melt DNA in each tube and shake to mix properly
94^{0}C for 2 mins summer cycle machine (heat shock 02)
Pour into the Analyzer box 96 wall reaction plate Placed in DNA sequencing analyzer
Collection of Data
Sequencing analysis was done by using Vector NTI Advance ver.11.5 software.
Construction of NJ tree and Maximum likelihood tree by using MEGA 6.0
Selection of the best fit model by MrModel test (PAUP b.0)
Bayesian analysis for phylogenetic study (MrBayes)
Haplotype network analysis by (TCS 2.1)
Ethanol Precipitation
Sequencing Analyzer
Construction of Phylogenetic tree

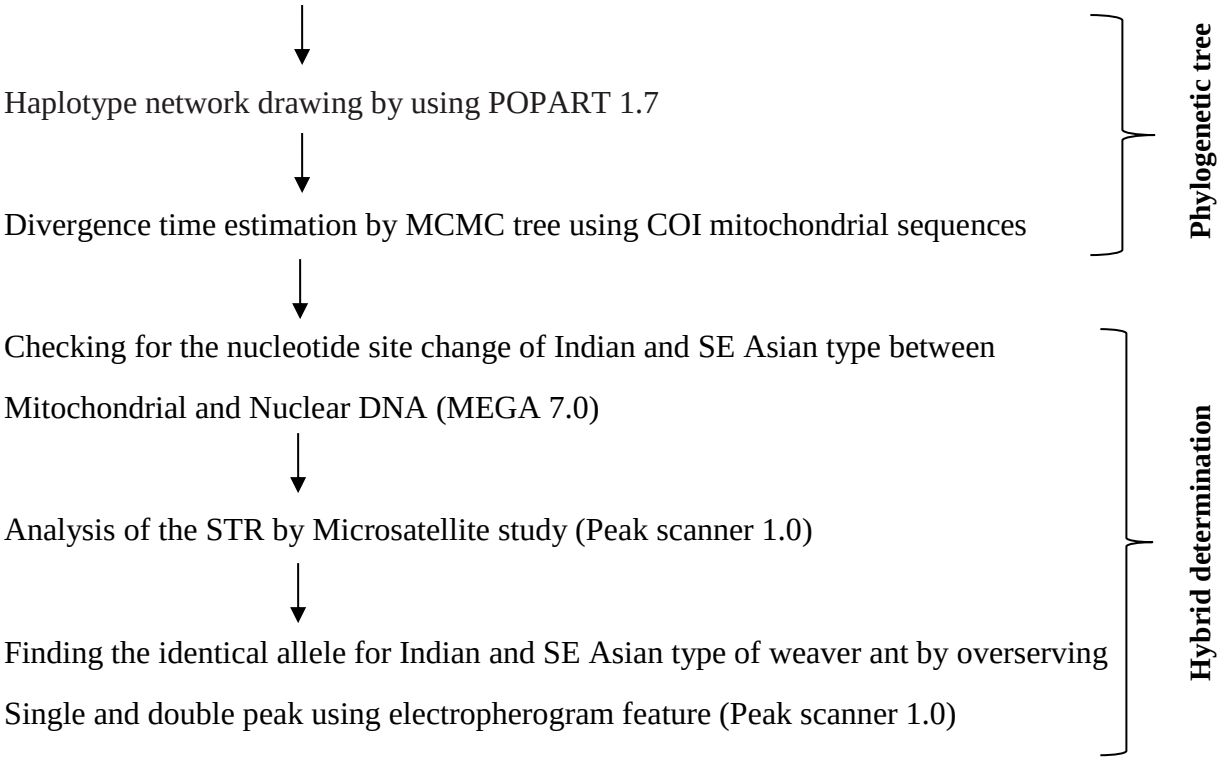


Fig. 3.3 Detailed flow chart of the experiments

CHAPTER 4

PHYLOGENETIC RELATIONSHIP INFERRED FROM MITOCHONDRIAL DNA OF *OECOPHYLLA SMARAGDINA* IN BANGLADESH

CHAPTER 4

Phylogenetic relationship inferred from mitochondrial DNA of *Oecophylla smaragdina* in Bangladesh

To reveal the molecular phylogeny of the Asian weaver ant in Bangladesh, the following experiments were conducted.

4.1 Revealing the distribution pattern of *O. smaragdina* in Bangladesh by phylogenetic study using mtDNA genes

4.2 Providing the haplotypes and distribution of the haplotype network of *O. smaragdina* in Bangladesh

In this chapter, we will discuss the results of each experiment in details.

4.1 Revealing the distribution pattern of *O. smaragdina* in Bangladesh

4.1.1 Introduction

The weaver ant, *O. smaragdina* is a broadly distributed genus and has the ability to disperse over ocean islands without human interventions. Azuma et al. (2002) first analyzed populations of *O. smaragdina* using molecular data and samples, including the *O. smaragdina* from Bangladesh. In addition, she added comprehensive samples of *O. smaragdina* from India, Southeast Asia and Australia, Azuma et al. (2006) proposed an outline of the phylogeography of *O. smaragdina* and categorized the sampled populations into 7 major clades: group 1 from India; group 2 from Southeast Asian mainland including the Indochinese and Malayan

Peninsulas, as well as the Greater Sunda Islands; group 3 from the Philippines; group 4 from Flores; group 5 from Sulawesi; group 6 from Halmahera; group 7 from Australia and New Guinea. Hereafter I will refer the Azuma et al. (2006) group 1 as Indian type and Azuma et al. (2006) group 2 as SE Asian type. Asaka (2010) extended the survey of *O. smaragdina* to South Asia and collected several samples from India and Sri Lanka. Her phylogenetic analysis showed that all analyzed samples belong to Indian clade with low levels of sequence divergence.

Azuma et al. (2006) characterized the mitochondrial sequence identity of the Bangladesh populations as belonging to the Southeast Asian clade in spite of the geographical proximity of Bangladesh to India. They hypothesized the existence of an Indian refuge for the independence of group 1 from group 2, and concluded that if the Indian population originated from the Indian refuge and Bangladesh Based on those data, Bangladesh is considered a major transition zone between Indian and Southeast Asian populations. This is the unique case of population boundaries without any distinguished geographical borders (e.g., deep sea or high mountains), although the seven groups of *O. smaragdina* based on haplotype grouping by Azuma et al. (2006) are geographically bordered by the sea. A recent study emphasized the site records for *O. smaragdina* in Bangladesh (Wetterer, 2017). Similar trends were also observed in the case of Asian elephants, where two highly divergent mtDNA of Asian elephants overlapped geographically due to secondary contact after glaciation during Pleistocene (Vidya et al. 2009).

In recent phylogeographic study Rahman et al. (2017b) identified the western Bangladeshi populations as Indian type. This is the first report of occurring Indian type in Bangladesh and this evidence proved that in Bangladesh, there is no such border to restrict the occurrence of Indian type in Bangladesh.

The purpose of the present experiment was to examine the phylogenetic relationship of weaver ant populations from northern, eastern and central part of Bangladesh for covering its distribution range in the country. The previous sampling by Azuma et al. (2006) was limited to only one site of Nurbag, Gazipur, located at the central part of Bangladesh, distinguished as Southeast Asian clades. However, *O. smaragdina* from the western parts of Bangladesh were identified as Indian clade. This result suggests the importance of phylogenetic study of central and eastern Bangladeshi populations to identify the nature of distribution.

4.1.2 Materials and method

Sampling and preparation of specimens

In 2013 to 2017 we collected adult *Oecophylla smaragdina* workers from 95 colonies at 87 localities in 47 districts belonging to 8 divisions of Bangladesh. The locality information in details are presented in Table 3.1 in the general methodology chapter. The specimens were preserved in 99% ethanol prior to DNA extraction.

Molecular study

Genomic DNA was extracted from the legs of specimens that were preserved in alcohol by using *QIAGEN DNeasy Blood and Tissue kit* (Qiagen, Maryland, USA). Amplification of both mitochondrial and nuclear DNA was done by polymerase chain reaction (PCR). The primers used for amplification are identical to primers reported by Crozier et al. (1994), Lunt et al. (1996), Azuma et al. (2002), and Azuma et al. (2006). For, mitochondrial DNA analysis, Primers for the Cytb gene fragment were Cb1 (5‘TATGTACTACCATGAGGACAAATATC’3) and tRs (5’TATTTCTTTATTATGTTTTCAAAAC’3). For the COI gene fragment, COI 1–3

(5'ATAATTTTTTTTATAGTTATACC'3) and COI 2–4 (5'TCCTAAAAAATGTTGAGGAAA'3) were used as forward and reverse primers, respectively by Crozier and Crozier (1993). The thermal cycling parameters for Cytb and COI basically followed the protocols established by Crozier and Crozier (1993) and Sameshima et al. (1999), including 95°C for 5 min for initial denaturation, 35 cycles of dissociation (92°C, 1 min), annealing (50°C for Cytb and 54°C for COI, 1 min), and extension (70°C, 2 min). Illustra ExoProStar was followed according to the instruction of the manufacturer GE Healthcare. For cycle sequencing, ABI PRISM Big Dye Terminator v3.1 cycle sequencing kits from Applied Biosystems were used in an automated sequencer. Sequencing reactions were performed by using ABI 3100 Avant DNA Sequencer (Applied Biosystems).

Phylogenetic inference

For the phylogenetic analysis of *O. smaragdina* populations, combination of mitochondrial cytochorome b and cytochorome oxidase subunit-1 gene of 1143 bp were used in the analysis. Of which, 63 samples for Cytb and 72 samples for COI genes have been used with 504 bp and 639 bp, respectively. In addition, sequence data of both COI and Cytb were used from Azuma et al. (2002), Azuma et al. (2006) and Asaka (2010). The sequence data of both COI and Cytb of *Oecophylla longinoda* from Cameroon were used as outgroup in this analysis. The sequencing alignment was done by using Vector NTI Advance ver. 11.5 software. The sequences of Cytb and COI were aligned by using MEGA 6.0 software (Tamura et al. 2013). Phylogenetic trees were inferred from 106 concatenated matrix sequences of both COI and Cytb genes, conducted by MrBayes 3.1.2 (Nascimento et al. 2017; Ronquist and Huelsenbeck, 2003) with 1,000,000 generations (Felsenstein, 1981). For the selection of best–fit model, MrModeltest 2.3 was performed with PAUP*4.0b10. (Nylander, 2004; Posada and Crandall, 1998). The substitution model, GTR + I + G was used in mitochondrial COI and Cytb

genes were used the alignment was partitioned into 1st, 2nd and 3rd nucleotide positions. The nucleotide sequences for both Cytb and COI were deposited in the GenBank with accession number are corresponding to Appendix 1.

4.1.3 Results and discussions

Bayesian phylogenetic tree inferences

Among identified 197 variable characters, 133 were parsimony informative. The Bayesian analysis of the mitochondrial concatenated matrix dataset of 1143 bp showed that the Bangladeshi *O. smaragdina* samples were nested into two distinct clades (posterior probability > 90%) (Fig. 4.1). Bangladeshi 48 weaver ant samples were nested with Indian clade of *O. smaragdina*, whereas 39 samples were nested with the Southeast Asian clades. Therefore, the occurrence of both the Indian and SE Asian types was found within Bangladesh. Based on the phylogenetic tree obtained, the Bangladeshi populations showed the overlapping distribution of the Indian and SE Asian types of *O. smaragdina* (Fig. 4.1).

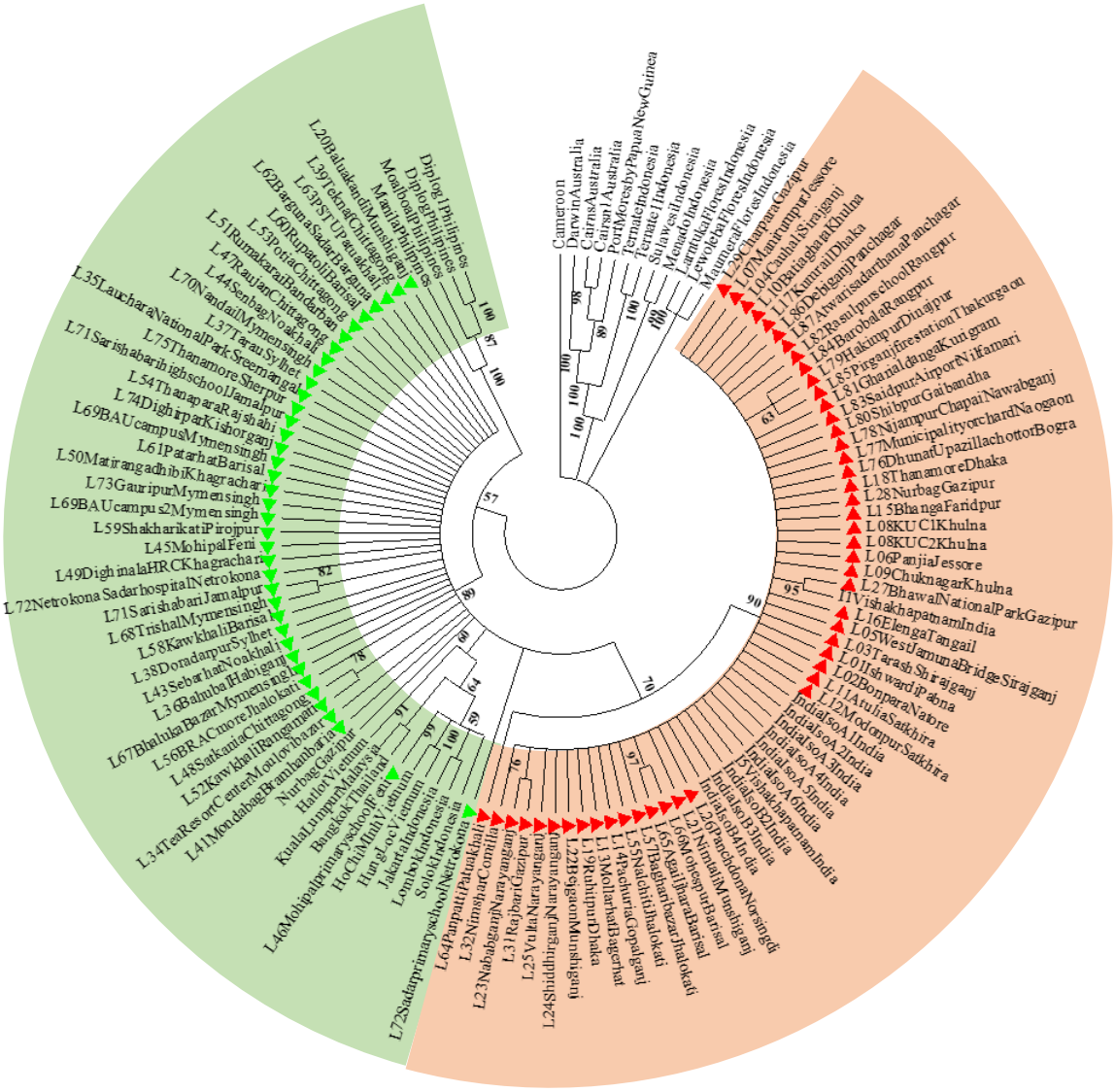


Fig. 4.1 Bayesian phylogenetic tree of Bangladeshi *O. smaragdina* populations as inferred from the mitochondrial gene fragments (1143bp) of the COI (639 bp) and the Cytb (504 bp) genes (substitution model: GTR + I + G 1000000 generations used in MrBayes 3.1.2 MrModeltest 2.3,PAUP*4.0b10). Number adjacent to internal nodes represent bootstrap values (%). Green rectangle indicates the samples from Bangladesh in the present study. Additional DNA sequence data were downloaded from DDBJ GenBank. The number ahead of each locality indicates the locality number. Red and green triangle denotes the samples from Bangladesh clustering with Indian and SE Asian types, respectively. Two shaded color represents the two types of *Oecophylla* population in Bangladesh.

Summary of the distribution pattern

The western part of Bangladesh was mainly occupied by Indian types, the Eastern part was dominated by SE Asian types, while, the mixture of two clades was found in the central parts of Bangladesh. (Fig. 4.2). The detailed samples results based on type are showed in Table 4.1. Out of 87 localities, we failed to detect 3 locality types of *Oecophylla*. The samples from rest 47 localities were identified as Indian type and 37 locality samples as SE Asian types (Table 4.1). According to those results, Bangladesh can be considered as a transitional zone of both the two clades. Divergence time of the ant genus *Oecophylla* is thought to be a significant factor of such distribution. Diversification within groups in this continent was recorded from the Middle Pliocene to Early Pleistocene (Azuma et al. 2002). After this period, world has encountered a significant climatic change.

It might also affect the distribution of *Oecophylla* in different parts of the world. Lokkers (1986) suggested two limiting factors, low temperature and humidity for distribution range of *Oecophylla* in Australia. During Last Glacial Maximum (LGM), the tropic region shifted southward and it retained northward after glaciation. The present study suggested that the Indian and Southeast Asian clades of *O. smaragdina* expanded their distribution northward along suitable regions with high temperature and humidity, and then the two types supposedly encountered and overlapped in central Bangladesh.

Table. 4.1 Detailed summary of the distribution pattern of weaver ant in Bangladesh

Sampled area	Surveyed district	Sampled colonies	Sampled localities	Type (in locality)		Failed
				Indian	SE Asian	
Area 01	14	18	18	17	1	
Area 02	11	23	22	15	7	
Area 03	10	30	25	13	11	1
Area 04	7	15	13	2	10	1
Area 05	5	9	9	0	8	1
Total	47	95	87	47	37	3

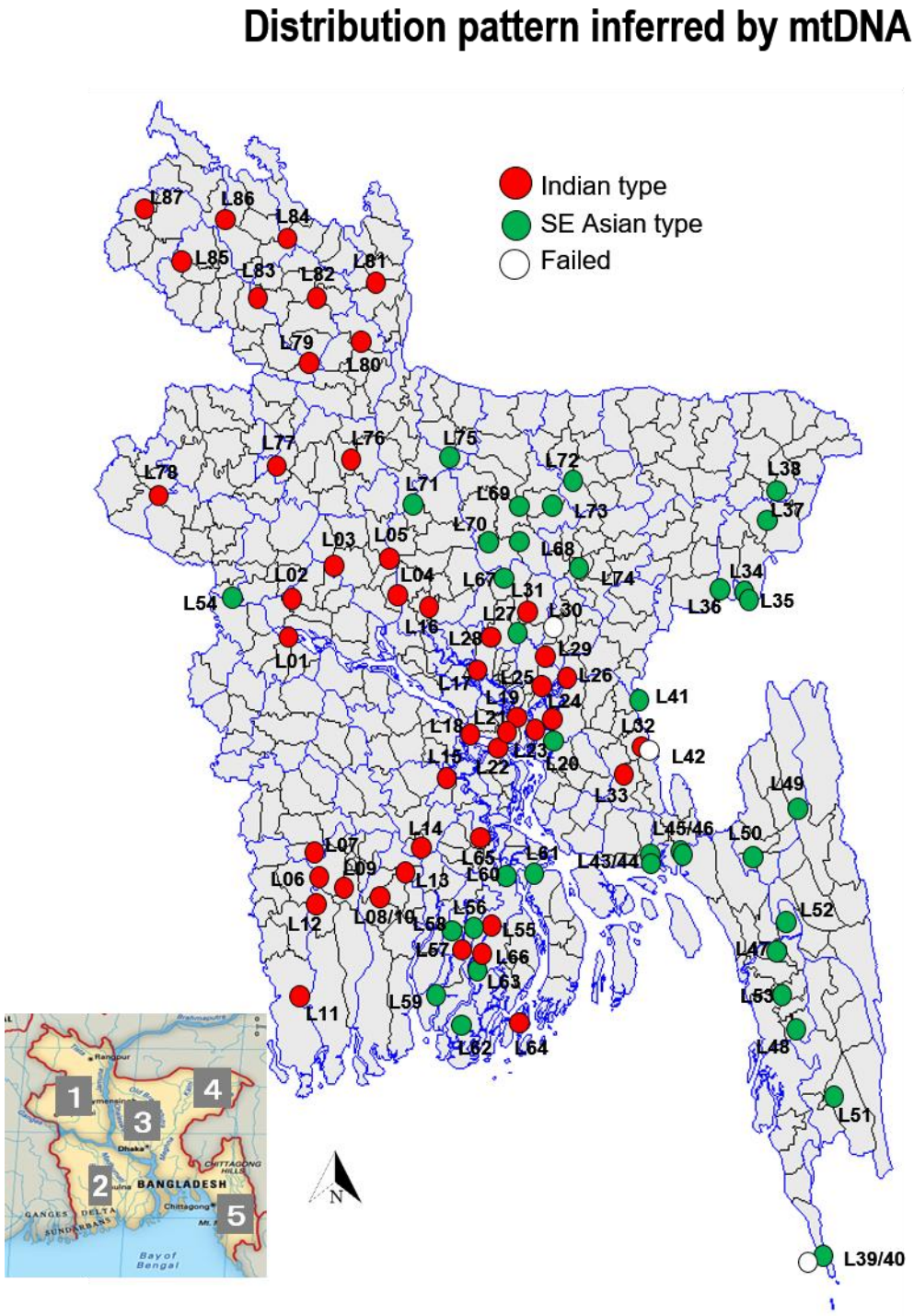


Fig. 4.2 Distribution pattern of Indian and SE Asian types of *O. smaragdina* in Bangladesh.

4.2 Haplotypes and its network distribution of *O. smaragdina* in Bangladesh

4.2.1 Introduction

Due to widespread distribution and evolutionary history, *O. smaragdina* was considered to be a valuable material for biogeographic study in tropical Asia. Azuma et al. (2006) proposed an outline of the phylogeography of *O. smaragdina* and they revealed the identity of *Oecophylla* population of Bangladesh as South East Asian type that distinct from the Indian types by means of mitochondrial DNA analysis of COI and Cytb genes. Similar findings were observed from Asaka (2010) who found a large genetic gap of *Oecophylla* populations from Bangladesh from India and Sri Lanka where Indian population originated from the Indian refuge and Bangladesh population originated from the Indochina refuge. However, Rahman et al. (2017b) in their phylogeographic study based on mitochondrial Cytb and COI genes, identified the western Bangladeshi population of *Oecophylla* as Indian type. Based on this interesting result, a details comprehensive phylogeographic study including eastern, northern and central part of Bangladesh were conducted by Rahman et al., (2017a). They identified that the western Bangladesh population were mainly Indian type whereas the Eastern part was dominated by South East Asian type, although the middle part of the country were occupied by overlapping populations of both Indian and South East Asian type. Climatic oscillations during the Pleistocene period have a strong effect on the genetic diversity and distribution of extant species (Hewitt, 2004). Increased aridity and decreased temperatures during the glacial led to a fragmentation of tropical environments and thus influenced the genetic diversity of *Oecophylla* in these tropics as well. Phylogeographic study with diversified haplotype

distribution network precisely congregate the genetic diversity phenomena in an particular geographical area.

The purpose of this experiment was to identify the available haplotypes and analyze haplotype network for better understanding the phylogeographic results of *O. smaragdina* in Bangladesh. Our goal was to get insights into the genetic structure and geographical patterns of genetic variation of *Oecophylla* across much of its distribution based on this haplotype distribution in Bangladesh.

4.2.2 Materials and methods

Sampling and preparation of specimens

The mitochondrial COI genes were extracted from 72 individuals collected from 71 localities of Bangladesh. The localities used in haplotype network analysis are mentioned in Table 3.1 in general methodology chapter.

Molecular data collection

For the molecular study of haplotype network, we followed the protocol as mentioned in the general methodology in chapter 3. We have used the mitochondrial COI data for revealing the haplotype network.

Haplotype network analysis

For haplotype network analysis of *O. smaragdina* populations, a total 93 sequences have been analyzed including 71 concatenated sequences of COI genes from 67 localities of Bangladesh have been used with 639 bp, In addition, referential sequence data of COI was used from Azuma et al. (2002), Azuma et al. (2006) and Asaka (2010). The sequencing analysis was done by using Vector NTI Advance ver. 11.5 software. The sequences of COI were aligned by using MEGA 6.0 software (Tamura et al. 2013). Haplotype network were determined by using TCS 1.21 software and the network figure has been generated based on TCS results by POP ART- 1.7 (Clement et al. 2000). The nucleotide sequences of COI were deposited in the GenBank with accession number were corresponding to appendix 1.

4.2.3 Results and discussions

Oecophylla smaragdina haplotypes in Bangladesh

Forty-two haplotypes were recorded from 93 concatenated sequences of COI mitochondrial gene of 639 bp. The nucleotide diversity was 0.0248709 and a total 89 segregating sites were recorded. The total number of parsimony informative sites was 50. Seventy two concatenated sequence of COI genes have been used from the samples of *Oecophylla* in Bangladesh resulted 25 haplotypes. The 25 haplotypes identified from Bangladesh are presented in Fig. 4.3. In this figure, only the variable sites and its position were displayed.

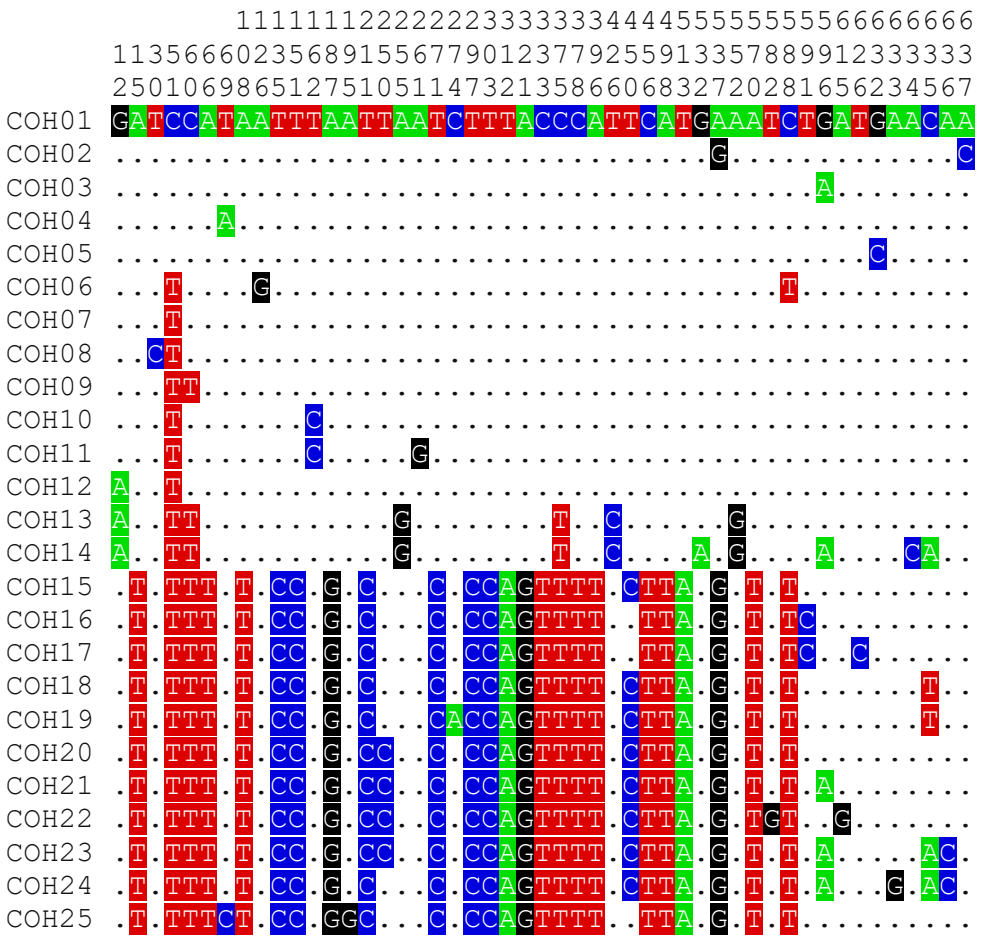


Fig. 4.3 Twenty five haplotypes in Bangladesh and their variable sites with changed position. The upper three numeric lines denotes the position on longitudinal basis of the each sequence. Each dot represent the identical sequence.

Haplotype network study

Bangladeshi haplotypes were marked from COH01 to COH25. COH26 to COH27 are considered as Indian type, COH28 to COH35 are from Indonesia, COH36 to COH38 from Philippines, COH39 to COH40 from Vietnam, COH41 from Malaysia and COH42 from Thailand (Fig.4.4). The detailed haplotype list were presented in Table 4.2. Bangladeshi haplotypes were clustered into two distinct zone on the network tree. Haplotype COH01 to COH13 were closely associated with the haplotypes from the Panjab state of India with COH26 and COH27. Moreover, COH10 and COH12 includes the haplotypes from Indian isolates along with the Bangladeshi haplotypes. On the other hand the Bangladeshi haplotypes from COH14 to COH25 are found to be clustered with the haplotypes associated from SE Asian clades. They were well connected with the haplotypes from Indonesia and Vietnam. Like other ant species, *Oecophylla smaragdina* were profoundly influenced by Pleistocene glaciation. This also happened in this South Asian continent.

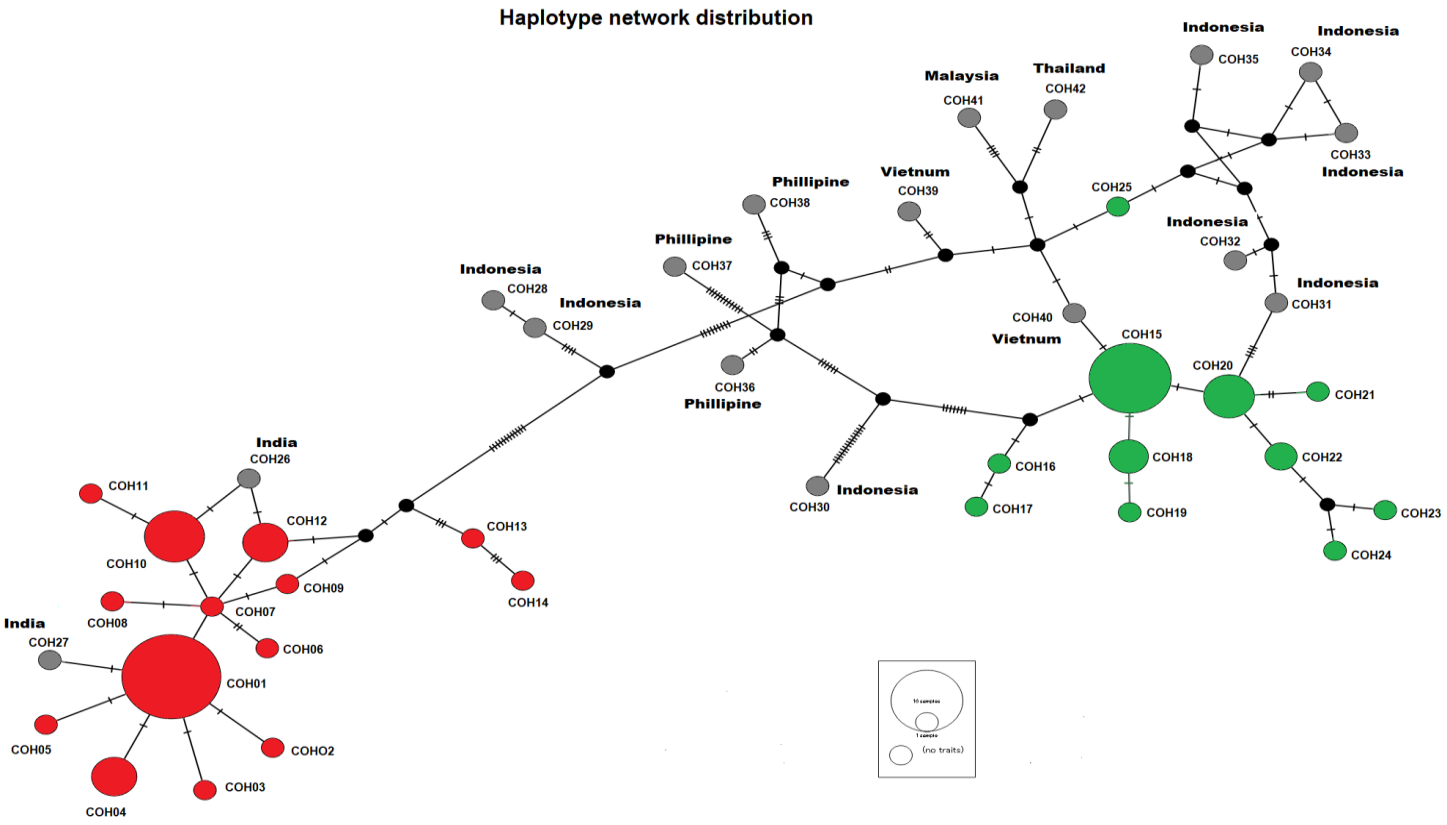


Fig. 4.4 Haplotype network of *O. smaragdina*. Solid colored circles indicate individual haplotype. A single bar connecting two haplotypes corresponds to a single base pair mutation. Each hatch mark on the bar indicate single base pair mutations. Haplotypes belonging to Indian clades are separated from the SE Asian clades by one of the largest number of mutational steps (27) inferred in the network. The locality information corresponding to each haplotype are represented in table 4.2.

Table 4.2 List of haplotypes inferred from COI gene sequences

SI No.	Haplotype No.	Corresponding locality (Country)
1	COH01	L01, L02, L05, L07, L09, L10, L11, L14, L15, , L55, L57 L76, L77, L78, , L80, L81, L83, L86, L87 (Bangladesh)
2	COH02	L64 (Bangladesh)
3	COH03	L04 (Bangladesh)
4	COH04	L18, L21, L26, L32 (Bangladesh)
5	COH05	L82 (Bangladesh)
6	COH06	L27 (Bangladesh)
7	COH07	L16 (Bangladesh)
8	COH08	L29 (Bangladesh)
9	COH09	L12
10	COH10	L08, L08 (02), L03, L79, L84 (Bangladesh) ISOA2 (India)
11	COH11	L28
12	COH12	L06 (Bangladesh), ISOA3, ISOA4, ISOA5 (India)
13	COH13	L65 (Bangladesh)
14	COH14	L66 (Bangladesh)
15	COH15	Nurbag, L34, L67, L36, L43, L38, L45, L73, L50, L69, L74, L75, L35 (Bangladesh)
16	COH16	L52 (Bangladesh)
17	COH17	L48, L39 (Bangladesh)
18	COH18	L58, L59, L61 (Bangladesh)
19	COH19	L54 (Bangladesh)
20	COH20	L72 (2), L72, L41, L49, L37 (Bangladesh)
21	COH21	L69(2), L71(2) (Bangladesh)
22	COH22	L56 (Bangladesh)
23	COH23	L71 (Bangladesh)
24	COH24	L68 (Bangladesh)
25	COH25	L46(Bangladesh)
26	COH26	ISOA6 (India)
27	COH27	ISOA1 (India)
28	COH28	Larantuka (Indonesia)
29	COH29	Lewleba (Indonesia)
30	COH30	Sulawesi (Indonesia)
31	COH31	Bogor (Indonesia)
32	COH32	Bali (Indonesia)
33	COH33	Rakata (Indonesia)
34	COH34	Jakarta (Indonesia)
35	COH35	Lombok (Philippine)
36	COH36	Manilla (Philippine)
37	COH37	Diplog (Philippine)
38	COH38	Moalboal (Philippine)
39	COH39	Hunloc (Vietnam)
40	COH40	Hatlot (Vietnam)
41	COH41	Kualalumpur (Malaysia)
42	COH42	Bangkok (Thailand)

The haplotype network showed a clear distribution of Indian and SE Asian haplotypes in Bangladesh. This shape of network seems bottleneck-type. The two types (groups) are connected with many missing haplotypes (longer branch); it is suggesting survived populations remained in refugia. Star-like subnetworks suggest recent expansion of populations. In the geographical distribution suggested that the western haplotypes are relatively less diverse and is connected mainly with the Indian haplotypes while the haplotypes from eastern part showed more genetic diversification.

Vidya et al. (2009) have found the similar results in their phylogeographic study of distribution of Asian elephants. This sorts of haplotype network distribution revealed that there was no effective barrier of separating Indian and SE Asian haplotypes in Bangladesh, hence Bangladesh is considered as a transitional zone of genetic diversification of *Oecophylla* populations. In the previous phylogeographic study by Azuma et al. (2002, 2006), the diversification of *Oecophylla* occurred during Pleistocene in this continent and the effects of last glacial maximum (LGM) might have an influential phenomenon of such distribution. This can be supported by the findings of Seal et al. (2015) who mentioned in their phylogeography of two *Trachymyrmex* species along the south eastern coastal plain of North America and characterized the both species by reduced haplotypic variation that may indicate recent expansion and / or bottleneck associated with changed climatic condition.

CHAPTER 5

DIVERGENCE AND CONTACT OF INDIAN AND SE ASIAN TYPES OF *OECOPHYLLA SMARAGDINA* IN BANGLADESH

CHAPTER 5

Divergence and contact of Indian and SE Asian types of *Oecophylla smaragdina* in Bangladesh

5.1 Divergence time estimation of *O. smaragdina* in Bangladesh

5.1.1 Introduction

Oecophylla smaragdina, based on the fossil record might have originated in the early Paleogene (ca. 60 Ma) in the Palaearctic region, and dispersed during the climatic changes of the Eocene–Oligocene transition at ca. 43 Ma (Dlussky et al. 2008). The abrupt cooling during this period had great impacts on biodiversity (Katz et al. 2008). During this period, Earth's climate shifted from a relatively ice-free world to one with glacial conditions in polar regions characterized by substantial ice sheets (Bowen, 2007). Recently, Blamier et al. (2015) estimated the divergence time of the genus *Oecophylla* based on the fossil records and ultra conserved elements (UCEs). They estimated that *Oecophylla* crown group evolved during Oligocene at ca. < 30 Ma and stem-group evolved during early Eocene at ca.50 Ma. Wetterer (2017) gave a glimpse of distribution of *O. smaragdina* in this continent with some interesting evidence of distribution. *Oecophylla smaragdina* and *O. longinoda* have diverged in 13.3 to 11.3Ma ago, in the late Miocene. Diversification of seven groups occurred between the middle of Miocene to early Pliocene. While the diversification within groups was recorded between middle Pliocene to early Pleistocene (Azuma et al. 2006). There was no evidence regarding the divergence time of group 1 or Indian types. Due to widespread distribution, *O. smaragdina*

was considered valuable materials for biogeographic study in tropical Asia. In the same period of Eocene-Oligocene transition, the emergence of South-East (SE) Asia, caused by the collision of the Eurasian and Australian plates (Buerki et al. 2011), already formed a large emergent land area by the late Cretaceous (including, for example, the older parts of Malaysia and Southwest Borneo), were created from this period onwards, with a peak of tectonic activity during the Miocene (Hall, 2009). Azuma et al. (2006) estimated the divergence time of *Oecophylla smaragdina*. Based on that estimation, *O. smaragdina* and *O. longinoda* diverged 13.3- 11.3 Ma, in the late Miocene. Diversification within the seven groups have been estimated between 7.8 and 3.6 Ma (Fig. 5.1). As Bangladesh has the overlapping populations of *O. smaragdina* from group 1 and group 2, so, the estimation of the divergence history of that two types would be an important biogeographical evidence of divergence history. The purpose of this study is to get insights of the estimation of the time of divergence of Indian and SE Asian haplotypes in Bangladesh.

5.1.2 Materials and methods

In this analysis, a total of seven localities nucleotide sequence data of Cytochrome oxidase subunit 1 (COI) and Cytb data were used. Those seven localities includes L07, L08, L26, L32, L41, L49, L66, respectively. The detailed locality information are presented in Table 3.1. in the general methodology chapter. The sequencing techniques were described in the general methodology chapter. In addition, in this analysis, sequence data of both COI and Cytb were used from Azuma et al. (2002), Azuma et al. (2006) and Asaka (2010) retrieved from DDBJ GenBank. Sequence data of both COI and Cytb of *Oecophylla longinoda* from Cameroon were used as outgroup in this analysis. The sequencing analysis was done by using Vector NTI Advance ver. 11.5 software. Haplotypes of Cytb and COI were aligned by using MEGA 6.0

software (Tamura et al. 2013). Out of these seven localities, five localities were Indian type and two localities were of SE Asian type, inferred by both mitochondrial and nuclear DNA analysis. A total of 1149 bp of COI and Cytb nucleotide sequences of the samples collected from those 7 localities were used along with the samples from Malaysian and Cameroon as references. A Bayesian Markov chain Monte Carlo (MCMC) packages (Drummond et al. 2006), which relay on a relaxed molecular clock approach, were used to estimate the divergence time. Optimal nucleotide substitutional model were chosen by Mr. ModelTest.

5.1.3 Results and discussion

The results of divergence time are shown in Fig. 5.2 in the MCMC divergence tree. In this tree, sequences of the haplotypes from Cameroon is considered as outgroup, and Malaysian and Indian haplotypes were used as reference. Although these divergence tree is not showing quite a good resolution due to lack of so many resolved sister groups but we can get some interesting evidence.

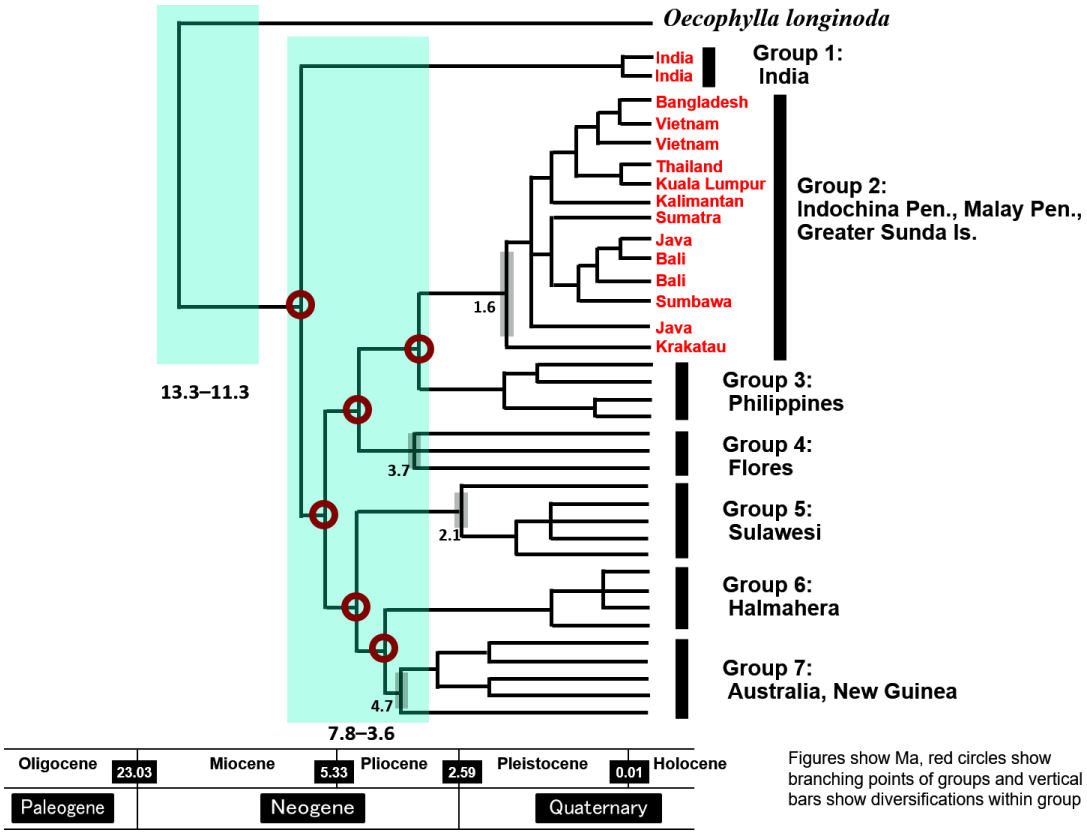


Fig. 5.1 Divergence time of *Oecophylla smaragdina* modified from Azuma et al. (2006). The red text of the area indicating the Indian and SE Asian group of *Oecophylla* calculated by Azuma et al. (2006).

According to this divergence tree, Indian type diverged ca. 2.2 Ma and SE Asian type diverged ca. 0.20 Ma corresponding to early to late Pleistocene. From this tree, we get the information regarding the divergence time of the Indian type that was missing in the previous study by Azuma et al. (2006). The quaternary paleo-geographic history of the SE Asia during the ice age showed some significant evidence of the divergence pattern. Vidya et al. (2009) discussed the distribution of the two clades of Asian elephants and proved the effect of allopatrically in different glacial refugia, the alpha clade in the Myanmar region and the beta clade possibly in southern India–Sri Lanka, 1.6–2.1 Ma ago. Results from nested clade and dispersal–vicariance analyses indicate a subsequent isolation and independent diversification of the b clade in both Sri Lanka and the Sunda region, followed by northward expansion of the clade.

Pleistocene glaciation has significant influential effects on temperature and rainfall that favored the diversification of several ant species including *Oecophylla* distribution (Lokkers, 1986). Rainfall moderate the vegetation density while low temperature inhibiting larval development. As Pleistocene glaciation had the effect on both these two limiting factors of *O. smaragdina* distribution so the divergence resulted (Lokkers, 1986).

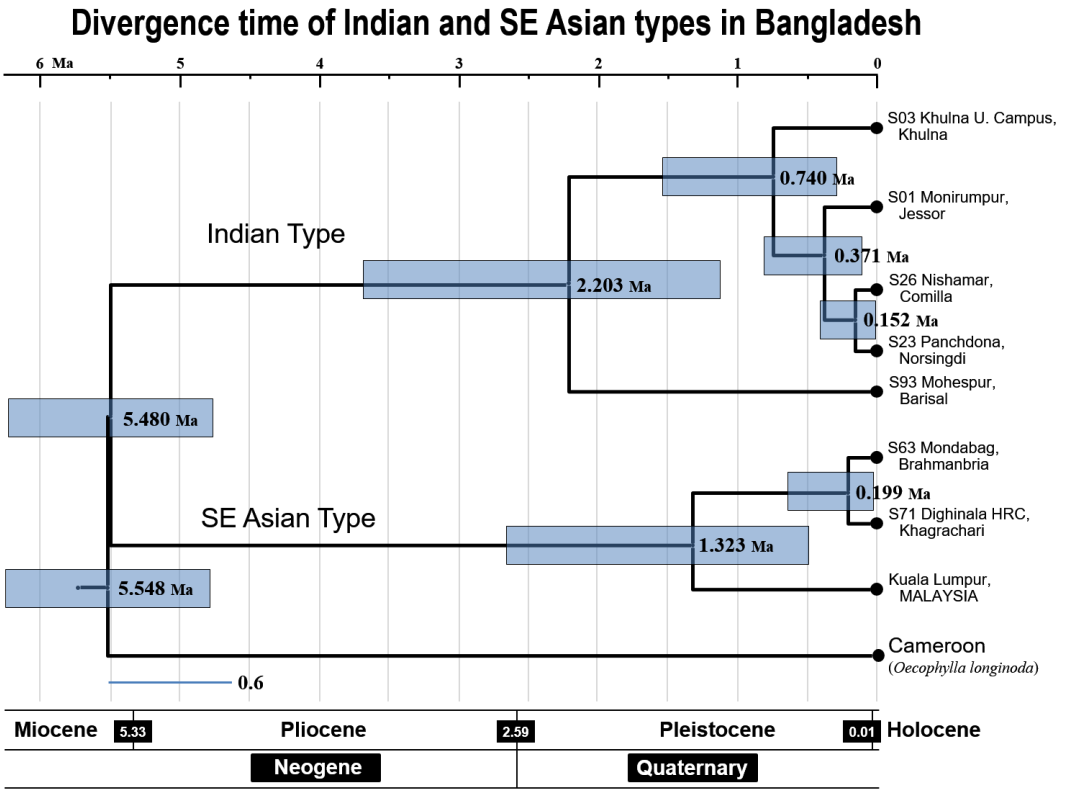


Fig. 5.2 Divergence time of Indian and SE Asian types of *O. smaragdina* in Bangladesh.

Bangladesh's geology began 350 million years ago when the Pangean supercontinent broke apart (Mannan, 2002). The Bengal Basin began 127 million years ago when the Indian Plate rifted away from Antarctica at 18 cm per year for 20 million years. Basin began 127 million years ago when the Indian Plate rifted away from Antarctica at 18 cm per year for 20 million years. This rapid velocity stopped 55 million years ago and was followed by a period in which little or no spreading took place west of the ninety East ridge for 20 million years. Formation of Himalaya due to continental collision with the following figures (Aitchison et al. 2008). The Indian subcontinent lies atop the Indian tectonic plate, a minor plate within the Indo-Australian Plate (Ali, 2005).Its defining geological processes began a north eastwards drift lasting fifty million years across the then unformed Indian Ocean. The subcontinent's subsequent collision with the Eurasian Plate and subduction under it, gave rise to the Himalayas, the planet's highest mountains (Aitchison et al. 2008). In the former seabed immediately south of the emerging Himalayas, plate movement created a vast trough, which, having gradually been filled with sediment borne by the Indus and its tributaries and the Ganges and its tributaries, now forms the Indo-Gangetic Plain (Uddin and Lundberg, 1999).

According to our study, diversification of both the types occurred in Pleistocene. There is a rapid fluctuation of temperature from late Pliocene to Pleistocene. It might influenced the distribution of *Oecophylla* in different part of the world. During that time, fragmentation and expansion of *Oecophylla* population happened. During LGM, the tropic changed from North to southward and it retained to north after glaciation. Thus, Bangladesh become vacant after glaciation with the tropical climate and hence the occurrence of the both the types have been found subsequently.

5.2 Hybridization scenario of Indian and SE Asian types of weaver ant using long wavelength rhodopsin (LW *Rh*) and microsatellite markers

5.2.1 Introduction

Previous phylogeographic study on *O. smaragdina* based on mitochondrial Cytb and CO1 genes identified two major types where Indian types occurred mainly in India and Sri Lanka while the Southeast Asian (SE Asian) types have been observed in most of the SE Asian countries including Bangladesh (Azuma et al. 2006). However, recent phylogenetic study revealed the occurrence and dominancy of Indian type in the western part (Rahman et al. 2017b) and SE Asian types in the eastern part and overlapping population of *O. smaragdina* at the central part of Bangladesh (Rahman et al. 2017a). In Bangladesh, the occurrence of different types implies the chance of hybridization. Recently, for inferring the evidence of hybridization a comprehensive view of evolutionary history by analyzing nuclear and mitochondrial DNA was found effective and has been using extensively (Roos et al. 2011). The nuclear long-wavelength rhodopsin gene (LW *Rh*) belongs to a family of visual pigment genes and has been regarded as a useful marker for the between-species level phylogeny of insects, especially Hymenoptera (Ascher et al. 2001; Cameron and Williams, 2003). In the LW *Rh* analysis, Azuma et al. (2006) categorized the Indian and SE Asian type of *O. smaragdina* population as Smaragdina B and Smaragdina A by comparing the nucleotide sequences. This analysis suggested that SE Asian are derived and monophyletic while the Indian type is the ancestral. Discordant genetic relationships between mtDNA and nuclear DNA results from mitochondrial introgression or the incomplete lineage sorting (Eto et al. 2013). Therefore, for confirming the validity of phylogenetic study LW *Rh* nuclear DNA sequence of *O. smaragdina*

colonies from some randomly selected localities in Bangladesh need to be compared with the results of mtDNA sequences.

In the recent time, the 'microsatellite' markers, also known as simple sequence repeats (SSRs) are considered as the codominant as it identifies the polymorphism along with detection of the high level of heterozygosity and has high mutation rate (Loxdale and Lushai, 1998; Hancock, 1999). The microsatellite markers consist of short but repeated units of around two to six base pairs in length and including both coding and non-coding regions can be up to 200 bp (Beukeboom and Zwaan, 2005). By combining the alleles from each parental species in all loci, microsatellites can indicate F1 hybrids as microsatellite is considered as nuclear DNA markers, although the event of backcrossing has the significant effect for detecting hybridization (Goodman et al. 1999). Through mtDNA the maternal identity of the species might be possible as mtDNA is maternally inherited, but mtDNA cannot solely detect hybridization. Hybridization can be revealed through the inconsistency result of mt DNA and nuclear DNA analysis when back crossing with the parental species occures. However by only microsatellite study, hybridization can be undetected if backcrossing occurred repeatedly because back crossing sometimes causes the complete elimination of the nuclear genome of the maternal species from hybrid (Steiner et al. 2011).

Mallet (2005) reported that hybridization often take place among the closely related species because of insufficient evolvement of reproductive barriers. In that sense there was a great chance of occurring hybrid that needed to be trace out. This may lead the inconsistency between nDNA and mtDNA types within the cell of a hybrid.

The main objectives of this study were to analyze the nuclear LW *Rh* gene for detecting possible hybridization by pointing out the inconsistency of nucleotide sequences. The occurrence of both Indian and SE Asia types, created the possibility of a hybrid colony of *O.*

smaragdina, especially in the overlapping zone in Bangladesh. In the present context, there are four possible combinations can happened within the cell if the crossing between the male and female of Indian and SE Asian type occurred. The occurrence of either Indian or SE Asian in both mtDNA and nDNA is consistent, i.e., both the mtDNA and nDNA are Indian type or both are the SE Asian type. The inconsistency might be find out if the nDNA are Indian type and mtDNA are SE Asian type or the vice-versa (Fig. 5.3a). In the case, where mtDNA are SE Asian type, the crossing might to be expected between the male of Indian type and female of SE Asian type and mtDNA are maternally inherited. Through, comparing the results of the nucleotide sequences obtained from nDNA analysis by LW *Rh* and mtDNA and nDNA and if there any inconsistency was observed, it can partially reveals the hybrid phenomenon but cannot fully detect the hybridization status of the colony. In addition, in the diploid cell, the nucleus can contain both the Indian and SE Asian types. The mtDNA types of that cell with both Indian and SE Asian types will detect the crossing pattern either the male or female are of Indian or SE Asian type as shown in Fig. 5.3b. Microsatellite markers could identify the heterozygosity in the above-mentioned condition. The detailed explanation about the justification of choosing nuclear DNA and microsatellite analysis for revealing the hybridization status were described in Fig. 5.3. Therefore, the another purpose of this study was to facilitate further confirmation of heterozygosity along with the actual hybrid condition within the diploid nucleus by detecting identical allele between Indian and SE Asian type within the colony.

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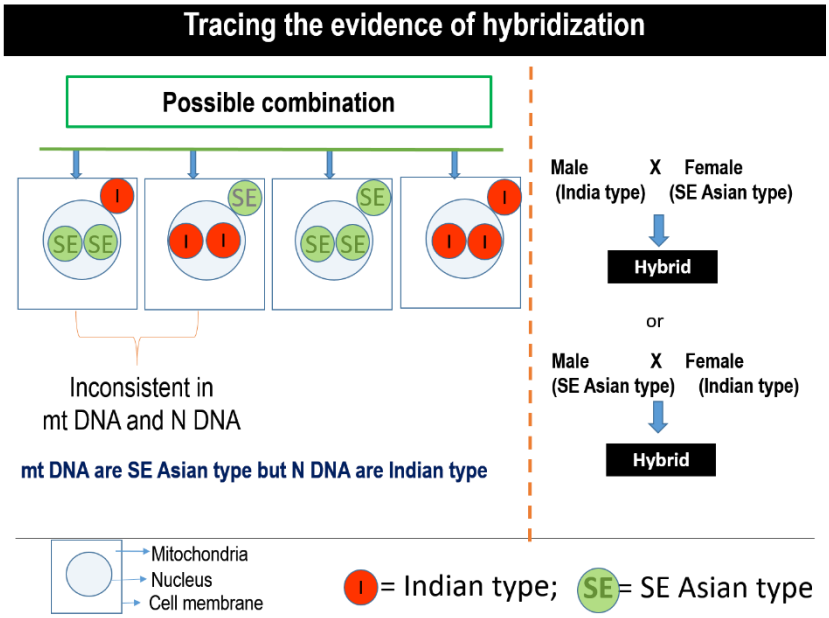

Fig. 5.3a. Consistent and inconsistent status of mitochondrial and nuclear DNA in cell

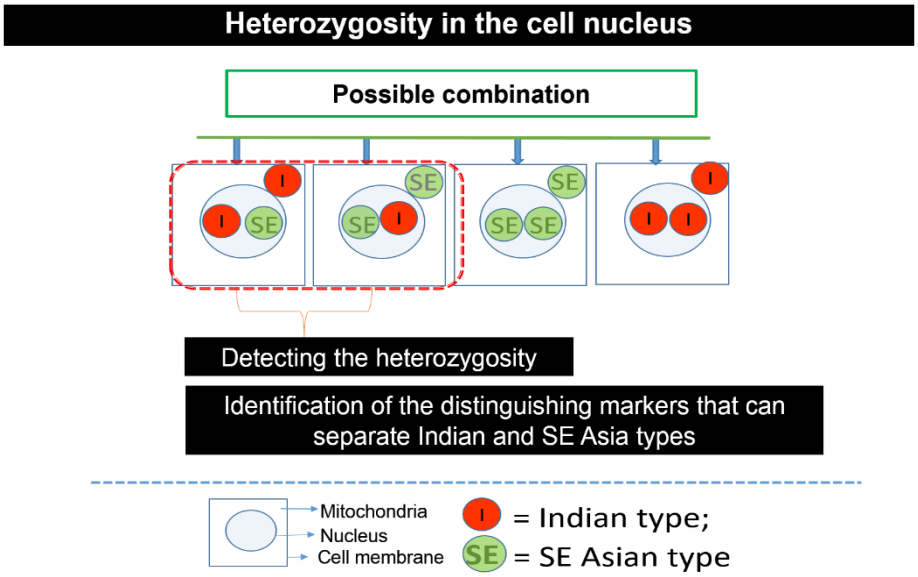

Fig. 5.3b. Heterozygous status of the cell nucleus

Fig. 5.3 mtDNA and nDNA inconsistency pattern and the heterozygous status within the cell due to crossing of male and female of different Indian and SE Asian types.

5.2.2 Materials and methods

LW *Rh* analysis

Adult *Oecophylla smaragdina* workers from 40 colonies of 39 localities of Bangladesh were used for performing this study in 2013 to 2017. The detailed procedure of nDNA analysis by Long wave length Rhodopsin (LW *Rh*) were described in the chapter III in the detailed methodology section. The locality information with their corresponding locality code are presented in chapter 3, Table 3.1.

The sequencing results of Nuclear DNA analysis from 40 samples of 39 localities in Bangladesh have been deposited to GenBank. The locality information with accession number was corresponding to the tables in the general methodology. For finding the inconsistency, the comparison was done between the previously sequenced results of a phylogeographic study by mtDNA analysis (COI and Cytb genes) from (Rahman et al. 2017b) and nuclear DNA among those 39 localities. For the two localities (locality 34& locality 46), a total of 40 individuals were analyzed and the sequence data was deposited to GenBank. The nucleotide sequences of LW *Rh* by Azuma et al. (2006) were used as references. The intron region was identified by comparing the 528-bp sequence with LW *Rh* mRNA of the Saharan silver ant (*Cataglyphis bombycinus*, DDBJ accession no. U32501), carpenter ant (*Camponotus abdominalis*, U32502), and large earth bumblebee (*Bombus terrestris*, AF091722). Whereas insect opsin genes comprise many paralogous copies, the determined sequences of *Oecophylla* were more similar to the three LW Rh sequences than to any others based on a homology search using FASTA in DDBJ. This homology search also proved that the amplified region was LW *Rh*. After identifying the intron regions, the introns were removed and the sequences were aligned in MEGA 7.0

Microsatellite study

Six *Oecophylla smaragdina* colonies from 6 localities of Bangladesh had been selected including two inconsistent locality L34 and L46 as detected by nuclear DNA Analysis. The samplings sites for microsatellite study were corresponding to Table 3.1 in the general methodology chapter.

DNA extraction

For molecular analyses, specimens were randomly chosen from 07 colonies of 07 different localities. 10 individuals from each colony were selected randomly for extraction of DNA. For the convenient discussion, we grouped sample no 133 to 142 from L34, 143 to 152 from L46, 153 to 160 from L27, 163 to 170 from L67, 173 to 180 from L13, 181 to 188 from L50 and 189 to 195 from L66. Genomic DNA was extracted from the fore, middle and hind legs of specimens that were preserved in alcohol by using *QIAGEN DNeasy Blood and Tissue kit* (Qiagen, Maryland, USA) following manufacturer's instruction. Samples were vortexed by adding 180 µl Buffer ATL and 20 µl proteinase K. Sample were incubated at 55^0 C for 48 hours. DNA extraction was completed by adding two wash buffer AW1 and wash buffer AW2 and Buffer AE and lysis buffer AL and elusion buffer AE, as per manufacturer instruction. All centrifugation steps were completed at room temperature. The colony mates of the specimens used for DNA analysis were preserved in the laboratory of Institute of Tropical Agriculture, Kyushu University after DNA extraction.

Microsatellites PCR condition and primer setting objectives

The primers used in PCR was mentioned in Table 3.5. PCR was performed in a 15 µl reaction volume containing 1 µl × reaction buffer (10XEx7Eq buffer), 1 µl × dNTP with 1.8 mM MgCl2, 1 µl of each primer, 0.1 µl of Ex Taq DNA polymerase (TAKARA, USA), and about 1 µl template DNA. Forward primers were 5'-end labeled with a fluorescent dye either 6-FAM.

Cycling conditions were mentioned in table 5.1. Loci and primers used are given in table 5.2. Fragments were run on an ABI PRISM 310 genetic analyzer (Applied Biosystems). For gene mapping by fragment analysis and data processing, Peak Scanner software vr 1.0 (Applied Biosystems) was used.

Table 5.1 PCR parameters used in microsatellite analysis

PCR for Microsatellite study					
Steps	Times (X)	^{0}C	M:S	Go to	Loops
1		95.0	05:00		
2		92.0	0:45		
3	35	48.0	0:45		
4		72.0	01:00	2	34
5		72.0	05:00		
6		4.0	Pause		

Table 5.2 List of loci used in microsatellite analysis of *O. smaragdina* in Bangladesh modified from (Azuma et al. 2004)

Locus Name	Primer Sequence (5′−3′)	Repeat motif	*Ta(°C)*	References
MS2.2.2	F:GTCTTATGTGTGGCCACTGCGA R: GTGAAATGAACGTGACTTG	(GCA)6	*48*	
MS2.3	F: TCCAGGTGACCGTCGTGT R: CATACACATTCGCGTACG	(GT)5GC(GT)5	*48*	
MS2.14	F: TCTACGTGTCTAACCCAAC R:GCGAGTCTACTCCATCGTATAG	(CGA)4CGT(CGA)6CAA–(CGA)10TGA(CGA)3	*52*	
MS3.2	F: GTGACATTGTCGGCGA R: CGAGCGCGAAAATTTCGTC	(GA)6	*52*	
MS5.2	F: AATTACAGTTCGGTCTCG R:ATCGAACTTCGCTTCGGTTGTA	(CT)11(CTTT)3GTTT(CTTT)2	*48*	
MS5.10	F: GAGAGGAAGTGCACCACAATG R: CGAACCGTGAGGAAATGTCGA	(GA)3AA(GA)5A8(GA)4	*52*	
MS6.29	F: CAATCCAGTTGCACGGCTA R: GTAACTTCGAGTTCGC	(GA)3AA(GA)4A(GA)3	*49*	Azuma et al. (2004)
MS6.45	F: GGTCGTTGCTGACCGT R: CAGATACAGGCAATGCT	(GTT)3GCT(GTT)9(GCT)4(GTT)2(GCT)4	*49*	
MS6.47	F: AGCCCTCTTGTTCATGA R: TTAAATTCGGCCGCA	(GA)9	*45*	
MS7.1	F: AAAGGACGTTGACGCGAC R: ACGTGCAATCCATTCACG	(GAT)8	*52*	
MS8.24	F: GCAGACAATGGCTATTTGT R: CGATGTGATTTAGCCGA	(CTT)5TCT(CTT)3–(GA)3AA(GA)11AATA(GA)9	*50*	

5.2.3 Results and discussions

Nuclear DNA study by LW *Rh*

Among 39 localities, 27 locality samples were found as Indian type and 12 localities are of SE Asian type (Table 5.3). Between these two types, there was only one substitution: site 27 contains thymine in SE Asian type and cytosine in Indian type (Fig. 5.4.). This substitution is in a coding region but is synonymous and transitional. Since all the other haplotypes, including *O. longinoda*, had a cytosine at site 27, this thymine substitution of SE Asian type is parsimoniously considered to be derived, suggesting strong monophyly (Azuma et al. 2006).

Table 5.3 List of Nuclear DNA haplotypes corresponding to locality

LW *Rh* haplotypes	Locality No.
Indian types	L08, L55, L13, L22, L05, L21, L66, L26, L33, L28, L29, L02, L34, L46, L86, L87, L82, L84, L79, L81, L85, L83, L80, L78, L76, L77, L64
SE Asian types	L38, L58, L35, L54, L10, L56, L43, L49, L67, L69, L70, L71

Locality No.	mtDNA types	nDNA types	Nucleotide at site 27	Locality No.	mtDNA types	nDNA types	Nucleotide at site 27
L38	SE Asian	SE Asian	T	L33	Indian	Indian	C
L35	SE Asian	SE Asian	T	L28	Indian	Indian	C
L58	SE Asian	SE Asian	T	L29	Indian	Indian	C
L54	SE Asian	SE Asian	T	L02	Indian	Indian	C
L10	SE Asian	SE Asian	T	L08	Indian	Indian	C
L56	SE Asian	SE Asian	T	L55	Indian	Indian	C
L43	SE Asian	SE Asian	T	L86	Indian	Indian	C
L49	SE Asian	SE Asian	T	L87	Indian	Indian	C
L67	SE Asian	SE Asian	T	L82	Indian	Indian	C
L69	SE Asian	SE Asian	T	L84	Indian	Indian	C
L70	SE Asian	SE Asian	T	L76	Indian	Indian	C
L71	SE Asian	SE Asian	T	L77	Indian	Indian	C
L34	SE Asian	Indian	C	L64	Indian	Indian	C
L46	SE Asian	Indian	C	L78	Indian	Indian	C
L13	Indian	Indian	C	L80	Indian	Indian	C
L22	Indian	Indian	C	L83	Indian	Indian	C
L05	Indian	Indian	C	L81	Indian	Indian	C
L21	Indian	Indian	C	L85	Indian	Indian	C
L66	Indian	Indian	C	L79	Indian	Indian	C
L26	Indian	Indian	C	L26	Indian	Indian	C

Site 27: Cytosine (C) = Indian type; Thiamine (T)= SE Asian type

Fig. 5.4 In Bangladesh, *O. smaragdina* types inferred from nDNA by LW *Rh* compared with the results of the types inferred from mtDNA. L34 and L46 showing the inconsistency in mtDNA and nDNA types.

Presence of heterozygous colony in L34 and L46

We have identified the inconsistent mitochondrial and nuclear DNA type in the colony located in L34 and L46 (Fig. 5.5). As in the mitochondrial DNA analysis by (Rahman et al. 2017b), these two localities were identified as SE Asian type. For further confirmation, we have analyzed the nuclear DNA of additional 40 individuals from those two colonies, 24 individuals from L34 and 16 individuals from L46. Among those 24 individuals from L34, we recognized 1 individual is exactly sharing the same nucleotide sequence as Indian type and 23 individuals are true to SE Asian type. However, we have checked the 16 individuals from the colony of locality 45 and identified 05 and 11 individuals as Indian and SE Asian type, respectively. This finding indicated that in both colonies of that two localities have the mixture of both Indian and SE Asian type of *Oecophylla smaragdina*, which can be treated as the heterozygous colony often, used for the evidence of hybridization.

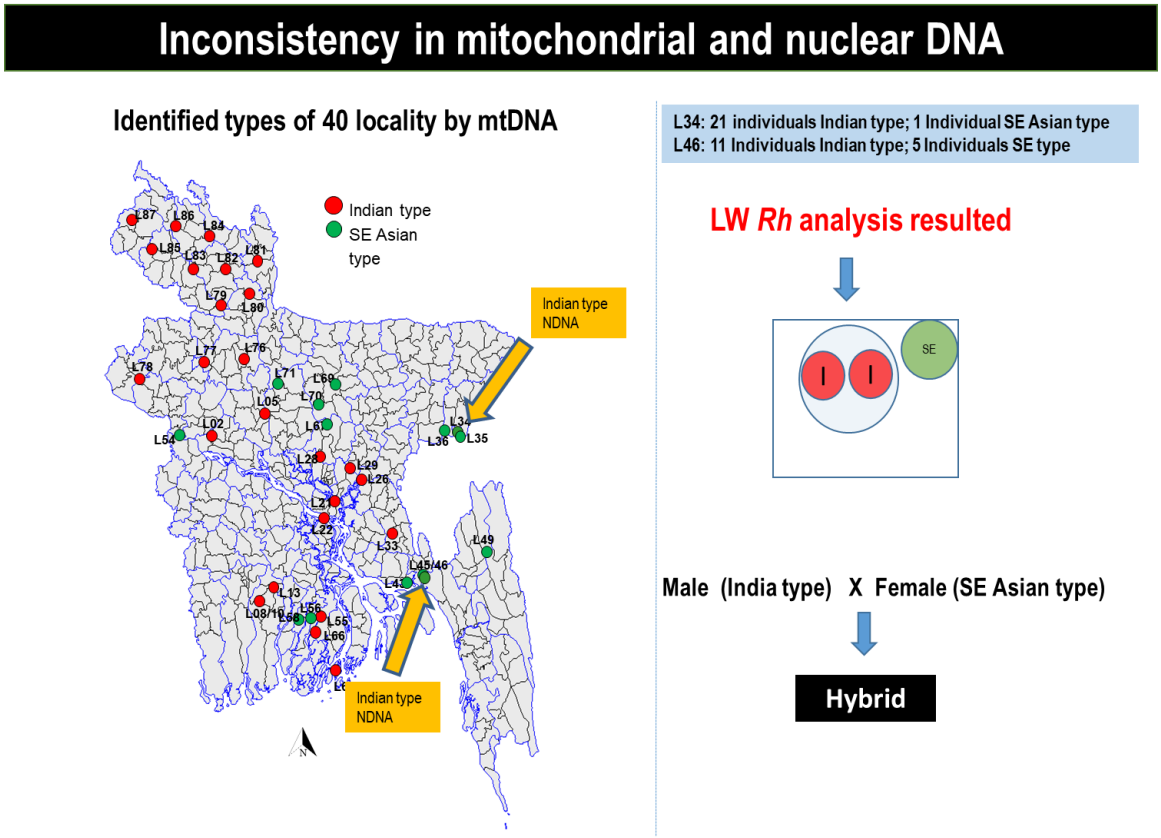

Fig. 5.5 The inconsistency of the distribution pattern of Indian and SE Asian type of *O. smaragdina* in Bangladesh inferred from mitochondrial and nuclear DNA analysis. Distribution pattern inferred by mitochondrial DNA analysis were retrieved from (Rahman et al. 2017b). The locality information is the same as mentioned in Table 3.1

Microsatellite study

In this study, 11 microsatellite primers (MS 2.2.2, MS 2.3, MS 5.10, MS 2.14, MS 3.2, MS 6.45, MS 6.29, MS 5.2, MS 7.1, MS 8.24 and MS 6.47) (Azuma et al. 2004) . However, we failed to analyze the samples using MS 5.2 microsatellite primer. For identifying the heterozygosity by the observation of number of peak by sharing the locus number were determined by electropherogrum study as mentioned in Fig. 5.6.

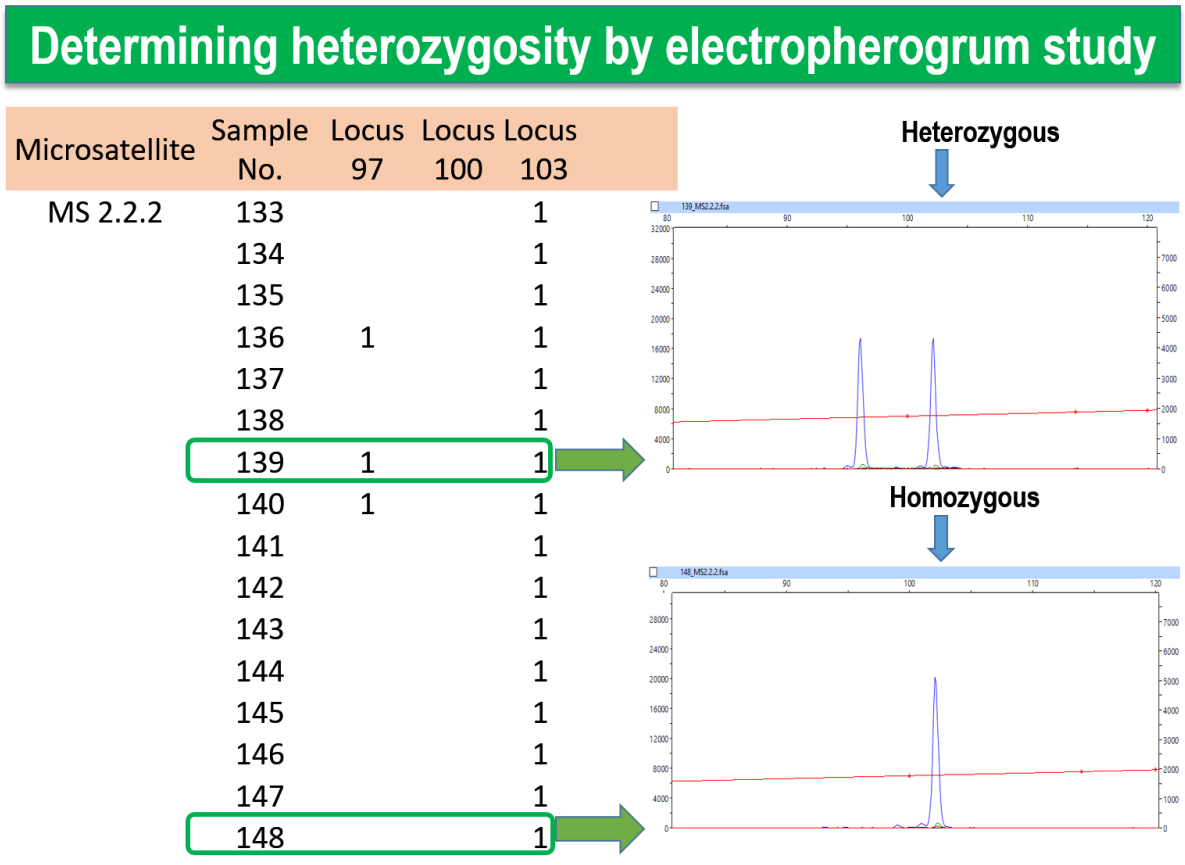

Fig. 5.6 Electropherogrum study to find out the sharing allele position of each individuals per colony.

5.3.1 Microsatellite markers failed to detect identical allele

Microsatellite primer MS 5.10

With this primer, we analyzed a total of 68 samples from 7 localities. Among them, all the samples from the locality L4, L46, L27, L67 L50 and L66 were sharing both the locus 98 and locus 99 while the samples from locality 13 sharing both the locus 96 and locus 97. L13 was identified as Indian type of *Oecophylla smaragdina* by both mitochondrial and nuclear DNA analysis. However, L34, L46, L67, L50 was determined as SE Asian type and L27 and L66 was characterized as Indian type. So, in this case for the primer MS 5.10 we failed to distinguish identical alleles for Indian and SE Asian type although we found heterozygosity as it showed the two peak in almost all the samples.

Microsatellite primer MS 2.14

This primer detected several allele positions for Indian and SE Asian types. The samples from L46, L27 shared the locus position 231 and 234 whereas sample from L34 shared the locus 221, in addition, 1 Sample from L50 shared the locus 262 with locus 231 and locus 234. The samples from locality L13 and L66 showing a quite different pattern of sharing allele frequencies. The samples from L13, shared the locus 245, locus 247, locus 262, locus 270 and locus 273. The samples from L66 shared the locus 238, locus 252 and locus 270 (Table 5.4). So, no distinguished locus position sharing by Indian and SE Asian types of samples were observed.

Microsatellite primer MS 3.2

For identifying the identical allele, I tested 32 samples from 4 localities. The samples from L27 and L67 shared the locus 81 while the samples from L13 shared the locus 86. The samples from locality 50 are sharedboth locus 81 and locus 86 (Table 5.4).

Microsatellite primer MS 7.1, MS 6.29 and MS 6.47

Eight samples from each of two localities (L67 and L13) were tested by microsatellite primer MS 7.1, MS 6.29 and MS 6.47. All 08 samples shared the locus 100, locus 128 and locus locus 172 for MS 7.1, MS 6.47 and MS 6.29, respectively (Table 5.4). So, no locus was identified that can separate Indian and SE Asian type of *Oecophylla* in Bangladesh.

Microsatellite primer MS 2.3 and MS 2.2.2

60 samples from 7 locaities were tested each for MS 2.3 and MS 2.2.2. All the samples from MS 2.3 shared the locus 144, while two samples from L34 and L46 shared both the loci 100 and 144. In the case of MS 2.2.2, all the samples sharing the locus 103 except the samples from L13 and L66 that were sharing the locus 97. The samples from other locality that were identified as Indian type also shared the locus position 97 along with some samples from L34 which was identified as SE Asian type. So, with these two primers the identical allele position was not possible to distinguish between Indian and SE Asian types (Table 5.4).

5.3.2 Detection of identical allele

The identical allele between Indian and SE Asian type of *Oecophylla* population in Bangladesh were possibly detected with the microsatellite primer MS 6.45 and MS 8.24. For MS 6.45, four samples from the locality L67 were analyzed and all 04 samples shared the locus 238. However, the samples from locality L13 shared the locus 244 and locus 250. As the colony of L13 locality was identified as Indian type and L67 was SE Asian type so, these locus position was identical. Similarly, the samples with 8.24, among 08 samples, 4 samples from L67 shared the loucs 277 while the rest 4 samples from locality L13 shared the locus 262 (Table 5.4)

Table 5.4 Allele record for 10 microsatellite loci on 7 localities. n indicating the number of individuals/ locality

Microsatellite	Allele	L34 (n=8)	L46 (n=8)	L27 (n=8)	L67 (n=8)	L13 (n=8)	L50 (n=8)	L66 (n=8)
mt DNA type		SE Asian	SE Asian	Indian	SE Asian	Indian	SE Asian	Indian
MS 2.2.2	97	3		4		8	1	8
	100							
	103	8	8	4	8		8	
% Heterozygosity		37.5	0	50	0	0	12.5	0
MS 2.3	100	1	1					
	144	8	8	8	8	8	8	8
% Heterozygosity		12.5	12.5	0	0	0	0	0
MS 5.10	96					8		
	97					8		
	98	8	8	8	8		8	8
	99	8	8	8	8		8	8
% Heterozygosity		100	100	100	100	100	100	100
MS 2.14	221	4						
	231	3	6	8			5	
	234	5	8	5	8		8	
	238							4
	245					5		
	247					3		
	252							3
	258					4	1	
	262							8
	270					1		
	273					3		
% Heterozygosity		50	62.5	62.5	0	100	75	87.5
MS 3.2	81			8	8		7	
	86					8	8	
% Heterozygosity				0	0	0	87.5	
MS 6.45	238				4			
	244					4		
	250					2		
% Heterozygosity					0	50		
MS 6.29	172				4	4		
% Heterozygosity					0	0		
MS 7.1	100				4	4		
% Heterozygosity					0	0		
MS 8.24	262				4			
	277					4		
% Heterozygosity					0	0		
MS 6.47	128				4	4		
% Heterozygosity					0	0		
MS 5.2	Failed							

There was not too many evidence of such heterozygous condition within the colony of *Oecophylla* in India or any other SE Asian country and this is the first report of such mixed colony in Bangladesh as well. Similarly, Roos et al. (2011) studied tracing the evolution and hybridization of colobine monkey in the Asian continent, found several hybridization patterns by testing the mitochondrial and nuclear DNA. This hybridization among ancestral lineages most likely causes for the observed phylogenetic incongruences, due to the presence of potential contact zones like today's Bangladesh, Myanmar and the northeast of India, which is suggested as hybridization area (Karanth et al., 2008). However, several big mountains and big rivers in the border region of today's Myanmar, India and China might have been a possible diversification hotspot (Chakraborty et al. 2007) which leads to develop such hybridization pattern.

Divergence time is also an important factor for many ant genera. *Oecophylla* is thought to be a significant factor in such distribution. Diversification within groups in this continent was recorded from the middle Pliocene to early Pleistocene (Azuma et al. 2002). After this period, the world has encountered a significant climatic change. It might also affect the distribution of *Oecophylla* in different parts of the world. During Last Glacial Maximum (LGM), the tropic region shifted southward and it retained northward after glaciation. This study suggested that the Indian and Southeast Asian clades of *O. smaragdina* expanded their distribution northward along suitable regions with high temperature and humidity, and then the two clades supposedly encountered and overlapped in central Bangladesh. Similar trends were also observed in the case of study of the origin of Asian elephants (Vidya et al. 2009). They suggested a contraction-expansion scenario during climatic oscillation leads to geographical overlaps of two mtDNA clades created the allopatric population of Asian elephants in India, Sri Lanka, and Myanmar. In the case of weaver ant in Bangladesh, as both the Indian and SE Asian types were dominated

in Western part and Eastern part of the country, respectively, and there was not such a big border of separating those two populations (Rahman et al. 2017a). The probability of the contact of both the types was considered very usual. Pusch et al. (2006) reported that ant colony with heterozygous produce hybrid workers. This type of gene introgression may increase the genetic diversity of the hybrid relative to its parental species and can lead to hybrid vigor. In this case, the *Oecophylla* colony from the locality 11 and 15 in Bangladesh might have the influence of the arising of new evolutionary lineages. This hybridization therefore, can be considered as a process of the evolutionary significance of *Oecophylla smaragdina* in Bangladesh.

Limitation of the study

For testing the degree of mixture of Indian and SE Asian type within the colony from the locality 34 and locality 46, I needed to analyze number of individuals from that colony. However, due to a shortage of sufficient individuals from that colony, we could not perform the study a bit more details with more samples to confirm the degree of heterozygosity or introgression of gene flow.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 6

General discussion and conclusions

6.1. General discussion

The present study started by checking the occurrence of SE Asian type of mtDNA in Bangladesh reported in previous studies (Azuma et al. 2002, 2006). When the survey began in the western Bangladesh, all the samples collected from Rajshahi, Khulna and Dhaka Divisions showed the Indian type of mtDNA. The following survey confirms the occurrence of SE Asian type in Chittagong and Sylhet Divisions. These were answer of the first question whether all the *O. smaragdina* populations in Bangladesh belong to SE Asian type.

The occurrence of both types leads to the second question where the boundary of SE Asian type and Indian type is. As Bangladesh is a riverine country with three main rivers the Ganges, Jamuna, and Meghna, the river might have some influence of separating the Indian type and the SE Asian type in Bangladesh. To check the hypothesis the study covers 87 sites of 47 districts which are divided into 7 major areas separated by the Ganges, Jamuna, and Meghna Rivers. As Bangladesh is a riverine country with three main rivers the Ganges, Jamuna, and Meghna crisscrossed throughout the mostly flat territories of the country, the river might have some influence of separating the Indian clade and the Southeast Asian clade in Bangladesh. However, the recent study revealed that the western Bangladesh population of *O. smaragdina* was dominated by Indian type (Rahman et al. 2017b). With the recognition of Indian type in western part of Bangladesh, it is clear that Indian mitochondrial haplotypes occurred on both

sides of Ganges river. *Oecophylla* species may disperse via nuptial flight of queens and/or rafting method which is very effective between island to island dispersal (Peng et al. 1998).

The result of distribution of mtDNA types shows that the western parts of Bangladesh is predominantly occupied by the Indian type, whereas the eastern part by the SE Asian type, and that the central parts is the mixture of both types. The study does not show the clear boundary, but suggests the central part as a transition zone. The haplotype network analysis implys the diversity within each of the types: the Indian type includes 14 haplobtypes and the SE Asian type includes 11 haplotypes.

Effects of temperature and rainfall influence the distribution of *O. smaragdina* as the combination of high temperature and high rainfall is necessary to continue the weaver ant growth rate and distribution, and to a limited extent, higher temperatures can compensate for lower than optimal rainfall levels, and vice versa (Lokkers, 1986). During the glaciation period the temperature had fall down nearly 10 ^{o}C in Vostok (Lowe and Walker, 1997) might have significant influence of the distribution of several species like *Oecophylla*, which is markedly affected by low temperature for their colony development. The haplotype network distribution pattern (in chapter 4) also showed that the bottleneck effects might be responsible for such distribution. Due to several glaciation during Pleistocene could be a key factor of such effects. Weterrer (2017) showed a comprehensive distribution pattern of *O. smaragdina* in the arid, tropical and subtropical climatic region that also shed lighted the effects of temperature on the distribution.

A hypothesis forming the distribution pattern

The occurrence of two types (Indian type and SE Asian type) in Bangladesh will be made by the following dispersal scenario: The disjunction of populations would occur several times as in shown of divergent time in 7 major groups of mtDNA, and the isolated populations may develop their unique haplotypes in India, in SE Asia, or others during the history of *O. smaragdina* which was estimated to diverged from *longinoda* about 13 - 11 Ma (Azuma et al. 2006; Blamer et al. 2015).

Before the Last Glacial Maximum (LGM: 25,000 - 15,000 BP) the haplotypes observed in Bangladesh would be already established. During the LGM, the Bengal Delta supposed to be vacant for *Oecophylla smaragdina* distribution because the northern limit of the species would be located down to the south. After the LGM, the populations expanded to north, and because of the geographical shape the east and west populations met in the Bengal Delta. When the two types of population groups met again in the Bengal Delta, each of the types already contained several haplotypes of mtDNA.

This vacant-reunion hypothesis requires a temperature drop in LGM, and diversification of populations before LGM. The present distribution of *Oecophylla smaragdina*, in particular, the northern limit of distribution is almost agree with the isothermal line of 10 ^{o}C in the average temperature of January. Bangladesh has a distinct monsoonal season, with an average temperature in January of 18 ^{o}C in Dhaka. Thus if the mean temperature of the coldest month (MTCO) drops 8 ^{o}C or more, *O. smaragdina* cannot distribute in Bengal Delta. Braconnot et al. (2012) estimated the mean temperature of the coldest month (MTCO) in Asia is around 10 ^{o}C below. Although northern limit of distribution of *O. smaragdina* in LGM is not clear, the

Bengal Delta is supposed to be out of the limit. After LGM, as the climate changed to warmer, the populations would extended to northward and come to Bengal Delta simultaneously. This is based on the result from the present study which revealed that there are14 haplotypes in Indian type and 11 haplotypes in SE Asian type. It is almost certain that the Indian type from west or southwest, and SE Asian type from east or southeast met in Bengal Delta. The populations extended to Bengal Delta were not uniform, because each of mtDNA types have a history of diversification in somewhere south in India and SE Asia. The diversification time was estimated in the Pliocene to Pleistocene.

The recent phylogeographic studies on insects, plants, amphibians or fishes shown the significance of Pleistocene climate changes and biogeographic barriers like mountains, rivers, seas and desserts alter the diversification, radiation and isolation of new genetic lineages within many species (Riddle et al. 2000). This climatic oscillation played the major role of forming such distribution of species by changing the genetic structure and diversity (Avise, 1994). Regarding the regions of East Asia, the glacial influence were not so extensive due to influence of monsoons formed by the Pacific Ocean and the presence of biotic zone at higher northern latitude. However, the fossil and biogeographic evidence showed the dramatic effects of climate changes thorough Asia and its impact on the distribution of several animal species is under consideration. The case of the distribution pattern of *O. smaragdina* in Bangladesh can be correlated with the distribution pattern of Asian elephants. Vidya et al. (2009), explained that in the case of the elephant haplotypes in Myanmar, rather suggested tha these haplotypes did not arise within Myanmar, but instead resulted from a northward range expansion of beta clade haplotypes during warm period from both Sri Lanka and the Sunda region followed by subsequent admixture in this region. The geological history of Bangladesh revealed that the formation has begun 350 million years ago when the Pangean supercontinent broke apart

(Mannan, 2002). The Bengal Basin, which is the major basin of Bengal formation, began 127 million years ago when the Indian Plate rifted away from Antartica at 18 cm per year for 20 million years. So, after the age of glaciation, as a new vacant area with newly growing vegetation favored by the tropical climate facilitate the chance of occurrences of both the types.

6.2 Conclusions

Oecophylla smaragdina has the evolutionary importance in Bangladesh. Based on previous phylogeographic study I hypothesized that the species has some barriers in the geology of Bangladesh and India that inhibiting the Indian types to enter into Bangladesh. Taking the view that Ganges river might be the barrier, we checked the *Oecophylla smaragdina* population from the western part of Bangladesh and identified as Indian type. This was the first report of occurring Indian type in Bangladesh. So, there was not such a barrier for separating the populations.

Using *O. smaragdina* samples from 5 broad areas in Bangladesh based on three main rivers, the phylogenetic study of mitochondrial COI and Cytb genes revealed the western Bangladesh population of *O. smaragdina* is dominated by Indian type, the eastern Bangladesh population are of SE Asian types and the mixture of the two types were found in the central parts of Bangladesh.

Twenty four *O. smaragdina* haplotypes from Bangladesh were recognized. Among them, 13 haplotypes were identical to Indian types and 11 haplotypes were similar to SE Asian types. Haplotype distribution patterns was found to be similar as the general distribution. Longest mutational steps (27) determined that many missing haplotypes may remain in the refugia.

I analyzed the divergence time of occurring Indian and SE Asian type in Bangladesh. diversification of both the types was suspected to occurr in Pleistocene. Indian type diverged ca. 2.3 Ma and SE Asian type diverged ca. 0.20 Ma corresponding to early to late Pleistocene.

The occurrence of two different lineages have the opportunity to make a hybridized colony. The overlapping zone of Indian and SE Asian type leaded the chance of this opportunity. In the nuclear DNA analysis by LW *Rh*, discordance between mitochondrial and nuclear DNA genes from two localities were identified. These phenomenon suggested the possibility of hybridization. Eleven microsatellite primers on *O. smaragdina* were implied to find out the identical allele of Indian and SE Asian types. Among them, two microsatellite loci were identified to treat it as identical allele recognition.

Based on Phylogeography study we can concluded Bangladesh is a transitional zone for evolutionary significance of *O. smaragdina* distribution.

6.3 Limitations of the study

In this study, I focused on the *O. smaragdina* population in Bangladesh by collecting the samples from different areas of Bangladesh and phylogeographic study was conducted at the Institute of Tropical Agriculture laboratory, Kyushu University, Japan. We couldn't afford to bring all samples from the designated area of Bangladesh due to certain regulations. Size of sampling in a colony resulted limited number of the analyzed individuals. We do not have sufficient data from sexual castes of females and male. Lack of information on nuptial flight and the effects of seasonal variation, mode was not observed in my experiment. Lack of paleoclimate information of LGM in Bangladesh was another major limiting factors to interpret the data to some extents.

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Md Mamunur Rahman

Fukuoka, Japan

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APPENDIX

APPENDIX

Appendix 1. Detailed locality information with GenBank accession number of sequencing data

Locali-ty No.	Locality Name	# of colonies	Upazila	District	Division	Collection Date	Area Code	Accession number			GPS Coordination	
								COI	Cytb	Nuclear DNA	N Latitude	E Longitude
L01	Ishwardi	[1]	Ishwardi	Pabna	Rajshahi	18 Mar. 2014	A01	KX385842	KX430217		24°06'52"	89°04'52"
L02	Bonpara	[1]	Baraigram	Natore	Rajshahi	19 Mar. 2014	A01	KX385843	KX430218	KY934248	24°16'29"	89°05'19"
L03	Tarash	[1]	Tarash	Sirajganj	Rajshahi	18 Mar. 2014	A01	KX385841	KX430216		24°21'59"	89°21'40"
L04	Chauhali	[1]	Belkuchi	Sirajganj	Rajshahi	19 Mar. 2014	A01	KX389168	KX398946		24°30'61"	89°72'73"
L05	w side of Jamuna Bridge	[1]	Sirajganj sadar	Sirajganj	Rajshahi	18 Mar. 2014	A01	KX385840	KX430215	KY906977	24°23'43"	89°44'44"
L06	Panjia	[1]	Keshabpur	Jessore	Khulna	04 Mar. 2014	A02	KX371575	KX398943		24°15'	89°30'
L07	Monirampur	[1]	Monirampur	Jessore	Khulna	14 Sep. 2013	A02	KX355139	KX430212		24°15'	89°30'
L08	Khulna Univ. Campus	[2]	Batiaghata	Khulna	Khulna	03 Mar. 2014	A02	KX379493/ KX379494	KX398942/	KY906981	24°46'	89°31'
L09	Chuknagar	[1]	Dumuria	Khulna	Khulna	04 Mar. 2014	A02	KX385837	KX398944		22°49'	89°18'
L10	Batiaghata	[1]	Batiaghata	Khulna	Khulna	15 Sep. 2013	A02	KX389167		KY906986	24°45'	89°31'
L11	Atulia	[1]	Shyamnagar	Satkhira	Khulna	24 Mar. 2014	A02	KX385844	KX398947		24°16'	89°03'
L12	Modonpur	[1]	Tala	Satkhira	Khulna	25 Mar. 2014	A02	KX385845	KX430219		24°46'	89°03'
L13	Mollahat Bazar	[1]	Mollahat	Bagerhat	Khulna	29 Oct. 2014	A02		KX430220	KY906980	24°01'16.6"	90°17'53.7"
L14	Pachuria	[1]	Gopalganj Sadar	Gapalgonj	Dhaka	29 Oct. 2014	A02	KY618816			23°00'3.6"	89°49'13.8"
L15	Bhanga	[1]	Bhanga	Faridpur	Dhaka	09 Nov. 2014	A02	KX389172			23° 23′ 0″	89° 59′ 0″
L16	Elenga	[1]	Kalihati	Tangail	Dhaka	18 Mar. 2014	A03	KX385839	KX398945		24°23'01"	89°50'05"
L17	Kumrail	[1]	Dharmrai	Dhaka	Dhaka	19 Oct. 2014	A03	KX389169			23°13'26.1"	90°13'26.1"
L18	Thanamore	[1]	Dohar	Dhaka	Dhaka	21 Oct. 2014	A03	KX389170			23°36'49.4"	90°07'13.3"
L19	Ruhitpur	[1]	Keraniganj	Dhaka	Dhaka	21 Oct. 2014	A03		KY562567		23°40'07"	90°17'54"

L20	Baluakandi	[1]	Gazaria	Munshiganj	Dhaka	21 Oct. 2014	A03	MG967475			23°38'57.2"	90°35'8.2"
L21	Nimtali	[1]	Sherajdikhan	Munshiganj	Dhaka	21 Oct. 2014	A03	KY628426		KY906976	23°36'55.3"	90°19'55.6"
L22	Bejgaon	[1]	Sreenagar	Munshiganj	Dhaka	21 Oct. 2014	A03		KY583087	KY906978	23°32'21.3"	90°17'48.2"
L23	Nababganj Bazar	[1]	Narayanganj	Narayanganj	Dhaka	21 Oct. 2014	A03		MG967471		23°39'39.2"	90°09'41.9"
L24	Shiddirganj	[1]	Siddhirganj	Narayanganj	Dhaka	21 Oct. 2014	A03		KY562571		23°41'41.8"	90°29'26.5"
L25	Vulta	[1]	Rupganj	Narayanganji	Dhaka	20 Oct. 2014	A03		KY562573		23°46'50"	90°34'16.5"
L26	Panchdona	[1]	Norsingdi	Norsingdi	Dhaka	20 Oct. 2014	A03	KY657490	KY562569	KY906973	23°53'36.2"	90°34'16.5"
L27	Bhawal National Park	[2]	Joydebpur	Gazipur	Dhaka	17 Mar. 2014	A03	KX385838	KX430214		24°04'43"	90°24'06"
L28	Nurbag	[1]	Kaliakoir	Gazipur	Dhaka	22 Oct. 2014	A03	KX389171	KX430221	KY906971	24°01'16.6"	90°17'53.7"
L29	Charpara	[1]	Kaliganj	Gazipur	Dhaka	20 Oct. 2014	A03	KY628425		KY906970	23°55'31.9"	90°36'0.8"
L30	Zamirarchala	[1]	Kapasia	Gazipur	Dhaka	20 Oct. 2014	A03				24°06'6.2"	90°28'25.8"
L31	Rajbari	[1]	Sreepur	Gazipur	Dhaka	20 Oct. 2014	A03		KY562568		24°03'5.7"	90°23'17.8"
L32	Nimshar	[1]	Burichang	Comilla	Chittagong	21 Oct. 2014	A05	MG967473	MG967472		23°06'6.2"	90°28'25.8"
L33	Madhoyia	[1]	Chandina	Comilla	Chittagong	21 Oct. 2014	A05			KY906972	23°30'08.5"	91° 0′ 30″
L34	Tea Resort Center	[3]	Sreemangal	Moulovibazar	Sylhet	14 Nov. 2014	A04	KY618809	KY583084	MF345826 , KY906974, KY906969	24°18'7.2"	91°45'34.92
L35	Lauachra National Park	[1]	Sreemangal	Moulovibazar	Sylhet	15 Nov. 2014	A04	KY618815	KY583085		24°18'05.7"	91°45'41.0"
L36	Bahubal	[1]	Bahubal	Habiganj	Sylhet	14 Nov. 2014	A04	KY618818	KY583083	KY906983, KY906979	24°22'49"	91°24'46.0"
L37	Tarau	[1]	Balaganj	Sylhet	Sylhet	15 Nov. 2014	A04	KY618817		KY906984	24°19'42.6"	91°47'05.2"
L38	Doradarpur	[1]	Dokshin Surma	Sylhet	Sylhet	15 Nov. 2014	A04	KY618810	KY657484	KY906988	24°46'40.0"	91°47'01.8"
L39	Marishbunia	[1]	Teknaf	Cox's Bazar	Chittagong	8 Sep. 2014	A05	MG967474			21°05'25"	92°20'
L40	Noakhali para	[1]	Teknaf	Cox's Bazar	Chittagong	9 Sep. 2014	A05				24°46'40.0"	91°47'01.8"
L41	Mondabag	[1]	Kasba	Bramhanbaria	Chittagong	10 Aug. 2015	A04	KY608802	KY657492		23° 44′ 0″	91° 10′ 0″
L42	Jogotpur	[1]	Burichang	Comilla	Chittagong	12 Aug. 2015	A04				23° 33′ 0″	91° 7′ 36″
L43	Sebarhat	[1]	Senbag	Noakhali	Chittagong	13 Aug. 2015	A04	KY608803	KY550396		22° 58′ 59.88″	91° 13′ 59.88″
L44	Senbag Upozilla Hospital	[1]	Senbag	Noakhali	Chittagong	13 Aug. 2015	A04		KY657493		22° 58′ 59.18″	91° 13′ 59.08″
L45	Mohipal	[1]	Feni sadar	Feni	Chittagong	14 Aug. 2015	A04	KY618811	KY550397	MF345827 (S65)	23° 1′ 0.89″	91° 23′ 30″

L46	Mohipal Primary School	[1]	Feni sadar	Feni	Chittagong	14 Aug. 2015	A04	KY628427	KY550398		23° 1′ 1.10″	91° 23′ 50″
L47	Raujan Bazar	[1]	Raujan	Chittagong	Chittagong	16 Aug. 2015	A05		KY550399		22° 32′ 0″	91° 56′ 0″
L48	Satkania	[1]	Satkania	Chittagong	Chittagong	16 Aug. 2015	A05	KY657489	KY550400	MF345830	22° 6′ 12″	92° 4′ 50″
L49	Dighinala HRC	[1]	Dighinala	Khagrachari	Chittagong	12 Aug. 2015	A05	KY608804	KY550401	KY934247	23° 15′ 30″	92° 3′ 30″
L50	Matiranga Dhibi	[1]	Matiranga	Khagrachari	Chittagong	18 Aug. 2015	A05	KY608805	KY550402		23° 2′ 30.12″	91° 52′ 30″
L51	Ruma Karai	[1]	Ruma	Bandarban	Chittagong	20 Aug. 2015	A05	KY657488	KY550403		22° 3′ 0″	92° 25′ 0.12″
L52	Kawkhali Bazar	[1]	Kawkhali	Rangamati	Chittagong	21 Aug. 2015	A05	KY583089		MF345828	22° 32′ 0″	92° 1′ 0″
L53	Patia	[1]	Patia	Chittagong	Chittagong	02 Sep. 2015	A05		KY562572		22° 18′ 0″	91° 59′ 0″
L54	Thanapara Sadah	[1]	Charghat	Rajshahi	Rajshahi	23 Nov. 2015	A01	KY628429			24° 17′ 0″	88° 46′ 30″
L55	Nalchiti prim. sch. field	[1]	Nalchiti	Jhalokati	Barisal	15 Feb. 2016	A02	KY618814	KY657585		22° 37′ 19.92″	90° 16′ 14.88″
L56	BRAC more	[1]	Jhalokati Sadar	Jhalokati	Barisal	15 Feb. 2016	A02	KY657491	KY550389		22° 38′ 36″	90° 12′ 0″
L57	Baghribazar	[1]	Rajapur	Jhalokati	Barisal	16 Feb. 2016	A02	KY618813	KY550388	KY906985	22° 40′ 0.12″	90° 8′ 30.12″
L58	Kawkhali Upz P Chottor	[1]	Kawkhali	Pirojpur	Barisal	16 Feb. 2016	A02	KY583090	KY550390	KY906988	22° 37′ 13.08″	90° 4′ 9.84″
L59	Shakharikathi	[1]	Mathbaria	Pirojpur	Barisal	16 Feb. 2016	A02	KY583091	KY550391	KY906987	22° 17′ 12.84″	89° 58′ 0.12″
L60	Rupatoli	[1]	Barisal sadar	Barisal	Barisal	17 Feb. 2016	A02		KY550395		22° 48′ 0″	90° 30′ 0″
L61	Patarhat	[1]	Mehendiganj	Barisal	Barisal	18 Feb. 2016	A02	KY583092	KY550392	KY906982	22° 49′ 54.84″	90° 31′ 59.88″
L62	Barguna Sadar bus stand	[1]	Barguna sadar	Barguna	Barisal	08 Feb. 2016	A02		KY550393		22° 9′ 2.88″	90° 7′ 35.04″
L63	PSTU	[1]	Dumki	Patuakhali	Barisal	11 Feb. 2016	A02		KY550394		22° 26′ 0″	90° 22′ 0″
L64	Panpatti	[1]	Golachipa	Patuakhali	Barisal	10 Feb. 2016	A02	KY583093	KY583088		22° 9′ 48″	90° 25′ 48″
L65	Agailjhara Uni P office	[1]	Agailjhara	Barisal	Barisal	10 Feb. 2016	A02	KY628430			22° 58′ 0″	90° 9′ 0″
L66	Mohespur	[1]	Bakerganj	Barisal	Barisal	10 Feb. 2016	A02	KY618812	KY657486		22° 33′ 0″	90° 20′ 18″
L67	Bhaluka Bazar	[1]	Bhaluka	Mymensingh	Mymensingh	12 Nov. 2016	A03	KY657499		MF345829	24° 22′ 30″	90° 22′ 42″
L68	Trishal Primary School	[1]	Trishal	Mymensingh	Mymensingh	12 Nov. 2016	A03	KY657500			24° 34′ 30″	90° 23′ 30″
L69	BAU Campus	[1]	BAU Sadar	Mymensingh	Mymensingh	13 Nov. 2016	A03	KY657501		KY906975	24°45'8.39"	90°24'6.59"

L69	BAU, Sesh Matha More	[1]	BAU Sadar	Mymensingh	Mymensingh	14 Nov. 2016	A03	KY657502		MF345831	24°45'8.39"	90°24'6.59"
L70	Nandail	[1]	Muktagacha	Mymensingh	Mymensingh	30 Oct. 2016	A03	KY657503	KY657505		24° 34′ 0″	90° 41′ 0″
L71	Sarisha Bari High School	[1]	Sarishabari	Jamalpulr	Mymensingh	02 Nov. 2016	A03	KY657504	KY657506	MF345832	24° 44′ 30″	89° 50′ 0″
L71	Bazar Pukur	[1]	Sarishabari	Jamalpur	Mymensingh	02 Nov. 2016	A03		KY65707	MF345833	24° 44′ 32″	89° 50′ 01″
L72	Sadar Hospital	[1]	Netrokona Sadar	Netrokona	Mymensingh	03 Nov. 2016	A03	KY657494		MF345834	24° 52′ 30″	90° 44′ 0″
L72	Sadar Primary School	[1]	Netrokona Sadar	Netrokona	Mymensingh	04 Nov. 2016	A03	KY657495		MF345835	24° 52′28″	90° 44′ 03″
L73	Gauripur Upz Complex	[1]	Gauripur	Mymensingh	Mymensingh	10 Nov. 2016	A03	KY657496			24° 45′ 30″	90° 34′ 30″
L74	Higher par	[2]	Kishorgonj	Kishorgonj	Mymensingh	01 Nov. 2016	A03	KY657497			24° 25′ 59.88	90° 46′ 59.88″
L75	Sadar Thana more	[1]	Sherpur Sadar	Sherpur	Mymensingh	03 Nov. 2016	A03	KY657498			25° 0′ 0″	90° 1′ 0″
L76	Dhunat Upz. Chattor	[1]	Dhunat	Bogra	Rajshahi	04 Jan. 2018	A01	MG873538	MG886852		24°41'10.00"	89°32'0.03"
L77	Municipality Orchard	[1]	Naoga Sadar	Naogaon	Rajshahi	04 Jan. 2018	A01	MG873539	MG886851		24°48'18.00"	88°57'0.18"
L78	Nijampur	[1]	Nachole	Chapainawab ganj	Rajshahi	03 Jan. 2018	A01	MG873540	MG886850		24°43'48.03"	88°25'12.13"
L79	Hakimpur Nursery	[1]	Hakimpur	Dinajpur	Rangpur	29 Dec. 2017	A01	MG873541	MG886847		25°16'59.88"	89°1'0.12"
L80	Shibpur	[1]	Gabindagan j sadar	Gaibandha	Rangpur	02 Jan. 2018	A01	MG873542	MG886849		25°07'59.88"	89°23'30.12"
L81	Gharialdanga	[1]	Rajarhat	Kurigram	Rangpur	30 Dec.2017	A01	MG873543			25°48'00"	89°33'0.02"
L82	Rasulpur School ground	[1]	Pirganj	Rangpur	Rangpur	27 Dec. 2017	A01	MG873544	MG886845		25°24'54"	89°19'0.10"
L83	Saidpur airport surrounding	[1]	Saidpur	Nilfamari	Rangpur	01 Jan. 2018	A01	MG873545			25°47'0.04"	88°54'0.02"
L84	Barobala	[1]	Mithapukur	Rangpur	Rangpur	28 Dec.2017	A01	MG873546	MG886846		25°32'30"	89°17'0.00"
L85	Pirganj fire station orchard	[1]	Pirganj	Thakurgaon	Rangpur	31 Dec. 2017	A01	MG873547	MG886848		25°51'15.12"	88°22'0.12"
L86	Debiganj bus stand	[1]	Debiganj	Panchagar	Rangpur	25 Dec. 2017	A01	MG873548	MG886844		26°07'9.89"	88°45'33.47"
L87	Atwari sadar thana more	[1]	Atwari	Panchagar	Rangpur	26 Dec. 2017	A01	MG873549			26°18'29.88"	88°27'29.88"