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ABSTRACT

Morphologies and morphometries as diagnostic characters are shown statistically to be able to differentiate between species and intraspecific variants. Intraspecific variants or morphovariants exist in nature but the variations they possessed are not enough to consider them as species (differentiation index values for morphovariants is 50% less than the values for species). These variations are due to genetic difference resulting from cross-fertilisations between different individuals of the same species. Such variations are necessary tools for future species diversification.

The 28S rDNA are able to group members of the major dactylogyridean families together showing the monophyly of these families within the Dactylogyridea. The phylogenetic trees also indicate the heterogeneity of the monogeneans currently placed under the Ancyrocephalidae, dividing them into two groups according to their macro-environment, freshwater and marine ecosystems with the exception of *Cichlidogyrus* and *Scutogyrus* spp. It is proposed that the two groups are separated with the freshwater members (*Ancyrocephalus*, *Actinocleidus*, *Cleidodiscus*, *Urocleidus* and *Onchocleidus*) remaining in Ancyrocephalidae and a new family be created to accommodate the marine members, i.e. *Haliotrema*, *Bravohollisia, Caballeria, Pseudohaliotrema, Metahaliotrema, Euryhaliotrema, Euryhaliotrematoides, Tetrancistrum, Haliotrematoides, Ligophorus* and *Aliatrema* as well as freshwater members from the cichlids, i.e. *Cichlidogyrus, Scutogyrus* and *Onchobdella*. The cichlid hosts have been postulated to have a marine origin. This analysis seems to confirm the familial status of Heteronchocleididae, Ancylodiscoididae, Neocalceostomatidae and Pseudodactylogyridae. However the positions of the different monogeneans in phylogenetic trees do not correspond with the hypothesized evolutionary history of the

morphological characteristics of the different dactylogyridean groups in particular the 2- and 4-anchor monogeneans.

The host relationships based on Cytochrome *b* and monogenean relationships based on 28S rDNA support the well established parasitophyletic rule that related host species harbours related monogenean parasites. The specificity of some monogenean species suggests they have co-evolved and co-speciated within their host groups. However there are cases of monogenean being acquired via host transfer: for example *Dactylogyrus* spp. from a cyprinid host are probably acquired by an anadromous marine fish and the subsequent speciation of the *Dactylgoyrus* spp. on the marine fish *Lateolabrax* sp. give rise to the present distribution patterns of *Dactylogyrus*. Besides host transfer, some monogeneans species might have failed to speciate and this is probably what happened in the case of some species of heteronchocleidids on the anabantoid-channid fish group.

This study shows that for a good statistical differentiation of the species and morphovariants, large morphometric data sets are necessary. The same is true when molecular data is used, one of the limitation in this thesis is the absence of some genera (*Dogielus* and *Thaparogyrus* are not represented in the Dactylgoyridae) and the lack of species representation in some (Pseudomurraytrematidae is represented by one species.). This study also notes the limitation of depending solely on 28S rDNA for reconstructing phylogenetic relationships.

ABSTRAK

Morfologi dan morfometri telah ditunjuk dengan kaedah statistik bahawa ia boleh digunakan sebagai ciri diagnostik untuk membezakan species dan variasi intraspesifik. Kumpulan variasi intraspesifik wujud dalam alam semulajadi tetapi tidak mempunyai variasi yang mencukupi untuk membolehkan mereka dipertimbangkan sebagai spesies benar (nilai indeks pembezaan untuk variasi intraspesifik adalah 50% kurang daripada nilai untuk spesies). Variasi ini besar kemungkinan disebabkan oleh perbezaan genetik yang berpunca daripada pembiakan silang antara individu spesies yang sama. Variasi ini adalah diperlukan untuk diversifikasi spesies. Pokok filogenetik yang dibina daripada 28S rDNA dapat membezakan ahli-ahli dari famili utama dactylogyridean dengan menujukkan ómonophylyô famili-famili ini dalam Dactylogyridea. Pokok filogenetik juga menunjukkan kepelbagaian monogenean yang kini diletakkan dalam Ancyrocephalidae, membahagikan mereka kepada dua kumpulan mengikut persekitaran makro mereka, iaitu ekosistem air tawar dan air masin dengan pengecualian seperti spesies *Cichlidogyrus* dan *Scutogyrus*. Ia adalah dicadangkan bahawa dua kumpulan ini dibahagikan dengan ahli-ahli air tawar (*Ancyrocephalus*, *Actinocleidus*, *Cleidodiscus*, *Urocleidus* and *Onchocleidus*) kekal dalam Ancyrocephalidae dan satu family baru harus dibina untuk ahli-ahli air masin seperti *Haliotrema*, *Bravohollisia, Caballeria, Pseudohaliotrema, Metahaliotrema, Euryhaliotrema, Euryhaliotrematoides, Tetrancistrum, Haliotrematoides, Ligophorus* dan *Aliatrema* serta ahli-ahli air tawar dari cichlid, i.e. *Cichlidogyrus*, *Scutogyrus* dan *Onchobdella*. Adalah dipostulasikan bahawa cichlid mempunyai origin air masin. Analisis ini juga mengesahkan status famili Heteronchocleididae, Ancylodiscoididae, Neocalceostomatidae dan Pseudodactylogyridae. Walau bagaimanapun, posisi monogenean-monogenean dalam pokok filogenetik adalah tidak serasi dengan hipotesis sejarah evolusi ciri-ciri morfologi kumpulan-kumpulan dactylogyridean, teruatamnya

bagi monogenean dengan 2- dan 4-anchor. Hubungan perumah berdasarkan Cytochrome *b* dan hubungan monogenean berdasarkan 28S rDNA menyokong hokum parasitophyletik di mana spesies perumah yang berhubungan rapat akan mempunyai parasit monogenean yang juga berhubungan rapat. Spesifisiti sesetengah spesies monogenean mencadangkan mereka telah menjalani koevolusi bersama dengan perumah mereka. Walau bagaimanapun, terdapat kes di mana monogenean dapat diperolehi melalui ópemindahan perumahô sebagai contoh *Dactylogyrus* spp. dari perumah cyprinid kemungkinan besar telah diperolehi oleh satu spesies ikan air masin dan proses spesiasi *Dactylgoyrus* spp. yang seterusnya dalam ikan air masin *Lateolabrax* sp. telah menyumbang kepada corak taburan *Dactylogyrus* hari ini. Selain ópemindahan perumahô, sesetengah spesies monogenean mungkin mengalami kegagalan untuk menjalani spesiasi dan ini mungkin telah berlaku pada kes sesetengah heteronchocleidid dalam ikan anabantoid-channid. Kajian ini juga menunjukkan bahawa data set morfometri yang besar adalah diperlukan untuk pembezaan spesies dan variasi intraspesifik menggunakan kaedah statistik. Ini juga didapati benar dalam penggunaan data molekular, salah satu had limitasi dalam thesis ini ialah kekurangan data molekular dari sesetengah genera (*Dogielus* dan *Thaparogyrus* tidak diwakili dalam Dactylgoyridae) dan spesies (Pseudomurraytrematidae hanya diwakili oleh satu spesies). Kajian ini juga mendapati had limitasi dalam membina semula hubungan filogenetik dengan berdasarkan 28S rDNA sahaja.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Monogenea Carus, 1863

Monogenea along with the Cestoda, Trematoda (Digenea and Aspidogastrea) and the highly diverse, heterogenous and mainly free-living turbellarians are members of the Platyhelminthes. The Monogenea, one of the most diverse classes of Platyhelminthes is currently grouped together with the Cestoda under Cercomeromorpha (ôhooked larvaõ) which with the Trematoda form the Neodermata (new dermis, referring to the syncytial epidermis found in these 3 group) (Cavalier-Smith, 1998).

Monogeneans are characterized by having a body proper and haptor with sizes ranging from <0.5mm to 1-2cm long, four eyespots, head organs (except in the Polystomatoidea), one or more than one testes e.g. *Myxinidocotyle* spp. (Gyrodactylidea: Acanthocotylidae), *Choricotyle* spp. (Mazocraeidea: Diclidophoridae), *Nasicola* spp. (Capsalidea: Capsalidae) and a single ovary. Monogeneans have a direct life cycle without any intermediate hosts. They are soft-bodied and cannot withstand desiccation and are hence found mainly as parasites on aquatic or aquatic-related organisms such as fish (freshwater and marine), frogs (see Lim & Du Preez, 2001, Vande Vusse, 1976), turtles (see Du Preez & Lim, 2000; Pichelin, 1995; Richardson & Brooks, 1987), squids and octopus (see Llewellyn, 1984), copepods (Bychowsky, 1957) and even on the eyes of the aquatic mammal, the hippopotamus (see Thurston & Law, 1965). Monogeneans can be found on body surface, scales, fins, gills and nasal cavity, pharyngeal cavity and stomach of fishes (see Gusev & Fernando, 1973; Ergens, 1988;

Bychowsky, 1957; Paperna, 1963; Pariselle, Lambert & Euzet, 1991), and in the cloaca, urinary bladder, conjunctival cavity, oral cavity and intestine of turtles (Du Preez & Lim, 2000; Richardson & Brooks, 1987; Rohde & Pearson, 1980; Rohde, 1965; Rohde, 1963) as well as the urinary bladder of frogs (see Lim & Du Preez, 2001; Du Preez & Kok, 1995).

Monogeneans have their greatest diversity on fishes. Currently in Malaysia, over 200 species of monogeneans have been described from 59 species of fish (35 and 25 species of freshwater and marine fish, respectively), 3 species of turtles and 1 species of frog (see Lim, 1998; 2002; 2003; 2006; Lim & Gibson, 2007; 2008a; 2008b; 2009; 2010; Lim, Tan & Gibson, 2010; Tan & Lim, 2009; Du Preez & Lim, 2000; Lim & Du Preez, 2001; Pariselle, Lim & Lambert, 2001a; 2001b; 2002a; 2002b; 2003; 2004; 2005a; 2005b; 2006). To date monogenean species belong to three subclasses, the Polystomatoinea, the Oligonchoinea and the Polyonchoinea (Lim, pers. com.; Lim, 1998). The subclass Polystomatoinea consists of monogeneans from turtles and amphibians while the other two subclasses, Oligonchoinea and Polyonchoinea consist of monogeneans from marine and freshwater fishes. The Polyonchoinea, with 3 orders, 8 families, 32 genera and 204 species, is the largest of the three subclasses. Within this subclass, the order Dactylogyridea Bychowsky, 1937 is the most diverse order. In Malaysia, the Dactylogyridea make up about 90% of the total monogenean species described to date. This is probably because Lim & Furtado (1983, 1984, 1985, 1986a, 1986b), Lim (1986, 1987a, 1987b, 1989, 1990, 1991, 1992, 1994, 1995a. 1995b, 1995c, 1996, 2002; 2003; 2006), Lim & Gibson (2007; 2008a; 2008b; 2009; 2010), Pariselle, Lim & Lambert (2001a; 2001b; 2002a; 2002b; 2003; 2004; 2005a; 2005b; 2006), Du Preez & Lim (2000), Lim & Du Preez (2001), Tan & Lim (2009) and Lim, Tan & Gibson (2010) have concentrated on this group of monogenean. There are still many

more species from the Polyonchoinea, as well as from Polystomatoinea and Oligonchoinea waiting to be discovered, described and documented (Lim, 1998).

To date not all the available host species have been examined for monogeneans. For instance it has been estimated that in Peninsular Malaysia, there are 272 freshwater fish species (Lim *et al.*, 1993), 294 marine fish species (Scott, 1959), 15 species of turtles (see Gregory & Sharma, 1997) and 155 species of frogs (see Kiew, 1984) but the host species examined for monogeneans so far represent only about 12%, 7%, 20% and 0.6% of freshwater and marine fish species, turtles and frogs, respectively (Lim, 1998; Lim & Gibson, 2009; Lim, unpublished data). Lim (1998) further estimated that 83%, 92% and 94% of monogeneans are yet to be described from these hosts, respectively.

Up till the 1960s, Bychowsky (1957) and Yamaguti (1965; 1966) had recorded approximately 957 and 1,350 described monogenean species in the world, respectively. During 1980s, an estimated number of 2200 spp. of monogeneans has been documented (Kurochkin, 1985). Since then, the number of known monogeneans species have been increasing gradually as more research are done and many more species have been described. Many authors (Lim, 1998; Poulin, 2002; Whittington, 1998; Justine, 2007; Kritsky, 2007) had estimated that only 10-20% of the monogenean species have been described and several thousands are yet to be discovered and described. Although amphibians and turtles are also hosts to the monogeneans, these are left out from previous estimates focusing only on those parasitizing fishes. Current numbers of valid fish species, amphibian species and turtle species worldwide are estimated to be approximately 32,000 (Froese & Pauly, 2011), 6,771 (Frost, 2011) and 452 (Rhodin *et al.*, 2010), respectively. Since most of the monogeneans species are highly host specific,

a conservative prediction of the monogenean species can be done by assuming that each fish, amphibian and turtle species is parasitized by three, one and three species of monogenean, respectively, giving an estimated number of 32,000 x 3 = 96,000 monogenean spp. for fish, 6,771 monogenean spp. for amphibians and 452 x 3 = 1,356 monogenean spp. for turtles (Lim, unpublished data). Rohde (2005) estimated that there are 10,000 known monogenean species. Lim (unpublished data) based on these estimates by Rohde (2005) noted that the known monogenean species are only approximately 9 % of the total estimated monogenean species.

1.2 Current status in the study of monogeneans

To date, most of the studies on monogeneans are concentrated mainly in taxonomy (e.g. Lim, 1995a; 1995b; 1995c; 1996; 1998; 2002; 2003, 2006; Lim & Gibson, 2007; Du Preez & Lim, 2000; Lim & Du Preez, 2001; Tan & Lim, 2009; Lim, Tan & Gibson, 2010), with some studies on ecological distribution (e.g. Wootten, 1974; Koskivaara & Voltonen, 1992; Simkova *et al.*, 2001b, Simkova *et al.*, 2000), physiology, diet and nutrition (e.g. Halton *et al.*, 1998; Buchmann *et al.*, 1987), functional morphology (e.g. Kearn *et al.*, 1995; Halton & Gustafasson, 1996; Wong *et al.*, 2006a), ontogeny (e.g. Muñoz & Zamora, 2011; Malmberg, 1990), histopathology (e.g. Morrison *et al.*, 2001; Bullard *et al.*, 2001; Tinsley *et al.*, 2002), control/treatment of diseases (e.g. Yoshinaga *et al.*, 2000; Cowell *et al.*, 1993; Ellis & Wanatabe, 1993; Liang & Leong, 1992; Bondadreantaso *et al.*, 1995; see also Lio-Po & Lim, 2002). There are also studies on characterisation of biomaterial from monogeneans (e.g. Wong *et al.*, 2006a; 2008), on relationships of monogeneans using molecular data (e.g. Mollaret *et al.*, 2000a; Mollaret *et al.*, 2000b; Mollaret *et al.*, 1997; Jovelin & Justine, 2001), geometric morphometrics of monogeneans (e.g. Vignon & Sasal, 2010; Vignon,

2011) and describing new methods for the collection and preservation of monogeneans (e.g. Justine *et al.*, 2012; Koskova *et al.*, 2010; Wong *et al.*, 2006b).

1.3 Approaches in taxonomic investigations on monogeneans

The most extensive work done on monogeneans remains in the area of taxonomy. However, only 9% of the estimated numbers of monogeneans are known. A quick review of the current taxonomic literature within the last 20 years revealed that the approaches used in characterisation and classification of organisms includes observations of morphological and anatomical character, cytological investigation (karyotyping), biochemical determination (analysis of immunological data and isozymes and allozymes banding) and more recently the use of molecular data (RAPD, AFLP, RFLP and PFGE profiles, protein and DNA sequences) (see also Quicke, 1993). Of these, morphological and anatomical characters are most commonly used in species identifications.

This is also true for the monogeneans, where the characterisation and classification of monogeneans are mainly done by using morphological characters obtained from light microscopy (e.g. Bychcowsky, 1957; Yamaguti, 1965; 1966; Gusev, 1985; Lim & Furtado, 1984, 1986a, 1986b, Lim, 1987; 1991; 1992; 1994; 1996; 2002; 2003; 2006) with occasional information from SEM (e.g. Malmberg & Fernholm, 1991; Antonelli *et al.*, 2010; Hodova *et al.*, 2010; Williams & McKenzie, 1995; Shinn *et al.*, 1993; Wong *et al.*, 2006a; Wong *et al.*, 2008), TEM (e.g. Arafa, 2011; Wong *et al.*, 2006a; Wong *et al.*, 2008; Shinn *et al.*, 2003; Harris *et al.*, 1997) and confocal scanning laser microscopy (e.g. Arafa *et al.*, 2007; Zurawski *et al.*, 2003; Cable *et al.*, 1996).

Although morphological characters are most commonly used, ontogenic characters (e.g. Llewellyn, 1963; Lambert, 1980; Malmberg, 1990), spermatozoon ultrastructure and spermiogenesis (e.g. Justine, 1992; Justine *et al.*, 1993; Fournier & Justine, 1994; Mollaret *et al.*, 1998; Quilichini *et al.*, 2009), distribution pattern in/on host, host specificity, pathogenicity (e.g. Jorgensen *et al.*, 2007; Simkova *et al.*, 2006a; Sterud *et al.*, 2002; Simkova *et al.*, 2001a; Simkova *et al.*, 2001b; Lim, 1987a) have also been used.

More recently there are increasing number of DNA sequences from monogenean being uploaded into the GenBank and currently there are approximately 3236 sequences on monogeneans in the GenBank (from year 1991 to May 2012) (see later; Section 1.3.2). Essentially there is thus a need to re-examine the use of morphologies and examine the use of molecular data in determining species validity and relationships (in particular phylogenetic relationships) based on reconstructed relationships trees.

1.3.1 Morphological characters

The most commonly used diagnostic characters in taxonomy is morphology and species have been described almost exclusively on these visually observable morphological features as noted in a quick search done on publications in systematic journal such as Systematic Parasitology (Lim, pers. com.). This use of morphological characters has resulted in the ósplittersô who use even the slightest differences in morphological characters to ócreateô new species and at the other end of the spectrum are the ólumpersô who lumps even remotely similar species some with good diagnostic characters as one species. The use of morphological characters seems to depend on the

researchers, creating subjectivity in this important field of taxonomy that very much needs objectivity. To overcome issues based on too much reliance on morphologies such as divergence and convergence (Mayr & Ashlock, 1991), researchers have included information from developmental biology, physiology, biochemistry and lately molecular biology to augment, confirm or refute interpretations based on morphological features (Cavalier-Smith, 1998). One good example is the relationships of monogeneans which have shifted from being a subclass within Trematoda (together with Digenea), to a class of its own but closely allied to the Trematoda in the 1960s and finally based on developmental biology the Monogenea become associated as a sister group with the Cestoda under the Cercomeromorpha based on having larvae with hooks (see Section 1.1).

The validity and the reliability of morphologies as diagnostic features is an on-going debate. Problems related to the use of morphological characters become especially crucial in the use of morphological characters in tracking evolutionary history (see Gusev, 1978; Kritsky & Boeger, 1989) prior to the advent of PCR and also in use even now (Domingues & Boeger, 2008). Taxonomists are looking for ways to ensure that morphological characters are used objectively (Soo & Lim, 2012; Tan *et al.*, 2010; Dmitrieva *et al.*, 2007; Rubtsova *et al.*, 2007; Sarabeev & Balbuena, 2004).

Questions related to the use of morphologies include: Are the morphological characters species-specific, generic-specific and family-specific diagnostic characters or are they just intraspecific differences? Related to this is the question of whether similar morphologies are due to convergence in evolution or as a result of common descent?

And also whether differences in morphological features are caused by divergence and adaptation to environmental factors rather than a result of gene differences?

Metric data are always given in descriptions of new species and the general practice is to provide average (min-max range) of metric data. However these data are usually not analysed resulting in a loss of information. Lim (1987a) noticed wide ranges in metric data in species descriptions whether many or few specimens are measured, raising question on the significance of these wide metric ranges and also whether the observed variations are intraspecific or interspecific variations. Lim (1987a) noted the presence of morphovariants amongst the dactylogyrids. In fact morphological variations within a species have resulted in the interpretation of the same species as different (see Lim, 1987a). If they are intraspecific variants, how common are such variants within a community? Are they simple continuum of metric variations or are they well defined variants? What are the implications of the presence of these variants in terms of evolution? These morphovariants usually give taxonomists a big headache deciding whether such species are different. Is there a reliable way to determine whether the observed variations amongst morphologically similar co-existing congeners are intraspecies variations or interspecies variations?

Since the use of morphological characters in taxonomy have raised the question of subjectivity and information from morphological characteristics such as in the form of morphometric measurements which usually consist of data sets with multiple variables, an objective method is very much needed to analyse such information. Principal Component Analysis (PCA) can be used in attempts to remove subjectivity in the use of morphological characters in taxonomy and solve the problems of analyzing

multivariate data sets as it can extract significant information and identify patterns from data sets with multiple dimensions (multiple variables) by highlighting their similarities and dissimilarities (Jolliffe, 2002). PCA is also one of the most widely employed and useful tools in the field of exploratory analysis.

Since patterns in data such as multivariate morphometric data sets can be hard to find, PCA is a powerful tool for detecting these patterns in such data by reducing the number of dimensions without much loss of information. Thus, PCA method is suitable to be used to provide the objectivity which is especially necessary in the cases to analyse monogenean species with very similar morphologies. In fact, PCA have been used to lend some objectivity in discriminating morphologically similar species (e.g. Sarabeev & Balbuena, 2004; Rubtsova *et al.*, 2006; Dmitrieva *et al.*, 2007; Rubtsova *et al.*, 2007) as well as to detect morphovariants within species (e.g. Mariniello *et al.*, 2004; Tan *et al.*, 2010; Poisot & Desdevises, 2010). However there are still limitations in the published data (cf Tan *et al.*, 2010; Marinello *et al.*, 2004) of this type of PCA analysis where there is a need to measure a large number of specimens so that conclusions can be valid. Prior to this study, the number of specimens used to represent one species are variable and can be as low as only 5 specimens (see Marinello *et al.*, 2004).

1.3.2 Molecular characters

The issues caused by morphologies (Section 1.3.1) have resulted in a shift to the use of molecular data (DNA sequences). For studies related to monogenean, molecular data in the form of DNA sequences are most commonly used for inferring relationships (Mollaret *et al.*, 1997; Justine *et al.*, 2002; Jovelin & Justine, 2001; Mollaret *et al.*, 2000a; Mollaret *et al.*, 2000b; Verneau *et al.*, 2002; Olson & Littlewood, 2002; Sinnappah *et al.*, 2001; Whittington *et al.*, 2004; Ġimková *et al.*, 2003; Ġimková *et al.*,

2004; Plaisance *et al.*, 2005; Ġimková *et al.*, 2006b; Wu *et al.*, 2006; Wu *et al.*, 2007a; 2007b; Wu *et al.*, 2008; Mendlova *et al.*, 2010; Mendlova *et al.*, 2012; Tan *et al.*, 2011) and differentiating species with high degree of morphological similarities or cryptic species (Desdevises *et al.*, 2000; Glennon *et al.*, 2008; Hansen *et al.*, 2003; Huyse & Volckaert, 2002; Kuusela *et al.*, 2008; Wu *et al.*, 2005).

Molecular data in inferring relationship

Molecular data are used in greater frequencies but mainly in reconstructing the relationships of monogeneans (Table 1.1). Is the information derived from molecular data such as DNA sequences which have been widely used to determine relationships at higher taxonomic levels be able to help us understand and resolve relationships in different group of monogeneans? In order to answer this question, DNA sequences from dactylogyridean monogeneans (see later; Sections 1.4 & 1.5) obtained in the duration of this study and DNA sequences deposited in the GenBank will be analysed to determine relationships of monogeneans within the Dactylogyridea (see Materials & Methods; Chapter 2).

Molecular data for species differentiation

As already noted molecular data have been used to differentiate species with high degree of morphological similarities. Besides using DNA sequences to infer relationships, this study also examine whether the DNA data can offer more information concerning the species. For example can the DNA data such as 28S rDNA be used to assist in differentiating morphologically closely related species? In other words can molecular biology be used to assist in decision making whether the morphologically

similar species are the same or different species? This is based on the premise that 28S rDNA sequences are basically highly conserve then it will suggest that members of the same species will have 100% similar 28S rDNA even if variations are present. In the course of this study an opportunity arose which enables this assumption to be put to the test (see Chapter 6) (also Lim, Tan & Gibson, 2010 & Appendix E).

However, to date the use of molecular data for species characterisation is hampered by the current lack of enough monogeneans with enough DNA sequences. Currently there are only approximately 3,236 DNA sequences on 819 monogenean species available in the GenBank compared to the estimated number of over 2,200 known monogenean species and the global estimated total number of 104,127 monogenean species (see Section 1.2; Lim, pers. com.; unpublished data). Furthermore the sequences known for monogeneans are usually short and limited to the partial conserve regions such as the 28S rDNA (most available data), 18S rDNA, internal transcribed spacers (ITS) regions, Cytochrome *b* and Cytochrome Oxidase I (COI) (Table 1.2) (see also GenBank). This give rise to controversial interpretations based on DNA sequences used are usually representing only a partial portion of a single gene present in the organism studied. By examining these partial gene sequences, one is therefore looking at a very small portion of the information that goes into making an organism what it is (Unnasch & Zimmerman, 1995).

Table 1.1 Previous studies on the relationships of monogeneans at different taxonomic levels based on molecular data

Title & Authority	**Type of molecular data used**	**Groups studied and species used**	**Overall conclusions**
Phylogenetic analysis of the Monogenea and their relationships with Digenea and Eucestoda inferred from 28S rDNA sequences (Mollaret *et al.*, 1997)	partial 28S rDNA	**Groups studied: Monogenea: Monopisthocotylea:** 1 *Acleotrema* sp., 1 *Tetrancistrum* sp., 1 *Haliotrema* sp., 1 *Troglocephalus* sp., 1 *Neoheterocotyle* sp., 1 *Merizocotyle* sp., 1 *Entobdella* sp., 1 *Benedenia* sp., 1 *Encotyllabe* sp.; **Polyopisthocotylea:** 1 *Zeuxaptera* sp., 1 *Gotocotyla* sp., 1 *Pricea* sp.; **Digenea:** 1 *Schistosoma* sp., 1 *Heterobilharzia* sp., 1 *Echinostoma* sp., 1 *Lepidapedon* sp.; **Eucestoda:** 1 *Hymenolepis* sp., 1 *Proteocephalus* sp., 1 *Caryophyllaeus* sp. **Outgroup: Tricladida:** 1 *Polycelis* sp., 1 *Bipalium* sp.	- the Digenea and not the Monogenea (Monopisthocotylea & Polyopisthocotylea) form the sister group of the cestodes - the Monopisthocotylea & Polyopisthocotylea are each monophyletic but the Monogenea do not form a monophylum - the sister group of the Digenea + Cestoda is the Polyopisthocotylea & Monopisthocotylea are the sister group of all other parasitic flatworm
Phylogenetic position of the monogeneans *Sundanonchus*, *Thaparocleidus* and *Cichlidogyrus* inferred from 28S rDNA sequences (Mollaret *et al.*, 2000b)	partial 28S rDNA	**Groups studied:** 1 *Sundanonchus* sp., 1 *Thaparocleidus* sp., 1 *Cichlidogyrus* sp. **Other species used: Ancyrocephalidae:** 1 *Tetrancistrum* sp., 1 *Ligophorus* sp., 1 *Haliotrema* sp.; **Diplectanidae:** 1 *Furnestinia* sp., 1 *Acleotrema* sp. **Outgroups: Capsalidae:** 1 *Trochopus* sp., 1 *Encotyllabe* sp., 1 *Benedenia* sp., 1 *Capsala* sp., 1 *Tristoma* sp., 1 *Entobdella* sp.; **Monocotylidae:** 1 *Troglocephalus* sp., 1 *Neoheterocotyle* sp., 1 *Calicotyle* sp., 1 *Merizocotyle* sp.; **Udonellidae:** 1 *Udonella* sp.	- Diplectanidae were the sister-group to a clade including *Sundanonchus* and the Ancyrocephalinae - *Sundanonchus* was the sister-group to the Ancyrocephalidae suggesting the validity of Sundanonchidae

Phylogeny of the Monopisthocotylea and Polyopisthocotylea (Platyhelminthes) inferred from 28S rDNA sequences (Mollaret *et al.*, 2000a)	partial 28S rDNA	**Groups studied: Monopisthocotylea:** Ancyrocephalidae (3 spp.), Diplectanidae (2 spp.), Capsalidae (6 spp.), Monocotylidae (4 spp.), Udonellidae (1 sp.); **Polyopisthocotylea:** Polystomatidae (6 spp.), Hexabothriidae (2 spp.), Mazocraeidae (2 spp.), Hexostomatidae (1 sp.), Plectanocotylidae (1 sp.), Diclidophoridae (3 spp.), Octomacridae (1 sp.), Gastrocotylidae (2 spp.), Neothoracocotylidae (1 sp.), Gotocotylidae (1 sp.), Microcotylidae (5 spp.), Heteraxinidae (1 sp.), Axinidae (1 sp.) **Outgroups:** Gyrocotylidea (1 sp.), Cestoda (2 spp.), Digenea (6 spp.), Aspidogastrea (2 spp.), Turbellaria (7 spp.), Catenulida (1 sp.)	- Within Monopisthocotylea, Ancyrocephalidae, Diplectanidae, Capsalidae, Monocotylidae, Udonellidae are found to be monophyletic - Within Polyopisthocotylea, the polystomatids were the sister-group of all others; Hexobothrium was the most basal and the mazocraeids were the sister groups of all other studied polyopisthocotyleans
A paedomorphic parasite associated with a neotenic amphibian host: phylogenetic evidence suggests a revised systematic position for Sphyranuridae within Anuran and turtle Polystomatoineans (Sinnappah *et al.*, 2001)	partial 18S rDNA	**Groups studied: Polystomatidae:** 2 *Polystoma* spp., 1 *Eupolystoma* sp., 1 *Protopolystoma* sp., 1 *Pseudodiplorchis* sp., 1 *Polystomoides* sp., 1 *Neopolystoma* sp.; **Sphyranuridae:** 1 *Sphyranura* sp. **Other species used: Diclidophoridae:** 1 *Diclidophora* sp., 1 *Choricotyle* sp.; **Microcotylidae:** 1 *Microcotyle* sp. **Outgroups: Bothriocephalidae:** 2 *Bothriocephalus* spp.; **Ancistrocephalidae:** 1 *Triaenophorus* sp.	- Polystomatoineans were shown to be monophyletic and consist of two clades, the amphibian monogeneans clade and the turtle polystomatids clade. - Polystomatoineans may have coevolved with amphibian hosts. - the genus *Sphyranura* initially assigned to the family Sphyranuridae is found nested within polystomatids, suggesting its systematic status must be revised.
Phylogenetic relationships within the Polyopisthocotylean monogeneans (Platyhelminthes) inferred from partial 28S rDNA sequences (Jovelin & Justine, 2001)	partial 28S rDNA	**Groups studied: Polyopisthocotylea:** Chimaericolidae (1 sp.), Discocotylidae (1 sp.), Diplozoidae (1 sp.), Diclidophoridae (2 spp.), Gastrocotylidae (2 spp.), Gotocotylidae (1 sp.), Plectanocotylidae (3 spp.), Microcotylidae (5 spp.), Pyragraphoridae (1 sp.) **Outgroups:** Mazocraeidae (2 spp.), Polystomatidae (5 spp.)	- the polytomy between Gastrocotylinea, Discocotylinea and Microcotylinea is partially resolved: Gastrocotylinea are the sister group of an unresolved group including the Microcotylinea, Discocotylinea and Plectanocotylidae. - Inclusion of Plectanocotylidae in the suborder Mazocraeinea is rejected. - Monophyly of Microcotylinea and Plectanocotylidae is confirmed but monophyly

			of Discocotylinea is questioned by the exclusion of *Diplozoon*.
A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea: Polystomatidae) (Verneau *et al.*, 2002)	partial 18S rDNA	**Groups studied: Polystomatidae:** 2 *Eupolystoma* spp., 9 *Polystoma* spp., 1 *Metapolystoma* sp., 1 *Sundapolystoma* sp., 1 *Neodiplorchis* sp., 2 *Protopolystoma* spp., 1 *Pseudodiplorchis* sp., 1 *Sphyranura* sp., 3 *Neopolystoma* spp., 4 *Polystomoides* spp., 1 *Concinnocotyla* sp. **Other species used: Microcotylidae:** 1 *Microcotyle* sp.; **Diclidophoridae:** 1 *Diclidophora* sp., 1 *Choricotyle* sp. **Outgroup: Cestoda:** 2 *Bothriocephalus*, 1 *Triaenophorus* sp.	- the monophyly of the polystomatid lineages from chelonian and lissamphibian hosts indicate that polystomatids from turtles are switched from an aquatic amniote - within polystomatids from lissamphibians, polytomy is observed for caudatan, neobatrachian, pelobatid and pipid polystomatid lineages - this suggest the first polystomatids of amphibians originated during the evolution and diversification of lissamphibian orders and suborders
Phylogenetics of the Monogenea ï evidence from a medley of molecules (Olson & Littlewood, 2002)	partial 28S rDNA, partial 18S rDNA	**Groups studied:** 27 families of Monogenea: **Chimaericolidea:** Chimaericolidae (1 sp.); **Diclybothriidea:** Hexabothriidae (2 spp.); **Mazocraeidea: Discocotylinea:** Discocotylidae (1 sp.); Diplozoidae (2 spp.); Octomacridae (1 sp.); **Gastrocotylinea:** Allodiscocotylidae (1 sp.); Gastrocotylidae (2 spp.); Gotocotylidae (4 spp.); Neothoracocotylidae (3 spp.); Protomicrocotylidae (1 sp.); **Hexostomatinea:** Hexostomatidae (1 sp.); **Mazocraeinea:** Mazocraeidae (2 spp.); Plectanocotylidae (3 spp.); **Microcotylinea:** Diclidophoridae (6 spp.); Heteraxinidae (3 spp.); Microcotylidae (10 spp.); Pyragraphoridae (1 sp.); **Polystomatidea:** Polystomatidae (7 spp.); **Capsalidea:** Capsalidae (11 spp.); **Dactylogyridea: Dactylogyrinea:** Dactylogyridae (7 spp.); Diplectanidae (2 spp.); Pseudomurraytrematidae (1 sp.); **Tetraonchinea:** Sundanonchidae (1 sp.); **Gyrodactylinea:** Gyrodactylidae (1 sp.); Anoplodiscidae (1 sp.); Udonellidae (1 sp.); **Monocotylidea:** Monocotylidae (33 spp.) **Outgroups: Cestoda: Gyrocotylidea:** Gyrocotylidae (2 spp.); **Eucestoda:** Lytocestidae (1 sp.); Echinobothriidae (1 sp.); Haplobothriidae (1 sp.); Diphyllobothriidae (1 sp.); Spathebothriidae (1 sp.); Eutetrarhynchidae (1 sp.); Tentacularidae (1 sp.)	- Maximum parsimonyand minimum evolution trees were rooted against sequences from the Cestoda, forcing the Monogenea to appear monophyletic - The Polyonchoinea showed greatest resolution with a general pattern of ((Monocotylidae(Capsalidae(Udonellidae + Gyrodactylidea)))((Anoplodiscidae+Sundanonchidae)(Pseudomurraytrematidae 1 Dactylogyridae))) - The Heteronchoinea readily split into the Polystomatoinea + Oligonchoinea, and Chimaericolidae & Hexabothriidae were successively the most basal of oligonchoinean taxa

Phylogenetic positions of the Bothitrematidae and Neocalceostomatidae (Monopisthocotylean Monogeneans) inferred from 28S rDNA sequences (Justine *et al.*, 2002)	partial 28S rDNA	**Groups studied: Bothitrematidae:** 1 *Bothitrema* spp.; **Neocalceostomatidae:** 1 *Neocalceostoma* sp. **Other species used: Ancyrocephalidae:** 1 *Tetrancistrum* sp., 1 *Haliotrema* sp., 1 *Ligophorus* sp., 1 *Thaparocleidus* sp., 1 *Cichlidogyrus* sp., 1 *Pseudohaliotrema* sp., 1 *Bravohollisia* sp.; **Pseudodactylogyridae:** 1 *Pseudodactylogyrus* sp.; **Anoplodiscidae:** 1 *Anoplodiscus* sp.; **Sundanonchidae:** 1 *Sundanonchus* sp. **Outgroup: Diplectanidae:** 1 *Acleotrema* sp., 1 *Furnestinia* sp.	- *Bothitrema*, *Anoplodiscus* and *Sundanonchus* formed a very robust clade that was the sister group to a group that included all other species examined - Molecular results that suggest inclusion of the families Bothitrematidae, Anoplodiscoididae and Sundanonchidae in the same group partially contradict a previous morphological analysis of Boeger & Kritsky in which the first 2 were placed in the Gyrodactylidea and the third in the Dactylogyridea.
Phylogenetic relationships of the Dactylogyridae Bychowsky, 1933 (Monogenea: Dactylogyridea): the need for the systematic revision of the Ancyrocephalinae Bychowsky, 1937 (Simkova *et al.*, 2003)	partial 18S rDNA	**Groups studied: Ancyrocephalinae:** 1 *Thylacicleidus* sp., 1 *Pseudohaliotrema* sp., 1 *Cleidodiscus* sp., 1 *Ancyrocephalus* sp., 1 *Urocleidus* sp.; **Dactylogyrinae:** 2 *Dactylogyrus* spp.; **Ancylodiscoidinae:** 2 *Thaparocleidus* spp.; **Pseudodactylogyrinae:** 3 *Pseudodactylogyrus* spp., 1 *Pseudodactylogyroides* sp.; **Pseudomurraytrematidae:** 1 *Pseudomurraytrema* sp.; **Diplectanidae:** 1 *Diplectanum* sp., 2 *Lamellodiscus* spp., 1 *Furnestinia* sp. **Other species used: Tetraonchinea:** 1 *Tetraonchus* sp., 1 *Sundanonchus* sp., 1 *Anoplodiscus* sp. **Outgroups: Monocotylidea:** 1 *Calicotyle* sp., 1 *Leptocotyle* sp., 1 *Dictyocotyle* sp., 1 *Troglocephalus* sp.; **Capsalidea:** 1 *Capsala* sp., 1 *Encotyllabe* sp., 1 *Benedenia* sp.; **Gyrodactylidea:** 1 *Gyrodactylus* sp., 1 *Udonella* sp.; **Trematoda:** 1 *Fasciola* sp.; **Tricladida:** 1 *Girardia* sp.	- relationships of Diplectanidae and Dactylogyridae with Pseudomurraytrematidae are not resolved - relationships between the Pseudodactylogyrinae, Ancyrocephalinae, Ancylodiscoidinae & Dactylogyrinae indicate paraphyly of the Ancyrocephalidae sensu Bychowsky & Nagibina (1978) - the non-monophyly of the Ancyrocephalinae, previously suggested by Kritsky & Boeger (1989) using morphological characters, indicates that classification of the Dactylogyridae needs to be revised

Molecular phylogenetic analysis of the genus *Gyrodactylus* (Platyhelminthes: Monogenea) inferred from rDNA ITS region: subgenera versus species groups (Matejusova *et al.*, 2003)	partial 18S rDNA, ITS region	**Groups studied:** 37 *Gyrodactylus* spp., 1 *Gyrdicotylus* sp., 1 *Macrogyrodactylus* sp., 1 *Gyrodactyloides* sp. **Other species used:** 1 *Udonella* sp., 1 *Encotyllabe* sp., 1 *Benedenia* sp., 1 *Capsala* sp., 1 *Dictyocotyle* sp., 1 *Calicotyle* sp., 1 *Leptocotyle* sp., 1 *Troglocephalus* sp., 1 *Pseudohaliotrema* sp., 1 *Pseudodactylogyrus* sp., 1 *Pseudomurraytrema* sp. **Outgroup:** 1 *Sundanonchus* sp., 1 *Anoplodiscus* sp.	- The genus *Gyrodactylus* appeared to be a monophyletic group and Within the genus, there were 3 major groups recognized - None of the 6 subgenera appeared to be monophyletic, and the most basal subgenus *G.(Gyrodactylus)* was paraphyletic - The grouping of species based on the morphology of the ventral bar and marginal hooks seems to have sufficient power to infer relationships between the *Gyrodactylus* species
A preliminary phylogenetic analysis of the Capsalidae (Platyhelminthes: Monogenea: Monopisthocotylea) inferred from large subunit rDNA sequences (Whittington *et al.*, 2004)	partial 28S rDNA	**Groups studied: Capsalidae: Benedeniinae:** 4 *Benedenia* spp., 2 *Neobenedenia* spp.; **Encotyllabinae:** 2 *Encotyllabe* spp.; **Trochopodinae:** 1 *Trochopus* sp.; **Entobdellinae:** 5 *Entobdella* spp.; **Capsalinae:** 2 *Capsala* spp., 1 *Tristoma* sp. **Outgroups: Monocotylidae:** 1 *Dendromonocotyle* sp., 1 *Calicotyle* sp.; **Udonellidae:** 1 *Udonella* sp.	- Capsalinae, Encotyllabinae, Entobdellinae and Trochopodinae are monophyletic but Benedeniinae is paraphyletic. - *Neobenedenia*, currently in the Benedeniinae, should perhaps be placed in a separate subfamily.
Molecular phylogeny of congeneric monogenean parasites (*Dactylogyrus*): a case of intrahost speciation (Simkova *et al.*, 2004)	partial 18S rDNA & ITS1	**Groups studied: Dactylogyridae:** 51 *Dactylogyrus* spp. **Other species used: Pseudodactylogyrinae:** 2 *Pseudodactylogyrus* spp., 1 *Pseudodactylogyroides* sp.; **Ancyrocephalinae:** 1 *Thylocicleidus* sp., 1 *Pseudohaliotrema* sp. **Outgroup: Ancylodiscoidinae:** 1 *Thaparocleidus* sp.; **Ancyrocephalinae:** 1 *Cleidodiscus* sp.	- 3 main *Dactylogyrus* lineages were recognized, i.e. *Dactylogyrus* of Cyprininae, *Dactylogyrus* of Gobioninae, Rasborinae, Cyprininae and *Dactylogyrus* of Leuciscinae, Alburninae and Cyprininae - Cyprininae could be the plesiomorphic hosts for *Dactylogyrus*.

			- *Dactylogyrus* diversification can be mainly explained by sympatric intrahost speciation.
Phylogenetic position of the monogeneans *Pseudodactylogyrus*, *Heteronchocleidus* & *Trianchoratus* inferred from the 5ô terminal sequences of 28S rDNA (Ding & Liao, 2005)	partial 28S rDNA	**Groups studied:** 1 *Pseudodactylogyrus* sp., 1 *Heteronchocleidus* sp., 1 *Trianchoratus* sp. **Other species used:** 1 *Onchocleidus* sp., 1 *Quadriacanthus* sp., 1 *Thaparocleidus* sp., 1 *Haliotrema* sp., 1 *Ancyrocephalus* sp., 1 *Pseudohaliotrema* sp., 1 *Tetrancistrum* sp., 4 *Dactylogyrus* spp., 1 *Pseudodactylogyroides* sp.	- the *Heteronchocleidus* and *Trianchoratus* are sister groups - the genera *Heteronchocleidus*, *Trianchoratus*, *Ancyrocephalus* & *Pseudodactylogyrus* display a close relationship - the *Heteronchocleidus*, *Trianchoratus* & *Pseudodactylogyrus* should belong to the Ancyrocephalidae
A molecular phylogeny of the Dactylogyridae *sensu* Kritsky & Boeger (1989) (Monogenea) based on the D1-D3 domains of large subunit rDNA (Simkova *et al.*, 2006b)	partial 28S rDNA	**Groups studied: Ancyrocephalinae:** 1 *Pseudohaliotrema* sp., 1 *Tetrancistrum* sp., 3 *Haliotrema* spp., 1 *Euryhaliotrema* sp., 7 *Euryhaliotrematoides* spp., 1 *Aliatrema* sp., 1 *Urocleidus* sp., 1 *Cleidodiscus* sp., 1 *Actinocleidus* sp., 1 *Ancyrocephalus* sp.; **Pseudodactylogyrinae:** 2 *Pseudodactylogyrus* spp.; **Dactylogyrinae:** 9 *Dactylogyrus* spp.; **Pseudomurraytrematidae:** 1 *Pseudomurraytrema* sp. **Other species used: Tetraonchinea:** 1 *Anoplodiscus* sp., 1 *Tetraonchus* sp. **Outgroup: Monocotylidea:** 1 *Dendromonocotyle* sp., 1 *Clemacotyle* sp., 1 *Decacotyle* sp., 1 *Troglocephalus* sp., 1 *Dictyocotyle* sp., 1 *Calicotyle* sp., 1 *Merizocotyle* sp., 1 *Empruthotrema* sp.	- Dactylogyridae *sensu* Kritsky & Boeger (1989) is monophyletic - Ancyrocephalidae & Ancyrocephalinae are polyphyletic - Freshwater species of Ancyrocephalinae & Ancylodiscoidinae were positioned at the base of Dactylogyridae - Dactylogyrinae formed a monophyletic group, sister to a clade including the Pseudodactylogyrinae and the tropical and subtropical Ancyrocephalinae

The radiation of *Haliotrema* (Monogenea: Dactylogyridae: Ancyrocephalinae): molecular evidence and explanation inferred from LSU rDNA sequences (Wu *et al.*, 2006)	partial 28S rDNA	**Groups studied:** 9 *Haliotrema* spp. **Other species used: Dactylogyridae:** 1 *Euryhaliotrema* sp., 1 *Ligophorus* sp., 2 *Metahaliotrema* spp., 1 *Scutogyrus* sp., 1 *Bravohollisia* sp., 1 *Cichlidogyrus* sp., 1 *Ancyrocephalus* sp., 4 *Protogyrodactylus* spp., 1 *Pseudodactylogyrus* sp.; **Ancylodiscoididae:** 4 *Thaparocleidus* spp. **Outgroup: Diplectanidae:** 1 *Murraytrema* sp., 1 *Sinodiplectanotrema* sp., 2 *Pseudorhabdosynochus* spp., 2 *Diplectanum* spp.	- *Haliotrema* is non-monophyly where 9 *Haliotrema* spp. were dispersed to form 4 clades with species from other genera -3 major groups were defined to explain the radiation of *Haliotrema* spp. - propose to transfer *H. spirotubiforum* & *Haliotrema* sp. ZHDDb to *Euryhaliotrema* as new combination based on molecular results & morphology of male copulatory organ (MCO) - propose to erect a new genus to accommodate the *Haliotrema* spp. with horn-liked shaped MCO.
The radiation of *Thaparocleidus* (Monogenoidea: Dactylogyridae: Ancylodiscoidinae): phylogenetic analyses and taxonomic implications inferred from ribosomal DNA sequences (Wu *et al.*, 2008)	partial 28S rDNA	**Groups studied:** 14 *Thaparocleidus* spp. **Other species used: Ancylodiscoididae:** 1 *Bychowskyella* sp., 3 *Pseudancylodiscoides* spp., 1 *Quadriacanthus* sp. **Outgroup: Diplectanidae**: 2 *Diplectanum* spp., 2 *Pseudorhabdosynochus* spp.	- *Thaparocleidus* is not a monophyletic group - 3 clades can be observed for *Thaparocleidus* spp. from *Silurus astus*, which is consistent with results of previous morphological analyses - *Pseudancylodiscoides* spp. were more closely related to *Thaparocleidus* spp. from *S. astus* - propose to erect a new genus to accommodate *Thaparocleidus* from *S. astus* and *Pseudancylodiscoides*

The evaluation for generic-level monophyly of Ancyrocephalinae (Monogenea, Dactylogyridae) using ribosomal DNA sequence data (Wu *et al.*, 2007a)	partial 28S rDNA, partial 18S rDNA, ITS1	**Groups studied: Ancyrocephalinae:** 1 *Aliatrema* sp., 5 *Bravohollisia* spp., 1 *Caballeria* sp., 19 *Cichlidogyrus* spp., 2 *Euryhaliotrema* spp., 8 *Euryhaliotrematoides* spp., 18 *Haliotrema* spp., 2 *Ligophorus* spp., 2 *Metahaliotrema* spp., 4 *Protogyrodactylus* spp., 4 *Scutogyrus* spp. **Outgroup: Ancylodiscoidinae:** 6 *Thaparocleidus* spp.	- 18 *Haliotrema* spp. were highly dispersive to form several clades - based on molecular evidence & MCO characters, it is proposed to transfer *H. kurodai*, *H. spirotubiforum*, *H. anguiformis* to the *Aliatrema* as new combinations and to combine *Bravohollisia* & *Caballeria* into one genus - *Scutogyrus* is polyphyletic and its status should be questioned - the vagina characters make little contribution for understanding the generic-level monophyly but useful for species determination - since phylogenetically closely related species from the same or closely related host species may have similar MCO characters but distinct haptoral characters, it is dangerous to erect a genus mainly based on different haptoral characters
A preliminary phylogenetic analysis of the Diplectanidae inferred from the C1-D2 domains of 28S rDNA sequences (Wu *et al.*, 2007b)	partial 28S rDNA	**Groups studied: Diplectanidae:** 1 *Acleotrema* sp., 3 *Calydiscoides* spp., 7 *Diplectanum* spp., 2 *Lamellodiscus* spp., 1 *Lepidotrema* sp., 1 *Lobotrema* sp., 5 *Pseudorhabdosynochus* spp. **Other species used:** 1 *Euryhaliotrema* sp., 2 *Haliotrema* spp., 2 *Sinodiplectanotrema* spp., 1 *Pseudomurraytrema* sp. **Outgroup: Monocotylidae:** 1 *Calicotyle* sp., 1 *Clemacotyle* sp., 1 *Decacotyle* sp., 1 *Dendromonocotyle* sp., 1 *Dictyocotyle* sp.	- *Sinodiplectanotrema* should be transferred from the Ancyrocephalidae to Diplectanidae as new combination of the subfamily Murraytrematoidinae - Murraytrematoidinae is not monophyletic and should be abolished, *Lobotrema* and *Murraytrema* from the Murraytrematoidinae should be transferred to Diplectaninae - the monophyly of the new combined subfamily Diplectaninae *sensu* Domingues,

			2004 could not be confirmed but monophyly of Lamellodiscinae was confirmed
Looks can deceive: Molecular phylogeny of a family of flatworm ectoparasites (Monogenea: Capsalidae) does not reflect current morphological classification (Perkins *et al.*, 2009)	partial 28S rDNA, Histone 3, Elongation Factor 1 ŭ	**Groups studied: Capsalidae:** 6 Benedenia spp., Dioncopseudobenedenia sp., Pseudonitzschia sp., Neobenedenia sp., Megalobenedenia sp., Encotyllabe sp., Interniloculus sp., Mediavagina sp., Allobenedenia sp., Capsala sp., Tristoma sp., Capsaloides sp., Nasicola sp., Nitzschia sp., Entobdella sp., Macrophyllida sp., Neoentobdella sp., Benedeniella sp., Listrocephalos sp. **Other species used:** Gyrodactylus sp., Udonella sp., Acanthocotyle sp., Asthenocotyle sp., Pseudoleptobothrium sp., Dermophthirius sp., Calicotyle sp., Dendromonocotyle sp., Microcotyloides sp.	- the Capsalidae was monophyletic, forming sister group with Gyrodactylidae and Udonellidae - the Capsalinae was monophyletic, but not for the Benedeniinae, Entobdellinae and Trochopodinae - Monophyly was supported for *Capsala*, *Entobdella*, *Listrocephalos*, *Neobenedenia* and *Tristoma*, but *Benedenia* and *Neoentobdella* were polyphyletic
Molecular phylogeny of monogeneans parasitizing African freshwater Cichlidae inferred from LSU rDNA sequences (Mendlova *et al.*, 2010)	partial 28S rDNA	**Groups studied:** 13 *Cichlidogyrus* spp., 3 *Enterogyrus* spp., 2 *Onchobdella* spp. & 1 *Scutogyrus* sp. **Other species used: Ancyrocephalidae:** 1 *Actinocleidus* sp., 1 *Ancyrocephalus* sp., 2 *Bravohollisia* spp., 1 *Cleidodiscus* sp., 2 *Euryhaliotrematoides* spp., 1 *Haliotrema* sp., 1 *Ligophorus* sp., 1 *Urocleidus* sp.; **Ancylodiscoididae:** 3 *Thaparocleidus* spp.; **Pseudodactylogyridae:** 2 *Pseudodactylogyrus* spp.; **Protogyrodactylogyridae:** 2 *Protogyrodactylus* spp.; **Dactylogyridae:** 4 *Dactylogyrus* spp. **Outgroup: Tetraonchinea:** 1 *Tetraonchus* sp.; 1 *Anoplodiscus* sp.	- Both *Enterogyrus* and *Onchobdella* were found to be monophyletic - The phylogenetic position of *Scutogyrus longicornis* was placed within the *Cichlidogyrus* species, suggesting the non-monophyly of *Cichlidogyrus* & therefore, taxonomical revision of the species recently considered to be *Scutogyrus* was proposed - *Cichlidogyrus*, *Enterogyrus*, *Onchobdella* & *Scutogyrus* do not form a monophyletic group, *Enterogyrus* and *Onchobdella* form a clade with *Protogyrodactylus*, i.e., the parasite species does not live in cichlids, which suggests that endoparasitism in cichlid monogeneans is not

			an ancestral feature
Relationships of the Heteronchocleidids (*Heteronchocleidus*, *Eutrianchoratus* & *Trianchoratus*) as inferred from ribosomal DNA nucleotide sequence data (Tan *et al.*, 2011)	partial 28S rDNA	**Groups studied:** 10 *Trianchoratus* spp., 2 *Eutrianchoratus* spp. & 1 *Heteronchocleidus* sp. **Other species used: Dactylogyridae:** 5 *Dactylogyrus* spp., 1 *Dactylogyroides* sp.; **Pseudodactylogyridae:** 4 *Pseudodactylogyrus* spp.; **Ancyrocephalidae:** 1 *Ancyrocephalus* sp.; **Ancylodiscoididae:** 4 *Thaparocleidus* spp., 1 *Quadriacanthus* sp., 1 *Bychowskyella* sp., 1 *Cornudiscoides* sp. **Outgroups: Diplectanidae:** 3 *Diplectanum* spp.; **Gyrodactylidae:** 3 *Gyrodactylus* spp.	- members of *Heteronchocleidus*, *Eutrianchoratus* & *Trianchoratus* form a monophyletic clade and Heteronchocleidinae is raised to family status as Heteronchocleididae - there are 2 lineages, the *Heteronchocleidus*-*Eutrianchoratus* clade with retention of bars & *Trianchoratus* clade with no bar - the ancestral heteronchocleidids could be present on both the ancestral forms of their fish hosts, anabantoids and channids, and subsequent speciation and extinction of some of the heteronchocleidids on different hosts gave rise to the present-day distribution patterns of the heteronchocleidids.

Table 1.2 Different types and number of DNA sequences from monogeneans available in the GenBank

Type of DNA sequences available in Genbank	**Number of sequences available**	**Number of monogenean species from which sequences are obtained**
Partial 28S rDNA	558	415
Partial 18S rDNA	334	241
Internal Transcribed Spacer (ITS) regions (ITS1 and /or ITS2)	439	125
Cytochrome Oxidase I (COI)	620	79
Cytochrome *b*	135	8
Others (different combination of partial 18S, ITS1, ITS2 & 28S rDNA, complete mitochondrial genome, histone 3 gene, elongation factor 1 alpha gene, Hox4 gene, NADH dehydrogenase subunit 4 gene, etc.)	1150	279

1.4 Dactylogyridea Bychowsky, 1937

Prior to identifying the research questions concerning the issues to be investigated and hypotheses to be tested (Section 1.5), a short account of the current taxonomic status of the focus group of this study, the order Dactylogyridea Bychowsky, 1937 (Subclass Polyonchoinea) will be given below. The order Dactylogyridea is chosen as the focus of this study because this order is probably the most problematic since it is the most diverse order in Polyonchoinea and also harbours most of the smaller and smallest monogenean species. The order Dactylogyridea is also the most investigated monogenean groups especially in Malaysia.

Taxonomic status of Dactylogyridea Bychowsky, 1937

Dactylogyridea is the most diverse order within Polyonchoinea Bychowsky, 1937 (Bychowsky, 1957). The status and relationships of the different families and subfamilies within the Dactylogyridea are still in constant debate (see Kritsky & Boeger, 1989; Lim, pers. com.; Lim *et al.*, 2001). The number of families and subfamilies and their status and generic compositions within the Dactylogyridea vary according to researchers (e.g. Yamaguti, 1963; Lebedev, 1988; Bychowsky & Nagibina, 1978; Gusev, 1978, 1985; Kritsky & Boeger, 1989; Boeger & Kritsky, 1993; Lim, 1998; Lim *et al.*, 2001). The family compositions in Dactylogyridea according to different authors are summarised in Table 1.3.

Table 1.3 Classification of different families/subfamilies of Dactylogyridea Bychowsky, 1937 according to different authors.

Superfamily Dactylogyroidea Yamaguti, 1963 **(*sensu* Yamaguti, 1963)**

- Family Protogyrodactylidae Johnston & Tiegs, 1922
- Family Calceostomatidae Parona & Perugia, 1890
- Family Diplectanidae Bychowsky, 1957
- Family Bothitrematidae Bychowsky, 1957
- Family Dactylogyridae Bychowsky, 1933
  - Subfamily Dactylogyrinae Bychowsky, 1933
  - Subfamily Ancyrocephalinae Bychowsky, 1937
  - Subfamily Geneticoenterinae Yamaguti, 1963
  - Subfamily Linguadactylinae Bychowsky, 1957

Superfamily Tetraoncoidea Yamaguti, 1963

- Family Tetraoncidae Bychowsky, 1957
- Family Tetraoncoididae Bychowsky, 1951

Superorder Dactylogyria Lebedev, 1988 **(*sensu* Lebedev, 1988)**

- Order Dactylogyridea Bychowsky, 1937
  - Suborder Dactylogyrinea Bychowsky, 1957
    - Family Dactylogyridae Bychowsky, 1933
    - Family Diplectanidae Bychowsky, 1957
    - Family Ancyrocephalidae Bychowsky, 1937
    - Family Neodactylodiscidae Kamegai, 1972
  - Suborder Calceostomatinea Gusev, 1977
    - Family Calceostomatidae Parona & Perugia, 1890
- Order Tetraonchidea Bychowsky, 1957
  - Family Tetraonchidae Bychowsky, 1937
  - Family Amphibdellatidae Carus, 1885
  - Family Tetraonchoididae Bychowsky, 1951
  - Family Bothitrematidae Bychowsky, 1957

Superoder Pedunculanchorea Malmberg, 1990 **(*sensu* Malmberg, 1990)**

Family Pseudodactylogyridae Gusev, 1965

Family Linguadactylidae Bychowsky, 1957

Family Ancyrocephalidae Bychowsky, 1937

Family Diplectanidae Bychowsky, 1957

Family Dactylogyridae Bychowsky, 1933

Family Amphibdellatidae Carus, 1885

Family Tetraonchidae Monticelli, 1903

Superorder Anchorea Malmberg, 1990

Family Ooegyrodactylidae Harris, 1983

Family Gyrodactylidae Cobbold, 1864

Family Tetraonchoididae Bychowsky, 1951

Family Bothitrematidae Bychowsky, 1957

Family Sundanonchidae Malmberg, 1990

Order Dactylogyridea Bychowsky, 1937 **(*sensu* Kritsky & Boeger, 1989; Boeger & Kritsky, 1993, 1997)**

Suborder Amphibdellatinea Boeger & Kritsky, 1993

Family Amphibdellatidae Carus, 1885

Suborder Tetraonchinea Bychowsky, 1937

Family Tetraonchidae Monticelli, 1903

Family Neotetraonchidae Bravo-Hollis, 1968

Suborder Dactylogyrinea Bychowsky, 1937

Family Dactylogyridae Bychowsky, 1933

Subfamily Dactylogyrinae Bychowsky, 1933

Subfamily Heterotesinae Euzet & Dossou, 1979

Subfamily Ancyrocephalinae Bychowsky & Nagibina, 1978

Subfamily Ancylodiscoidinae Gusev, 1961

Subfamily Pseudodactylogyrinae Ogawa, 1986

Subfamily Linguadactylinae Bychowsky, 1957

Subfamily Linguadactyloidinae Thatcher & Kritsky, 1983

Subfamily Hareocephalinae Young, 1968

Subfamily Anacanthorinae Price, 1967

Family Diplectanidae Monticelli, 1903

Family Pseudomurraytrematidae Kritsky, Mizelle & Bilqees, 1978

Suborder Neodactylodiscinea Boeger & Kritsky, 1993

Family Neodactylodiscidae Kamegai, 1972

Suborder Calceostomatinea Gusev, 1977

Family Calceostomatidae Parona & Perugia, 1890

Order Dactylogyridea Bychowsky, 1937 **(*sensu* Lim, 1998; Lim, Timofeeva & Gibson, 2001)**

Suborder Dactylogyrinea Bychowsky, 1937

Family Ancyrocephalidae Bychowsky, 1937

Subfamily Ancyrocephalinae Bychowsky, 1957

Subfamily Heteronchoclidinae Price, 1968

Family Ancylodiscoididae Gusev, 1961

Family Dactylogyridae Bychowsky, 1933

Family Diplectanidae Monticelli, 1903

Family Neocalceostomatidae Lim, 1995

Family Pseudodactylogyridae Le Brun, Lambert & Justine, 1986

Suborder Tetraonchoinea Bychowsky, 1937

Family Sundanonchidae Malmberg, 1990

In 1957, Bychowsky included three families, Calceostomatidae, Diplectanidae and Dactylogyridae in suborder Dactylogyrinea Bychowsky, 1937 within the order Dactylogyridea Bychowsky, 1937, with three subfamilies (Linguadactylinae Bychowsky, 1957, Dactylogyrinae and Ancyrocephalinae Bychowsky, 1937) within the Dactylogyridae. Later, Ancylodiscoidinae Gusev, 1961, Heteronchocleidinae Price, 1968 and Anacanthorinae Price, 1967 were included in Dactylogyridae by Gusev (1961) and Price (1968), respectively. In 1978, Bychowsky and Nagibina removed Ancyrocephalinae from Dactylogyridae and raised it to full family status and re-assigned Ancylodiscoidinae, Linguadactylinae Bychowsky, 1957 and Hareocephalinae Young, 1968 which were originally grouped in the Dactylogyridae as subfamilies within Ancyrocephalidae. This move was also supported by Gusev (1978).

In 1978, Kritsky, Mizelle & Bilqees assigned the subfamily Pseudomurraytrematinae into Dactylogyridae, which was later raised to family status by Beverley-Burton (1984). Thatcher & Kritsky (1983) erected Linguadactyloidinae Thatcher & Kritsky, 1983 as a subfamily within the Dactylogyridae to accommodate the new genus *Linguadactyloides*. Heterotesiidae Euzet & Dossou, 1979 was included in Dactylogyrinea by Euzet & Dossou (1979). Ogawa (1986) proposed Pseudodactylogyrinae with *Pseudodactylogyrus* Gusev, 1965 as type-genus in Dactylogyridae, which was raised to family Pseudodactylogyridae by Le Brun *et al.* (1986). By 1989, there are seven families in Dactylogyridea (i.e. Calceostomatidae, Diplectanidae, Dactylogyridae, Ancyrocephalidae, Pseudomurraytrematidae, Heterotesiidae and Pseudodactylogyridae).

However, in 1989 Kritsky & Boeger proposed two options to resolve the paraphyly of Ancyrocephalidae based on cladistic analysis of morphological characters. One of their proposed options was to raise all the subfamilies of Ancyrocephalidae, viz., Linguadactyloidinae, Linguadactylinae, Hareocephalinae, Ancylodiscoidinae, Anacanthorinae (see revision by Bychowsky & Nagibina, 1978) to family status. However they opted for the option to reduce Ancyrocephalidae, Heterotesiidae and Pseudodactylogyridae to subfamily status and to include them (Ancyrocephalinae, Heterostesiidinae and Pseudodactylogyrinae) and 5 other subfamilies formerly listed within them (Linguadactylinae, Linguadactyloidinae, Hareocephalinae, Ancylodiscoidinae and Anacanthorinae) into Dactylogyridae including of course Dactylogyrinae. This option of theirs essentially revert the status of these monogeneans back to their status prior to the revision by Bychowsky & Nagibina in 1978 (see Kritsky & Boeger, 1989; Table 1.1). Therefore by 1989, the Dactylogyridea has four families viz. Dactylogyridae (with 9 subfamilies) (*sensu* Kritsky & Boeger, 1989), Calceostomatidae, Pseudomurraytrematidae and Diplectanidae (with 4 subfamilies) (*sensu* Oliver, 1987). The proposal of Kritsky & Boeger (1989) (see also Boeger & Kritsky, 1993, 1997) found support in Ġimková *et al.* (2003, 2006b) and Wu *et al.* (2007a).

Lim (1998, pers. com.) and Lim *et al.* (2001) on the other hand, vehemently disagree with the inclusion of members of the Ancyrocephalidae into Dactylogyridae by Kritsky & Boeger (1989) and suggested Dactylogyridae and Ancyrocephalidae be left intact within the Dactylogyridea. Lim *et al.* (2001) suggested that more óweightô should be given to the presence of four anchors and absence of ñneedle-like structureò in the members of the Ancyrocephalidae. Furthermore, Dactylogyridae (*sensu* Bychowsky & Nagibina, 1978) is typified by *Dactylogyrus* Diesing, 1850 with two anchors and two

unique ñneedle-like structureò and should be exclusive to monogenean species with two anchors, two ñneedle-likeò structures, 14 marginal hooks and one or two bars such as *Dactylogyrus*, *Dogielus* Bychowsky, 1936, *Dactylogyroides* Gusev, 1963, and *Thaprogyrus* Gusev, 1976 (Lim, pers. com.; unpublished data).

In 1995 the family Neocalceostomatidae Lim, 1995 was included in Dactylogyridea, and in 2001, Lim, Timoofeeva & Gibson, raised Ancylodiscoidinae to family status within Dactylogyidea. By 2001 therefore the Dactylogyridea accepted by Lim (pers. com.) is as listed in Table 1.1 which is essentially that of Bychowsky & Nagibina (1978) with extra families of Ancylodiscoididae and Neocalceostomatidae Lim, 1995. As a result of this present study (see Appendix B) Heteronchocleidinae has been raised to family status within the Dactylogyridea. The status of the Dactylogyridae and Ancyrocephalidae is thus an issue in the study of monogeneans.

1.5 Objectives and scope of study

1.5.1 Objectives

The overall idea of this thesis is to examine the information obtainable from morphological and molecular characteristics of monogeneans in order to understand the morphological and molecular diversities at different levels of organismatic organisation from population to species. In order to do this it is necessary to obtain morphometric data from individuals from different groups of organisms and molecular data from different species and individuals.

In this study, morphometric data from two types of species populations, different congeneric species from a group of related host species (*Trianchoratus* Price & Berry, 1966 from channid species) and a group of co-existing monogenean species (*Caballeria* Bychowsky & Nagibina, 1970 and *Bravohollisia* Bychowsky & Nagibina, 1970) from *Pomadasys hasta* (Bloch) are collected and analysed in order to answer the aforementioned questions about whether the use of morphologies are reliable species diagnostic characters and how to distinguish intraspecies variations or interspecies differences (see Chapter 2; Materials & Methods).

As already noted molecular characterisations have been used notably for determining relationships at higher taxonomic levels and the use of molecular data for species differentiations are limited mainly because such studies will warrant molecular data from different individuals of the same species. In this study, molecular data are used to examine relationships of the different monogenean species within the Dactylogyridea Bychowsky, 1957 (see Section 1.4) and an attempt to use molecular data to distinguish between two closely related species (see Chapter 6; Appendix E).

1. In this study the following questions are examined: Amount of morphometric variations within and between different species and the factors influencing these variations. The existences of variations are noted in the wide ranges of measurements observed in taxonomic descriptions (see Section 1.3.1).

    (a) Can these variations be objectively differentiated statistically? What do they indicate? How much variations exist between species and within species? In other words, how much differences must exist before morphovariants can be considered as different species?

(b) Do intraspecific morphovariants have a specific distribution pattern? What are the possible factors influencing the occurrences and number of intraspecific variants? Do intraspecific morphovariants have a genetic basis?

2. Currently most studies on the relationships of dactylogyrideans based on molecular data have limited number of DNA sequences (in the Genbank there are currently approximately 558 partial 28S rDNA sequences belonging to various species of monogeneans; see Table 1.2). This study attempts to use as many DNA sequences as possible. Previously the main limiting factor was the computational time taken to build and generate the relationships trees. We were able to purchase and use the Linux version of PAUP*4.0b10 (Swofford, 2002) in HPC (high performance computer) which have shortened the computational time considerably (see Chapter 2; Materials & Methods). In this section the following questions are examined:

   (a) How the different members of the dactylogyrideans will be grouped based on molecular data (28S rDNA)? What are the relationships of dactylogyrideans based on molecular data? Can relationships tree generated based on molecular data explain the evolutionary diversification of morphological characteristics among dactylogyrideans?

   (b) Are there any correlation between the dactylogyrideans and their fish hosts based on molecular information? If correlations exist what is the basis for the correlations? Will such data reveal how monogeneans are acquired ï through inheritance or host transfer?

1.5.2 Scope and delimitation of study

This study is delimited to the Dactylogyridea (Section 1.4). As already noted this Order of monogenean is probably the largest group comprising of monogeneans with 2 to 4 anchors, 14-16 marginal hooks and 0-2 bars (which if present can be single bar or separate ósegmentedô bar) (Lim, pers. com.). This delimitation is partly due to the need to optimize the techniques for molecular biology and also time spent is needed for the collection of fresh materials, morphometric data and molecular data (see Chapter 2; Materials & Methods). This study has been limited by the number of DNA sequences to 28S rDNA mainly because the need to use external sequences from the Genbank meaning that only the most available sequences from the Genbank for monogenean, which is the 28S rDNA will be used.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter provides the methodology used to accomplish the objectives of this study which is primarily to investigate the morphometric and molecular characterisations of monogenean species. The sites and methods of fish collection, the methods used for the collection and preparation of monogeneans for morphological and molecular studies as well as the methods used to analyse morphological and molecular data are given in this chapter.

2.2 Collection of fish hosts

Fish hosts were collected from different localities in Peninsular Malaysia from freshwater and marine habitats such as riverine system, freshwater lake/reservoir, inshore coastal area, brackish mangrove area, offshore islands and aquaculture farm (Table 2.1).

2.2.1 Fish collection sites

a) Freshwater systems

i) Selai River (Endau-Rompin) (2^{o} 21' N; 103^{o} 18' E)

Endau-Rompin is a national forest reserve (national park) in the state of Johor, Peninsular Malaysia which contains one of the world oldest tropical rainforests. The Selai river region is located at the southwestern to south-central portion of Johorôs Endau-Rompin National Park. Fish specimens were caught from the Selai river using hook and lines, electro-fishing methods and cast net.

ii) Sangga Besar River (Kuala Sepetang) (4^o 85' N; 100^o 55' E)

Kuala Sepetang, a fishing village in the state of Perak, is located within the Matang Mangrove Forest Reserve area. The forested area of Matang mangrove consists of six major islands, Pulau Kalumpang, Pulau Selinsing, Pulau Sangga Kecil, Pulau Sangga Besar, Pulau Terong and Pulau Pasir Hitam. The major rivers draining these areas are Selinsing river, Sangga Besar river, Sangga Kecil river, Larut river, Terong river and Jarum Mas river. Fish specimens were collected from the brackish water of Sangga Besar river using trawl net.

iii) Bukit Merah Reservoir (5^o 1' N; 100^o 39' E)

Bukit Merah is originally a 7,000 acre freshwater lake where a reservoir is being built. It is located at the north-west of Peninsular Malaysia in the state of Kedah. Fish specimens were caught from the reservoir lake using hook and line, gillnet and various traps.

iv) Freshwater aquaculture farm in Sungai Bakau (3^o 19' N; 101^o 32' E)

Fish specimens were bought from a freshwater fish farm in Sungai Bakau, Rawang which are one of the freshwater aquaculture farms located at the outskirts of Kuala Lumpur. Freshwater fishes are cultured in small ponds measuring about 3-4 acres and also big ponds measuring up to 18 acres which are disused mining pools. There are approximately 100 ponds in this farm but not all are utilised.

b) Marine systems

i) Off Langkawi Island (6^o 28' N; 99^o 47' E)

Langkawi Island is situated in the Andaman Sea, some 30 km off the mainland coast of northwestern Peninsular Malaysia. Fish hosts were collected from the brackish mangrove area in the riverine system around the island as well as from the offshore sea. Gillnets were used in catching the fish specimens.

ii) Off Carey Island (2^o 47' N; 101^o 24' E)

Carey Island is an island in Selangor, Peninsular Malaysia. Carey Island is located to the south of Port Klang and north of Banting town. It is a huge island separated from the Selangor coast by the Langat River, connected by a bridge from Chondoi and Teluk Panglima Garang near Banting. Fish specimens were caught using gillnet from the brackish mangrove area, along the Langat river until the river mouth which connect to the Straits of Malacca.

iii) Marine aquaculture farm off Pulau Ketam (6^o 24' N; 100^o 7' E)

Fish specimens were obtained from a commercial cage culture farm off Pulau Ketam, which is located off the southwestern coast of Malaysia in the Straits of Malacca. Floating cages are widely used in coastal areas around Pulau Ketam since these sites are protected from strong winds, rough weather and have sufficient water movements with appropriate water quality.

Table 2.1 Fish host species collected from different localities of freshwater and marine habitats in Peninsular Malaysia

Locality	Fish host family	Fish species
Endau-Rompin	Anabantidae	*Anabas testudineus*
	Osphronemidae	*Betta* sp.
		Trichogaster trichopterus
	Bagridae	*Hemibagrus nemurus*
		Mystus nigriceps
		Mystus sp.
	Balitoridae	*Homaloptera orthogoniata*
		Vaillantella sp.
	Channidae	*Channa gachua*
		Channa lucius
	Clariidae	*Clarias teijsmanni*
		Clarias batrachus
		Clarias sp.
	Cyprinidae	*Cyclocheilichtys apogon*
		Hampala macrolepidota
		Labiobarbus sp.
		Luciosoma setigerum
		Mystacoleucus marginatus
		Osteochilus hasseltii
		Osteochilus microcephalus
		Osteochilus wandersii
		Osteochilus sp.
		Parachela oxygastroides
		Poropuntius deauratus
		Puntius binotatus
		Puntius gonionotus
		Puntius lateristriga
		Puntius sp.
		Rasbora elagans
		Rasbora marginatus
		Rasbora sp.
		Tor sp.
		Parachela oxygastroides
		Mystacoleucus marginatus
	Hemiramphidae	*Hemirhamphodon pogonognathus*
		Hemirhamphodon sp.
	Mastacembelidae	*Mastacembelus* sp.
	Notopteridae	*Notopterus notopterus*
	Pristolepidae	*Pristolepis fasciatus*
	Siluridae	*Silurichthys hasselti*
		Silurichthys sp.
Kuala Sepetang	Plotosidae	*Plotosus canius*
	Ariidae	*Arius maculatus*
		Arius caelatus
		Arius sagor
Bukit Merah	Channidae	*Channa lucius*
		Channa striata
		Channa micropeltis
	Helostomatidae	*Helostoma temminckii*
	Osphronemidae	*Trichogaster leeri*
		Belontia hasseltii
	Anabantidae	*Anabas testudineus*
	Eleotridae	*Oxyeleotris marmoratae*

	Notopteridae	*Notopterus notopterus*
	Bagridae	*Mystus nigriceps*
Sungai Bakau	Cyprinidae	*Hypophthalmichthys nobilis*
		Ctenopharyngodon idella
Langkawi Island	Carangidae	*Alepes melanoptera*
		Carangoides armatus
		Carangoides praeustus
		Scomberoides commersonnianus
		Scomberoides tol
		Carangoides sp.
	Triacanthidae	*Triacanthus biaculeatus*
	Mugilidae	*Liza vaigiensis*
		Valamugil seheli
	Gerreidae	*Gerres filamentosus*
		Gerres abbreviatus
	Scombridae	*Rastrelliger kanagurta*
	Engraulididae	*Stolephorus* sp.
	Mullidae	*Upeneus sulphureus*
	Leiognathidae	*Leiognathus brevirostris*
		Leiognathus equulus
		Secutor sp.
	Clupeidae	*Anodontostoma chacunda*
	Pristigasteridae	*Ilisha megaloptera*
	Pomadasyidae	*Pomadasys hasta*
	Pomacentridae	*Abudefduf vaigiensis*
	Siganidae	*Siganus canaliculatus*
		Siganus javus
	Sciaenidae	*Johnius carutta*
		Gelama sp.
		Dendrophysa russelli
		Pennahia anea
	Lutjanidae	*Lutjanus johnii*
		Lutjanus russeli
		Lutjanus vita
	Toxotidae	*Toxotes jaculator*
	Scatophagidae	*Scatophagus argus*
	Serranidae	*Cephalopholis boenak*
	Hemiramphidae	*Hemiramphus far*
	Dasyatidae	*Dasyatis* sp.
	Centropomidae	*Lates calcarifer*
	Ephippidae	*Ephippus orbis*
	Tetraodontidae	*Tetraodon nigroviridis*
		Lagocephalus spadiceus
	Nemipteridae	*Nemipterus* sp.
	Ariidae	*Arius venosus*
		Arius sagor
		Arius maculatus
		Arius caelatus
		Osteogeneiosus militaris
	Megalopidae	*Megalops cyprinoides*
	Sphyranenidae	*Sphyraena jello*
	Drepanidae	*Drepane punctate*
	Platycephalidae	*Platycephalus indicus*
	Sparidae	*Acanthopagrus berda*
		Strongylura strongyloides
	Ambassidae	*Ambassis gymnocephalus*
	Polynemidae	*Eleutheronema tetradactylum*

	Belonidae	*Tylosurus crocodilus*
	Bothidae	*Pseudorhombus natalensis*
Carey Island	Mugilidae	*Liza subviridis*
	Polynemidae	*Eleuteronema tetradactylum*
	Sciaenidae	*Johnius amblycephalus*
		Pennahia argentata
		Protonibea diacanthus
		Johnius vogleri
		Panna macrodon
		Pennahia aneas
	Terapontidae	*Terapon jarbua*
		Terapon theraps
	Echeneidae	*Echeneis naucrates*
	Tetraodonthidae	*Takifugu oblongus*
		Tetraodon nigroviridis
	Gerreidae	*Gerres filamentosus*
	Drepanidae	*Drepane punctata*
	Scatophagidae	*Scatophagus argus*
	Ambassidae	*Ambassis vachelli*
	Carangidae	*Scomberoides sp.*
Pulau Ketam	Haemulidae	*Pomadasys hasta*

2.3 Collections of monogeneans

The fish specimens were killed by severing the spinal cord with a sharp needle either at the collection site or after being transferred back to the laboratory. The gills were removed and placed in petri dishes containing clean local water. The freshly excised gills were gently scraped with a bent needle to dislodge the monogeneans. The dislodge monogeneans were collected under a dissecting microscope using a small fine pipette.

2.3.1 Preparation of monogeneans for morphological characterisation

For morphological study, the monogeneans specimens were prepared onto glass slides for investigations of their soft and hard parts. The monogeneans were dropped onto a clean glass slide with a small drop of water. A cover slip was gently dropped onto the monogenean specimen. Excess water on the slide was dried off. Some specimens were flattened and mounted in modified ammonium picrate glycerine (Lim, 1991) and later made into unstained permanent mounts in Canada balsam while some other specimens were flattened to varying degrees to best expose the soft anatomical structures and stained in Gomoriôs triple stain, following Lim (2006). There were also some specimens treated with SDS (Sodium Dodecyl Sulphate) to expose the hard anatomical structure of the reproductive organs and sclerotised structures of the haptor following Wong, Tan & Lim (2006) (Appendix F) to assist in the identification of the monogeneans. The collected monogeneans were identified mainly on the basis of the shapes and sizes of the hard sclerotised parts of the haptoral armaments and reproductive organs. The new species found in this study were also described (see also Chapter 3) (Tan & Lim, 2009; Appendix D).

2.3.2 Preparation of monogeneans for molecular characterisation

For molecular study, the collected monogenean specimens were preserved in 75% ethanol in micro-centrifuge tubes. Monogeneans were then removed from ethanol, transferred individually using fine pipette onto glass slide with a drop of distilled water and covered with a cover slip and examined and identified under a light microscope equipped with phase contrast and Leica image analysis software (Qwin Plus).

Protocol for DNA extraction from monogeneans

The identified monogenean specimens were removed from the glass slide and put individually in separate 0.5 ml Eppendorf tube and DNA extracted using DNEasy extraction kit from Qiagen. Five µl of lysate from the DNA extraction was used as template in PCR reactions to amplify the partial D1-D2 domain of the 28S rDNA, using either pair of primers, C1 (5ô-ACCCGCTGAATTTAAGCAT-3ô) and C2 (5ô-CTCTCTYTYCAAAGTTCTTTTC-3ô) (Justine *et al.*, 2002). PCR reactions (50 µl) were performed in 1.5 mM $MgCl_2$, PCR buffer (Fermentas), 200 µM of each deoxyribonucleotide triphosphate, 1.0 µM of each PCR primer, and 1U of *Taq* polymerase (Fermentas) in a thermocycler (Biometra) using the following conditions: an initial denaturation at 95ºC for 4 min, followed by 35 cycles of 95ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min, followed by a final extension at 72ºC for 10 min. Ten µl of aliquots from the amplicons were examined in 1.3% agarose gels, stained with ethidium bromide and view under a UV illuminator.

2.4 Data collection

Two types of data, i.e. morphological and molecular data were collected in this study to answer questions and achieve the objectives as outline in Section 1.5 (Chapter 1). Morphological data in the form of morphometric measurements were taken from two types of species populations, different congeneric species from a group of related host species (*Trianchoratus* Price & Berry, 1966 from channid host species) and a group of co-existing monogenean species (*Caballeria* Bychowsky & Nagibina, 1970 and *Bravohollisia* Bychowsky & Nagibina, 1970) from *Pomadasys hasta* (Bloch) to answer the questions such as how much variations must occur especially before two different groups of morphologically similar organisms are considered to be different species, how much variations are present within a species population and possible factors causing these variations within the populations (see Section 1.5.1).

DNA sequences were collected from monogeneans of the order Dactylogyridea to infer the relationships of dactylogyridean monogeneans from different families, genera and species based on molecular data (see Section 1.5.1).

2.4.1 Morphometric data

The morphometrical parameters taken for the 3 different groups of monogeneans are shown in Figs. 2.1 & 2.2. They are inner root (IR), outer root (OR), inner length (IL), outer length (OL) and point (pt) for the anchors, i.e. 4 developed anchors in *Bravohollisia* and *Caballeria* (Fig. 2.1) and 3 developed anchors in *Trianhcoratus* (Fig. 2.2). Parameters for the bars (width and length), copulatory organ (initial length and total length) and marginal hook (length) are also taken for *Bravohollisia* and *Caballeria* only (Fig. 2.1).

Morphometric data from 5 *Bravohollisia* spp. and 3 *Caballeria* spp.

Morphometric measurements were obtained from five species of *Bravohollisia* viz. *B.rosetta* Lim, 1995, *B.reticulata* Lim, 1995, *B. gussevi* Lim, 1995, *B. Kritskyi* Lim, 1995, *Bravohollisia* n. sp. and three species of *Caballeria* viz. *C. liewi* Lim, 1995, *C. intermedius* Lim, 1995 and *C. pedunculata* Bychowsky & Nagibina, 1970 from *Pomadasys hasta* (Bloch). *Bravohollisia* n. sp. is a previously unknown *Bravohollisia* species which can be observed in this study to possess highly similar haptoral sclerotised parts (anchors, bars and hooks) with the existing *B. kritskyi* but differ in the morphology of copulatory organ. These specimens of *Bravohollisia* and *Caballeria* were collected during a previous study to survey the distribution pattern of *Bravohollisia* and *Caballeria* on *Pomadasys hasta* from Pulau Ketam and deposited in the parasite collection of Fish Parasite Laboratory, University of Malaya (see also Chuan, J., Unpublished master thesis).

In this study, the morphometric measurements were taken from the sclerotised hard parts of *Bravohollisia* and *Caballeria* viz. four well-developed anchors (two dorsal anchors and two ventral anchors), two bars (dorsal and ventral bar), marginal hook as well as the copulatory organ of each of the *Bravohollisia* and *Caballeria* specimens. Five parameters were taken: inner root (IR), outer root (OR), inner length (IL), outer length (OL) and point (pt), for each of the four well-developed anchors, two parameters were taken: length (BL) and width (BW) for each of the two bars, one parameter was taken: length (ML) for marginal hook and two parameters were taken: initial length (CIL) and total length (CTL) for copulatory organ (Fig. 2.1) using Leica image analysis software (QWin Plus) resulting in a total of 27 variables per monogenean (the numbers attached to the parameters denote the position of the anchors and bars, 1 and 2 for the two dorsal anchors and 3 and 4 for the two ventral anchor; 1 for dorsal bar and 2 for ventral

bar). A total of 744 specimens of *Bravohollisia* and 295 specimens of *Caballeria* were measured (Table 2.2).

Morphometric data from 4 species *Trianchoratus* Price & Berry, 1966

Morphometric measurements were obtained from four *Trianchoratus* species found on two species of channid hosts. *T. malayensis* Lim, 1986 and *T. pahangensis* Lim, 1986 are found on *Channa lucius* (Cuvier) and *T. ophicephali* Lim, 1986 is on *C. striata* (Bloch). In 2009, *Trianchoratus longianchoratus* Tan & Lim, 2009 was collected and described from *C. lucius* during the course of this study and this species is similar to the previous 3 *Trianchoratus* species from channid hosts but different from the *Trianchoratus* species of the anabantoid hosts in possessing very similar morphological characteristics, especially the three developed anchors and copulatory organs (see Tan & Lim, 2009; Appendix D).

These four *Trianchoratus* species were collected from three different localities viz. the riverine swamp-lake Tasik Bera, Endau-Rompin and Bukit Merah Reservoir (see Table 2.3). These *Trianchoratus* specimens include type and voucher specimens of the *Trianchoratus* species collected in the current study (from Endau-Rompin & Bukit Merah: 101 specimens of *T. malayensis*, 136 specimens of *T. pahangensis*, 250 specimens of *T. ophicephali* and 29 specimens of *T. longianchoratus*, as well as from the specimens deposited in the Parasite Collection at the Zoological Museum of University of Malaya (UMZD) (from Tasik Bera: *T. malayensis* (31 specimens), *T. pahangensis* (50 specimens) and *T. ophicephali* (79 specimens).

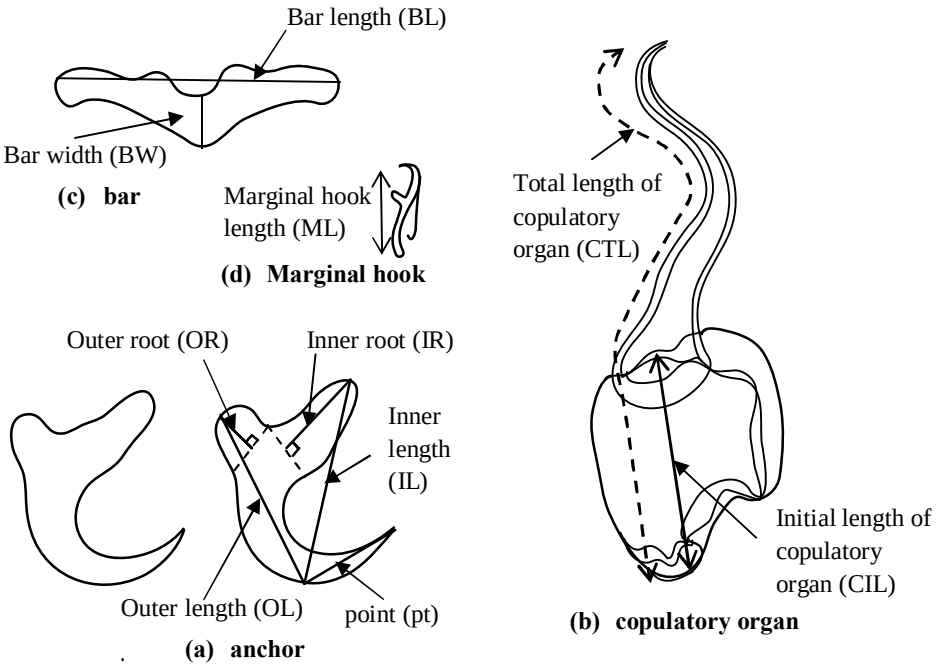


Figure 2.1 An example of parameters taken from (a) anchor, (b) copulatory organ, (c) bar and (d) marginal hook of *Bravohollisia* and *Caballeria* spp.

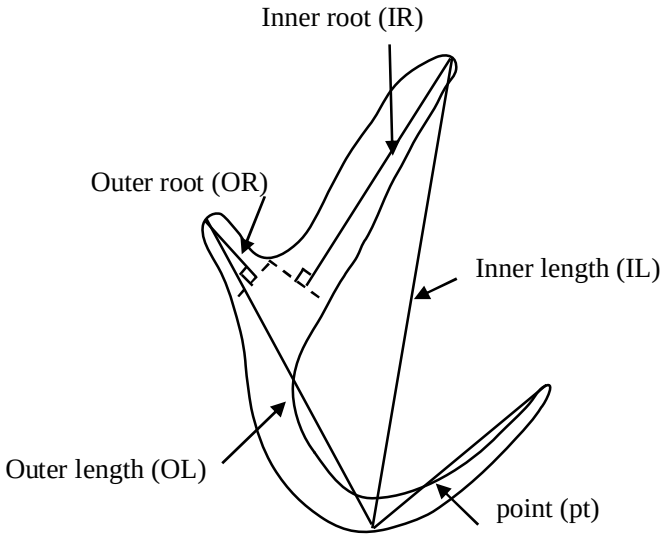


Figure 2.2 A well-developed anchor of *Trianchoratus* species showing the basic measurements taken for morphometric analysis.

Briefly, *Trianchoratus* species possess three well-developed anchors (two ventral anchors and one dorsal anchor), one comma-shaped vestigial dorsal anchor and 14 marginal hooks. Although the *Trianchoratus* species have morphologically similar copulatory organs which are different in terms of size they are subjected to distortions due to orientations and hence difficult to measure. They are therefore not included in the analysis. The vestigial anchor and marginal hooks are also not considered because they are either obscured by the larger well-developed anchors or are not disposed properly to be measured. In this study 5 parameters were taken: inner root (IR), outer root (OR), inner length (IL), outer length (OL) and point (pt), for each of the three well-developed anchors (Fig. 2.2) using Leica image analysis software (QWin Plus) resulting in a total of 15 variables per monogenean (the numbers attached to the parameters denote the position of the anchors, 1 and 2 for the two ventral anchors and 3 for the dorsal anchor). The measurements of the two ventral anchors are treated as separate datasets. A total of 448 specimens of *Trianchoratu*s specimens were measured (Table 2.3).

Table 2.2 Distribution patterns according to different host size (fish standard length: 40-100mm=small, 100-150mm=medium, 150-200mm=large) of the 744 individuals of *Bravohollisia* spp. and 295 individuals of *Caballeria* used in the morphometric analysis.

***Bravohollisia* and *Caballeria* spp.**	**No. of individuals used in this study according to host size**			
	Small host	**Medium host**	**Large host**	**Total**
B. rosetta	50	50	50	150
B. reticulata	80	50	50	180
B. gussevi	50	50	50	150
B. kritskyi	50	50	50	150
Bravohollisia n. sp.	14	50	50	114
C. liewi	50	50	50	150
C. intermedius	21	21	44	86
C. pedunculata	8	21	30	59
Grand total				**1039**

Table 2.3 Host and locality distribution patterns of the 448 individuals of *Trianchoratus* spp. used in the morphometric analysis.

***Trianchoratus* spp.**	**Host species**	**Locality**	**No. of individuals used in this study**
T. malayensis	*Channa lucius*	Bukit Merah	58
		Endau-Rompin	36
		Tasik Bera	25
Total			**119**
T. pahangensis	*Channa lucius*	Bukit Merah	113
		Endau-Rompin	4
		Tasik Bera	42
Total			**159**
T. longianchoratus	*Channa lucius*	Bukit Merah	25
T. ophicephali	*Channa striata*	Bukit Merah	95
		Tasik Bera	50
Total			**145**
Grand total			**448**

2.4.2 Molecular sequence data

Monogenean sequences from present study

The remaining 40 µl of each amplicon from PCR amplification (Section 2.3.2) was purified using DNA purification kit (Qiagen) and subjected to automated DNA sequencing (ABI 3730 DNA Sequencer, First Base Laboratories) using the same primers used for PCR amplification. The partial 28S rDNA of 62 monogenean species were sequenced (see Table 2.4).

Monogenean sequences (partial 28S rDNA) from GenBank

A survey was also done to identify partial 28S rDNA sequences of dactylogyridean monogenean species from GenBank which were related to this study. A total of 126 partial 28S rDNA sequences from monogenean of the Order Dactylogyridea which were comparable to the partial 28S rDNA sequences collected in this study (see section 2.6.1) were obtained from the Genbank (see Table 2.4).

Table 2.4 List of partial 28S rDNA sequences of dactylogyridean monogenean species used in this study with their host species, locality and GenBank accession numbers. (*sequences collected from present study) (**names used in original paper/GenBank).

Monogenean species	Host species	Locality	GenBank No.
Heteronchocleididae Price, 1968			
Trianchoratus malayensis	*Channa lucius*	Bukit Merah, Malaysia	HQ719218*
Trianchoratus pahangensis	*Channa lucius*	Bukit Merah, Malaysia	HQ719219*
Trianchoratus longianchoratus	*Channa lucius*	Bukit Merah, Malaysia	HQ719220*
Trianchoratus ophicephali	*Channa striata*	Bukit Merah, Malaysia	HQ719215*
Trianchoratus acleithrium	*Helostoma temminkii*	Peninsular Malaysia	HQ719214*
Trianchoratus leerium	*Trichopodus leerii (Trichogaster leerii**)*	Peninsular Malaysia	HQ719216*
Trianchoratus trichogasterium	*Trichopodus trichopterus (Trichogaster trichopterus**)*	Endau-Rompin, Malaysia	HQ719217*
Trianchoratus gussevi	*Anabas testudieus*	Bukit Merah, Malaysia	HQ719221*
Trianchoratus gussevi CHN	*Anabas testudieus*	Hainan, China	AY841875
Trianchoratus parvulus	*Anabas testudieus*	Bukit Merah, Malaysia	HQ719223*
Trianchoratus grandis	*Anabas testudieus*	Bukit Merah, Malaysia	HQ719222*
Eutrianchoratus inequalis	*Belontia hasselti*	Bukit Merah, Malaysia	HQ719225*
Eutrianchoratus cleithrium	*Belontia hasselti*	Bukit Merah, Malaysia	HQ719224*
Heteronchocleidus buschkieli	*Macropodus opercularis*	Guangdong, China	AY841876
Dactylogyridae Bychowsky, 1933			
Dactylogyrus apogonae	*Cyclocheilicththys apogon*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus aristichthys	*Hypophthalmichthys nobilis*	Rawang, Malaysia	Present study*
Dactylogyrus hampalai	*Hampala macrelipidota*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus sclerovaginalis	*Systomus binotatus* (*Puntius binotatus***)	Endau-Rompin, Malaysia	Present study*
Dactylogyrus cheligenitalis	*Osteochilus hasselti*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus elegani	*Rasbora elagans*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus quadribrachiatus	*Hampala macrelipidota*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus damansari	*Systomus binotatus* (*Puntius binotatus***)	Endau-Rompin, Malaysia	Present study*
Dactylogyrus laterstriga	*Systomus laterstriga* (*Puntius laterstriga***)	Endau-Rompin, Malaysia	Present study*
Dactylogyrus spirocopulatrium	*Systomus binotatus* (*Puntius binotatus***)	Endau-Rompin, Malaysia	Present study*
Dactylogyrus lamellatus	*Ctenopharyngodon idellus*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus sp. LAB	*Labiobarbus* sp.	Endau-Rompin, Malaysia	Present study*

Monogenean species	Host species	Locality	GenBank No.
Dactylogyrus inversus	*Lateolabrax japonicus*	Japan	Present study*
Dactylogyrus gotoi	*Lateolabrax japonicus*	Japan	Present study*
Dactylogyrus hemiramphodonus	*Hemiramphodon pogonognothus*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus temperasi	*Cyclocheilicththys apogon*	Endau-Rompin, Malaysia	Present study*
Dactylogyrus puntii	*Systomus gonionotus* (*Puntius gonionotus***)	Endau-Rompin, Malaysia	Present study*
Dactylogyrus sp.	*Rasbora* sp.	Endau-Rompin, Malaysia	Present study*
Dactylogyrus extensus	*Cyprinus carpio*	Czech Republic	AJ969944
Dactylogyrus cryptomeres	*Gobio gobio*	Czech Republic	AJ969947
Dactylogyrus hemiamphibothrium	*Gymnocephalus cernuus*	Czech Republic	AJ969946
Dactylogyrus inexpectatus	*Carassius auratus*	Czech Republic	AJ969945
Dactylogyrus nanus	*Rutilus rutilus*	Czech Republic	AJ969942
Dactylogyrus kikuchii	*Lateolabrax japonicus*	China	AY548929
Dactylogyrus petruschewskyi	*Megalobrama amblycephala*	China	AY548927
Dactylogyrus sphyrna	*Rutilus rutilus*	Czech Republic	AJ969943
Dactylogyrus pekinensis	*Megalobrama amblycephala*	Guangdong, China	EF100535
Dactylogyrus quanfami	*Cirrhinus moliorella*	Guangdong, China	EF100536
Dactylogyrus hypophalmichthys	*Hypophthalmichthys molitrix*	Chongqing, China	EF100532
Dactylogyrus parabramis	*Megalobrama terminalis*	Guangdong, China	EF100534
Dactylogyroides longicirrus	*Systomus sophore* (*Puntius sophore***)	India	GU903482
Pseudodactylogyridae Le Brun, Lambert & Justine, 1986			
Pseudodactylogyroides marmoratae	*Oxyeleotris marmoratae*	Bukit Merah, Malaysia	Present study*
Pseudodactylogyrus bini	*Anguilla anguilla*	Austria	AJ969949
Pseudodactylogyrus anguillae	*Anguilla anguilla*	Slovak Republic	AJ969950
Pseudodactylogyrus sp. UK	*Anguilla anguilla*	United Kingdom	AF382057
Pseudodactylogyrus sp. XHY	*Anguilla anguilla*	China	EF100540
Ancyrocephalidae Bychowsky, 1937			
Bravohollisia gussevi	*Pomadasys hasta*	Pulau Ketam, Malaysia	Present study*
Bravohollisia reticulata	*Pomadasys hasta*	Pulau Ketam, Malaysia	Present study*
Bravohollisia rosetta	*Pomadasys maculatus*	Guangdong, China	DQ537364

Monogenean species	Host species	Locality	GenBank No.
Bravohollisia maculatus	*Pomadasys maculatus*	Guangdong, China	DQ537363
Bravohollisia parvianchoratus	*Pomadasys maculatus*	Guangdong, China	DQ537362
Bravohollisia kritskyi	*Pomadasys hasta*	Pulau Ketam, Malaysia	Present study*
Bravohollisia sp.	*Pomadasys hasta*	Pulau Ketam, Malaysia	Present study*
Caballeria pedunculata	*Pomadasys hasta*	Pulau Ketam, Malaysia	Present study*
Caballeria liewi	*Pomadasys hasta*	Pulau Ketam, Malaysia	Present study*
Caballeria intermedius	*Pomadasys hasta*	China	DQ537366
Pseudohaliotrema sp.	*Siganus* sp.	Langkawi, Malaysia	Present study*
Pseudohaliotrema sphincteroporus	*Siganus doliatus*	Australia	AF382058
Ancyrocephalus paradoxus	*Stizostedion lucioperca*	Czech Republic	AJ969952
Haliotrema spirotubiforum	*Lutjanus stellatus*	Guangdong, China	DQ157656
Haliotrema anguiformis	*Lutjanus monostigma*	Guangdong, China	DQ537375
Haliotrema subancistroides	*Gerres filamentosus*	Guangdong, China	DQ157648
Haliotrema chenhsintaoi	*Branchiostegus auratus*	Guangdong, China	DQ537371
Haliotrema bihamulatum	*Upeneus quadrilineatus*	Guangdong, China	DQ537378
Haliotrema platycephali	*Platycephalus indicus*	Shangdong, China	DQ157662
Haliotrema johnstoni	*Upeneus luzonius*	Hainan, China	DQ157664
Haliotrema shenzhenensis	*Lutjanus argentimaculatus*	Guangdong, China	DQ537372
Haliotrema kurodai	*Sparus macrocephalus*	Guangdong, China	DQ537376
Haliotrema nanaoensis	*Lutjanus argentimaculatus*	Guangdong, China	DQ537373
Haliotrema eukurodai	*Acanthopagrus schlegelii*	Not stated in GenBank	EU836202
Haliotrema geminatohamula	*Leiognathus brevirostris*	Guangdong, China	DQ157649
Haliotrema digyroides	*Gerres macrosoma*	Guangdong, China	DQ537377
Haliotrema fleti	*Lethrinus nebulosus*	Guangdong, China	DQ157661
Haliotrema grossecurvitubus	*Sparus macrocephalus*	Not stated in GenBank	EU836204
Haliotrema cromileptis	*Cromileptes altivelis*	Hainan, China	DQ537379
Haliotrema epinepheli	*Epinephelus sexfasciatus*	Not stated in GenBank	EU836201
Haliotrema macasarensis	*Platycephalus indicus*	Not stated in GenBank	EU836207
Haliotrema aurigae	*Chaetodon auriga*	Australia	AY820621

Monogenean species	Host species	Locality	GenBank No.
Haliotrema leporinus	*Acanthurus nigrofuscus*	South China Sea	EU836206
Haliotrema angelopterum	*Chaetodon kleinii*	Palau	AY820620
Haliotrema scyphovagina	*Forcipiger flavissimus*	Polynesia, French	AY820622
Haliotrema ctenochaeti	*Ctenochaetus strigosus*	Not stated in GenBank	EU836199
Haliotrema macracantha	*Acanthurus nigroris*	Not stated in GenBank	EU836208
Haliotrema pratasensis	*Acanthurus olivaceus*	South China Sea	EU836209
Metahaliotrema geminatohamula	*Scatophagus argus*	Guangdong, China	DQ157646
Metahaliotrema mizellei	*Scatophagus argus*	Guangdong, China	DQ157647
Euryhaliotrema perezponcei	*Lutjanus guttatus*	Mexico	HQ615996
Euryhaliotrema johnii	*Lutjanus rhodopterus*	Guangdong, China	DQ157657
Euryhaliotrema sp. HBDD	*Lutjanus russelli*	Guangdong, China	DQ537374
Euryhaliotrematoides annulocirrus	*Chaetodon vagabundus*	Australia	AY820613
Euryhaliotrematoides sp. HQDD	*Lutjanus rhodopterus*	Guangdong, China	DQ537369
Euryhaliotrematoides triangulovagina	*Chaetodon kleinii*	Palau	AY820619
Euryhaliotrematoides pirulum	*Chaetodon lunula*	Polynesia, French	AY820618
Euryhaliotrematoides microphallus	*Heniochus chrysostomus*	Palau	AY820617
Euryhaliotrematoides grandis	*Chaetodon vagabundus*	Palau	AY820616
Euryhaliotrematoides berenguelae	*Chaetodon citrinellus*	Polynesia, French	AY820615
Euryhaliotrematoides aspistis	*Chaetodon vagabundus*	Australia	AY820614
Tetrancistrum sp.	*Siganus fuscescens*	Heron Island, Australia	AF026114
Cichlidogyrus pouyaudi	*Tylochromis intermedius*	Senegal, Africa	HQ010039
Cichlidogyrus falcifer	*Hemichromis fasciatus*	Senegal, Africa	HQ010024
Cichlidogyrus acerbus	*Sarotherodon galilaeus*	Senegal, Africa	HQ010036
Cichlidogyrus tilapiae	*Hemichromis fasciatus*	Senegal, Africa	HQ010029
Scutogyrus longicornis	*Oreochromis niloticus*	Senegal, Africa	HQ010035
Haliotrematoides plectridium	*Lutjanus guttatus*	Mexico	HQ615994
Haliotrematoides spinatus	*Lutjanus guttatus*	Mexico	HQ615995
Haliotrematoides guttati	*Lutjanus guttatus*	Mexico	HQ615993
Ligophorus vanbenedenii	*Mugil cephalus*	Guangdong, China	DQ157655

Monogenean species	Host species	Locality	GenBank No.
Ligophorus leporinus	*Mugil cephalus*	Guangdong, China	DQ537380
Aliatrema cribbi	*Chaetodon citrinellus*	Polynesia, French	AY820612
Onchobdella aframae	*Hemichromis fasciatus*	Senegal, Africa	HQ010033
Onchobdella bopeleti	*Hemichromis fasciatus*	Senegal, Africa	HQ010034
Actinocleidus recurvatus	*Lepomis gibbosus*	Slovak Republic	AJ969951
Cleidodiscus pricei	*Ameiurus nebulosus (Ictalurus nebulosus**)*	Czech Republic	AJ969939
Urocleidus similis	*Lepomis gibbosus*	Slovak Republic	AJ969938
Onchocleidus sp.	*Lepomis macrochirus*	China	AY841873
Calceostomatidae Parona & Perugia, 1890			
Calceostomatidae sp.	*Eugerres axillaris*	Mexico	FJ971977
Ancylodiscoididae Gusev, 1961			
Ancylodiscus malayensis	*Plotosus canius*	Matang, Malaysia	Present study*
Malayanodiscoides bihamuli	*Notopterus notopterus*	Bukit Merah, Malaysia	Present study*
Bifurcohaptor lanchangensis	*Mystus* sp.	Endau-Rompin, Malaysia	Present study*
Quadriacanthus kobiensis	*Clarias batrachus*	Endau-Rompin, Malaysia	Present study*
Bychowskyella pseudobagri	*Pseudobagrus fulvidraco*	Guangdong, China	EF100541
Cornudiscoides sp.	*Mystus* sp.	Endau-Rompin, Malaysia	Present study*
Cornudiscoides facicirrus	*Mystus nigriceps*	Bukit Merah, Malaysia	Present study*
Cornudiscoides proximus	*Mystus vittatus*	India	GQ925913
Thaparocleidus notopteri	*Notopterus notopterus*	Bukit Merah, Malaysia	Present study*
Thaparocleidus magnicirrus	*Silurus astus*	Guangdong, China	EF100549
Thaparocleidus obscura	*Silurus astus*	Chongqing, China	EF100551
Thaparocleidus mutabilis	*Silurus astus*	Guangdong, China	EF100550
Thaparocleidus omegavagina	*Silurus astus*	Guangdong, China	EF100552
Thaparocleidus infundibulovagina	*Silurus astus*	Chongqing, China	EF100548
Thaparocleidus vistulensis	*Silurus glanis*	Czech Republic	AJ969941
Thaparocleidus siluri	*Silurus glanis*	Czech Republic	AJ969940
Thaparocleidus asoti	*Silurus astus*	Chongqing, China	DQ157669
Thaparocleidus varicus	*Silurus astus*	Chongqing, China	DQ157668
Thaparocleidus cochleavagina	*Silurus astus*	Guangdong, China	EF100547

Monogenean species	Host species	Locality	GenBank No.
Thaparocleidus campylopterocirrus	*Pangasianodon hypophthalmus (Pangasius sutchi**)*	Guangdong, China	EF100546
Pseudancylodiscoides sp. HSY4	*Pseudobagrus fulvidraco*	Guangdong, China	EF100544
Pseudancylodiscoides sp. HSY3	*Pseudobagrus fulvidraco*	Guangdong, China	EF100543
Pseudancylodiscoides sp. HSY1	*Pseudobagrus fulvidraco*	Guangdong, China	EF100542
Hamatopeduncularia sp.	*Arius maculatus*	Langkawi, Malaysia	Present study*
Hamatopeduncularia papernai	*Arius maculatus*	Langkawi, Malaysia	Present study*
Hamatopeduncularia simplex	*Osteogeneiosus militaris*	Langkawi, Malaysia	Present study*
Hamatopeduncularia venosus	*Arius venosus*	Langkawi, Malaysia	Present study*
Hamatopeduncularia malayanus	*Arius caelatus*	Matang, Malaysia	Present study*
Hamatopeduncularia isosimplex	*Arius sagor*	Matang, Malaysia	Present study*
Chauhanellus osteogenosus	*Osteogeneiosus militaris*	Matang, Malaysia	Present study*
Chauhanellus digitalis	*Arius sagor*	Matang, Malaysia	Present study*
Chauhanellus poculus	*Arius maculatus*	Matang, Malaysia	Present study*
Chauhanellus pulutanus	*Arius maculatus*	Matang, Malaysia	Present study*
Neocalceostomatidae Lim, 1995			
Neocalceostoma sp. Malaysia	*Arius venosus*	Malaysia	AF387510
Neocalceostomoides hamatum	*Arius sagor*	Matang, Malaysia	Present study*
Pseudomurraytrematidae Kritsky, Mizelle & Bilqees, 1978			
Pseudomurraytrema sp. USA	*Catostomus ardens*	USA	AF382059
Diplectanidae Bychowsky, 1957			
Diplectanum penangi	*Lates calcarifer*	Hainan, China	DQ054821
Diplectanum grouperi	*Epinephelus coioides*	Guangdong, China	AY553628
Diplectanum umbrinum	*Johnius amblycephalus*	Guangdong, China	EF100560
Diplectanum blairense (=*Paradiplectanum blairense***)	*Sillago sihama*	Hainan, China	AY553627
Diplectanum sillagonum (=*Paradiplectanum sillagonum***)	*Sillago sihama*	Hainan, China	AY553626
Diplectanum veropolynemi	*Polynemus sextarius*	Guangdong, China	AY553625

Monogenean species	Host species	Locality	GenBank No.
Lamellodiscus japonicus	*Sparus macrocephalus*	Guangdong, China	EF100561
Lamellodiscus pagrosomi	*Pagrosomus major*	Hainan, China	EF100562
Lamellodiscus spari	*Lates calcarifer*	China	DQ054823
Lamellodiscus acanthopagri	*Acanthopagrus australis*	Not given in the original paper	DQ054822
Lepidotrema longipenis	*Terapon jarbua*	Guangdong, China	EF100563
Pseudorhabdosynochus lantauensis	*Epinephelus brunneus*	Guangdong, China	AY553624
Pseudorhabdosynochus coioidesis	*Epinephelus coioides*	Guangdong, China	AY553623
Pseudorhabdosynochus epinepheli	*Epinephelus brunneus*	Guangdong, China	AY553622
Pseudorhabdosynochus latesi (=*Pseudorhabdosynochus latesis***)	*Lates calcarifer*	Guangdong, China	AY553621
Pseudorhabdosynochus shenzhenensis	*Epinephelus coioides*	Not given in the original paper	DQ054830
Laticola seabassi (=*Pseudorhabdosynochus seabassi***)	*Lates calcarifer*	Guangdong, China	AY553620
Calydiscoides sp.	*Nemipterus bathybius*	Guangdong, China	EF100558
Calydiscoides indianus	*Nemipterus japonicus*	Guangdong, China	EF100557
Lobotrema sciaenae	*Nibea albiflora*	Guangdong, China	EF100556
Lobotrema sp.	*Johnius* sp.	Carey Island, Malaysia	Present study*
Laticola paralatesi	*Lates calcarifer*	China	DQ054826
Laticola lingaoensis	*Lates calcarifer*	China	DQ054825
Murraytrema bychowskyi (=*M. pricei***)	*Nibea albiflora*	Guangdong, China	DQ157672
Sinodiplectanotrema malayanum	*Pennahia anea*	Langkawi, Malaysia	GU573891*
Sinodiplectanotrema sp. HGY	*Nibea albiflora*	Guangdong, China	EF100778
Acleotrema sp.	*Kyphosus vaigienis*	Australia	AF026118
Sundanonchidae Malmberg, 1990			
Sundanonchus triradiacatus	*Pristolepis fasciatus*	Endau-Rompin, Malaysia	Present study*
Sundanonchus foliaceus	*Channa micropeltes*	Bukit Merah, Malaysia	Present study*
Sundanonchus tomanorum	*Channa micropeltes*	Bukit Merah, Malaysia	Present study*
Sundanonchus micropeltis	*Channa micropeltes*	Bukit Merah, Malaysia	Present study*
Tetraonchidae Bychowsky, 1937			
Tetraonchus monenteron	*Esox lucius*	Czech Republic	AJ969953

Monogenean species	Host species	Locality	GenBank No.
Outgroups			
Gyrodactylidae van Beneden & Hesse, 1863			
Gyrodactylus salaris	*Salmo salar*	Norway	FJ971996
Gyrodactylus derjavini	*Oncorhynchus mykiss*	Denmark	FJ971994
Gyrodactylus macracanthus	*Misgurnus anguillicaudatus*	Australia	FJ971995

Fish hosts sequence (partial Cytochrome *b*) from GenBank

Cytochrome *b* sequences of fish species were obtained from GenBank to determine the relationships of the fish hosts of the dactylogyrideans (see Table 2.5). It should be noted that only Cytochrome *b* sequences from the fish hosts of the dactylogyrideans analysed in this study (see Table 2.4) are obtained from GenBank to reconstruct the relationship trees of the hosts. It is also beyond the scope of the study to provide a full phylogeny of the fish species. Thus, this does not mean that the other fish groups which are not included in the current study do not possess monogeneans.

However, Cytochrome *b* sequences are not available in the GenBank for all the fish hosts of the dactylogyrideans analysed in this study (see Table 2.4) and in order to generate the host relationship trees, Cytochrome *b* sequences of related fish species are used. For example, Cytochrome *b* sequences are not available for *Lutjanus guttatus* (Steindachner), *Heniochus chrysostomus* (Cuvier), *Tylochromis intermedius* (Boulenger) and *Hemichromis fasciatus* (Peters) but available for *Lutjanus stellatus* (Akazaki), *L. argentimaculatus* (Forsskål), *Heniochus diphreutes* (Jordan), *Tylochromis polylepis* (Boulenger) and *Hemichromis bimaculatus* (Gill) and these latter sequences are used in this study (Table 2.5).

Table 2.5 List of partial Cytochrome *b* sequences of fish host species used in this study with their GenBank accession numbers. (*names used in original paper/GenBank).

Host order/family	Host species	GenBank No.
Order		
Perciformes		
Family		
Nemipteridae	*Scolopsis ciliate (Scolopsis ciliates*)*	AF240753
	Nemipterus marginatus	AF240754
Channidae	*Channa bleheri*	AY763770
	Channa maculate	FJ415743
	Channa asiatica	AF480933
	Channa marulius (Channa marulia)*	AY763771
	Channa striata	GU288564
	Channa micropeltes	GU288555
	Channa lucius	GU288553
	Parachanna obscura	AY763772
Anabantidae	*Anabas testudineus*	AY763727
	Ctenopoma acutirostre	AY763728
	Ctenopoma kingsleyae	AY763729
	Ctenopoma petherici	AY763733
	Microctenopoma ansorgii	AY763736
	Microctenopoma fasciolatum	AY763738
	Sandelia capensis	AY763741
Helostomatidae	*Helostoma temminkii (Helostoma temminckii*)*	AY763742
Osphronemidae	*Belontia hasselti*	AY763743
	Belontia signata	AY763744
	Trichogaster fasciata (Colisa fasciatus)*	AY763745
	Trichogaster labiosa (Colisa labiosus)*	AY763746
	Trichogaster lalius (Colisa lalia)*	AY763747
	Trichogaster leerii	AF519695
	Trichogaster pectoralis	AY763758
	Trichogaster trichopterus	AY763759
	Macropodus opercularis	AF519698
	Macropodus spechti (Macropodus concolor)*	AF763760
	Trichopsis pumila	AY763765
	Trichopsis vittata	AF519697
	Trichopsis schalleri	AY763766
Serranidae	*Epinephelus coioides*	DQ354156
	Epinephelus bruneus	FJ594964
	Epinephelus itajara	EU823102
	Anyperodon leucogrammicus	AY963557
	Promicrops lanceolatus	DQ486927
	Cromileptes altivelis	DQ683362
Latidae	*Lates calcarifer*	DQ010541
	Lates niloticus	AB117106
Terapontidae	*Terapon jarbua*	AM265626
	Rhynchopelates oxyrhynchus	AP011064
Sciaenidae	*Argyrosomus regius*	DQ197924

	Larimichthys crocea	EU363519
	Bairdiella ronchus	GQ220025
	Stellifer illecebrosus	GQ220023
	Nebris microps	GQ220022
	Pennahia argentata	HQ890946
	Nibea albiflora	HQ890947
	Seriphus politus	GQ220019
	Macrodon mordax	GQ220015
Sparidae	*Sparus aurata*	DQ198005
	Pagellus bogaraveo	DQ197972
	Lithognathus mormyrus	DQ197961
	Diplodus vulgaris	DQ197947
	Diplodus sargus	DQ197946
	Sparodon durbanensis	AF240733
	Rhabdosargus thorpei	AF240732
Haemulidae	*Pomadasys incises*	DQ197981
	Pomadasys maculatus	EF512297
	Pomadasys perotaei	EF456016
	Plectorhinchus mediterraneus	DQ197979
	Plectorhinchus octolineatum	DQ197977
Lutjanidae	*Lutjanus russellii*	DQ900671
	Lutjanus stellatus	DQ900662
	Lutjanus argentimaculatus	DQ900675
	Lutjanus johnii	DQ900683
Scatophagidae	*Scatophagus argus*	AB276967
Mullidae	*Mullus surmuletus*	DQ197965
	Mullus barbatus	EU036452
Siganidae	*Siganus fuscescens*	AB276833
	Siganus javus	AB276853
	Siganus canaliculatus	AB276851
	Siganus doliatus	AB276961
	Siganus virgatus	AB276949
Cichlidae	*Tylochromis polylepis*	AF370639
	Hemichromis bimaculatus	AF370635
	Sarotherodon galilaeus	AJ845008
	Oreochromis niloticus	AB018989
	Australoheros kaaygua	HQ197686
	Neolamprologus modestus	HM049954
Centrarchidae	*Lepomis gibbosus*	JF742829
	Lepomis cyanellus	JF742828
	Lepomis macrochirus	AY828968
	Ambloplites cavifrons	JF742823
	Pomoxis annularis	JF742839
	Pomoxis nigromaculatus	JF742840
Percidae	*Sander volgensis (Stizostedion volgense*)*	AY374292
	Sander lucioperca (Stizostedion lucioperca)*	AY374291
	Gymnocephalus cernua (Gymnocephalus cernuus)*	AF045356
	Percina stictogaster	AF045355

	Perca flavescens	AF045357
Gobiidae	*Bathygobius soporator*	JN575299
	Bathygobius lineatus	JN575300
	Bathygobius curacao	JN575297
	Bathygobius ramosus	JN575317
	Acentrogobius janthinopterus	AB253463
Eleotridae	*Oxyeleotris selheimi*	AY722238
	Oxyeleotris nullipora	AY722249
	Oxyeleotris marmorata	AY722251
	Oxyeleotris lineolata	AY722237
	Butis amboinensis	AB021232
Apogonidae	*Apogon doederleini*	EU380969
	Apogon maculatus	EU380971
	Apogon semilineatus	AB018995
	Flowleria aurita	EU380973
Lateolabracidae	*Lateolabrax japonicus*	AF240741
	Lateolabrax latus	AF240743
Malacanthidae	*Branchiostegus albus*	EU861053
	Branchiostegus argentatus	EU861054
	Branchiostegus japonicus	EU861052
Chaetodontidae	*Chaetodon ornatissimus*	HQ329584
	Chaetodon meyeri	HQ329544
	Chaetodon auripes	AP006004
	Chaetodon quadrimaculatus	EU823099
	Heniochus diphreutes	AP006005
Nandidae	*Badis ruber*	AY330953
	Dario hysginon	AY330962
	Nandus oxyrhynchus	AY330965
	Nandus nandus	AY330963
Order		
Osteoglossiformes		
Family		
Notopteridae	*Notopterus notopterus*	AY504822
	Papyrocranus afer	AY504823
	Chitala ornata	AF201583
	Xenomystus nigri	AF201614
Order		
Siluriformes		
Family		
Plotosidae	*Plotosus canius*	DQ119445
	Porochilus rendahli	DQ119425
	Plotosus lineatus	DQ119351
Ariidae	*Osteogeneiosus militaris*	FJ013168
	Arius oetik	FJ626195
	Arius maculatus	FJ626194
	Arius leptonotacanthus	FJ626193
	Hexanematichthys sagor (Arius sagor)*	FJ626203
Bagridae	*Mystus vittatus*	DQ119356

	Pelteobagrus fulvidraco	AY912321
	Pelteobagrus vachellii	AY912371
	Pelteobagrus nitidus	AY912357
	Mystus pulcher	DQ119441
	Mystus cavasius	DQ119437
	Bagrichthys macropterus	DQ119455
	Pseudobagrus tenuis	AY912391
	Pseudobagrus truncatus	AY912417
	Pseudobagrus pratti	AY912413
Siluridae	*Silurus asotus*	DQ119376
	Silurus microdorsalis	DQ321756
	Silurus meridionalis	AF416892
	Ompok bimaculatus	FJ711331
	Ompok pabo	FJ711292
	Ompok pabda	FJ711257
Clariidae	*Clarias gariepinus*	DQ646371
	Clarias fuscus	AF416885
	Prophagorus nieuhofii	DQ119377
	Gymnallabes typus	DQ119368
Order		
Cypriniformes		
Family		
Cyprinidae	*Cyclocheilichthys apogon*	JQ346138
	Hypophthalmichthys nobilis (Aristichthys nobilis)*	AF051855
	Hypophthalmichthys molitrix	AF051866
	Hampala macrolepidota	HM536790
	Osteochilus hasselti	JQ346148
	Rasbora elegans	HM224350
	Systomus sophore (Puntius sophore)*	EU241461
	Ctenopharyngodon idella	HM238042
	Labiobarbus lineatus	HM536789
	Gobio gobio	AY953007
	Carassius auratus	GU135604
	Rutilus rutilus	HM560167
	Megalobrama amblycephala	AF051867
	Megalobrama terminalis	AF051872
	Cirrhinus molitorella	GU086538
Order		
Anguilliformes		
Family		
Anguillidae	*Anguilla mossambica*	AF074864
	Anguilla marmorata	AF074863
	Anguilla anguilla	AF006714
	Anguilla japonica	AF006702
	Anguilla malgumora	AF006719

Order		
Mugiliformes		
Family		
Mugilidae	*Mugil cephalus*	EU036450
	Liza aurata	EF439540
	Chelon labrosus	EF427544
	Liza affinis	EU083808
	Liza ramada	EU224059
Outgroup		
Order		
Salmoniformes		
Family		
Salmonidae	*Salmo salar*	EF584212
	Oncorhynchus mykiss	FJ435601
	Oncorhynchus kisutch	FJ435609

There are also instances where the Cytochrome *b* sequences for some fish host species which are present in the GenBank but not suitable for current analysis due to the shorteness of the length of the overall comparable segment of the Cytochrome *b* sequences. For example, Cytochrome *b* sequences of *Nemipterus* spp., *Upeneus* spp., *Lethrinus nebulosus* (Forsskål) and *Silago sihama* (Forsskål) from the GenBank are not included in current analysis due to the above mentioned reason. It should also be noted that more species have been chosen to provide a stronger relationship tree for the hosts which are included in Table 2.5. For example, *Siganus javus* (Linnaeus), *S. canaliculatus* (Park) and *S. virgatus* (Valenciennes) are added for the Siganidae; *Oxyeleotris selhemi* (Macleay), *O. nullipora* (Roberts), *O. lineolata* (Steindachner) and *Butis amboinensis* (Bleeker) are added for the Eleotridae. Thus, a total of 176 partial Cytochrome *b* sequences were obtained from GenBank to infer the relationship of the fish hosts (Table 2.5).

2.5 Data analysis for morphometric data

2.5.1 Principal component analysis (PCA)

PCA was used to analysed morphometric data in this study based on its objectivity as a statistical method and its suitability in analysing multivariate data sets (Jolliffe, 2002) (see Section 1.4; Chapter 1). In this study, morphometric data with multiple variables were analysed using PCA available in R (Version 2.8.1; R Core Development Team 2008). R is a language and environment for statistical computing and graphics. It includes an effective data handling and storage facility, graphical facilities for data analysis and a well-developed, simple and effective programming language. R provides a wide variety of classical statistical tests viz. principal component analysis (PCA), discriminant analysis

(DA), multivariate analysis of variance (MANOVA), biplots, boxtest, time-series analysis, classification and clustering. PCA and biplots are the two main analyses used in this study.

PCA was first used to group and differentiate the morphologically highly similar *Trianchoratus* spp. (4 species), *Bravohollisia* spp. (5 species) and *Caballeria* spp. (3 species) based on their total morphometric data sets. The total data set of *Trianchoratus* analysed consist of 15 parameters collected from 448 individuals, whilst for the *Bravohollisia* and *Caballeria* spp., the total data set consist of 27 parameters measured from anchors, bars, marginal hook and copulatory organ from 1039 individuals (Section 2.4.1). The different morphometric data sets of *Bravohollisia* and *Caballeria* species, i.e. anchors, bars and copulatory organ data set were also analysed separately to test the effectiveness of the different morphological characters in grouping and differentiating the *Bravohollisia* and *Caballeria* species.

Within group analysis was also done for each of the four *Trianchoratus* spp., five *Bravohollisia* spp. and three *Caballeria* spp. based on their morphometric data sets to determine if there are any noticeable variations within each of these species (intraspecific variations). The possible factors influencing the occurrences of these intraspecific variations were also explored. The effects of locality and host factors on the occurrences and distribution patterns of intraspecific variations within *Trianchoratus* spp. were investigated. Similar analyses were done to determine if there exist any intraspecific variations among the individuals of *Bravohollisia* and *Caballeria* species collected from *Pomadasys hasta* of different sizes. This is to test if the presence and distribution of

intraspecific variations within *Bravohollisia* and *Caballeria* species are affetcted by host factors such as host size.

All the results from PCA were presented as scatterplots. Biplots were also used to aid interpretation of the principal component (PC) axes and determine the main distinguishing characters which differentiate among the different species or individuals. All the morphometric data in excel format were imported into R. The R programming scripts used to perform PCA and biplots are given in Appendix A.

2.5.2 Differentiation Index, Φ

Intraspecific and interspecific differences can be quantified as Differentiation Index, $\bar{u}$. The magnitude of this index provides a measure of the amount of differences among species and differences among morphometric variants. The Differentiation Index is calculated based on Euclidean distance which is represented by the two principal components, the horizontal and vertical components that provide clear clustering of the different species or morphometric variants on the PCA scatterplot using R. Euclidean distance is used as it is the simplest and most commonly used distance function which can be easily measured from the different points (representing different individuals) in the PCA scatterplots.

Comparison between the Differentiation Index of species and morphovariants provides a measure for the amount of differences that exist between species and their detected morphovariants. This information derived from the differentiation index calculated in this study can be used as an attempt to answer the question of how much differences must exist before morphovariants can be considered as different species.

The Differentiation Index, ū , for the different species of *Bravohollisia*, *Caballeria* and *Trianchoratus* and their morphovariants (Table 3.3 to 3.6) were calculated according to the method shown below:

Geometrically, $\bar{u}_{ij}$ is the ratio of the square induced by the Euclidean distance between the mean of species *i* and *j* to the average square induced by the Euclidean distance within species *i* and *j*. The horizontal and vertical components of the distance are represented by the two principal components that provide clear clustering of the different species on the PCA plot. The magnitude of $\bar{u}_{ij}$ therefore provides a measure of the amount of between species differentiation relative to within species differentiation.

$$\Phi_{ij} = \frac{(\mu_i - \mu_j)^2 + (\nu_i - \nu_j)^2}{(\sigma_i^2 + \sigma_j^2)/2},$$

where μ_i and g_i are the means of the principal component on the x-axis and y-axis for species *i*, respectively; $\mathring{u}_i^2$ is the sum of variances in the two principal components within species *i*.

The differentiation parameter $\bar{u}_{ij}$ can be estimated by replacing the population means and variances with their sample estimates:

$$\breve{\Phi}_{ij} = \frac{(\bar{x}_i - \bar{x}_j)^2 + (\bar{y}_i - \bar{y}_j)^2}{(\breve{\sigma}_1^2 + \breve{\sigma}_2^2)/2},$$

where the coordinate of the *k*th specimen in the *i*th species on the PC plot is indicated by (x_{ki},y_{ki}), the sample means of the PC on the x and y axes in the ith species by $\bar{x}_i$ and $\bar{y}_i$, and

$$\breve{\sigma}_i^2 = \frac{1}{n_i}\sum_{k=1}^{n_i}(x_{ik} - \bar{x}_i)^2 + (y_{ik} - \bar{y}_i)^2,$$

$$\breve{\sigma}_j^2 = \frac{1}{n_j}\sum_{k=1}^{n_j}(x_{jk} - \bar{x}_j)^2 + (y_{jk} - \bar{y}_j)^2.$$

2.6 Analysis of DNA sequences

The 191 partial 28S rDNA sequences from monogeneans and 176 partial Cytochrome *b* sequences of the fish hosts were edited and aligned with Clustal X (Thompson *et al.*, 1997) using default parameter and verified/edited visually by BioEdit version 7.0.5.3 (Hall, 1999). The aligned and edited sequences were tested for best-fit model of nucleotide substitution and model parameters using Akaike Information Criterion as implemented by Modeltest 3.7 (Posada & Crandall, 1998). Model parameters obtained include empirical base frequencies, proportion of invariable sites (pinvar), rate matrix (rmat) for the selected substitution model and the shape parameter of the gamma distribution.

2.6.1 Phylogenetic Analysis Using Parsimony (PAUP*)

The relationships of the dactylogyridean monogeneans and fish hosts were constructed using neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) method as implemented in PAUP* (version 4.0b10; Swofford, 2002) based on the aligned partial 28S rDNA and partial cytochrome *b* sequences respectively. PAUP*4.0b10 is the most widely used software package for the inference of evolutionary trees among phylogeneticists (Hall, 2001). Other than the parsimony method, PAUP*4.0b10 also support distance matrix and likelihood methods.

For MP analyses, all characters were unordered and equally weighted. For NJ and ML analyses, the selected best-fit model and parameters by Modeltest 3.7 (Posada & Crandall, 1998) were used to construct NJ and MP relationship trees. NJ, MP and ML relationship trees were built using a faststep search where tree searches in each replication were performed using one random-sequence-addition and with no branch swapping (Swofford, 2002). Bootstrap procedures (for NJ, MP, and ML analyses) were performed to

assess the robustness of the inferred relationships. For NJ and MP analyses, bootstrapping were conducted with 10,000 replications while for ML analysis, 100 replications were performed due to long computational time.

A total of 3 relationship trees (NJ, MP and ML) were generated for the dactylogyridean monogeneans and also for the fish hosts based on partial 28S rDNA and Cytochrome *b* respectively. The relationship trees generated for the monogenean were used to test if the relationships of the dactylogyrideans inferred from molecular data are congruent with their relationships based on morphological characteristics, whilst the relationship trees generated for the fish hosts are used to determine if there is any correlation between the relationships of the dactylogyridean monogeneans and the relationships of their hosts (see Section 1.7). All relationship trees were displayed and edited using TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html/).

The molecular data from the monogeneans and fish hosts obtained in this study and GenBank (Sections 2.4.2) were first analysed using PAUP* (version 4.0b10; Swofford, 2002) in personal computer (PC). Due to the large amount of molecular data where a total of 191 partial 28S rDNA sequences (monogeneans) and 176 partial Cytochrome *b* sequences (fish hosts) were analysed, the computational time needed has become unrealistically long which can take up to several weeks to months. Thus, high performance computing (HPC) services provided by the University of Malaya High Performance Computing (UMHPC) were used in this study to overcome the issue of long computational time. All the relationship trees in this study were constructed with the Linux version of PAUP*4.0b10 (Swofford, 2002) using a high performance computer (HPC) SGI Altix 1300 (32 Cores).

CHAPTER 3

RESULTS & DISCUSSION

MORPHOMETRIC CHARACTERS IN DACTYLOGYRIDEAN MONOGENEANS TAXONOMY

3.1 Introduction

Currently information on species diversity is based mainly on morphometric characters. Morphometric data is used in all species descriptions to date and this can be observed in many systematic journals such as Systematic Parasitology for instance. In fact almost all species descriptions include range of morphometric information denoting observed variations within the species population (Lim, pers. com.). Although there are few analyses done to determine if the variations can be used to statistically differentiate related species especially congeners (e.g. Geets *et al.*, 1999; Mariniello *et al.*, 2004; Dmitrieva *et al.*, 2007), it is not common in such studies to discuss or determine which variables are of species importance (interspecific variations) or which are merely displaying population variations (intraspecific variations).

In this study, PCA (see Section 2.5.1) has been used to determine if morphometries can be used to differentiate species and the important diagnostic morphometric features in differentiating species. The results of the PCA analyses (Section 2.5.1) are presented as scatterplots in Figures 3.1 to 3.8, 3.11, 3.12, 3.15, 3.17, 3.19 & 3.21 to 3.23. The main distinguishing features are given in Tables 3.1 & 3.2 and biplot figures (Figs. 3.9, 3.10, 3.13, 3.14, 3.16, 3.18 & 3.20). In order to decide whether the morphovariants within the species populations are not different species, it is necessary to determine the range of variations that are present for each morphovariant group within species population and

amount of variations between species in these natural population by calculating the Differentiation Indices, ū (see Section 2.5.2) and the results are tabulated in Tables 3.3 to 3.6. The spread of the scatterplots for each species is also examined to determine the number of morphovariant groups within the species population and the morphometric characteristics that delimit these morphovariant groups. Another task is to determine the factors (such as micro- and macro-environment) that give rise and affect the morphovariants within a species population. To do this the distribution of morphovariants in the different host individuals is mapped out as in Tables 3.7 & 3.8 and will be discussed in Section 3.4 of this chapter.

3.2 PCA scatterplots and biplots for 12 monogenean species based on morphometric data (Figs. 3.1 – 3.10)

The results of the PCA analyses (in R statistical software; see Section 2.5.1) of the morphometric data (see Section 2.4.1) from two types of species populations, co-existing monogenean species of *Bravohollisia* Bychowsky & Nagibina, 1970 and *Caballeria* Bychowsky & Nagibina, 1970 from *Pomadasys hasta* (Bloch) as well as congeneric monogenean species of *Trianchoratus* Price & Berry, 1966 from a group of related channid host species are presented as scatterplots and biplots (Figs. 3.1 ï 3.10).

3.2.1 Interspecies morphometric variations

The results of the scatterplots and biplots generated from the morphometric data of 744 individuals of *Bravohollisia*, 295 individuals of *Caballeria* and 448 individuals of *Trianchoratus* (see Section 2.4.1) are given in Figs. 3.1 to 3.10. In the analyses for the *Trianchoratus*, only the morphometric data for the anchors are used. The morphometric data for the other features are not used because bars are absent and the copulatory organs are subjected to distortion due to orientations and hence are difficult to measure (see Tan *et al.*, 2010).

3.2.1.1 PCA scatterplots for *Bravohollisia* and *Caballeria* species

3.2.1.1.1 PCA scatterplots for *Bravohollisia* and *Caballeria* species based on morphometric data of all hard parts

The 1039 individuals of *Bravohollisia* and *Caballeria* individuals are differentiated into eight groups, with five groups of *Bravohollisia* and three groups of *Caballeria* (Fig. 3.1). Four of the *Bravohollisia* groups correspond to four *Bravohollisa* spp., with 150 specimens as *B. rosetta*, 180 specimens as *B. reticulata*, 150 specimens as *B. gussevi* and 150 specimens as *B. kritskyi*. However, there is one group of *Bravohollisia* individuals (115 specimens) which does not correspond to any previously described *Bravohollisia* spp. from *Pomadasys hasta* (see Lim, 1995b). Current results have shown that these 115 specimens are a previously unknown *Bravohollisia* species and are different from *B. kritskyi*, *B. rosetta*, *B. reticulata* and *B. gussevi* (Fig. 3.1). Thus, this previously unknown *Bravohollisia* species from *P. hasta* is recognised as a new species and referred to as *Bravohollisia* n. sp. from here on.

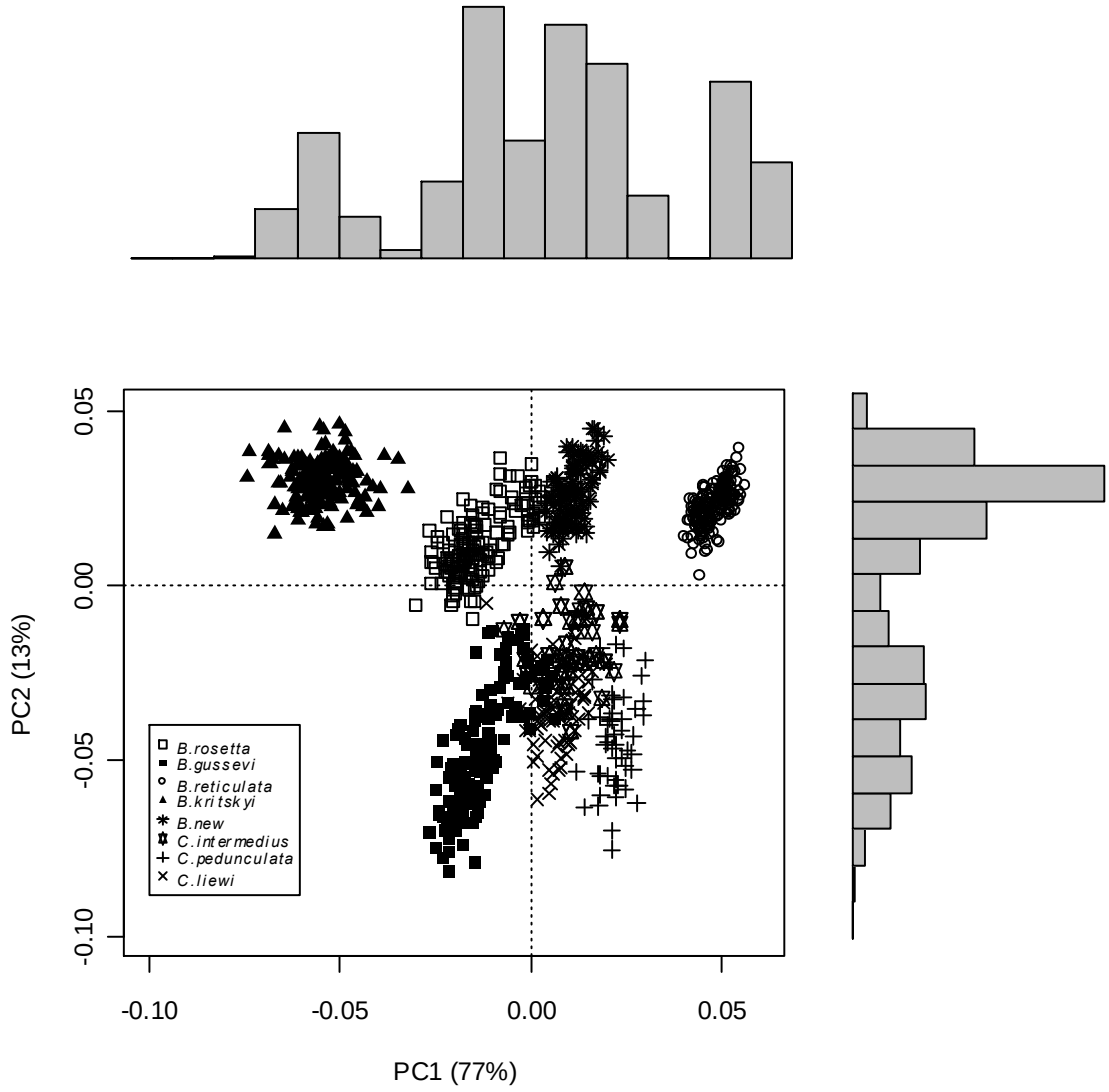


Figure 3.1 PCA plot of five *Bravohollisia* and three *Caballeria* species. The horizontal and vertical barplots indicate one-dimensional summary of the PC axes.

The other remaining three groups observed in the PCA scatterplot of Figure 3.1 correspond to the three *Caballeria* spp., *C. pedunculata*, *C. intermedius* and *C. liewi*. The *C. pedunculata* is clearly shown to be separated from the *C. liewi* and *C. intermedius* while the individuals of *C. liewi* and *C. intermedius* are shown to be partially overlapped. Nonetheless, current PCA result has clearly shown that the *Bravohollisia* species can be effectively differentiated from the *Caballeria* species based on their morphometric data. These results provide evidence which confirmed the observation by Lim (1995b) that *Bravohollisia* and *Caballeria* are two distinct and valid genera. Thus current results refute the suggestion by Wu *et al.* (2007a) to combine the *Bravohollisia* and *Caballeria* into one genus mainly based on their similar copulatory organ characters.

3.2.1.1.2 PCA scatterplots of *Bravohollisia* and *Caballeria* species based separately on anchors, bars and copulatory organ

The morphometric data from each of the different morphological characters, i.e. anchors, bars and copulatory organ of *Bravohollisia* and *Caballeria* species are analysed separately using PCA (see Section 2.5.1). This is to show the variations in each of these different morphological characters and their effectiveness in differentiating the *Bravohollisia* and *Caballeria* species.

By using morphometric data from anchors, the individuals of *Bravohollisia* spp. are separated into four groups on the scatterplots which correspond to *B. reticulata*, *B. rosetta*, *B. gussevi* and the *Bravohollisia* n. sp. – *B. kritskyi* group (Fig. 3.2). Thus, morphometric data from anchors did not manage to differentiate the *Bravohollisia* n. sp. – *B. kritskyi* group. From analysis of morphometric data of bars, the individuals of different *Bravohollisia* spp. are not fully resolved and no distinct groups are formed (Fig. 3.3).

Lastly, the analysis of morphometric data from copulatory organ is shown to differentiate the *Bravohollisia* individuals into five distinct groups which correspond to the each of the five respective *Bravohollisia* species, i.e. *B. rosetta*, *B. reticulata*, *B. gussevi*, *B. kritskyi* and *Bravohollisia* n. sp. (Fig. 3.4). Thus, this study shows that the copulatory organ is the only morphological character which can be effectively used to differentiate among all the five *Bravohollisia* species.

From the analysis of morphometric data of anchors, the *Caballeria* individuals are differentiated into three groups, i.e. *C. intermedius*, *C. liewi* and *C. pedunculata* (Fig. 3.5). However, analyses of morphometric data from bars (Fig. 3.6) and copulatory organ (Fig. 3.7) showed the individuals of *Caballeria* can only be separated into two groups, i.e. *C. liewi* group and the *C. intermedius – C. pedunculata* group from the analysis of morphometric data of bars while *C. pedunculata* group and the *C. intermedius – C. liewi* group can be observed from the analysis of morphometric data of copulatory organ. These results showed that anchors are the major distinguishing morphological character which can be effectively used to differentiate among these three *Caballeria* species.

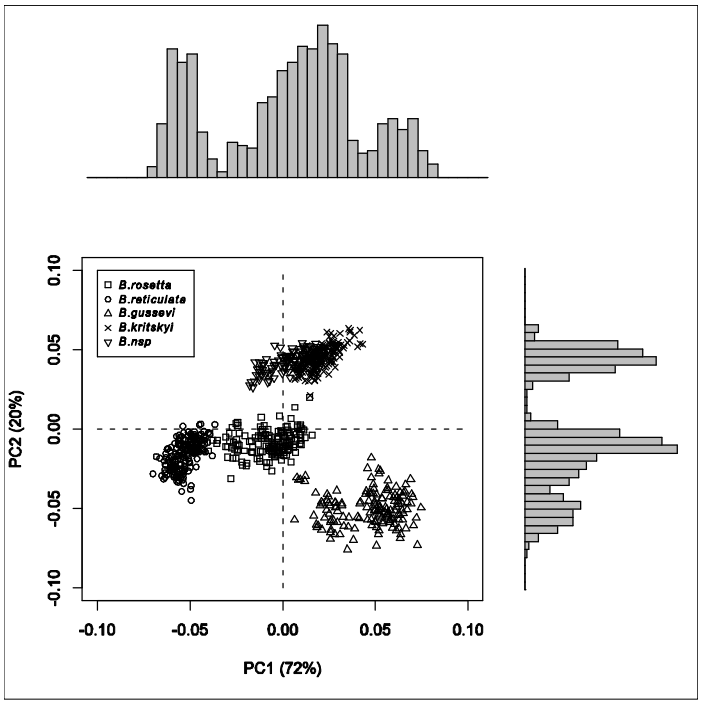


Figure 3.2 PCA plot of the five *Bravohollisia* species using morphometric data from **dorsal and ventral anchors**. The horizontal and vertical barplots indicate one dimensional summary of the PC axes.

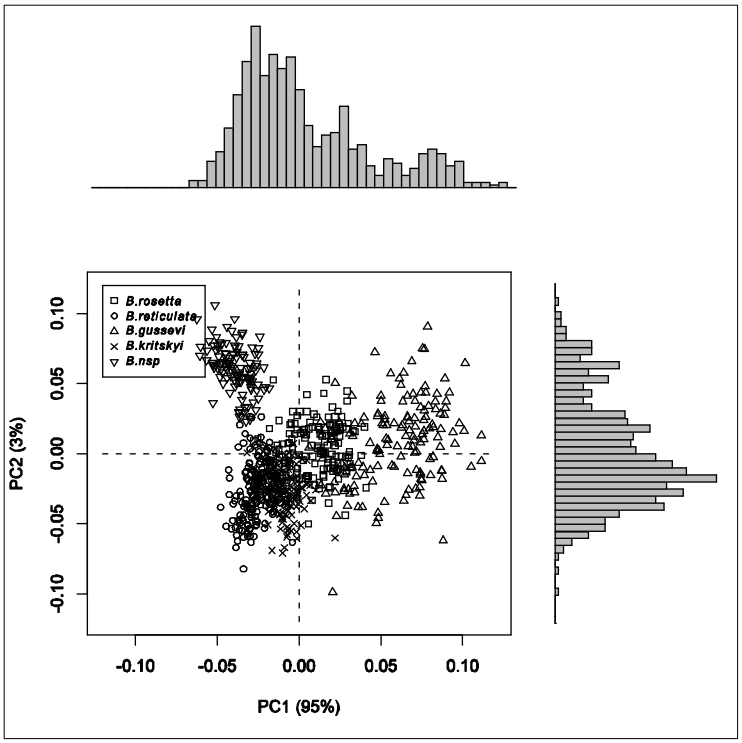


Figure 3.3 PCA plot of the five *Bravohollisia* species using morphometric data from **dorsal and ventral bars**. The horizontal and vertical barplots indicate one dimensional summary of the PC axes.

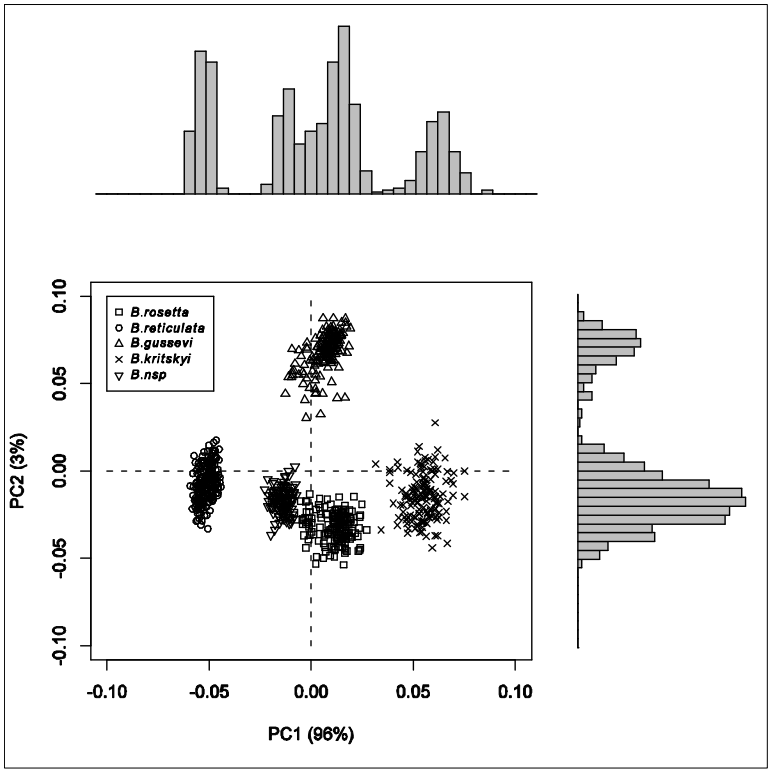


Figure 3.4 PCA plot of the five *Bravohollisia* species using morphometric data from **copulatory organ**. The horizontal and vertical barplots indicate one dimensional summary of the PC axes.

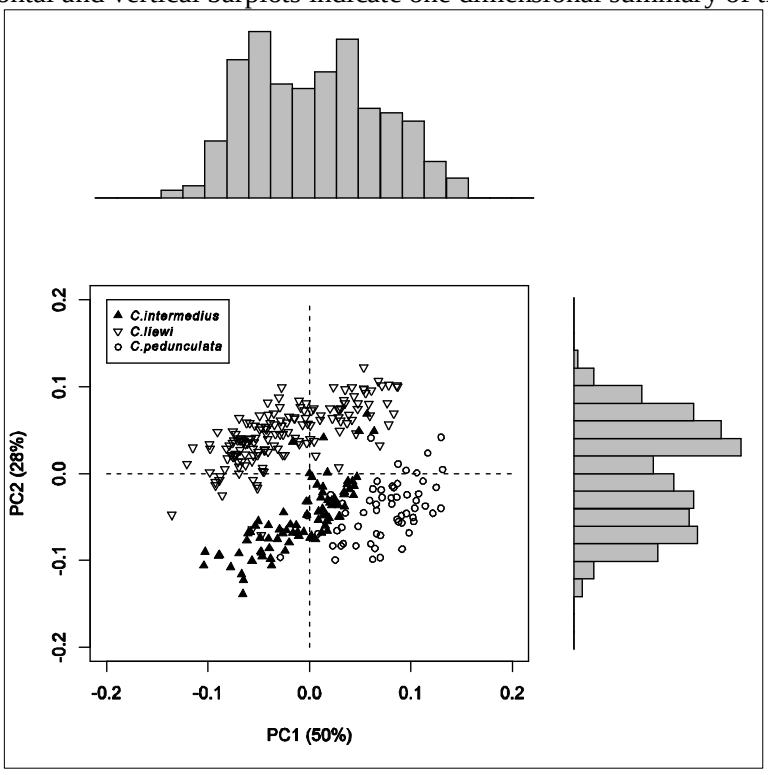


Figure 3.5 PCA plot of the three *Caballeria* species using morphometric data from **dorsal and ventral anchors**. The horizontal and vertical barplots indicate one dimensional summary of the PC axes.

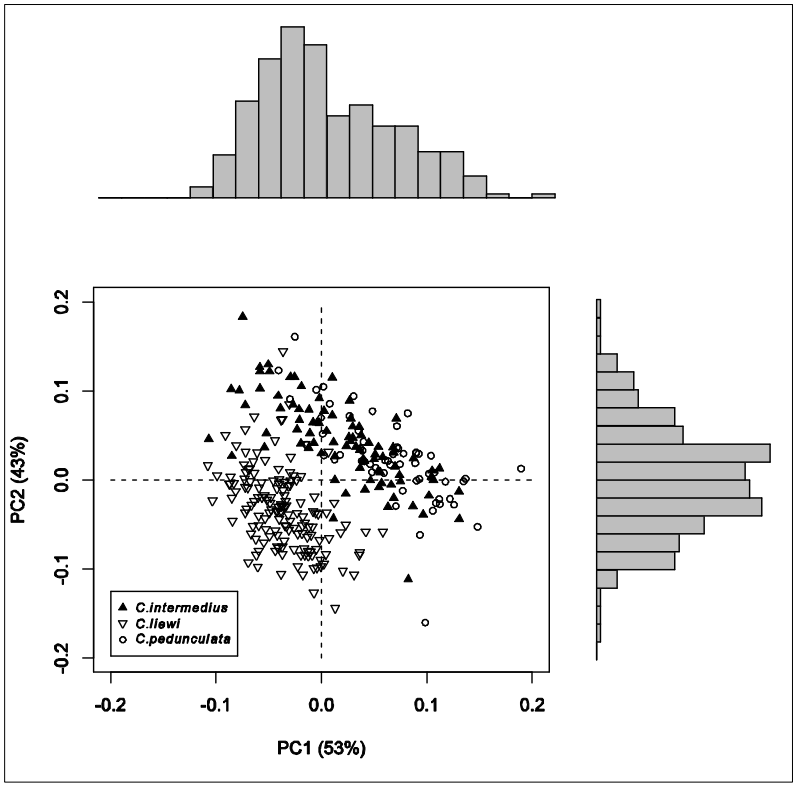


Figure 3.6 PCA plot of the three *Caballeria* species using morphometric data from **dorsal and ventral bars**. The horizontal and vertical barplots indicate one dimensional summary of the PC axes.

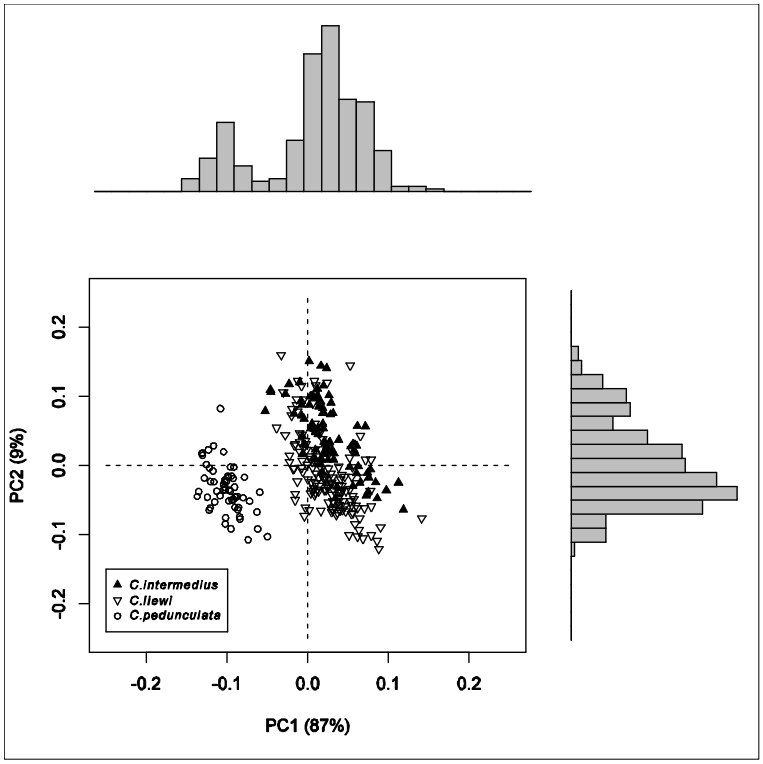


Figure 3.7 PCA plot of the three *Caballeria* species using morphometric data from **copulatory organ**. The horizontal and vertical barplots indicate one dimensional summary of the PC axes.

3.2.1.2 PCA scatterplots for *Trianchoratus* spp. based on morphometric data of 3-developed anchors

The PCA scatterplot (Fig. 3.8) shows the 448 individuals of *Trianchoratus* are differentiated into four groups which correspond to four different *Trianchoratus* species: 119 individuals as *T. malayensis*, 159 as *T. pahangensis*, 145 as *T. ophicephali* and 25 as *T. longianchoratus*.

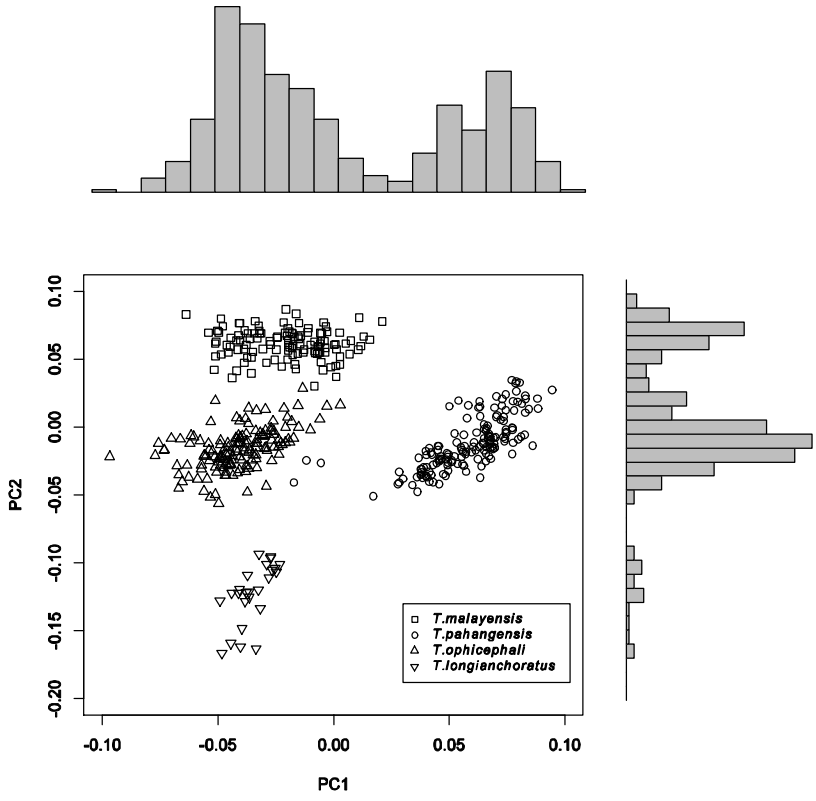


Figure 3.8 PCA plot of four species of *Trianchoratus*. The horizontal and vertical barplots indicate one-dimensional summary of the PC axes.

3.2.1.3 Biplots of morphometric data

The biplots indicate that morphometric measurements from the copulatory organ are the main distinguishing features for the five species of *Bravohollisia* and three species of *Caballeria* (Fig. 3.9; Table 3.1) while inner length and outer length of the three well-developed dorsal anchor (IL3 and OL3) are the main distinguishing features for the four species of *Trianchoratus* (Fig. 3.10; Table 3.2). Thus, current results from the scatterplots showed that morphometric data can be used to differentiate the five species of *Bravohollisia* and three species of *Caballeria* (Fig. 3.1), four species of *Trianchoratus* (Fig. 3.2) and the distinguishing or diagnostic features among the different species can also be determined statistically.

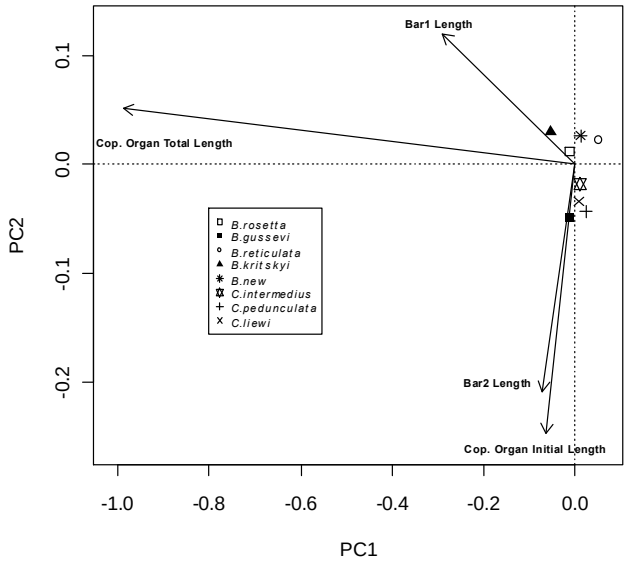


Figure 3.9 Biplot of the first two principal components for the five *Bravohollisia* and three *Caballeria* species with mean coordinates of the species indicated.

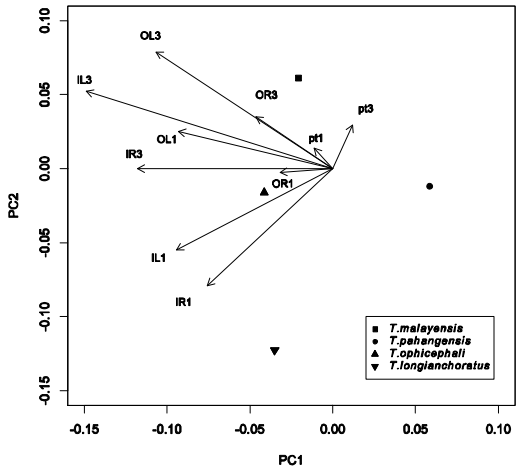


Figure 3.10 Biplot of the first two principal components for the four species of *Trianchoratus*, with mean coordinates of the species indicated. Only vectors (IL1, OL1, IR1, OR1 and pt1) from one ventral anchor are shown.

3.2.2 Intraspecific morphometric variations

3.2.2.1 *Bravohollisia* and *Caballeria* species using morphometric data of all hard parts

The PCA scatterplots resulted from the analysis of morphometric data from individuals within each *Bravohollisia* and *Caballeria* species are presented in Figure 3.11 and 3.12. From the PCA scatterplots, it can be observed that individuals within *Bravohollisia rosetta* (150 specimens), *B. reticulata* (180 specimens), *B. gussevi* (150 specimens), *Bravohollisia* n. sp. (115 specimens), *Caballeria liewi* (150 specimens), *C. intermedius* (86 specimens) and *C. pedunculata* (59 specimens) are separated into two groups along PC1. This shows two groups of intraspecific morphovariants are present within these *Bravohollisia* and *Caballeria* species. Individuals which formed these two groups of morphovariants are found to correspond to the size of the fish host. This indicates that the presence of these morphovariants are host-size dependent.

For *B. rosetta*, *B. reticulata* and *B. gussevi*, individuals from small fish host formed a group distinct from individuals from medium and large fish host (Fig.

3.11).The major distinguishing character for these two host dependent groups is the total length of copulatory organ (COTL) (Fig. 3.13; Table 3.1). For *C. intermedius*, *C. liewi* and *C. pedunculata*, individuals from large fish host formed a distinct group from individuals of small and medium fish host (Fig. 3.12). The major distinguishing characters for these two host dependent groups are the total length of copulatory organ (COTL) and the length of dorsal and ventral bars (BL1 & BL2) for *C. intermedius* and *C. liewi*, whilst for *C. pedunculata*, the major distinguishing character is dorsal and ventral bars (BL1 & BL2) (Fig. 3.14; Table 3.1).

Although it is shown that there are also two groups of morphovariants present within *Bravohollisia* n. sp., the distribution pattern of individuals from fish hosts of different sizes is different for *Bravohollisia* n. sp. where the individuals from small fish hosts and the individuals from large fish host form two distinct groups while individuals from medium fish hosts are found to overlap with individuals from both the small and large fish hosts (Fig. 3.11). This means that the medium fish host possesses both the two morphovariants present within *Bravohollisia* n. sp. According to biplot of the PCA scatterplot, the major distinguishing character for these two host dependent morphometric variants from *Bravohollisia* n. sp. is the total length of copulatory organ (COTL) (Fig. 3.13; Table 3.1).

Lastly for *Bravohollisia kritskyi*, no cluster is observed from the PCA scatterplot (Fig. 3.11). There are no distinct separation of individuals along both, the PC1 and PC2. This means morphometric variation is not present within individuals of *B. kritskyi* collected from fish hosts of different sizes.

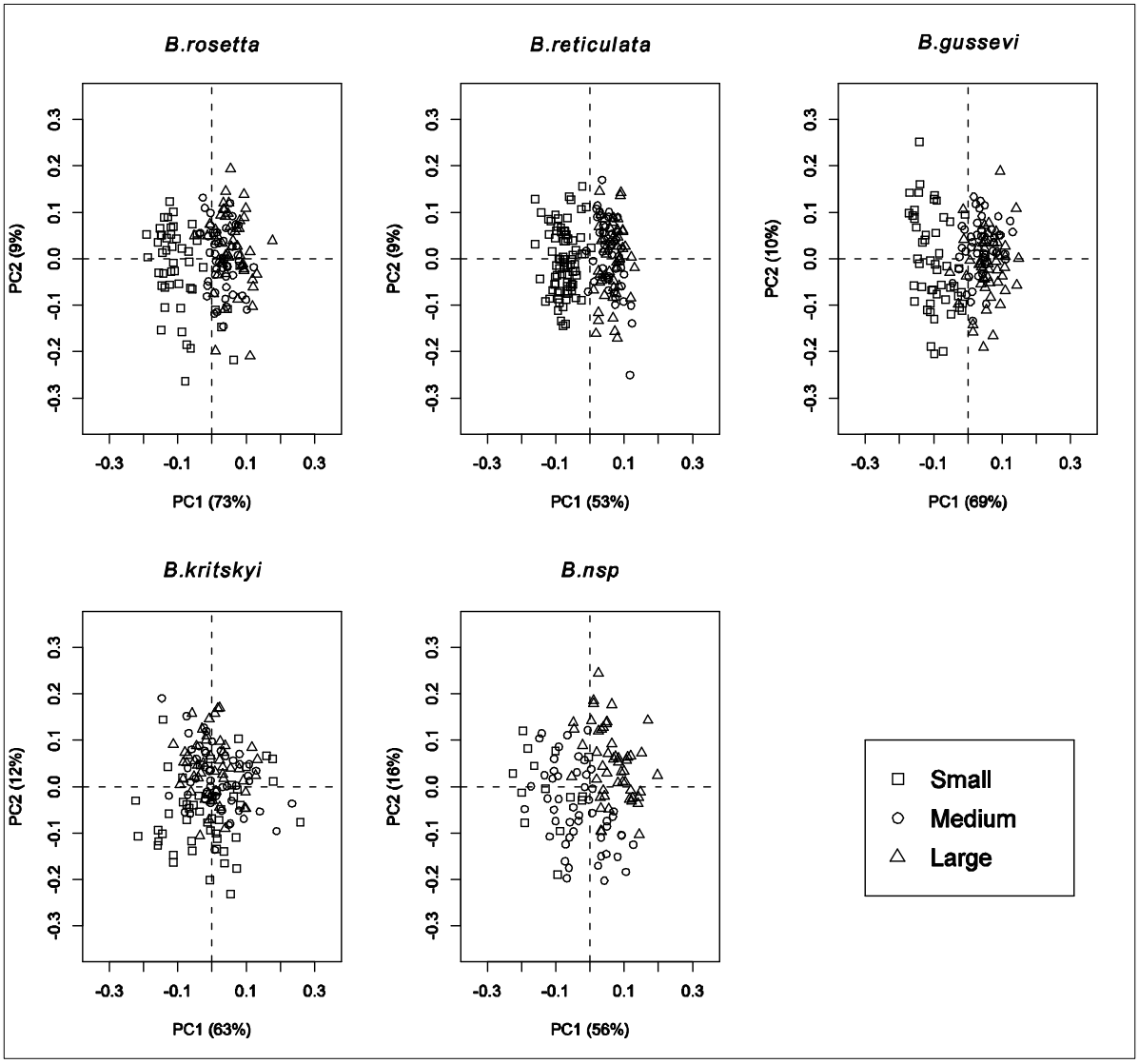


Figure 3.11 Individual PCA plots of the five *Bravohollisia* species, with host size information (Fish standard length: 40-100mm=small, 100-150mm=medium, 150-200mm=large). Except for *B.kritskyi*, individuals in the other four species generally show separation into two groups: small and medium-large along PC1, which is an index of overall size.

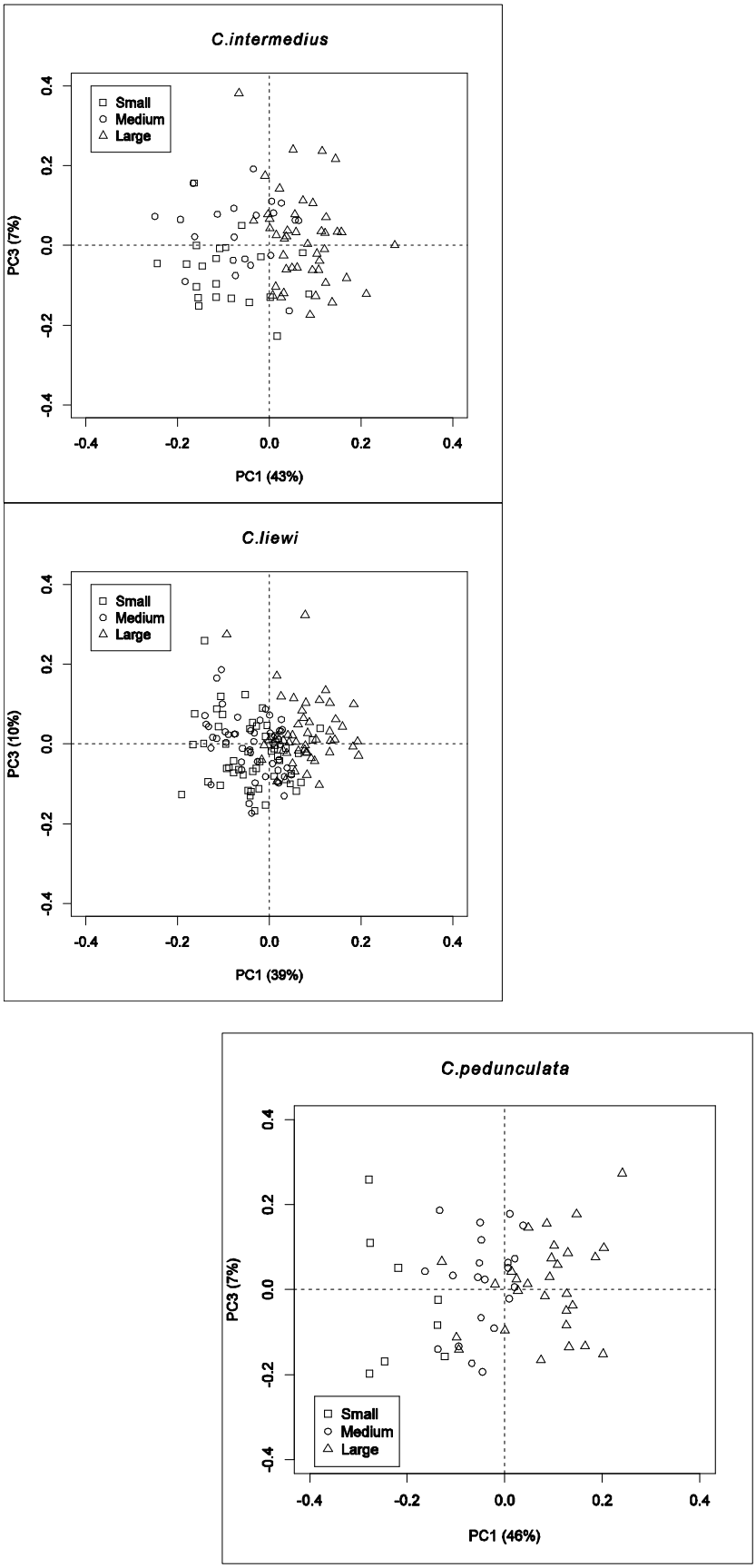


Figure 3.12 Individual PCA plots within each of the three *Caballeria* species, with host size information (Fish standard length: 40-100mm=small, 100-150mm=medium, 150-200mm=large).

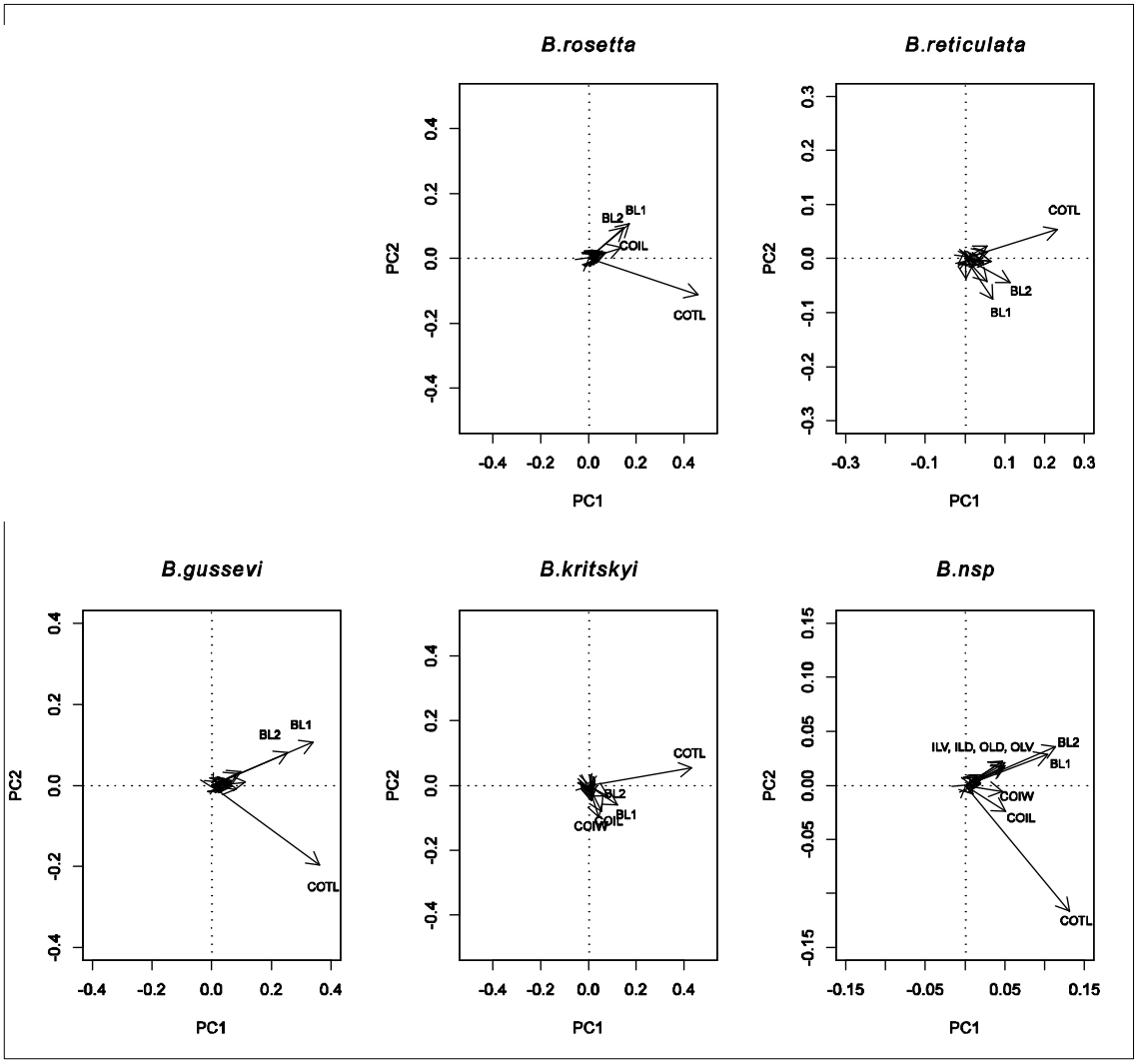


Figure 3.13 Biplots of the PCA plot for each of the five *Bravohollisia* species with host size variation.

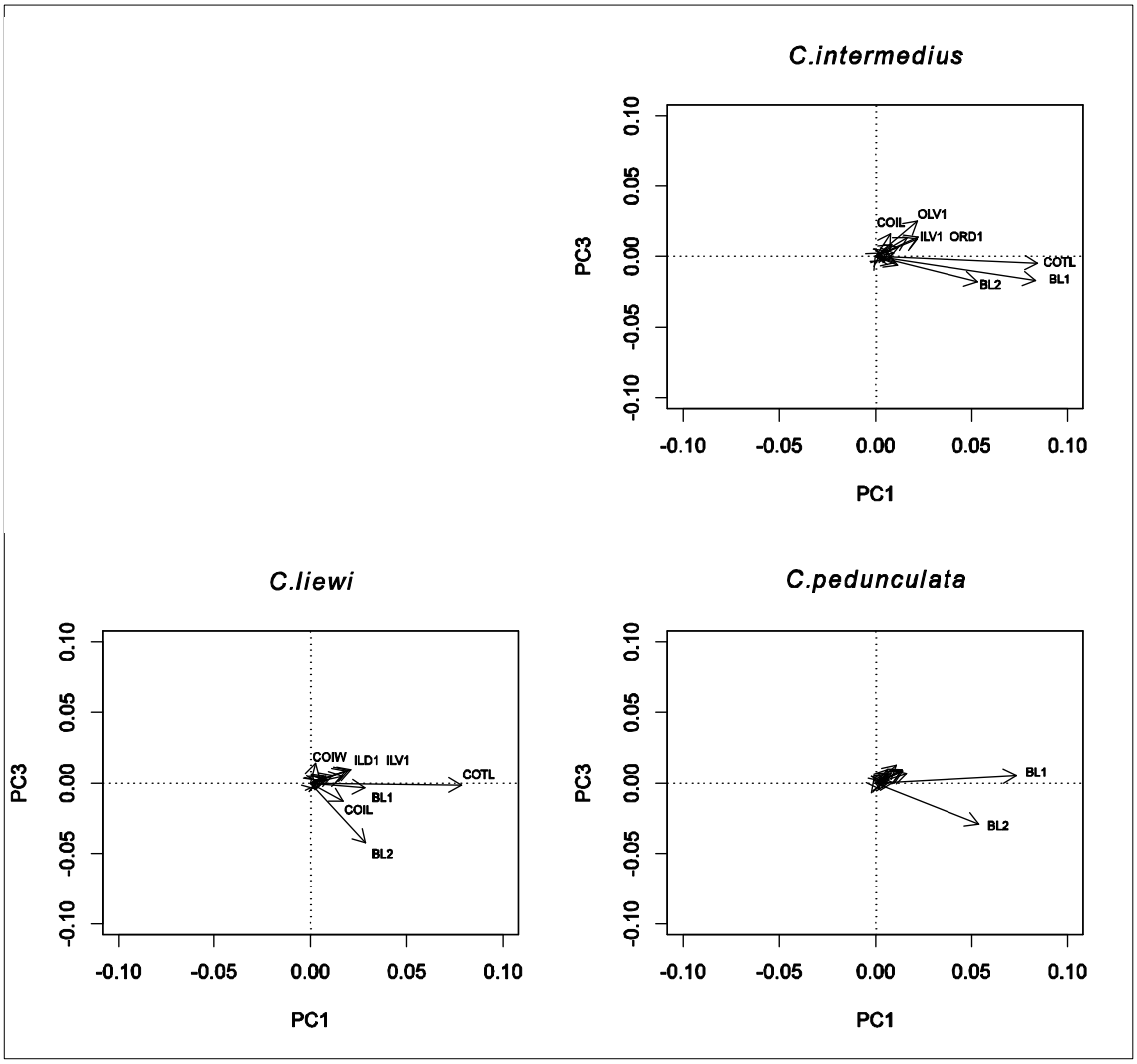


Figure 3.14 Biplots of the PCA plot for each of the three *Caballeria* species with host size variation.

Table 3.1 Interspecies and intraspecies grouping observed for the *Bravohollisia* and *Caballeria* species with their distinguishing morphometric characters and possible factors affecting the intraspecific variants.

Monogenean spp. (*Bravohollisia* and *Caballeria* spp.)	**Number of groups observed in PCA scatterplot**	**Distinguishing morphometric characters**	**Factors affecting intraspecific variants**
Interspecies variations: Total datasets (n=1039 specimens) : All 27 parameter measured **5 *Bravohollisia* spp.** : *B. rosetta* (n=150), *B. reticulata* (n=180), *B. gussevi* (n=150), *B. kritskyi* (n=150), *Bravohollisia* n. sp. (n=115) and **3 *Caballeria* spp.** : *C. liewi* (n=150), *C. intermedius* (n=86), *C. pedunculata* (n=59)	8 groups	Copulatory organ	-
Selected datasets: **5 *Bravohollisia* spp.:** *B. rosetta* (n=150), *B. reticulata* (n=180), *B. gussevi* (n=150), *B. kritskyi* (n=150), *Bravohollisia* n. sp. (n=115)			
i)Anchors, bars, marginal hooks	4 groups (*Bravohollisia* n. sp. - *B. kritskyi* group not resolved)	Inner length of dorsal anchors (ILD1 & ILD2)	-
ii)Bars	No distinct groups are formed	-	-
iii)Anchors	4 groups (*Bravohollisia* n. sp. - *B. kritskyi* group not resolved)	Inner length of dorsal anchors (ILD1 & ILD2)	-
iv)Copulatory organ	5 groups (all 5 *Bravohollisia* spp. are distinguished)	Total length of copulatory organ (COTL)	-

3 *Caballeria* spp. : *C. liewi* (n=150), *C. intermedius* (n=86), *C. pedunculata* (n=59)			
i)Anchors, bars, marginal hooks	2 groups (*C. intermedius* - *C. pedunculata* group not resolved)	Inner length of dorsal anchors (ILD1 & ILD2) and length of dorsal bar (BL1)	-
ii)Bars	2 groups (*C. intermedius* - *C. pedunculata* group not resolved)	length of dorsal bar (BL1)	-
iii)Anchors	3 groups (all 3 *Caballeria* spp. are distinguished)	Inner length of dorsal anchors (ILD1 & ILD2)	-
iv)Copulatory organ	2 groups (*C. intermedius* – *C. liewi* group not resolved)	Total length of copulatory organ (COTL)	-
Intraspecies variations:			
Bravohollisia rosetta (n=150)	2 groups	Total length of copulatory organ (COTL)	Host size
B. reticulata(n=180)	2 groups	Total length of copulatory organ (COTL)	Host size
B. gussevi (n=150)	2 groups	Total length of copulatory organ (COTL)	Host size
B. kritskyi (n=150)	No distinct groups are formed	-	-

Bravohollisia n. sp. (n=115)	2 groups	Total length of copulatory organ (COTL)	Host size
Caballeria liewi (n=150)	2 groups	Total length of copulatory organ (COTL) & length of dorsal and ventral bar (BL1 & BL2)	Host size
C. intermedius (n=86)	2 groups	Total length of copulatory organ (COTL) & length of dorsal and ventral bar (BL1 & BL2	Host size
C. pedunculata (n=59)	2 groups	Total length of copulatory organ (COTL) & length of dorsal and ventral bar (BL1 & BL2	Host size

3.2.2.2 *Trianchoratus* species using morphometric data of 3-developed anchors

The PCA scatterplots generated based on morphometric data of 3-developed anchors for each of the four *Trianchoratus* species show that there are 3 groups observed within *T. malayensis* and *T. pahangensis,* 2 groups within *T. ophicephali* and no distinct group can be observed for *T. longianchoratus* (Figs. 3.15, 3.17, 3.19 & 3.21). These results show intraspecific morphovariants are present within *T. malayensis, T. pahangensis* and *T. ophicephali*.

The intraspecific morphovariants of *T. malayensis* and *T. ophicephali* are shown to be locality dependent. In other words, individuals of *T. malayensis* and *T. ophicephali* from different locality possess three well-developed anchors of different sizes. For example, *T. malayensis* from Bukit Merah has the largest overall size of the three well-developed anchors, followed by *T. malayensis* from Tasik Bera with medium size anchors and *T. malayensis* from Endau-Rompin, which has the smallest size anchors (Fig. 3.15). The distinguishing character for these three locality-dependent morphovariants is the inner length of the well-developed dorsal anchor (IL3) (Fig. 3.16; Table 3.2). For *T. ophicephali*, the intraspecific morphovariants from the two localities, Bukit Merah and Tasik Bera (Fig. 3.17) are distinguished by two main traits: IL1 and IL3 (Fig. 3.18; Table 3.2).

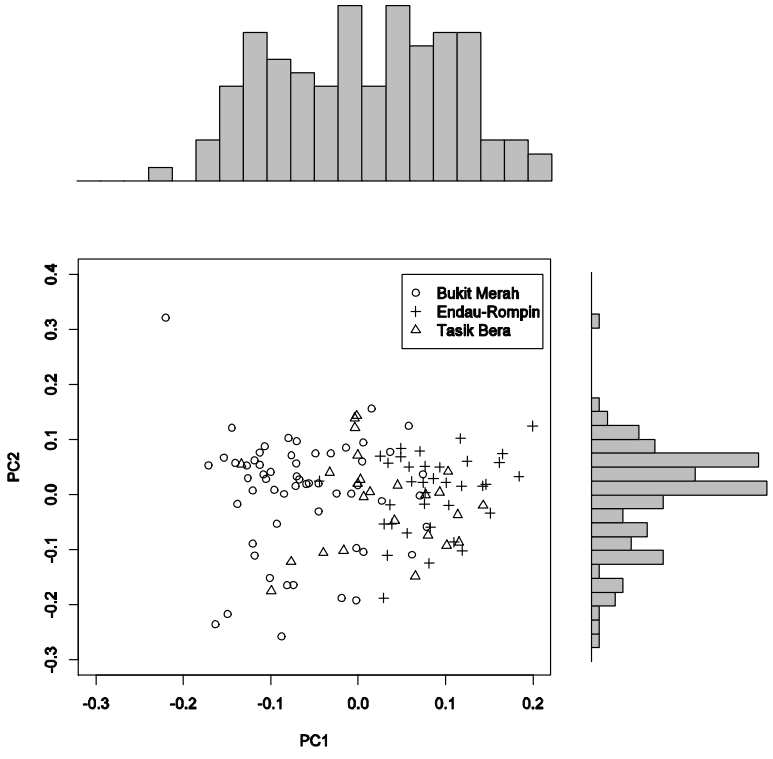


Figure 3.15 PCA plot of *T. malayensis*, with geographical origin of data indicated. The horizontal and vertical barplots indicate one-dimensional summary of the PC axes.

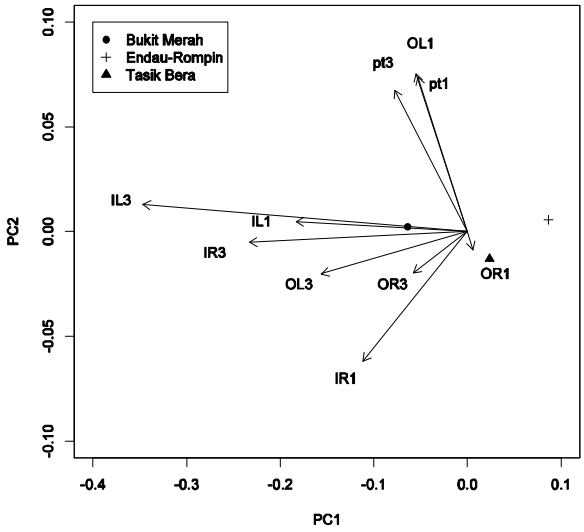


Figure 3.16 Biplot of the first two principal components for *T. malayensis* at three locations, with mean coordinates of the morphovariants. Only vectors (IL1, OL1, IR1, OR1 and pt1) from one ventral anchor are shown.

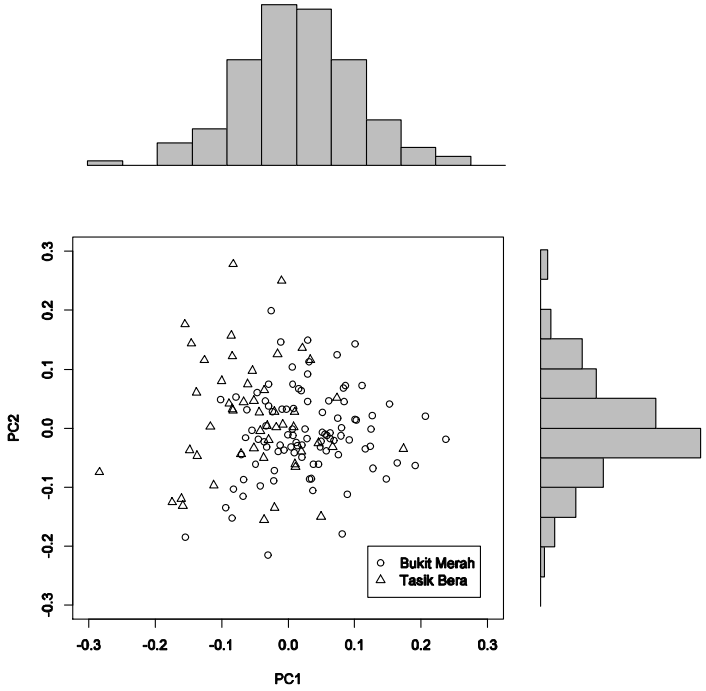


Figure 3.17 PCA plot of *T. ophicephali* with geographical origin of data indicated. The horizontal and vertical barplots indicate one-dimensional summary of the PC axes.

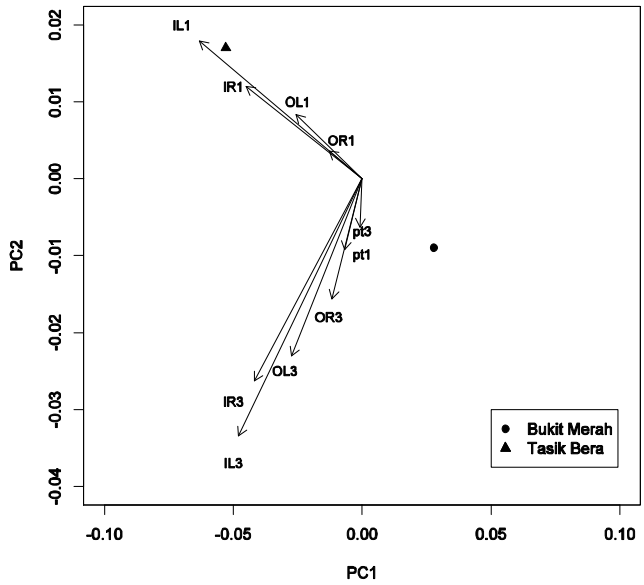


Figure 3.18 Biplot of the first two principal components for *T. ophicephali*, with mean coordinates of the morphovariants at two locations indicated. Only vectors (IL1, OL1, IR1, OR1 and pt1) from one ventral anchor are shown.

The three intraspecific morphovariants within *T. pahangensis* can be observed to be present in all three localities, Bukit Merah, Tasik Bera and Endau-Rompin (Fig. 3.19). This shows that the morphovariants are not dependent on locality. It should be noted that the *T. pahangensis* individuals from two *C. lucius* (Host 1 and Host 2) from Bukit Merah are colour-coded in Figure 3.19. The scatterplot (Fig. 3.19) shows that Host 1 possess variant 1 and variant 3 while Host 2 has variant 2 and variant 3. These results suggest that intraspecific morphovariants of *T. pahangensis* appear to be dependent on host factors. The biplot indicates that the outer length and inner root of the ventral anchors (OL1 and IR1) are the distinguishing characters (Fig. 3.20; Table 3.1) for these host dependent morphovariants.

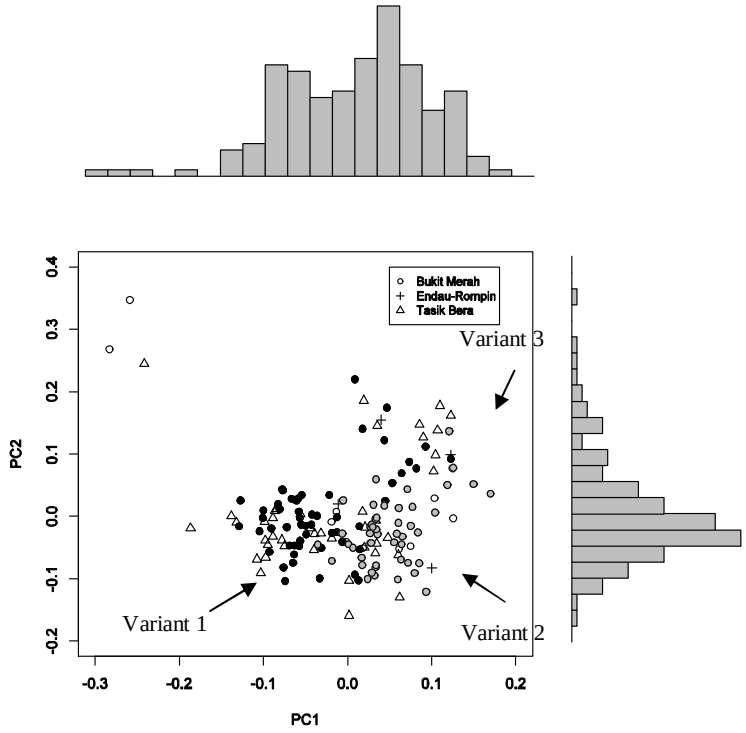


Figure 3.19 PCA plot of *T. pahangensis* with geographical origin of data indicated. Two host individuals from Bukit Merah are also labeled (Black dots = Host 1; Grey dots = Host 2) to show the distribution pattern of morphovariants.

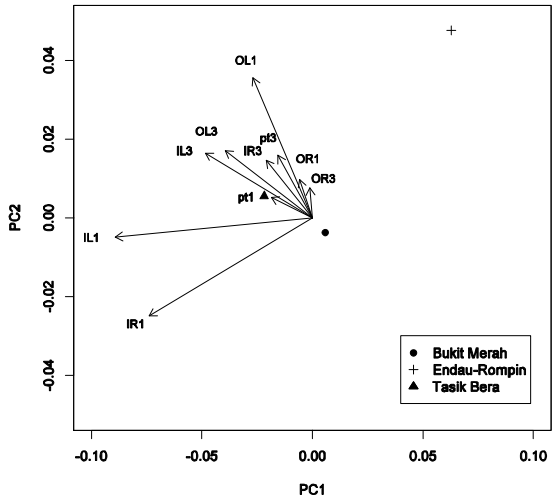


Figure 3.20 Biplot of the first two principal components for *T. pahangensis*, with mean coordinates of the morphovariants at three locations. Only vectors (IL1, OL1, IR1, OR1 and pt1) from one ventral anchor are shown.

Even though intraspecific morphovariants appear to be present within the PCA scatterplot of *T. longianchoratus* (Fig. 3.21), it is difficult to define the variants due to small sample size (25 specimens) and thus it would be premature to declare the number of intraspecific morphometric variants present until more samples of *T. longianchoratus* are analysed. Therefore, it is shown that larger sample size is a necessary requirement to statistically define intraspecific morphometric variants within species populations.

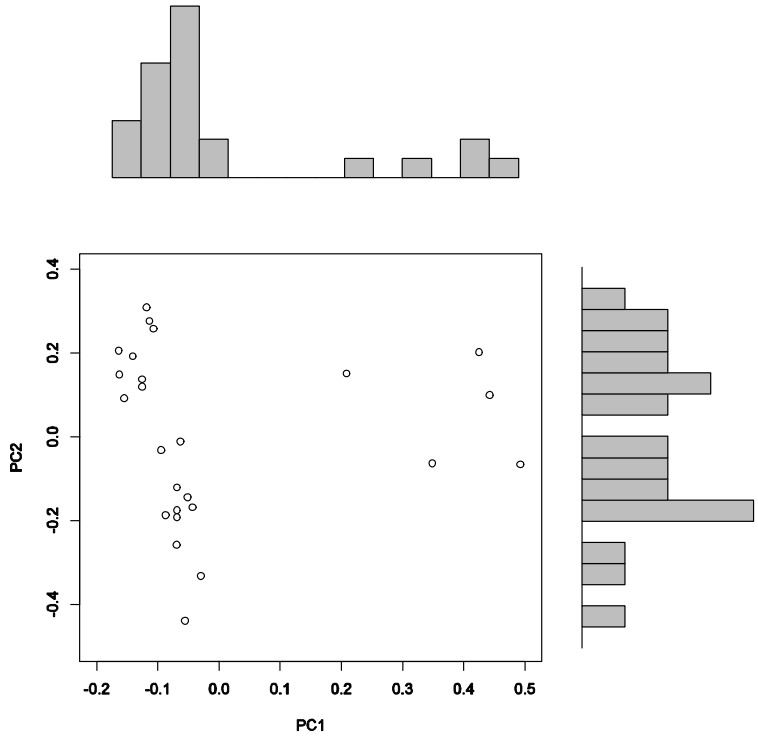


Figure 3.21 PCA plot of *T. longianchoratus*. The horizontal and vertical barplots indicate one-dimensional summary of the PC axes.

Table 3.2 Interspecies and intraspecies grouping observed for the four *Trianchoratus* species with their distinguishing morphometric characters and possible factors affecting the intraspecific variants.

Monogenean spp. (*Trianchoratus* spp.)	**Host spp.**	**Number of groups observed in PCA scatterplot**	**Distinguishing morphometric characters**	**Factors affecting intraspecific variants**
Interspecies variations: **4 *Trianchoratus* spp.** (n=448): *T. malayensis* (n=119), *T. ophicephali* (n=145), *T. pahangensis* (n=159), *T.longianchoratus* (n=25)	*Channa lucius* & *C. striata*	4 groups	Inner length (IL3) and outer length (OL3) of the well-developed dorsal anchor	-
Intraspecies variations:				
T. malayensis (n=119)	*Channa lucius*	3 groups	Inner length (IL3) of the well-developed dorsal anchor	Locality
T. ophicephali (n=145)	*C. striata*	2 groups	Inner length of the well-developed dorsal anchor(IL3) and ventral anchor (IL1)	Locality
T. pahangensis (n=159)	*C. lucius*	3 groups	Outer length (OL1)and inner (IR1) root of well-developed ventral anchor	Host
T.longianchoratus (n=25)	*C. lucius*	Needs further confirmation	-	-

3.3 Amount of variations between and within congeneric species (differentiation index, Φ)

In this section, the variations between different species and morphovariants of *Bravohollisia*, *Caballeria* and *Trianchoratus* are quantified respectively as interspecies and intraspecies differentiation index, ū (see Section 2.5.2). These interspecies and intraspecies differentiation indexes are compared to show the amount of variations which exist between species and morphovariant and these results can be used to answer the question of how much variation should be present amongst different groups of morphologically similar organisms before they can be considered to be different species.

3.3.1 *Bravohollisia* and *Caballeria* species

For *Bravohollisia* and *Caballeria* species, the value of interspecies differentiation index ranges from 14 to 192 (Tables 3.3 & 3.4). The smallest value of the interspecies differentiation index, ū = 14, is observed between *C. liewi* ï *C. intermedius* while the highest value, ū = 192 can be observed from *Bravohollisia* n. sp. ï *B. rosetta*. This result indicates that *C. liewi* and *C. intermedius* are morphologically most similar among the three *Caballeria* spp. while *Bravohollisia* n. sp. and *B. rosetta* are morphologically most different among the five *Bravohollisia* spp.

Table 3.3 Matrix of pairwise interspecies differentiation index, ū , measures among the five *Bravohollisia* species.

	B.rosetta	*B.reticulata*	*B.gussevi*	*B.kritskyi*	*B.nsp*
B.rosetta	0				
B.reticulata	77	0			
B.gussevi	38	70	0		
B.kritskyi	47	176	19	0	
B.nsp	192	185	47	122	0

Table 3.4 Matrix of pairwise interspecies differentiation index, ū , measures among the three *Caballeria* species.

	. intermedius	*. liewi*	*. pedunculata*
. intermedius	0		
. liewi	14	0	
. pedunculata	28	29	0

For the morphovariants of *Bravohollisia* and *Caballeria* species, the highest value for intraspecies differentiation index is only 7.3 (Tables 3.5). Comparison between the interspecies and intraspecies differentiation index shows the values of interspecies differentiation index among different *Bravohollisia* species (ranges from 19 to 192) and *Caballeria* species (ranges from 14 to 29) (Tables 3.3 & 3.4) are far greater than the intraspecies differentiation index among the morphovariants within *Bravohollisia* species (ranges from Ó1 to 7.3) and *Caballeria* species (ranges from Ó1 to 6.8) (Tables 3.5).

Table 3.5 Matrix of pairwise differentiation index, ū , measures among morphovariants within *Bravohollisia*, *Caballeria* and *Trianchoratus* species shown accordingly with variation in host size and locality.

	Small	*Medium*	*Large*
Small	0		
Medium	3.5	0	
Large	4.8	0.4	0

B. rosetta

	Small	*Medium*	*Large*
Small	0		
Medium	1.0	0	
Large	1.6	0.2	0

B. kritskyi

	Small	*Medium*	*Large*
Small	0		
Medium	5.5	0	
Large	6.1	0.1	0

B. reticulata

	Small	*Medium*	*Large*
Small	0		
Medium	1.5	0	
Large	7.3	3.4	0

***Bravohollisia* n. sp.**

	Small	*Medium*	*Large*
Small	0		
Medium	4.6	0	
Large	5.0	0.7	0

B. gussevi

	Small	*Medium*	*Large*
Small	0		
Medium	1.6	0	
Large	4.4	2.4	0

C. intermedius

	Small	*Medium*	*Large*
Small	0		
Medium	0.1	0	
Large	2.9	3.0	0

C. liewi

	Small	*Medium*	*Large*
Small	0		
Medium	2.8	0	
Large	6.8	1.7	0

C. pedunculata

Locality	*BukitMerah*	*Endau – Rompin*	*TasikBera*
BukitMerah	0		
Endau – Rompin	3.9	0	
TasikBera	1.1	0.9	0

T. malayensis

3.3.2 *Trianchoratus* species

For *Trianchoratus*, comparison between the interspecies and intraspecies differentiation index are done with the example of *T. malayensis*. Results which are similar to those for *Bravohollisia* and *Caballeria* species can be observed where the values of interspecies differentiation index among the four *Trianchoratus* species which ranges from 28 to 139 (Table 3.6) are found to be far greater than the intraspecies differentiation index among the morphovariants within *T. malayensis* which ranges from Ó1 to 3.9 (Table 3.5). Thus, there are also marked differences between species and morphovariants of *Trianchoratus*.

Table 3.6 Matrix of pairwise interspecies differentiation index, ū , measures among the four *Trianchoratus* species.

	T.malayensis	*T.pahangensis*	*T.pahangensis*	*T.longianchoratus*
T.malayensis	0			
T.pahangensis	40	0		
T.ophicephali	28	34	0	
T.longianchoratus	139	69	46	0

3.4 Mapping of the distribution patterns of the intraspecific morphovariants

Current results showed the presence of morphovariants within the community of 3 genera of monogenean species, i.e. *Bravohollisia*, *Caballeria* and *Trianchoratus* (Figs. 3.11, 3.12, 3.15, 3.17 & 3.18). The numbers of morphovariants present for each of these monogenean species are different and the distribution of the various morphovariants seems to be affected by different factors such as locality and host (host size) (Sections 3.2.1.2 & 3.2.2.2). For example, *Bravohollisia* n. sp., *B. rosetta*, *B. reticulata, B. gussevi*, *Caballeria intermedius*, *C. liewi* and *C. pedunculata* possess two morphovariants which are influenced by host size (Table 3.1), *Trianchoratus ophicephali* possess two locality dependent morphovariants while *T. malayensis* and *T. pahangensis* possess three morphovariants which are locality and host dependent, respectively (Table 3.2). For the non-locality dependent morphovariants from *T. pahangensis*, a breakdown of the different morphovariants according to host was done to examine if the different morphovariants can be found on the same host (with similar macro- and micro-environment). Similar analysis was also done for the *Bravohollisia* n. sp. where its medium size hosts are observed to possess the two types of morphovariants (see Section 3.2.2.2).

The breakdown of the different morphovariants according to host shows the presence of more than one morphovariant of *T. pahangensis* within a *Channa lucius* host. This is shown in Table 3.7 where Host 1 from Bukit Merah, Host 5 from Endau Rompin, Host 10 and Host 12 from Tasik Bera possess variant 1 and variant 3; Host 2 from Bukit Merah possesses variant 2 and variant 3; Host 11 and Host 13 from Tasik Bera possess variant 1 and variant 2; Host 4 from Bukit Merah possesses all 3 variants. Similar results can also be observed in *Bravohollisia* n. sp. from medium size hosts

where both the morphovariants of *Bravohollisia* n. sp. are present in a *Pomadasys hasta* host. This is exemplified by Host 1, Host 5 and Host 6 which possess variant 1 and variant 2 of *Bravohollisia* n. sp. (Table 3.8).

Table 3.7 Distribution patterns of morphovariants of *Trianchoratus pahangensis* found in fish individuals from different localities.

Host	Locality	*Trianchoratus pahangensis*		
		Variant 1	Variant 2	Variant 3
Channa lucius 1	Bukit Merah	ã	-	ã
Channa lucius 2	Bukit Merah	-	ã	ã
Channa lucius 3	Bukit Merah	-	ã	-
Channa lucius 4	Bukit Merah	ã	ã	ã
Channa lucius 5	Endau-Rompin	ã	-	ã
Channa lucius 6	Endau-Rompin	-	-	-
Channa lucius 7	Endau-Rompin	-	ã	-
Channa lucius 8	Endau-Rompin	-	-	-
Channa lucius 9	Tasik Bera	-	ã	-
Channa lucius 10	Tasik Bera	ã	-	ã
Channa lucius 11	Tasik Bera	ã	ã	-
Channa lucius 12	Tasik Bera	ã	-	ã
Channa lucius 13	Tasik Bera	ã	ã	-

Table 3.8 Distribution patterns of morphovariants of *Bravohollisia* n. sp. found in fish individuals of different sizes.

Host size (Fish standard length)	Host	*Bravohollisia* n. sp.	
		Variant 1	Variant 2
Medium (100-150mm)	*Pomadasys hasta* 1	√	√
	Pomadasys hasta 2	-	-
	Pomadasys hasta 3	-	√
	Pomadasys hasta 4	-	-
	Pomadasys hasta 5	√	√
	Pomadasys hasta 6	√	√
	Pomadasys hasta 7	-	-
	Pomadasys hasta 8	-	-
	Pomadasys hasta 9	-	-
	Pomadasys hasta 10	√	-
	Pomadasys hasta 11	√	-
	Pomadasys hasta 12	√	-
	Pomadasys hasta 13	√	-
	Pomadasys hasta 14	√	-

Thus, current results indicate that these morphovariants with similar macro- and micro-environment (within single host) could be genetically different. Despite being hermaphrodite, the monogeneans have been shown to prefer mating via cross fertilisation (Lim, 2002). This phenomenon most probably has provided the basis for variation in genetic materials which subsequently leads to the occurrences of morphovariants with possible genetic differences.

3.5 Summary of chapter

Current results show that morphometries of the sclerotised hard parts of the monogenans can be used for species differentiations as shown in the above statistical analyses (PCA). The important diagnostic features can also be detected statistically in the biplots (Section 3.2.1). PCA is also effective in removing subjectivity in species differentiation based on morphologies. This study is part of a database initiative which enables the storage of morphometric data and the potential use of the stored data for species differentiation can be done once the system is complete and such a system can serve as a convenient model of species classification tool in the future for assignments of species. However such an automatic system to aid species identification needs the authentication by large amount of data.

Morphometric variations within a species population or related group of species can be detected when large data sets are available. This is shown within the five species of *Bravohollisia*, three species of *Caballeria* and four species of *Trianchoratus* (Section 3.2.2). The present distribution patterns of the morphovariants (Tables 3.1 & 3.2) indicates that there are two morphovariants in *B. rosetta*, *B. reticulata*, *B. gussevi*, *Bravohollisia* n. sp, *C. liewi*, *C. intermedius*, *C. pedunculata*, *T. ophicephali* and three morphovariants in *T. malayensis* and *T. pahangensis*. The numbers of morphovariants present within each of these monogenean species vary possibly due the amount of genetic diversity within each species population (Lim, 2002; Lim pers. com.) (see General Discussion).

The differentiation indices, ū basically provides us with an estimated amount of variations that exist between different species and amongst different morphovariants within species population (Section 3.3). The differentiation indices thus suggested that the range for interspecific variations is 14 ï 192 and for intraspecific variations, it is 1 ï 7.3 (Tables 3.5 ï 3.8). These amount of variations existing between species and between morphovariants show that there are some minimum genetic differences must be achieved before complete speciation occurs (Lim, pers. com.) (see General Discussion).

The statistical analyses shown in this study requires morphometric data from many specimens for accurate results or else one can mistake morphovariants as different species. The information resulting from morphometric analysis of *Trianchoratus* is already published in Tan *et al.* (2010) (Appendix C). The results obtained for the co-existing congeners and non-congeners of *Bravohollisia* and *Caballeria* on *Pomadasys hasta* will be published soon.

3.6 Limitations

There are limitations to this chapter in (1) the number of specimens collected for morphometric measurements and (2) the lack of morphometric data to map the distribution patterns of the different morphovariants within a monogenean population.

These limitations are elaborated as below:

1. There is a need to collect morphometric data from larger sample size (more specimens). The statistical analyses shown in this study requires morphometric data from large sample size for accurate results, especially to determine the presence of morphovariants within a monogenean species population. As exemplified in this study, it is difficult to determine the presence of intraspecific morphovariants of *Trianchoratus longianchoratus* due to small sample size (Section 3.2.2.2).
2. In order to map the distribution patterns of morphovariants, morphometric data needs to be collected from all the monogeneans within a population. This has been done for the four *Trianchoratus* species in this study (Section 3.2.2.2). However, due to their large population size (with an average ranges from 500 to over 1000 individuals), not all the *Bravohollisia* and *Caballeria* individuals within a population are measured and analysed. Future studies which measure and analyse all the *Bravohollisia* and *Caballeria* individuals within a population should be carried out to fully map out the distribution patterns of morphovariants of *Bravohollisia* and *Caballeria* species.

CHAPTER 4

RESULTS & DISCUSSION

MOLECULAR CHARACTERISTICS (PARTIAL 28S rDNA) OF DACTYLOGYRIDEAN MONOGENEANS

4.1 Introduction

This chapter deals with the results of the analysis of molecular data (see Section 2.6) of monogenean species in the order Dactylogyridea (see Section 1.4 & Table 2.4). As already noted molecular data are mainly used to infer relationships amongst the different groups of organisms (see Section 1.3.2). Many of the previous molecular studies on relationships focus only on certain dactylogyridean groups (e.g. Mendlová *et al.*, 2011; Ġimková *et al.*, 2004; Wu *et al.*, 2006; see also Table 1.1). Even in the rare attempts to determine the relationships of various families and subfamilies within the Dactylogyridea, each subfamily and family was represented by very few species as exemplified by 2 *Dactylogyrus* spp. and 2 *Thaparocleidus* spp. were used to represent Dactylogyrinae Bychowsky, 1933 and Ancylodiscoidinae Gusev, 1961 respectively, in Ġimková *et al.* (2003) and Ġimková *et al.* (2006).

One of the aims of this study is to determine how the different members of the dactylogyrideans are grouped together based on molecular data (partial 28S rDNA). The relationships of the monogeneans and status of the different families within the Dactylogyridea are also examined using information from partial 28S rDNA. In this study, partial 28S rDNA (the most available sequences for monogeneans) (see Table 1.2) from 190 dactylogyridean species obtained in the course of this study and from the

GenBank (see Section 2.4.2) are used to reconstruct the relationship trees of the dactylogyrideans.

To avoid any controversies arising from the use of terms that might suggest support either for cladistic or evolutionary systematics (at least for the time being) neutral term such as relationship tree is used for the dendogram generated instead of cladogram or phylogram, although PAUP*4.0b10 (a software usually used by cladists) is used to generate dendogram in this study.

4.2 Partial 28S rDNA sequences of dactylogyrideans

In this study, sequence alignment of the partial 28S rDNA of 191 monogenean species with 306 alignable positions shows there are 218 variable sites and 192 of these variable sites are considered to be parsimony informative. Based on Modeltest 3.7, the current dataset of aligned sequences resulted in the best likelihood score for the Tamura-Nei model with invariable sites and rate heterogeneity (TrN+I+G). Base frequencies are unequal where A=0.2611, C =0.1631, G=0.2097, T=0.3662 and the estimated proportion of invariable sites (pinvar) is 0.2546. The rate matrix (rmat) for the selected substitution model is [A-C]=1.0000, [A-G]=3.3425, [A-T]=1.0000, [C-G]=1.0000, [C-T]=3.5677, [G-T]=1.0000. The shape parameter of the gamma distribution is Ŭ= 0.7627. This model and parameters are used in NJ and ML analyses (see Section 2.6.1).

4.3 Monogenean groups in MP, ML and NJ trees (Figs. 4.1, 4.2, 4.3 & 4.4)

Three relationship trees, i.e. MP, ML and NJ trees are generated in this study (Figs. 4.1, 4.2, 4.3). Eight major groups are observed in MP, ML and NJ relationship trees generated (Figs. 4.1, 4.2, 4.3 & 4.4). In MP and NJ relationship trees (Figs. 4.1 & 4.3), there are seven major nodes whilst in the ML relationship tree (Fig. 4.2), there are six major nodes (Fig. 4.4). To facilitate discussion, the groups formed in all the three relationship trees are named according to the families of the group (see below).

4.3.1 MP, ML and NJ trees (Figs. 4.1, 4.2 & 4.3; Table 4.1)

In MP, ML and NJ relationship trees, memberships for each of the eight observed groups are the same and the compositions of each group are found to correspond to the different dactylogyridean families (see also Table 4.1). The eight observed groups are the Sundanonchidaeï Tetraonchidae group, Diplectanidaeï Pseudomurraytrematidae group; Ancyrocephalidae I group, Ancyrocephalidae II group, Ancyrocephalidae IIIï Calceostomatidae group, Ancylodiscoididaeï Neocalceostomatidae group, Dactylogyridae group and Heteronchocleididaeï Pseudodactylogyridae group at different nodes in the relationship trees (Figs. 4.1, 4.2, 4.3 & 4.4).

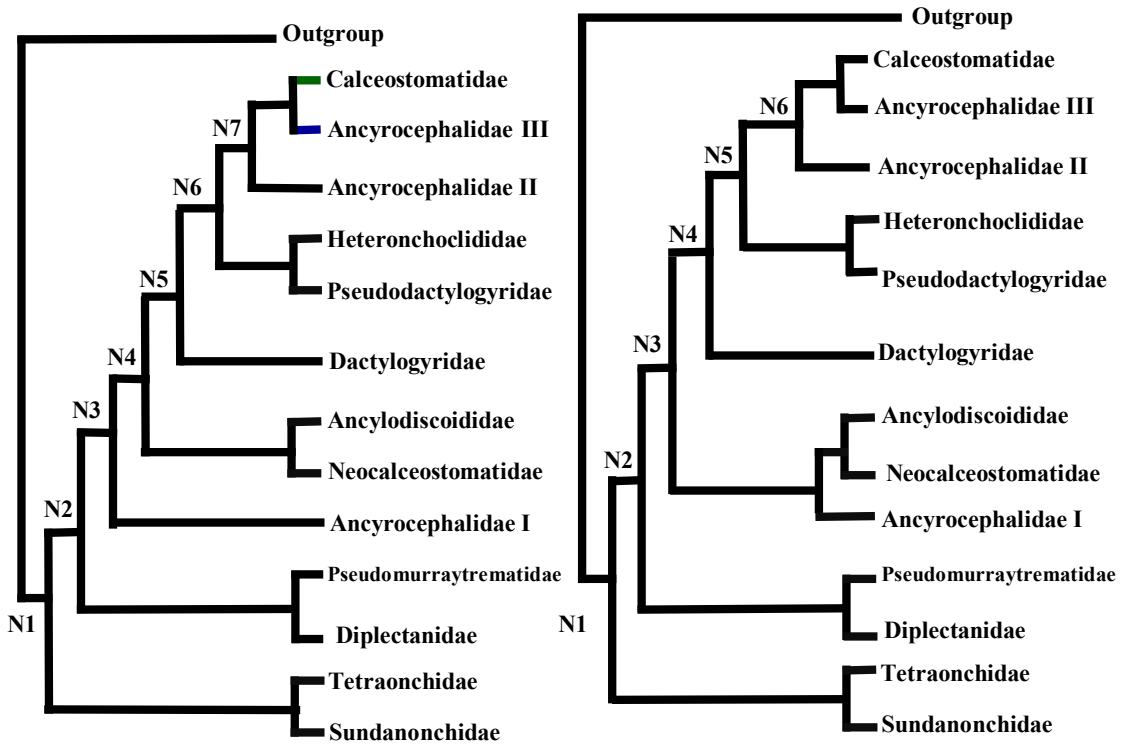
From the MP, ML and NJ relationship trees (Figs. 4.1, 4.2, 4.3 & 4.4), it can be observed that the Sundanonchidaeï Tetraonchidae group separated early at Node 1 (N1) while the Diplectanidaeï Pseudomurraytrematidae group is formed at Node 2 (N2). For the Ancylodiscoididaeï Neocalceostomatidae group, it is formed at Node 4 (N4) in MP and NJ trees (Figs. 4.1 & 4.3) while it can be observed to form sister group with the Ancyrocephalidae I group at Node 3 (N3) in ML tree (Fig. 4.2). The Dactylogyridae

group is shown to be separated at Node 5 (N5), Node 4 (N4) and Node 6 (N6) in MP, ML and NJ trees, respectively (Figs. 4.1, 4.2, 4.3 & 4.4). The Heteronchocleididaeï Pseudodactylogyridae group is formed at Node 5 (N5) in ML and NJ trees (Figs. 4.2 & 4.3) while in MP tree (Fig. 4.1) it is formed at Node 6 (N6).

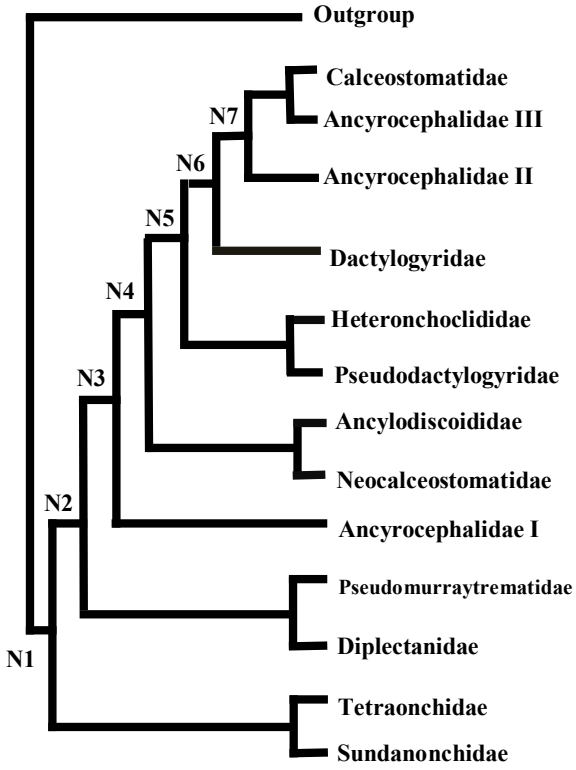
Three ancyrocephalid groups, i.e. Ancyrocephalidae I group, Ancyrocephalidae II group and the Ancyrocephalidaeï Calceostomatidae group, can be observed in the MP, ML and NJ relationship trees (Figs. 4.1, 4.2, 4.3 & 4.4). The Ancyrocephalidae I group is formed at Node 3 (N3) in MP, ML and NJ trees. The Ancyrocephalidae II group and Ancyrocephalidaeï Calceostomatidae group are formed at Node 7 (N7) in MP and NJ trees (Figs. 4.1 & 4.3) and at Node 6 (N6) in ML tree (Fig. 4.2).

4.4 Memberships in the different family groups in the relationship trees

A total of eight groups are formed in the MP, ML and NJ relationship trees. As noted above these groups are related in a similar manner in the MP, ML and NJ trees with only a few exceptions such as the Heteronchocleididaeï Pseudodactylogyridae group is sister group of Ancyrocephalidae II group and Ancyrocephalidaeï Calceostomatidae group in MP and ML trees while in NJ tree, Dactylogyridae group is sister group of Ancyrocephalidae II group and Ancyrocephalidaeï Calceostomatidae group. Also the Ancyrocephalidae I group and Ancylodiscoididaeï Neocalceostomatidae group only form sister group in ML tree but not in MP and NJ trees (Figs. 4.1, 4.2, 4.3 & 4.4). In the section below members of the family groupings will be discussed.



(a) Maximum parsimony (MP) (b) Maximum likelihood (ML)



Neighbour-joining (NJ)

Figure 4.4 Simplified tree of (a) maximum parsimony (MP), (b) maximum likelihood (ML) and (c) Neighbour-joining (NJ) showing the interrelationships among different families within the order Dactylogyridea Bychowsky, 1937 used in this present study. (N1-N7 = major nodes in the relationship trees).

Table 4.1 A summary of the major grouping observed in the maximum parsimony (MP), maximum likelihood (ML) and neighbor-joining (NJ) relationship trees with the memberships within each of the major group formed.

Nodes in relationship trees			**Groups separated at each node**	**Membership within the groups separated at each node**	**Monogenean family within the group**
MP	**ML**	**NJ**			
Node 1 (N1)	Node 1 (N1)	Node 1 (N1)	Sundanonchidae ï Tetraonchidae group	***Tetraonchus*****:** *T. monenteron* Diesing, 1858	Tetraonchidae
				Sundanonchus**:** *S. foliaceus* Lim & Furtado, 1985, *S. triradiacatus* Lim & Furtado, 1985, *S. tomanorum* Kritsky & Lim, 1995 and *S. micropeltis* Lim & Furtado, 1985	Sundanonchidae
Node 2 (N2)	Node 2 (N2)	Node 2 (N2)	Diplectanidaeï Pseudomurraytrematidae group	***Pseudorhabdosynochus*****:** *P. coioidesis* Bu, Leong, Wong, Woo & Foo, 1999, *P. latesi* Tripathi, 1955, *P. lantauensis* Beverley-Burton & Suriano, 1981, *P. shenzhenensis* Yang, Zeng & Gibson, 2005, *P. epinepheli* Kritsky & Beverley-Burton, 1986 ***Acleotrema*****:** *Acleotrema* sp. ***Laticola*****:** *L. paralatesi* Nagibina, 1976, *L. seabasi* Wu, Li, Zhu & Xie, 2005, *L. lingaoensis* Yang, Kritsky, Sun, Zhang, Shi & Agrawal, 2006 ***Diplectanum*****:** *D. veropolynemi* Nagibina, 1976, *D. grouperi* Bu, Leong, Wong, Woo & Foo, 1999 and *D. penangi* Liang & Leong, 1991, *D. umbrinum* Tripathi, 1955, *D. blairense* Gupta & Khanna, 1974 (=*Paradiplectanum blairense*), *D. sillagonum* Tripathi, 1957 (=*Paradiplectanum sillagonum*) ***Lobotrema*****:** *L. sciaenae* Bychowsky & Nagibina, 1977 and *Lobotrema* sp. ***Lepidotrema*****:** *L. longipenis* Yamaguti, 1934 ***Murraytrema*****:** *M. pricei* Bychowsky, 1977 (=*M. bychowskyi*) ***Sinodiplectanotrema*****:** *S. malayanum* Lim, Tan & Gibson, 2010, *Sinodiplectanotrema* sp.HGY ***Lamellodiscus*****:** *L. pagrosomi* Murray, 1931, *L. spari* Zhukov, 1970, *L. japonicus* Ogawa & Eugusa, 1978 and *L. acanthopagri* Roubal, 1981 ***Calydiscoides*****:** *C. indianus* Karyakarte & Das, 1978, *Calydiscoides* sp.	Diplectanidae
				Pseudomurraytrema**:** *Pseudomurraytrema* sp.	Pseudomurraytrematidae
Node 3	Node 3	Node 3	Ancyrocephalid	***Actinocleidus*****:** *A. recurvatus* Mizelle & Donahue, 1944	Ancyrocephalidae

(N3)	(N3)	(N3)	ae I group	***Urocleidus*****:** *U. similis* Mueller, 1936 ***Ancyrocephalus*****:** *A. paradoxus* Creplin, 1839 ***Onchocleidus*****:** *Onchocleidus* sp. ***Cleidodiscus*****:** *C. pricei* Mueller, 1936	
Node 4 (N4)		Node 4 (N4)	Ancylodiscoidid aeï Neocalceostoma tidae group	***Thaparocleidus*****:** *T. notopterus* Jain, 1955, *T. cochleavagina* Gusev & Strelkov, 1960, *T. omegavagina* Hwang, 1964, *T. obscura* Gusev & Strelkov, 1960, *T. mutabilis* Gusev & Strelkov, 1960, *T. asoti* Yamaguti, 1937, *T. magnicirrus* Gusev & Strelkov, 1960, *T. vistulensis* Siwak, 1932, *T. siluri* Zandt, 1924, *T. infundibulovagina* Yamaguti, 1942, *T. varicus* Akhmerov, 1952, *T. campylopterocirrus* Zeng, 1988 ***Cornudiscoides*****:** *C. proximus* Gusev, 1976, *C. facicirrus* Lim, 1987, *Cornudiscoides* sp. ***Chauhanellus*****:** *C. digitalis* Lim, 1994, *C. poculus* Lim, 1994, *C. osteogeneiosi* Lim, 1994, *C. pulutanus* Lim, 1994 ***Hamatopeduncularia*****:** *H. simplex* Bychowsky & Nagibina, 1969, *H. malayanus* Lim, 1996, *H. isosimplex* Lim, 1996, *H. venosus* Lim, 1996, *H. papernai* Lim, 1996, *Hamatopeduncularia* sp. ***Ancylodiscus*****:** A. *malayensis* Lim, 1994 ***Pseudancylodiscoides*****:** *Pseudancylodiscoides* sp.HSY1, *Pseudancylodiscoides* sp.HSY3, *Pseudancylodiscoides* sp.HSY4 ***Quadriacanthus*****:** *Q. kobiensis* Ha Ky, 1968 ***Bychowskyella*****:** *B. pseudobagri* Achmerow, 1952 ***Malayanodiscoides:*** *M. bihamuli* Lim & Furtado, 1986 ***Bifurcohaptor*****:** *B. lanchangensis* Lim, 1987	Ancylodiscoididae
				Neocalceostomoides**:** *N. hamatum* Lim, 1995 ***Neocalceostoma*****:** *Neocalceostoma* sp.	Neocalceostomatidae

Node 5 (N5)	Node 4 (N4)	Node 6 (N6)	Dactylogyridae group	***Dactylogyrus*****:** *D. hemiamphibothrium* Ergens, 1956, *D. pekinensis* Gusev, 1962, *D. petruschewskyi* Gusev, 1955, *D. parabramis* Akhmerov, 1952, *D. hypophalmichthys* Akhmerov, 1952, *D. cryptomeres* Bychowsky, 1934, *D. kikuchii* Gusev, 1965, *D. nanus* Dogiel & Bychowsky, 1934, *D. sphyrna* Linstow, 1878, *D. inversus* Goto & Kikuchi, 1917, *D. lamellatus* Akhmerov, 1952, *D. temperasi* Lim, pers. com., *D. gotoi* Gusev, 1965, *D. apogonae* Lim, pers. com., *D. aristichthys* Long & Yu, 1958, *D. quanfami* Ky, 1971, *D. hampalai* Lim, pers. com., *D. quadribrachiatus* Lim, pers. com., *D. sclerovaginalis* Lim & Furtado, 1986, *D. elegani* Lim, pers. com., *D. lampam* Lim, 1992, *D. laterstriga* Lim, pers. com., *D. spirocopulatrium* Lim, pers. com., *D. damansari* Lim, pers. com., *D. hemiramphodonus* Lim, pers. com., *D. cheligenitalis* Lim & Furtado, 1984, *D. extensus* Mueller & Van Cleave, 1932, *D. inexpectatus* Gusev, 1955, *Dactylogyrus* sp.LAB, *Dactylogyrus* sp. ***Dactylogyroides*****:** *D. longicirrus* Tripathi, 1959	Dactylogyridae
Node 6 (N6)	Node 5 (N5)	Node 5 (N5)	Heteronchocleididaeï Pseudodactylogyridae group	***Heteronchocleidus*****:** *H. buschkieli* Bychowsky, 1957 ***Eutrianchoratus*****:** *E. inequalis* Lim, 1989 and *E. cleithrium* Lim, 1989 ***Trianchoratus*****:** *T. pahangensis* Lim, 1986, *T. longianchoratus* Tan & Lim, 2009, *T. ophicephali* Lim, 1986, *T. malayensis* Lim, 1986, *T. acleithrium* Lim, 1986, *T. gussevi* Lim, 1986, *T. leerium* Lim, 1986, *T. trichogasterium* Lim, 1986, *T. parvulus* Lim, 1986, *T. grandis* Lim, 1986	Heteronchocleididae
				Pseudodactylogyrus**:** *Pseudodactylogyrus* sp. XHY, *Pseudodactylogyrus* sp. UK, *P. bini* Kikuchi, 1929 and *P. anguillae* Yin & Sproston, 1948 ***Pseudodactylogyroides*****:** *P. marmoratae* Lim, 1995	Pseudodactylogyridae
Node 7 (N7)	Node 6 (N6)	Node 7 (N7)	Ancyrocephalidae II group	***Bravohollisia*****:** *B. maculatus* Venkatanarasaiah, 1984, *B. gussevi* Lim, 1995, *B. parvianchoratus* Venkatanarasaiah, 1984, *B. kritskyi* Lim, 1995, *B. rosetta* Lim, 1995, *B. reticulata* Lim, 1995 and *Bravohollisia* sp. ***Caballeria*****:** *C. pedunculata* Bychowsky & Nagibina, 1970, *C. intermedius* Lim, 1995 and *C. liewi* Lim, 1995 ***Haliotrema*****:** *H. fleti* Young, 1968, *H. grossecurvitubus* Li & Chen, 2005, *H. cromileptis* Young, 1968, *H. epinepheli* Young, 1968, *H. chenhsintaoi* Chang, 2001, *H. macasarensis* Yamaguti, 1963, *H. platycephali* Yin & Sproston, 1948, *H. johnstoni* Bychowsky & Nagibina, 1970, *H. aurigae* Yamaguti, 1968, *H.*	Ancyrocephalidae

				leporinus Sun, Kritsky & Yang, 2007, *H. angelopterum* Plaisance, Bouamer & Morand, 2004, *H. bihamulatum* Zhang, 2001, *H. scyphovagina* Yamaguti, 1968, *H. ctenochaeti* Young, 1968, *H. macracantha* Yamaguti, 1968 and *H. pratasensis* Sun, Kritsky & Yang, 2007 ***Pseudohaliotrema*:** *P. sphincteroporus* Yamaguti, 1953 and *Pseudohaliotrema* sp. ***Tetrancistrum*:** *Tetrancistrum* sp.	
			Ancyrocephalidae IIIï Calceostomatidae group	***Euryhaliotrematoides*:** *E. annulocirrus* Yamaguti, 1968, *E. microphallus* Yamaguti, 1968, *E. berenguelae* Plaisance & Kritsky, 2004, *E. grandis* Mizelle & Kritsky, 1969, *E. aspistis* Plaisance & Kritsky, 2004, *E. triangulovagina* Yamaguti, 1968, *E. pirulum* Plaisance & Kritsky, 2004, *Euryhaliotrematoides* sp.HQDD ***Euryhaliotrema*:** *E. johnii* Tripathi, 1959, *E. perezponcei* Garcia-Vargas, Fajer-Avila & Lamothe-Argumedo, 2008, *Euryhaliotrema* sp. ***Haliotrema*:** *H. spirotubiforum* Zhang, 2001 and *H. anguiformis* Zhang, 2001, *H. kurodai* Ogawa & Egusa, 1978, *H. nanaoensis* Yao, Wang, Xia & Chen, 1998, *H. eukuro*dai Zhang & Ding, 1994, *H. subancistroides* Zhang, 2001, *H. geminatohamula* Bychowsky & Nagibina, 1970, *H. digyroides* Zhang, 2001, *H. shenzhenensis* Wang, Liu & Zhou, 2003 ***Ligophorus*:** *L. vanbenedenii* Parona & Perugia, 1890, *L. leporinus* Zhang & Ji, 1981 ***Aliatrema*:** *A. cribbi* Plaisance & Kritsky, 2004 ***Metahaliotrema*:** *M. geminatohamula* Pan, Zhang & Ding, 1995 and *M. mizellei* Venkatanarasaiah, 1981 ***Onchobdella*:** *O. aframae* Paperna, 1968 and *O. bopeleti* Bilong Bilong & Euzet, 1995 ***Cichlidogyrus*:** *C. pouyaudi* Pariselle & Euzet, 1994, *C. falcifer* Dossou & Birgi, 1984, *C. acerbus* Dossou, 1982 and *C. tilapiae* Paperna, 1960 ***Haliotrematoides*:** *H. plectridium* Kritsky & Mendoza-Franco, 2009, *H. spinatus* Kritsky & Mendoza-Franco, 2009 and *H. guttati* Garcia-Vargas, Fajer-Avila & Lamothe-Argumedo, 2008 ***Scutogyrus*:** *S. longicornis* Paperna & Thurston, 1969	Ancyrocephalidae
				Calceostomatidae sp.	Calceostomatidae

4.4.1 Sundanonchidae – Tetraonchidae group

In Sundanonchidae ï Tetraonchidae group, there are four *Sundanonchus* spp. (*Sundanonchus foliaceus* Lim & Furtado, 1985, *S. triradiacatus* Lim & Furtado, 1985, *S. tomanorum* Kritsky & Lim, 1995 and *S. micropeltis* Lim & Furtado, 1985) and *Tetraonchus monenteron* Diesing, 1858 which correspond to the family Sundanonchidae and Tetraonchidae respectively (Figs. 4.1, 4.2, 4.3). Based on the fact that these two families are sister group in all the three relationship generated, Tetraonchidae and Sundanonchidae are expected to be closely related. Morphologically the Tetraonchidae and Sundanonchidae possess similar copulatory complex, vagina apparatus, single intestinal track, 16 tetraonchid-gyrodactylid type of marginal hooks and the X-shaped vitellarian ducts (Lim & Furtado, 1985). Similar results were obtained from analyses by Kritsky & Lim (1995) and Boeger & Kritsky (1997) using morphological data and by Ġimková et al (2003) using and molecular data.

4.4.2 Diplectanidae – Pseudomurraytrematidae group (Figs. 4.5, 4.6 & 4.7)

There are 28 members within Diplectanidae ï Pseudomurraytrematidae group where 27 of them (from 10 genera) correspond to the family Diplectanidae and one member (*Pseudomurraytrema* sp.) corresponds to the family Pseudomurraytrematidae (Figs. 4.1, 4.2 & 4.3). In this section, only the interrelationships among the 27 members within the Diplectanidae will be discussed since Pseudomurraytrematidae is represented by one *Pseudomurraytrema* sp. in this study. Three subGroups can be observed within the Diplectanidae ï Pseudomurraytrematidae group (Figs. 4.5, 4.6 & 4.7).

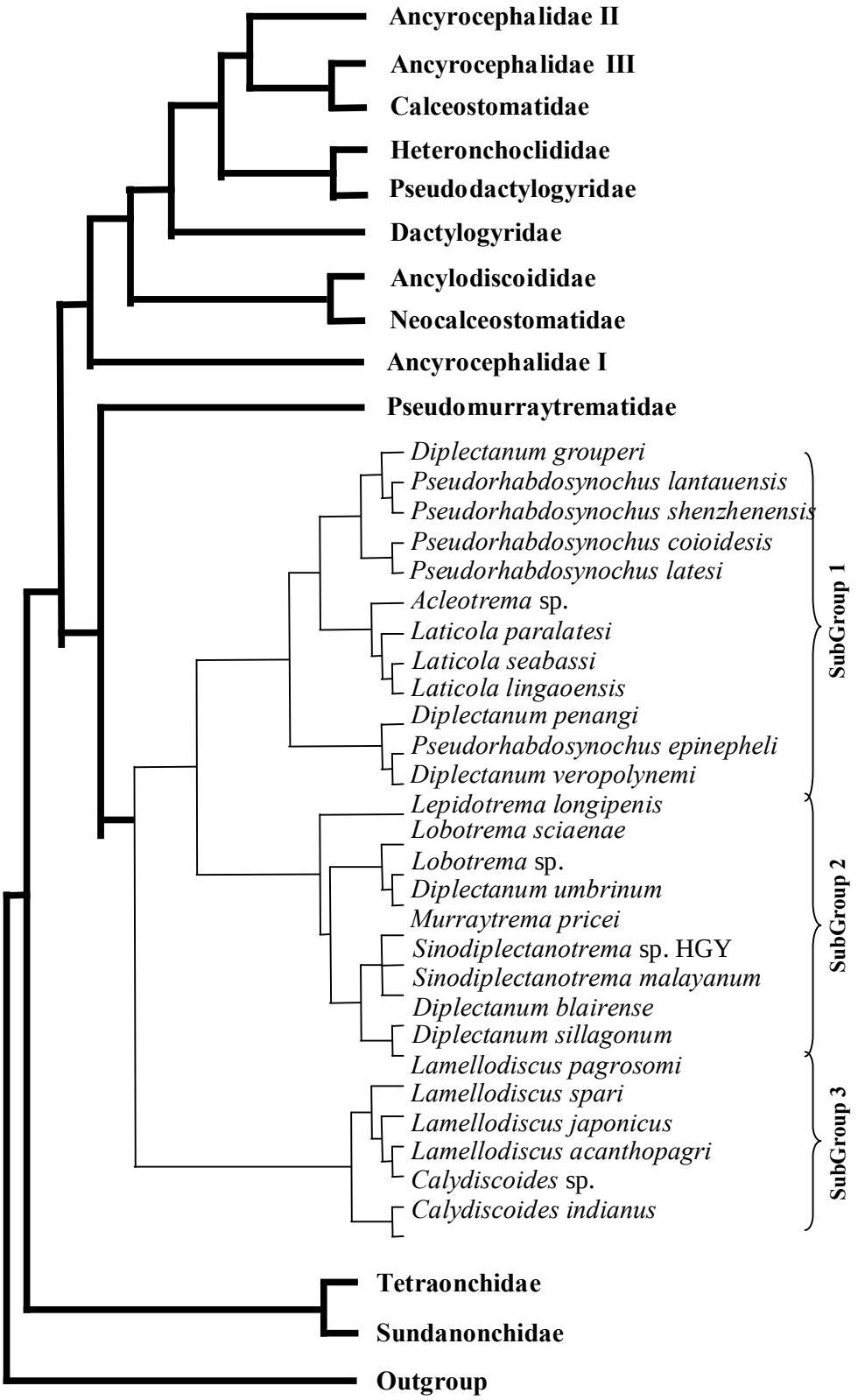


Figure 4.5 Maximum parsimony (MP) tree depicting the interrelationships within the Diplectanidae Bychowsky, 1957.

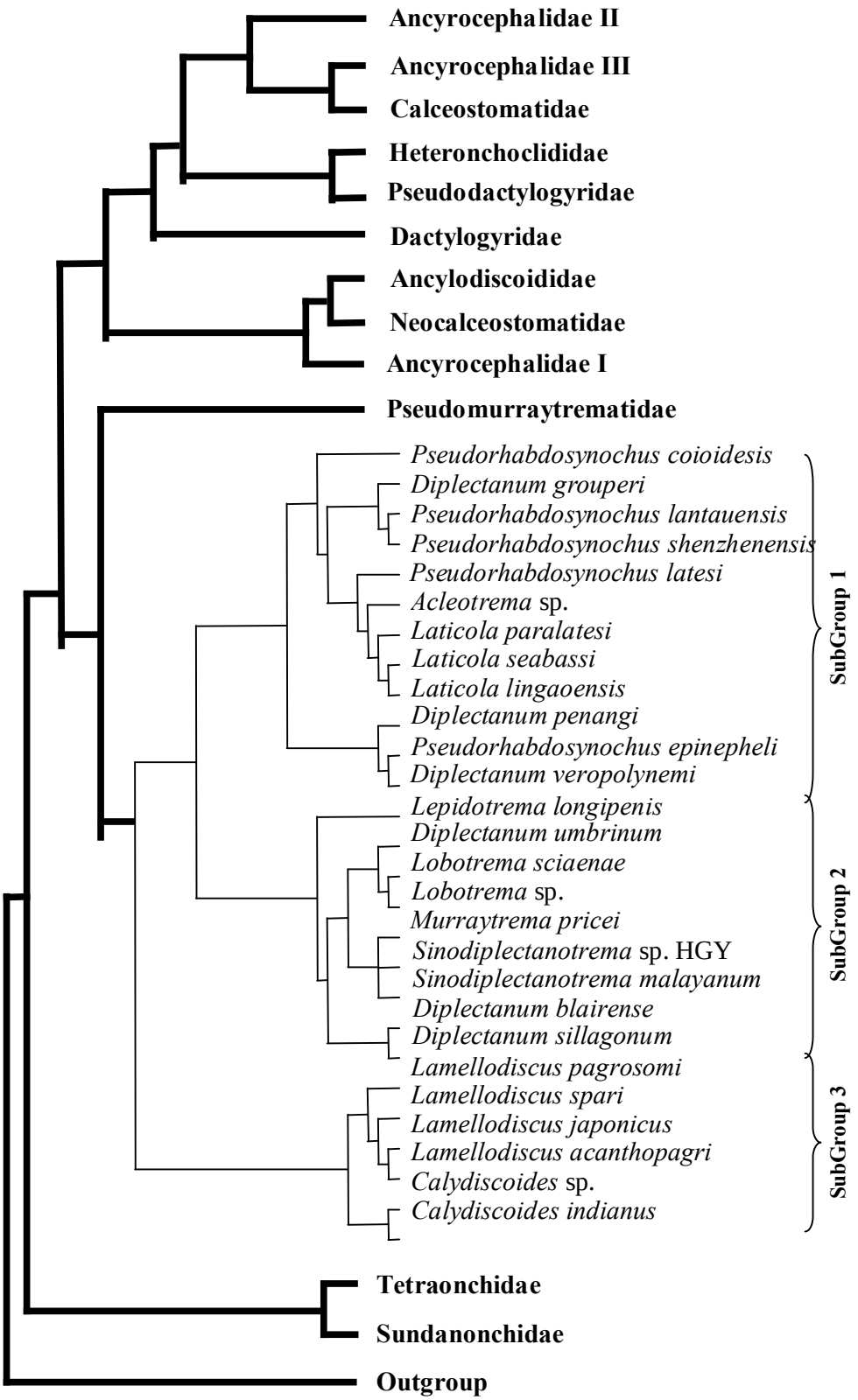


Figure 4.6 Maximum likelihood (ML) tree depicting the interrelationships within the Diplectanidae Bychowsky, 1957.

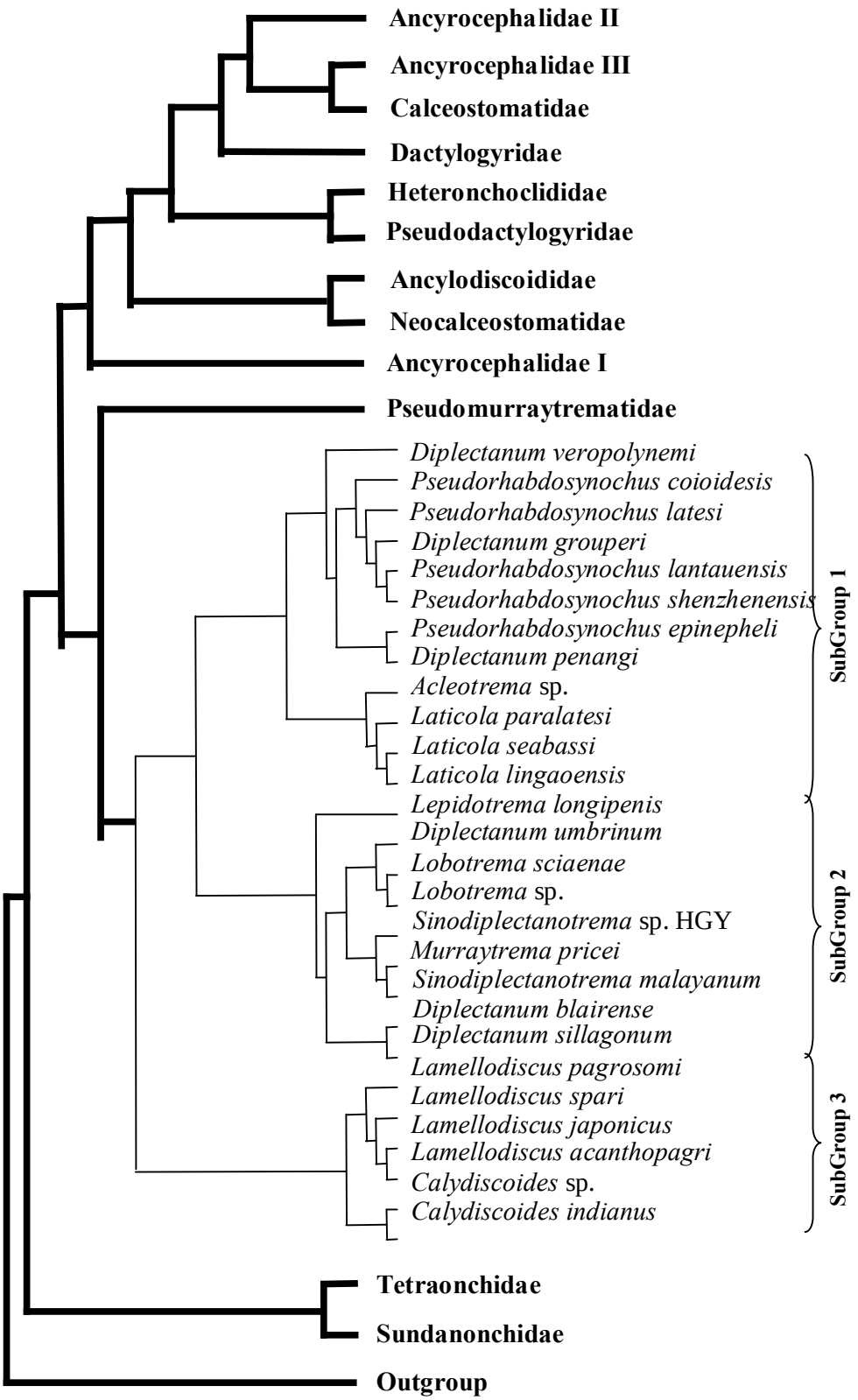


Figure 4.7 Neighbour-joining (NJ) tree depicting the interrelationships within the Diplectanidae Bychowsky, 1957.

4.4.2.1 *Pseudorhabdosynochus*, *Acleotrema*, *Laticola* & *Diplectanum* (SubGroup 1)

SubGroup 1 consists of five *Pseudorhabdosynochus* spp., *P. coioidesis* Bu, Leong, Wong, Woo & Foo, 1999, *P. latesi* Tripathi, 1955, *P. lantauensis* Beverley-Burton & Suriano, 1981, *P. shenzhenensis* Yang, Zeng & Gibson, 2005 and *P. epinepheli* Kritsky & Beverley-Burton, 1986, one *Acleotrema* sp., three species of *Laticola*, *L. paralatesi* Nagibina, 1976, *L. seabasi* Wu, Li, Zhu & Xie, 2005 and *L. lingaoensis* Yang, Kritsky, Sun, Zhang, Shi & Agrawal, 2006 and three species of *Diplectanum*, *D. veropolynemi* Nagibina, 1976, *D. grouperi* Bu, Leong, Wong, Woo & Foo, 1999 and *D. penangi* Liang & Leong, 1991.

Within SubGroup 1, *Pseudohabdosynochus* spp. with quadriloculate copulatory organ can be observed to cluster together while *Acleotrema* sp. and *Laticola* spp. with copulatory organ composed of two nested tube form sister group (Fig. 4.8). There are also *D. grouperi*, *D. penangi* and *D. veropolynemi* which clustered among the *Pseudohabdosynochus* spp. *Pseudorhabdosynochus*, *Acleotrema*, *Laticola* and *Diplectanum* in SubGroup 1 also correspond to members of the subfamily Diplectaninae Monticelli, 1903. The close relationship of *Laticola* and *Pseudorhabdosynochus* shown in SubGroup 1 was also shown by Yang *et al.* (2006) and Domingues & Boeger (2008) in their analyses based on morphological data.

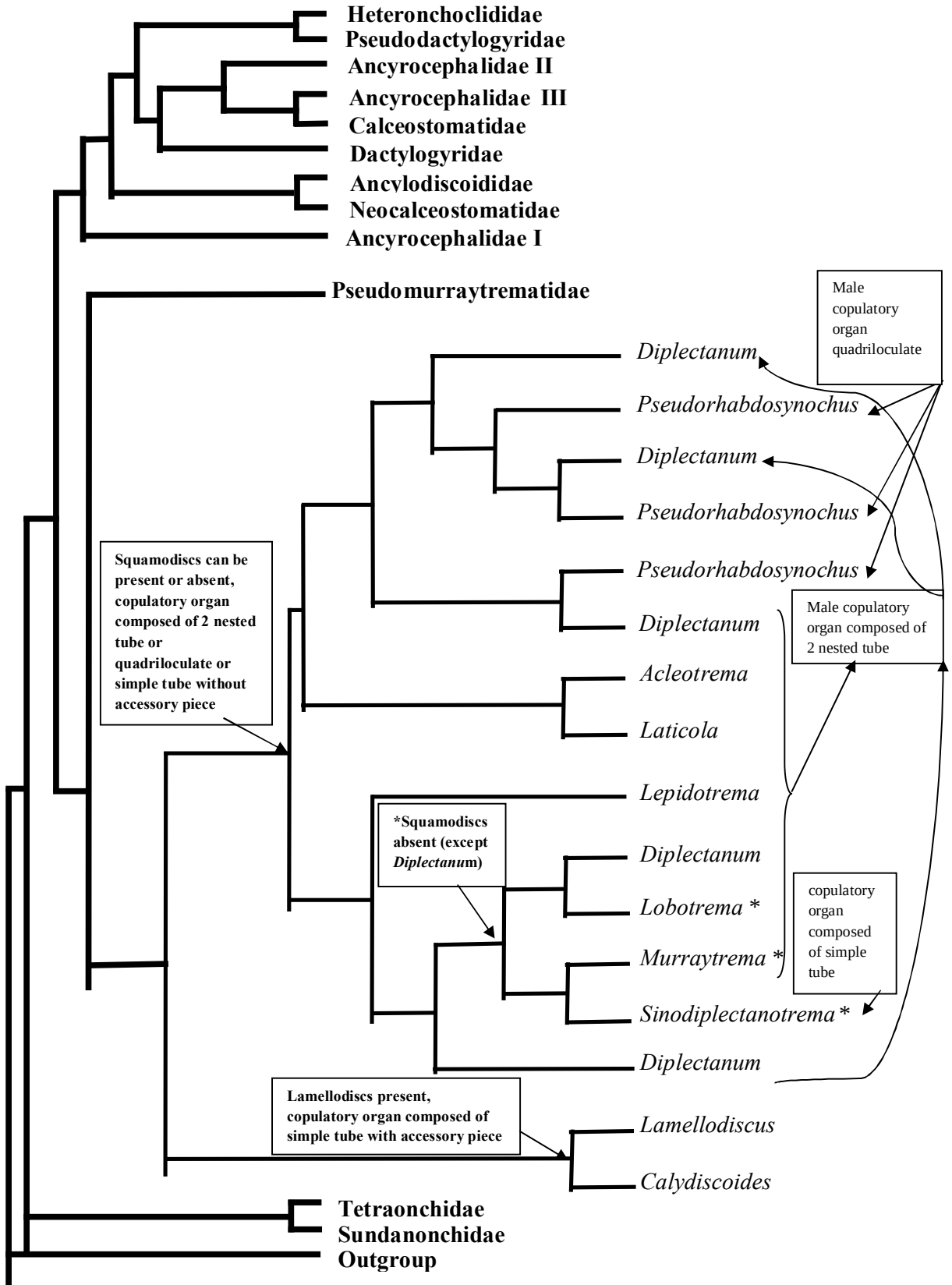


Figure 4.8 Interrelationships within the Diplectanidae Bychowsky, 1957 with morphological characteristics of different genera (*using NJ tree as example).

4.4.2.2 *Lobotrema*, *Lepidotrema*, *Murraytrema*, *Sinodiplectanotrema* & *Diplectanum* (SubGroup 2)

Within SubGroup 2, there are two species of *Lobotrema*, *L. sciaenae* Bychowsky & Nagibina, 1977 and *Lobotrema* sp., *Lepidotrema longipenis* Yamaguti, 1934, *Murraytrema pricei* Bychowsky, 1977 (=*M. bychowskyi*), three *Diplectanum* spp., *D. umbrinum* Tripathi, 1955, *D. blairense* Gupta & Khanna, 1974 (=*Paradiplectanum blairense*) and *D. sillagonum* Tripathi, 1957 (=*Paradiplectanum sillagonum*) and two *Sinodiplectanotrema* spp., *S. malayanum* Lim, Tan & Gibson, 2010 and *Sinodiplectanotrema* sp. HGY. It should be noted that *Sinodiplectanotrema* has been officially re-assigned to Diplectanidae (see Lim *et al.*, 2010) from Ancyrocephalidae (see Zhang, 2001). Although Wu *et al.* (2007) noted the possibility that *Sinodiplectanotrema* was a diplectanid based on molecular data, *Sinodiplectanotrema* was not re-assign to the Diplectanidae. Lim *et al.* (2010) has provided both morphological and molecular evidences that *Sinodiplectanotrema* belongs to the Diplectanidae (see also Appendix E).

Lobobtrema and *Murraytrema* in SubGroup 2 are members of the subfamily Murraytrematoidinae Oliver, 1982 which do not possess any accessory adhesive organs (squamodiscs or lamellodiscs). The two *Sinodiplectanotrema* species analysed in this study are shown to be closely related to *Murraytrema* and *Lobotrema* (see Figs. 4.5, 4.6 & 4.7). In a study on *Sinodiplectanotrema* based on morphological and molecular data, Lim *et al.* (2010) had also observed that *Sinodiplectanotrema* is morphologically similar to members of Murraytrematoidinae in lacking squamodiscs and lamellodiscs.

4.4.2.3 *Lamellodiscus* & *Calydiscoides* (SubGroup 3)

SubGroup 3 corresponds to the subfamily Lamellodiscinae (*sensu* Domingues & Boeger, 2008) where it consists of four species of *Lamellodiscus*, *L. pagrosomi* Murray, 1931, *L. spari* Zhukov, 1970, *L. japonicus* Ogawa & Eugusa, 1978 and *L. acanthopagri* Roubal, 1981 and two *Calydiscoides* spp., *C. indianus* Karyakarte & Das, 1978 and *Calydiscoides* sp. (Figs. 4.5, 4.6 & 4.7). These *Lamellodiscus* and *Calydiscoides* in SubGroup 3 are characterised by the presence of lamellodiscs instead of squamodiscs and copulatory organ of simple tube with accessory piece (Fig. 4.8).

4.4.3 Ancyrocephalidae groups (Figs. 4.9, 4.10 & 4.11)

In MP, ML and NJ relationship trees, the ancyrocephalid monogeneans are split into three groups which in Figs. 4.9, 4.10 & 4.11 are depicted as Ancyrocephalidae I, Ancyrocephalidae II and Ancyrocephalidae IIIï Calceostomatidae group. The members of the three different groups of ancyrocephalid are discussed below.

4.4.3.1 Ancyrocephalidae I

This group is made up of ancyrocephalid species of *Actinocleidus*, *Urocleidus*, *Ancyrocephalus*, *Cleidodiscus* and *Onchocleidus*. The members of Group 3 are all freshwater monogeneans but from different biogeographical regions (see Table 2.4). Could this close relationship be due to their freshwater origin? or are members of this group brought together because they are closely related. In this analysis only one species per genus is available for analysis (Figs. 4.9, 4.10 & 4.11) and more species are needed for a proper analysis of the relationships within this group.

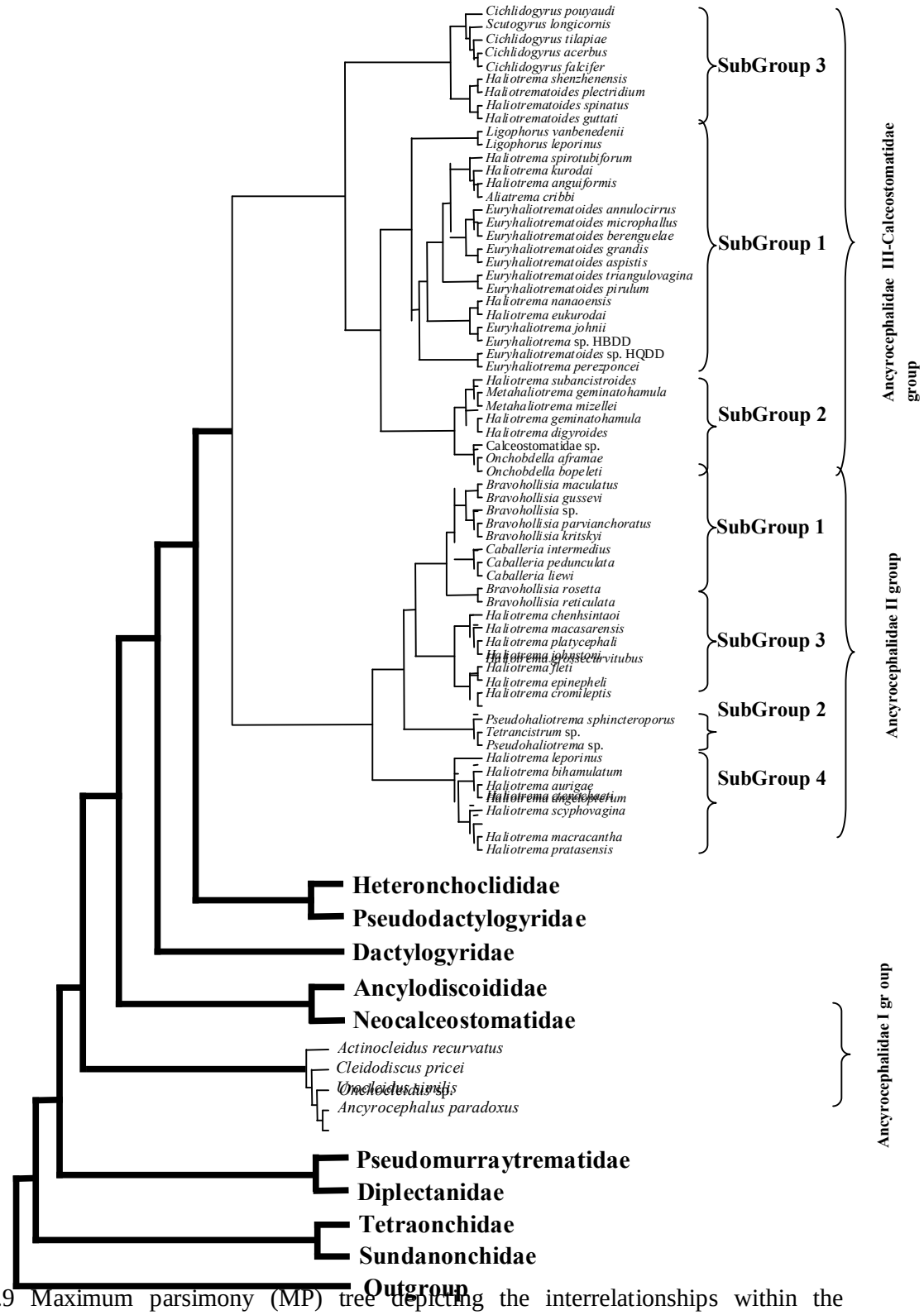


Figure 4.9 Maximum parsimony (MP) tree depicting the interrelationships within the Ancyrocephalidae groups (*sensu* Bychowsky & Nagibina, 1978; Gusev, 1978).

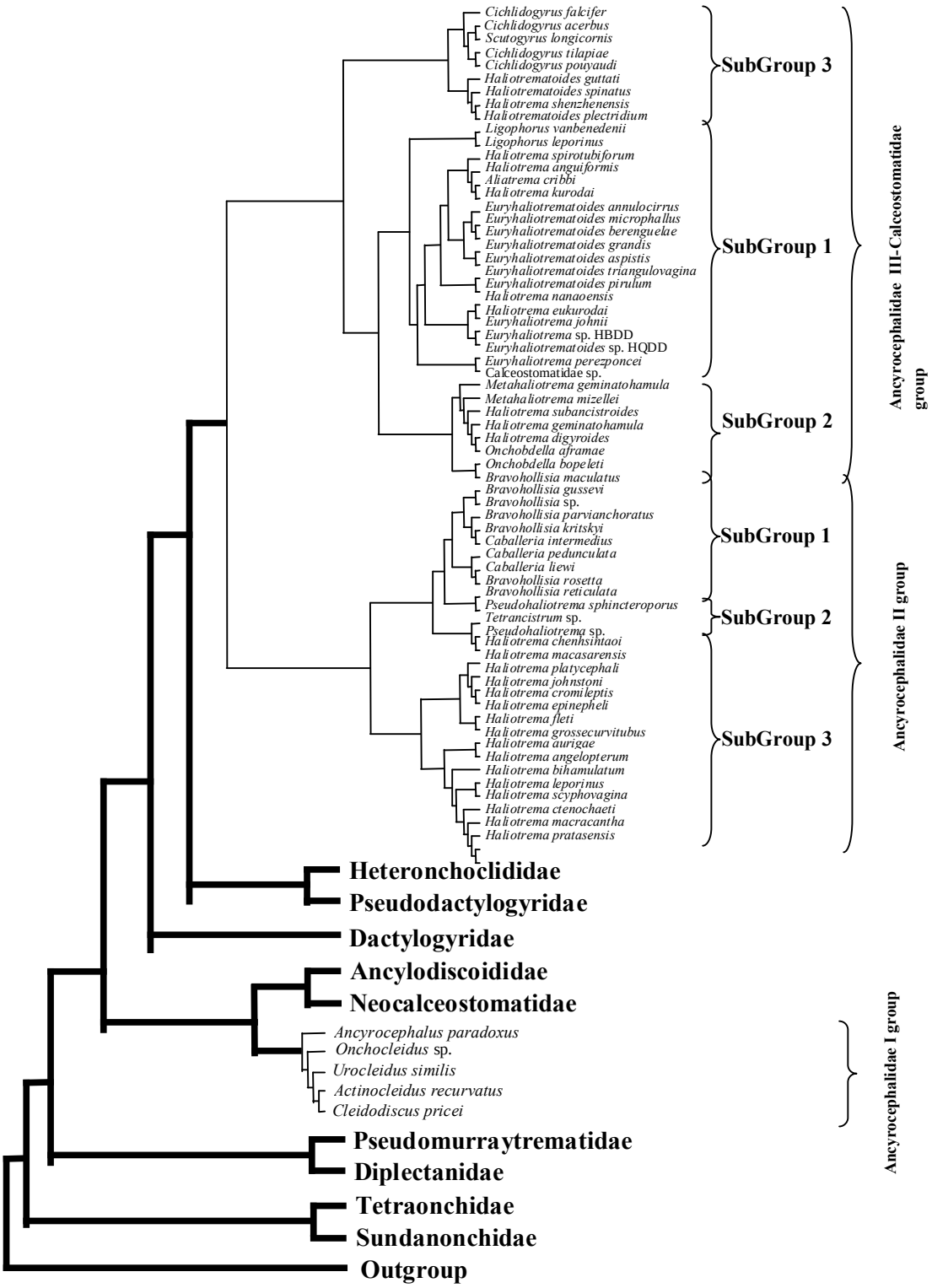


Figure 4.10 Maximum likelihood (ML) tree depicting the interrelationships within the Ancyrocephalidae groups (*sensu* Bychowsky & Nagibina, 1978; Gusev, 1978).

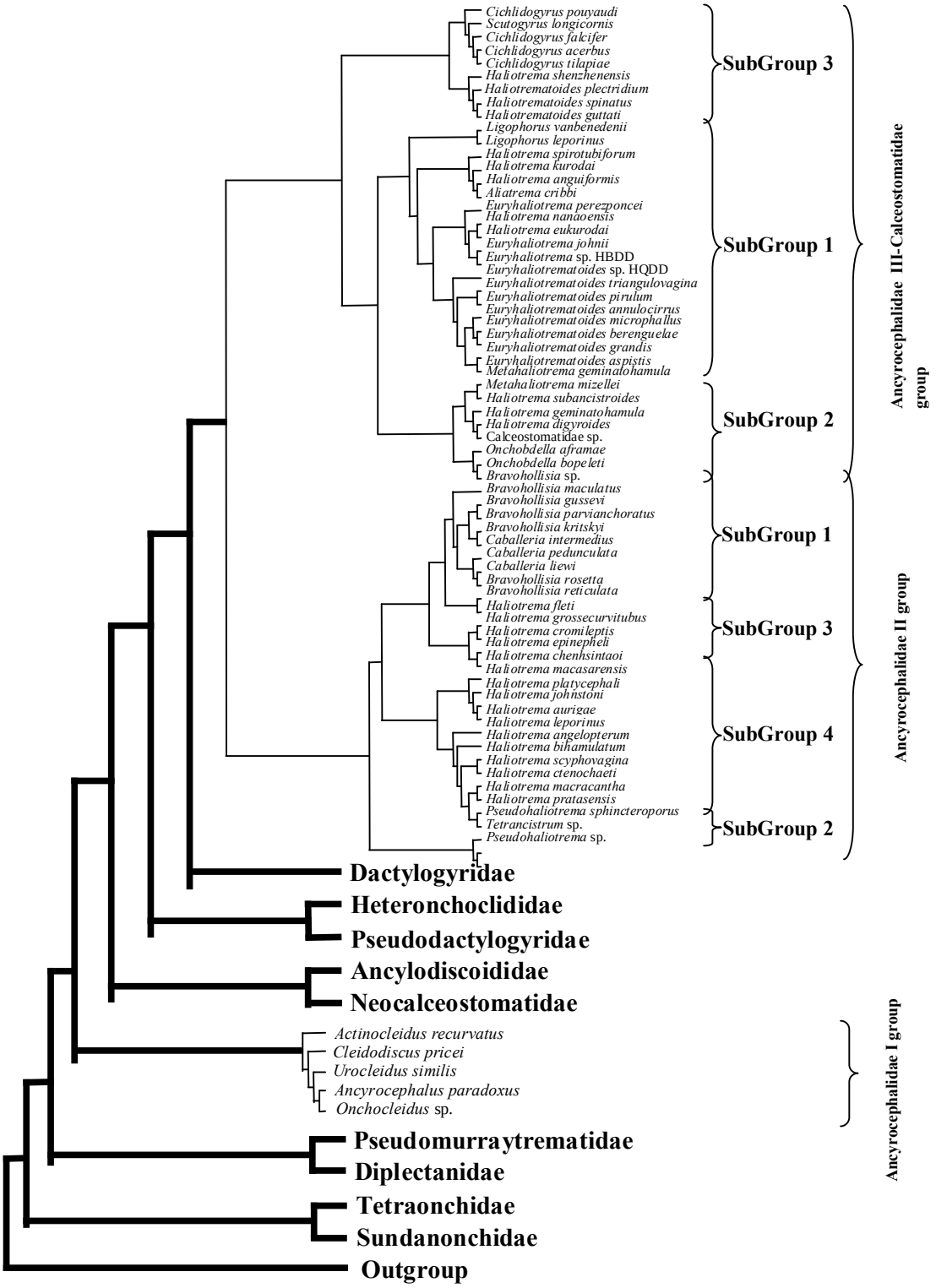


Figure 4.11 Neighbour-joining (NJ) tree depicting the interrelationships within the Ancyrocephalidae groups (*sensu* Bychowsky & Nagibina, 1978; Gusev, 1978).

4.4.3.2 Ancyrocephalidae II

The ancyrocephalids genera in this group include *Bravohollisia*, *Caballeria*, *Haliotrema*, *Pseudohaliotrema* and *Tetrancistrum*. Within Ancyrocephalidae II, four subGroups can be observed in MP and NJ trees (Figs. 4.9 & 4.11) while there are three subGroups in ML tree (Fig. 4.10).

4.4.3.2.1 *Bravohollisia* & *Caballeria* (SubGroup 1)

SubGroup 1 consists of seven *Bravohollisia* spp., *B. maculatus* Venkatanarasaiah, 1984, *B. gussevi* Lim, 1995, *B. parvianchoratus* Venkatanarasaiah, 1984, *B. kritskyi* Lim, 1995, *B. rosetta* Lim, 1995, *B. reticulata* Lim, 1995 and *Bravohollisia* sp. and three *Caballeria* spp., *C. pedunculata* Bychowsky & Nagibina, 1970, *C. intermedius* Lim, 1995 and *C. liewi* Lim, 1995. The close relationships of *Bravohollisia* and *Caballeria* have been noted by Lim (1995b) where these two genera share similar morphological characteristics with the presence of haptoral reservoirs, net like structure near tip of anchors and copulatory organ without accessory piece (Fig. 4.12).

Bravohollisia and *Caballeria* are also morphologically different as indicated by the presence of haptoral digits in *Caballeria* (see Lim, 1995b). The present results from analysis of the morphometric data have shown that the sclerotised hard parts can be effectively used to differentiate between the species of *Bravohollisia* and *Caballeria* (see Section 3.2.1.1) and this is supported by results from current molecular analysis showing the *Caballeria* spp. form a monophyletic group which is distinct from *Bravohollisia* spp. (Figs. 4.9, 4.10 & 4.11). Therefore, *Bravohollisia* and *Caballeria* are two distinct genera and suggestion by Wu *et al.* (2007a) to combine *Bravohollisia* and

Caballeria into one genus should be rejected. Wu *et al.* (2007a) also failed to realise *Caballeria* is different from *Bravohollisi*a in possessing haptoral digits (see Lim, 1995b).

4.4.3.2.2 *Pseudohaliotrema* & *Tetrancistrum* (SubGroup 2)

SubGroup 2 consists of members from two genera. They are two *Pseudohaliotrema* spp., *P. sphincteroporus* Yamaguti, 1953 and *Pseudohaliotrema* sp. as well as a *Tetrancistrum* sp. (Figs. 4.9, 4.10 & 4.11). The *Pseudohaliotrema* and *Tetrancistrum* can be observed to possess similar morphological characteristics in ventral anchors with broad, expanded and massive roots and copulatory organ with accessory piece (Fig. 4.12).

4.4.3.2.3 *Haliotrema* (SubGroup 3/SubGroup 3 and SubGroup 4)

Ancyrocephalidae II consists of 16 *Haliotrema* spp. which are either present in SubGroup 3 in ML tree (Fig. 4.10) or in SubGroup 3 and SubGroup 4 in MP and NJ trees (Figs. 4.9 & 4.11). These 16 *Haliotrema* spp. are *H. fleti* Young, 1968, *H. grossecurvitubus* Li & Chen, 2005, *H. cromileptis* Young, 1968, *H. epinepheli* Young, 1968, *H. chenhsintaoi* Chang, 2001, *H. macasarensis* Yamaguti, 1963, *H. platycephali* Yin & Sproston, 1948, *H. johnstoni* Bychowsky & Nagibina, 1970, *H. aurigae* Yamaguti, 1968, *H. leporinus* Sun, Kritsky & Yang, 2007, *H. angelopterum* Plaisance, Bouamer & Morand, 2004, *H. bihamulatum* Zhang, 2001, *H. scyphovagina* Yamaguti, 1968, *H. ctenochaeti* Young, 1968, *H. macracantha* Yamaguti, 1968 and *H. pratasensis* Sun, Kritsky & Yang, 2007.

This *Haliotrema* group in Ancyrocephalidae II could most probably be the true *Haliotrema* group as it consists of the highest number of *Haliotrema* spp. compare to

any other subGroups within the Ancyrocephalidae groups (Ancyrocephalidae I, Ancyrocephalidae II and Ancyrocephalidae IIIï Calceostomatidae group) (Figs. 4.9, 4.10 & 4.11). However, the DNA sequences of the type species of *Haliotrema*, *H. australe* Johnston & Tiegs, 1922 is needed to further confirm this.

4.4.3.3 Ancyrocephalidae III – Calceostomatidae group

In MP, ML and NJ relationship trees generated, the memberships within this group are consistent and three subGroups are observed (Figs. 4.9, 4.10 & 4.11).

4.4.3.3.1 *Euryhaliotrematoides*, *Euryhaliotrema*, *Ligophorus*, *Aliatrema* & *Haliotrema* (SubGroup 1)

SubGroup 1 includes all *Euryhaliotrematoides* spp., *Euryhaliotrema* spp., five species of *Haliotrema, H. spirotubiforum* Zhang, 2001 and *H. anguiformis* Zhang, 2001, *H. kurodai* Ogawa & Egusa, 1978, *H. nanaoensis* Yao, Wang, Xia & Chen, 1998, *H. eukuro*dai Zhang & Ding, 1994, two *Ligophorus* spp., *L. vanbenedenii* Parona & Perugia, 1890 and *L. leporinus* Zhang & Ji, 1981 and *Aliatrema cribbi* Plaisance & Kritsky, 2004. These genera in SubGroup 1 (with the exception of *Haliotrema* and *Ligophorus* spp.) can be observed to possess similar morphological characteristic where the base of copulatory organ expanded to form bulb or funnel shape, i.e. *Euryhaliotrema* (bulb shape), *Aliatrema* (funnel shape), *Euryhaliotrematoides* (funnel shape) (Fig. 4.12).

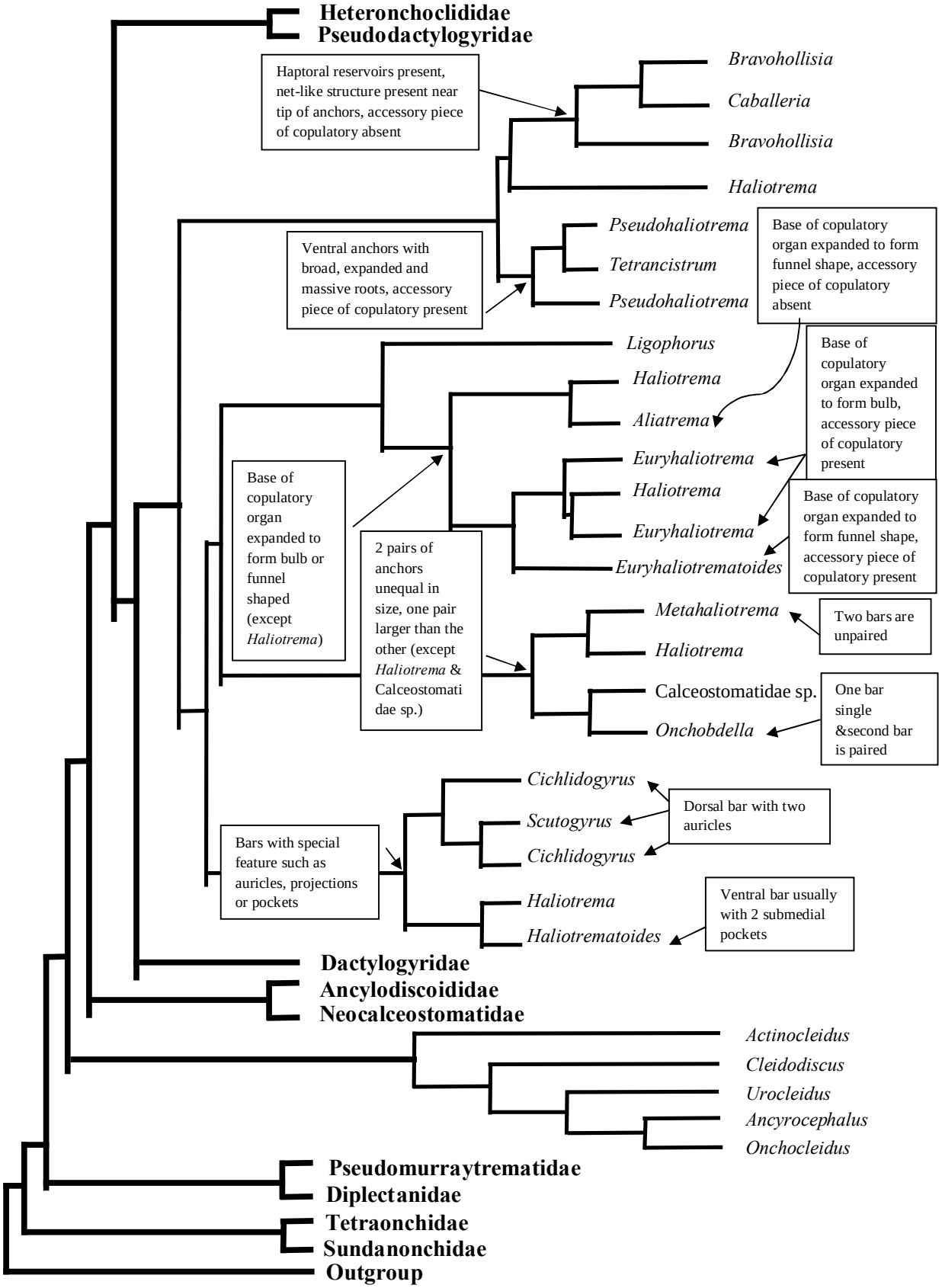


Figure 4.12 Interrelationships within the Ancyrocephalidae (*sensu* Bychowsky & Nagibina, 1978; Gusev, 1978) with morphological characteristics of different genera (*using NJ tree as example).

4.4.3.3.2 *Metahaliotrema*, *Onchobdella*, *Haliotrema* & Calceostomatidae (SubGroup 2)

SubGroup 2 consists of two *Metahaliotrema* spp., *M. geminatohamula* Pan, Zhang & Ding, 1995 and *M. mizellei* Venkatanarasaiah, 1981, two species of *Onchobdella*, *O. aframae* Paperna, 1968 and *O. bopeleti* Bilong Bilong & Euzet, 1995, three *Haliotrema* spp., *H. subancistroides* Zhang, 2001, *H. geminatohamula* Bychowsky & Nagibina, 1970 and *H. digyroides* Zhang, 2001 and a Calceostomatidae sp. The main morphological characteristic share by the members of SubGroup 2 (with the exception in *Haliotrema* spp. and Calceostomatidae sp.) are two pairs of anchors unequal in size where one pair is larger than the other as found in *Metahaliotrema* and *Onchobdella* spp. (Fig. 4.12).

4.4.3.3.3 *Cichlidogyrus*, *Haliotrematoides*, *Scutogyrus* & *Haliotrema* (SubGroup 3)

SubGroup 3 includes four species of *Cichlidogyrus*, *C. pouyaudi* Pariselle & Euzet, 1994, *C. falcifer* Dossou & Birgi, 1984, *C. acerbus* Dossou, 1982 and *C. tilapiae* Paperna, 1960, three *Haliotrematoides* spp., *H. plectridium* Kritsky & Mendoza-Franco, 2009, *H. spinatus* Kritsky & Mendoza-Franco, 2009 and *H. guttati* Garcia-Vargas, Fajer-Avila & Lamothe-Argumedo, 2008, *Scutogyrus longicornis* Paperna & Thurston, 1969 and *Haliotrema shenzhenensis* Wang, Liu & Zhou, 2003. All the members (except *Haliotrema shenzhenensis*) in SubGroup 3 share the main morphological characteristic where they possess bars with special feature such as auricles, projections or pockets, i.e. two auricles on dorsal bar of *Cichlidogyrus* and *Scutogyrus* and two submedial pockets on ventral bar of *Haliotrematoides* (Fig. 4.12).

4.4.3.3.4 Summation for Ancyrocephalidae III – Calceostomatidae group

The *Haliotrema* spp. in Ancyrocephalidae IIIï Calceostomatidae group are shown to cluster with members from genus *Aliatrema* Plaisance & Kritsky, 2004, *Euryhaliotrema* Kritsky & Boeger, 2002, *Haliotrematoides* Kritsky, Yang & Sun, 2009 and *Metahaliotrema* Yamaguti, 1953 (Figs. 4.9, 4.10 & 4.11). These genera are erected to accommodate species previously recognised as *Haliotrema* spp. in studies where revisions and transfer of *Haliotrema* spp. were done to restrict the size of the genus *Haliotrema* (Kritsky & Boeger, 2002; Plaisance & Kritsky, 2004; Kritsky, Yang & Sun, 2009).

The current clustering pattern of the *Haliotrema* spp. in Ancyrocephalidae III ï Calceostomatidae group with *Aliatrema*, *Euryhaliotrema*, *Haliotrematoides* and *Metahaliotrema* indicates a high possibility that these *Haliotrema* spp. could be misidentified. For instance, *H. spirotubiforum*, *H. kurodai* and *H. anguiformis* which are found clustered with *Aliatrema* could be *Aliatrema* species mistakenly identified as *Haliotrema*. Similarly, *H. nanaoensis* and *H. eukurodai* which are grouped with the *Euryhaliotrema* group could be *Euryhaliotrema* species whilst *H. subancistroides*, *H. geminahamula* and *H. digyroides* which are found clustered with *Metahaliotrema* group could be *Metahaliotrema* species. Lastly, *H. shenzhenensis* which is clustered with *Haliotrematoides* group could be a *Haliotrematoides* species. Thus, results from current molecular analyses indicate that there is a need to re-examine the status of these *Haliotrema* spp. mentioned above.

4.4.4 Ancylodiscoididae – Neocalceostomatidae group (Figs. 4.13, 4.14 & 4.15)

In this section, the interrelationships among the 33 members within the Ancylodiscoididae and two members from Neocalceostomatidae are discussed. Within the Ancylodiscoididaeï Neocalceostomatidae group, five subGroups can be observed in the MP, ML and NJ trees (Figs. 4.13, 4.14 & 4.15).

4.4.4.1 *Thaparocleidus* (SubGroup 1)

SubGroup 1 consists of *Thaparocleidus cochleavagina* Gusev & Strelkov, 1960, *T. omegavagina* Hwang, 1964, *T. obscura* Gusev & Strelkov, 1960, *T. mutabilis* Gusev & Strelkov, 1960, *T. asoti* Yamaguti, 1937, *T. magnicirrus* Gusev & Strelkov, 1960, *T. vistulensis* Siwak, 1932, *T. siluri* Zandt, 1924, *T. infundibulovagina* Yamaguti, 1942, *T. varicus* Akhmerov, 1952 and *T. notopterus* Jain, 1955 (only in NJ tree; Fig. 4.15).

4.4.4.2 *Cornudiscoides*, *Pseudancylodiscoides* & *Bifurcohaptor* (SubGroup 2)

SubGroup 2 includes *Cornudiscoides proximus* Gusev, 1976, *C. facicirrus* Lim, 1987 and *Cornudiscoides* sp., three *Pseudancylodiscoides* spp., and *Bifurcohaptor lanchangensis* Lim, 1987. These three genera in SubGroup 2 share similar morphological characteristics such as dorsal anchors either without roots (*Bifurcohaptor*) or only with inner root (*Cornudiscoides*), ventral anchor smaller than dorsal anchors and a blind sac-like seminal vesicle (Fig. 4.9). Members of SubGroup 1 (*Thaparocleidus* spp.; see above) and SubGroup 2 which possess blind sac-like seminal vesicle are closely related and form sister group in MP and NJ trees (Figs. 4.13 & 4.15).

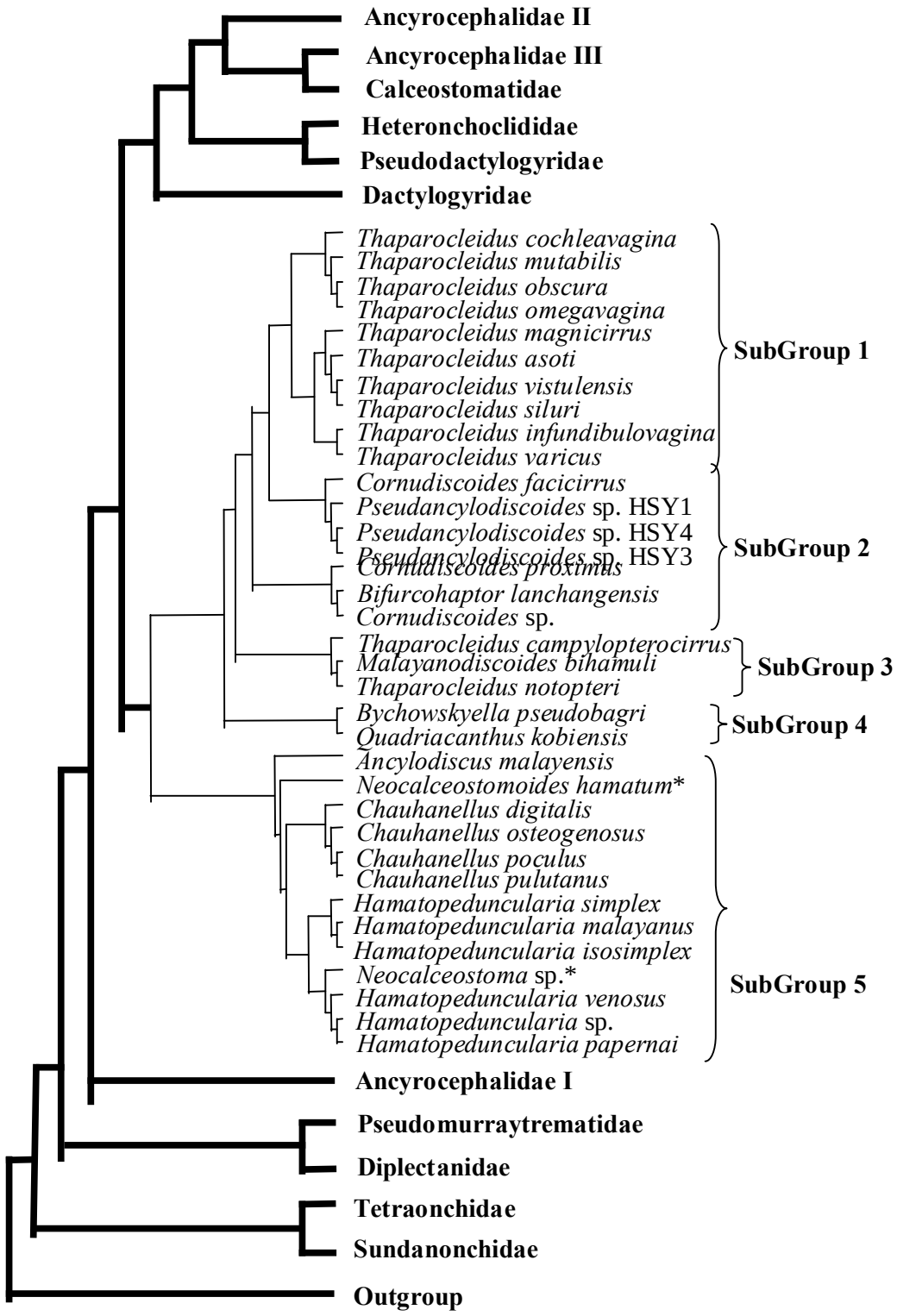


Figure 4.13 Maximum parsimony (MP) tree depicting the interrelationships within the Ancylodiscoididae-Neocalceostomatidae group (*sensu* Lim, Timofeeva & Gibson, 2001) (*=members of Neocalceostomatidae).

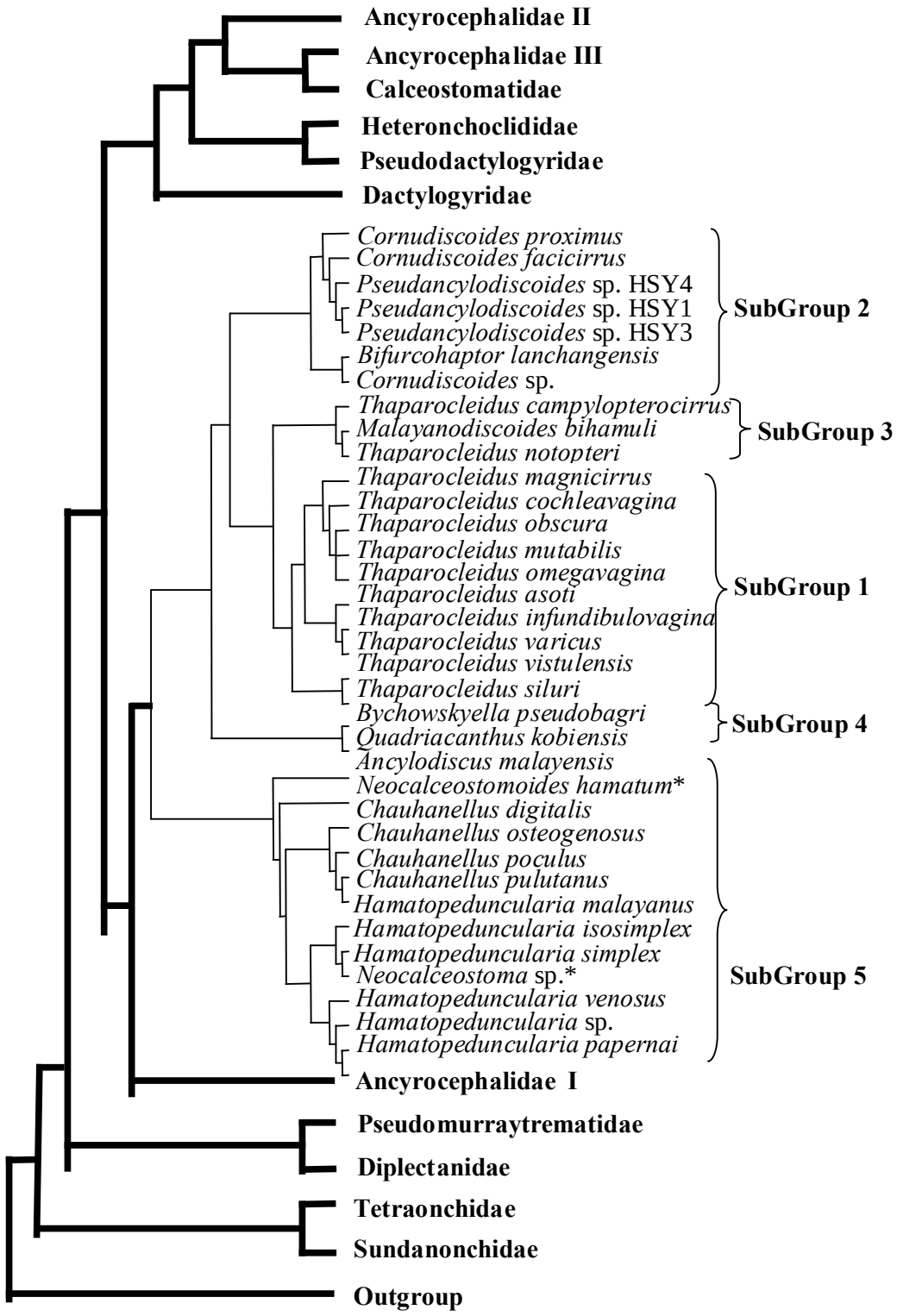


Figure 4.14 Maximum likelihood (ML) tree depicting the interrelationships within the Ancylodiscoididae-Neocalceostomatidae group (*sensu* Lim, Timofeeva & Gibson, 2001) (*=members of Neocalceostomatidae).

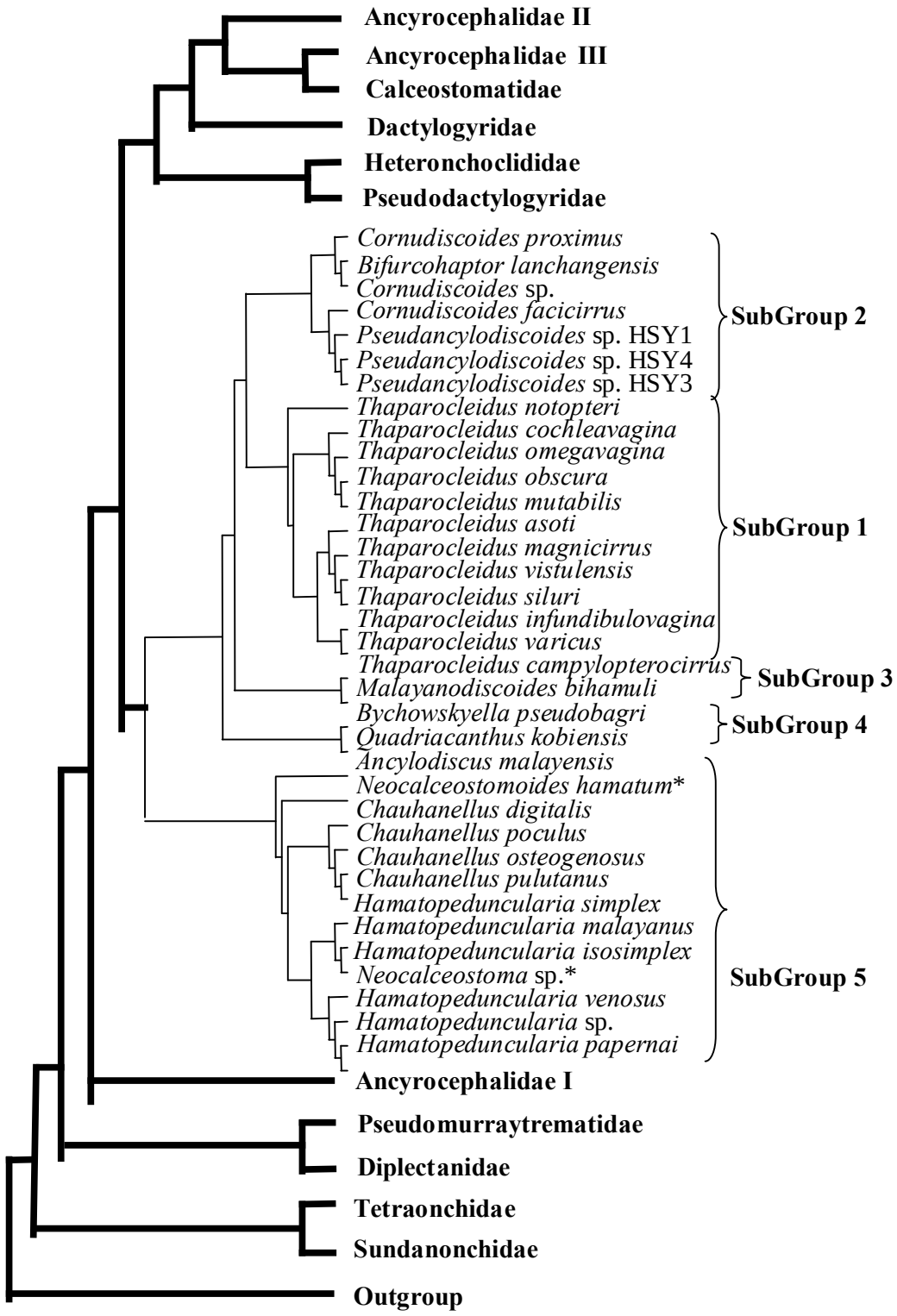


Figure 4.15 Neighbour-joining (NJ) tree depicting the interrelationships within the Ancylodiscoididae-Neocalceostomatidae group (*sensu* Lim, Timofeeva & Gibson, 2001) (*=members of Neocalceostomatidae).

4.4.4.3 *Malayanodiscoide*s & *Thaparocleidus* (SubGroup 3)

In SubGroup 3, there are two *Thaparocleidus* spp. (*T. campylopterocirrus* Zeng, 1988 and *T. notopterus*) in MP and ML trees (Figs. 4.13 & 4.14) while only *T. campylopterocirrus* is present in SubGroup 3 of NJ tree (Fig. 4.15). These *Thaparocleidus* spp. are clustered with *Malayanodiscoide*s *bihamuli* Lim & Furtado, 1986 (Figs. 4.13, 4.14 & 4.15). The presence of *T. campylopterocirrus* and *T. notopterus* in SubGroup 3 indicates the possible non-monophyly of the *Thaparocleidus* which need further investigation in future studies by analysing more sequences from *Thaparocleidus* spp. For *Malayanodiscoide*s *bihamuli* which is from a monotypic genus, its relationship with other ancylodiscoidids can only be further confirmed when more species from this genus are available.

4.4.4.4 *Bychowskyella* & *Quadriacanthus* (SubGroup 4)

The two members which consistently present in SubGroup 4 are *Bychowskyella pseudobagri* Achmerow, 1952 and *Quadriacanthus kobiensis* Ha Ky, 1968 (Figs. 4.13, 4.14 & 4.15). These two members are characterised by their dorsal and ventral anchors which are without roots and seminal vesicle a dialation of vas deferens (*Bychowskyella* and *Quadriacanthus*) (Fig. 4.16).

4.4.4.5 *Chauhanellus*, *Hamatopeduncularia*, *Ancylodiscus*, *Neocalceostomoides* & *Neocalceostoma* (SubGroup 5)

In SubGroup 5, there are four *Chauhanellus* spp., *C. digitalis* Lim, 1994, *C. poculus* Lim, 1994, *C. osteogeneiosi* Lim, 1994 and *C. pulutanus* Lim, 1994, six *Hamatopeduncularia* spp., *H. simplex* Bychowsky & Nagibina, 1969, *H. malayanus*

Lim, 1996, *H. isosimplex* Lim, 1996, *H. venosus* Lim, 1996, *H. papernai* Lim, 1996 and *Hamatopeduncularia* sp. and *Ancylodiscus malayensis* Lim, 1994 of Ancylodiscoididae and *Neocalceostomoides hamatum* Lim, 1995 & *Neocalceostoma* sp. from Neocalceostomatidae (Figs. 4.13, 4.14 & 4.15).

The *Chauhanellus* and *Hamatopeduncularia* spp. are shown to be closely related and form sister groups (Figs. 4.13, 4.14 & 4.15). This close relationship of *Chauhanellus* and *Hamatopeduncularia* is expected since both genera possess similar morphological characteristics where they possess anchors with expanded outer roots, bars with protuberances and seminal vesicle a dialation of vas deferens but differ in the presence of extensible haptoral digits on haptor of *Hamatopeduncularia* but not in *Chauhanellus* (Lim, 1995) (Fig. 4.16). Current results also showed that *Ancylodiscus malayensis* is not found clustered within the *Chauhanellus* and *Hamatopeduncularia* group. This is most probably due to *Ancylodiscus* being unique in having two seminal vesicles, both the blind sac-like seminal vesicle and the dactylogyrid type seminal vesicle (a dialation of vas deferens) (Fig. 4.16).

Neocalceostomoides hamatum and *Neocalceostoma* sp. from Neocalceostomatidae are also found in SubGroup 5 with the Ancylodiscoididae (Figs. 4.13, 4.14 & 4.15) although both families are morphologically different (Neocalceotsomatidae possess 2 anchors while Ancylodiscoididae possess 4 anchors). This current position of Neocalceostomatidae could be due to only two neocalceostomatids are included in this study (see Table 2.4). Thus more members of Neocalceostomatidae are needed to ascertain its relationships with the other dactylogyridean families in future studies.

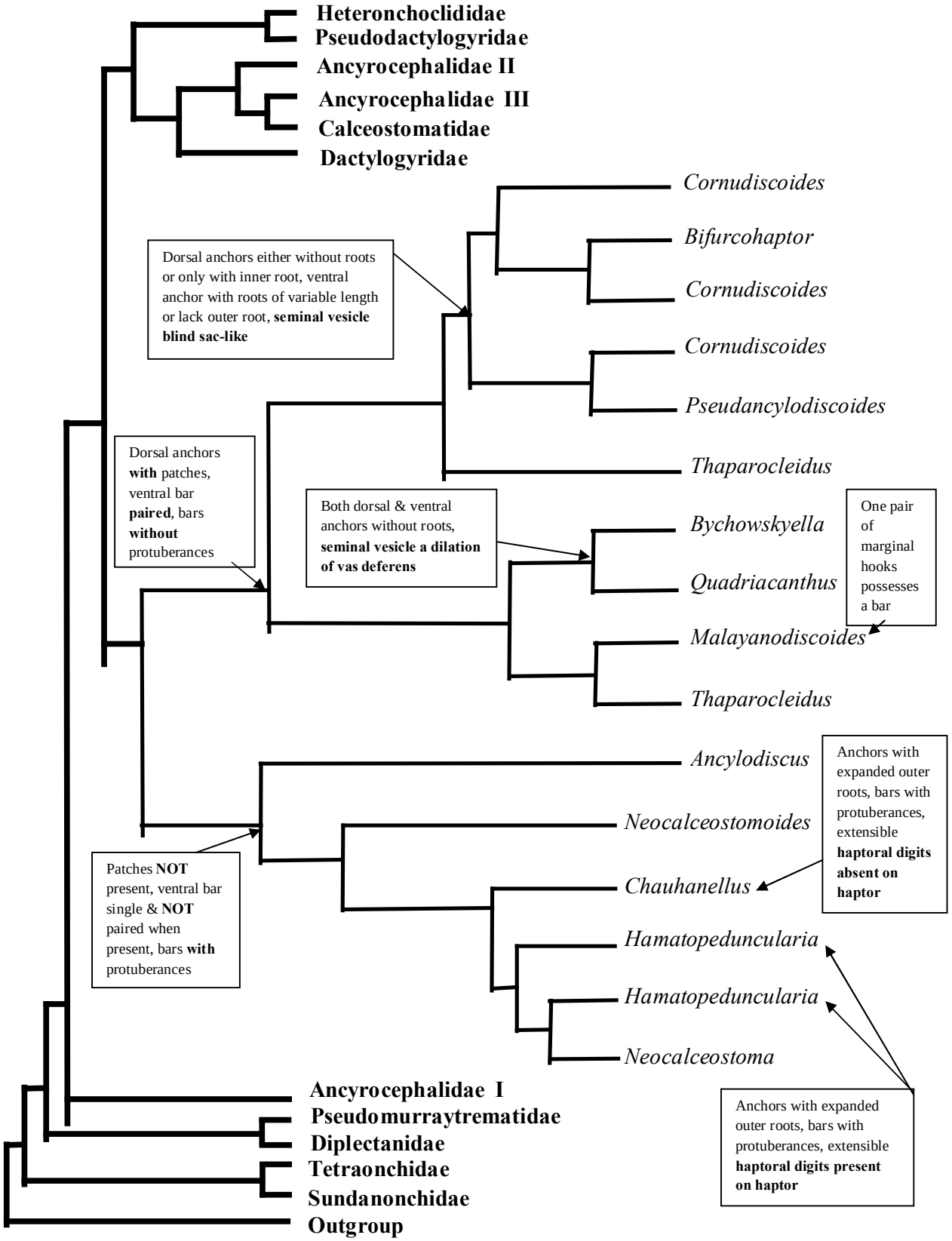


Figure 4.16 Interrelationships within the Ancylodiscoididae (*sensu* Lim, Timofeeva & Gibson, 2001) with morphological characteristics of different genera (*using NJ tree as example).

4.4.5 Dactylogyridae group (Figs. 4.17, 4.18 & 4.19)

This group consists of 30 *Dactylogyrus* spp. and one *Dactylogyroides longicirrus* Tripathi, 1959 from the family Dactylogyridae (*sensu* Bychowsky & Nagibina, 1978; Timofeeva, Gerasev & Gibson, 1997; Lim, 1998; Lim *et al.*, 2001). The other dactylogyrid genera such as *Dogielus* and *Thaprogyrus* are not included in this study due to their low numbers of species and to date there are no partial 28S rDNA sequences for these monogeneans in the Genbank.

Dactylogyroides longicirrus can be observed to consistently form sister group with all the *Dactylogyrus* spp. analysed in this study (Figs. 4.17, 4.18 & 4.19). The interrelationships among the *Dactylogyrus* spp. within the Dactylogyridae group are shown to be varied in the NJ, MP and ML relationship trees (Figs. 4.17, 4.18 & 4.19). In the NJ tree, there are four subGroups within the Dactylogyridae group (Fig. 4.19) while in MP and ML trees, five subGroups can be observed (see Figs. 4.17 & 4.18). In the MP and ML trees, all the *Dactylogyrus* spp. possess similar clustering except for the position of *D. quadribrachiatus* where it is found in different subGroups within the MP and ML trees (Figs 4.17 & 4.18).

4.4.5.1 Dactylogyridae in NJ tree

In NJ tree (Fig. 4.19), SubGroup 1 consists of *Dactylogyrus* spp. from Oriental region (*D. pekinensis* Gusev, 1962, *D. petruschewskyi* Gusev, 1955, *D. parabramis* Achmerow, 1952, *D. kikuchii* Gusev, 1965 and *D. hypophalmichthys* Achmerow, 1952) and Europe (*D. hemiamphibothrium* Ergens, 1956, *D. cryptomeres* Bychowsky, 1934, *D. nanus* Dogiel & Bychowsky, 1934 and *D. sphyrna* Linstow, 1878).

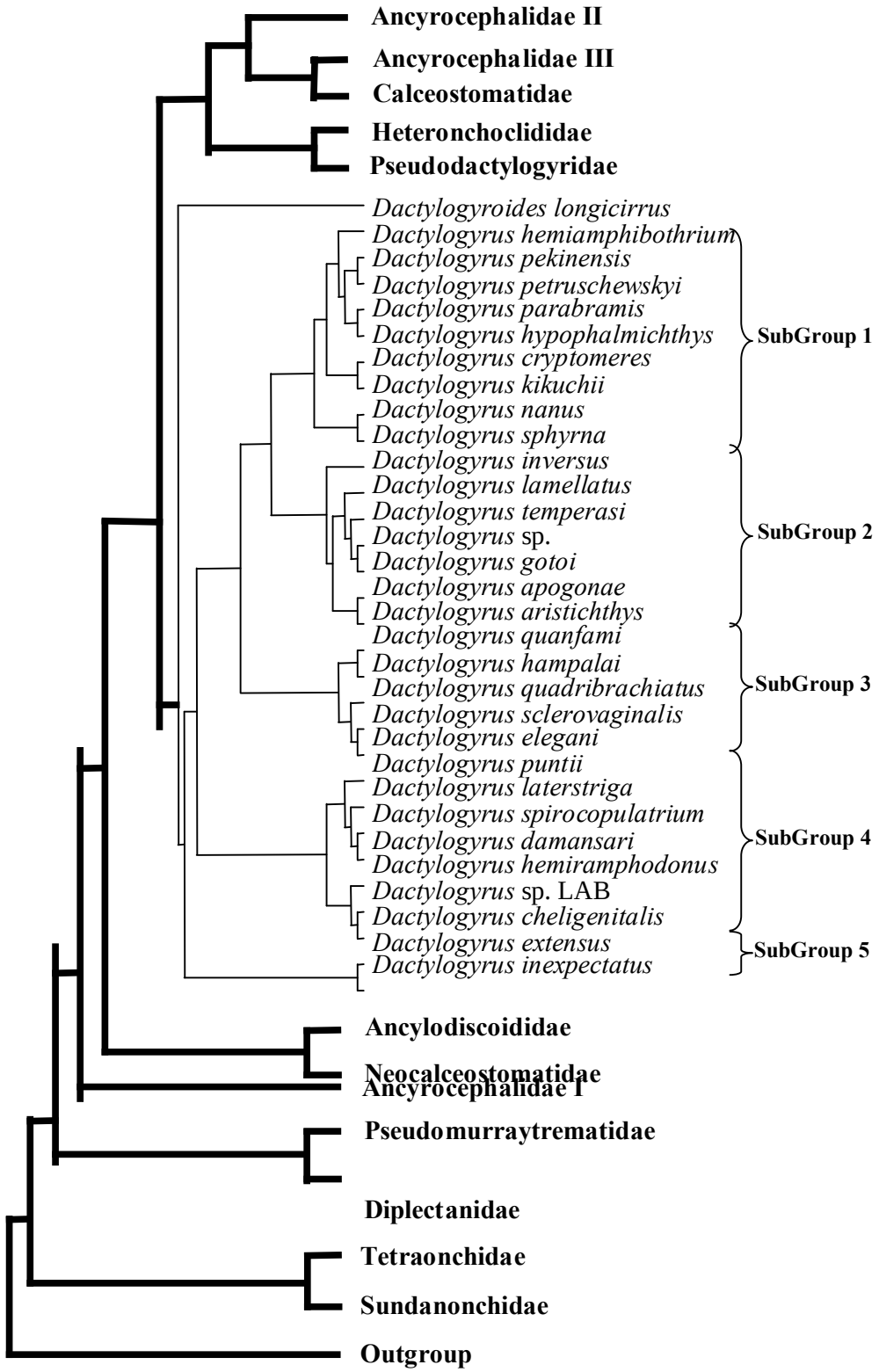


Figure 4.17 Maximum parsimony (MP) tree depicting the interrelationships within the Dactylogyridae group (*sensu* Bychowsky & Nagibina, 1978; Lim, 1998).

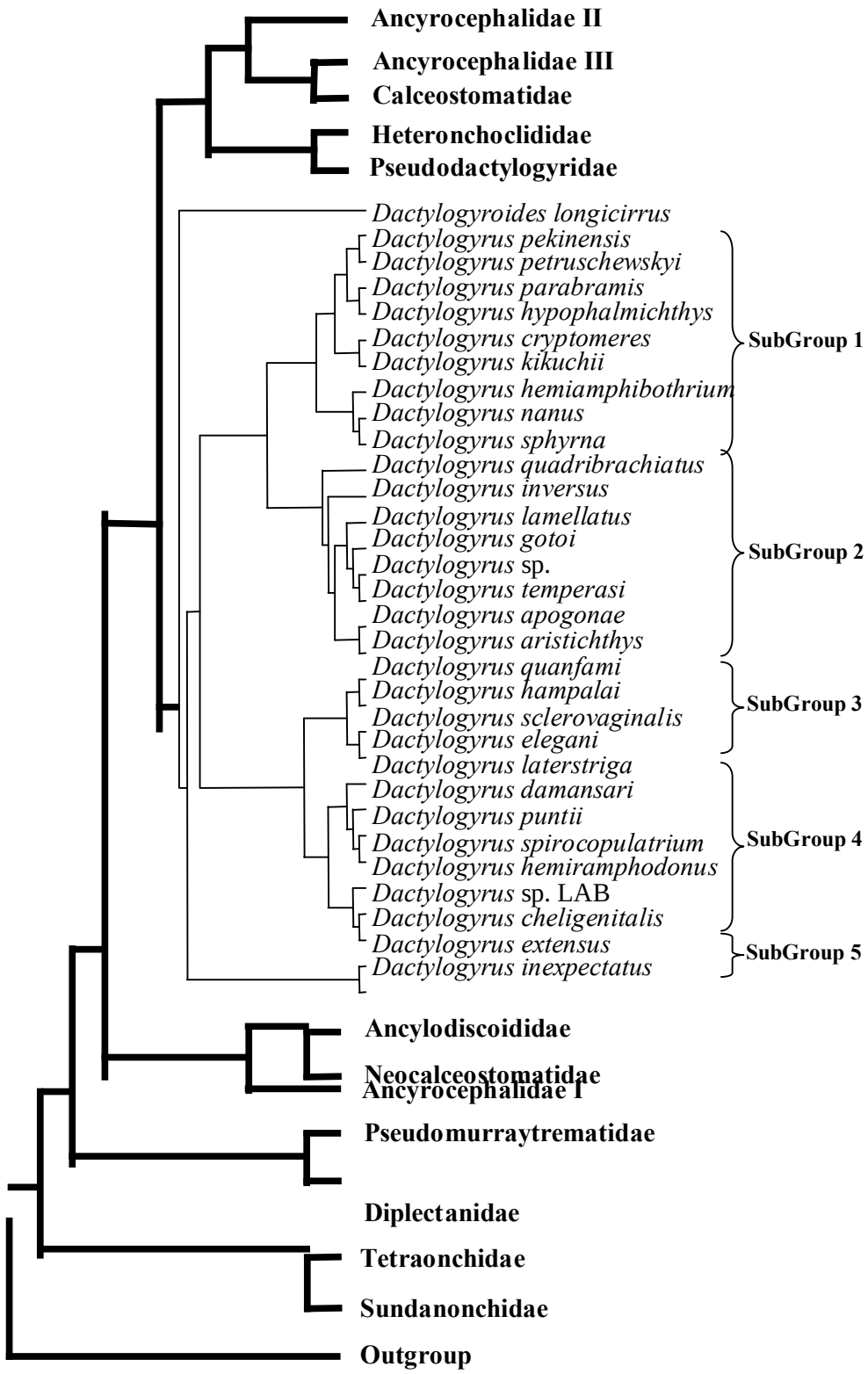
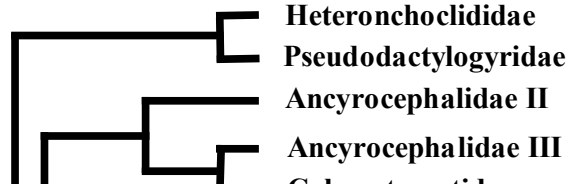


Figure 4.18 Maximum likelihood (ML) tree depicting the interrelationships within the Dactylogyridae group (*sensu* Bychowsky & Nagibina, 1978; Lim, 1998).



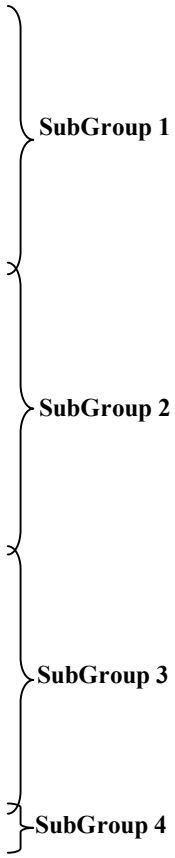


Figure 4.19 Neighbour-joining (NJ) tree depicting the interrelationships within the Dactylogyridae group (*sensu* Bychowsky & Nagibina, 1978; Lim, 1998).

All the *Dactylogyrus* spp. in SubGroups 2 & 3 are from Peninsular Malaysia except for *D. quanfami* Ky, 1971, *D. inversus* Goto & Kikuchi, 1917 and *D. gotoi* Gusev, 1965 which are from China and Japan. *D. extensus* Muller & Van Cleave, 1932 and *D. inexpectatus* Izjumova, 1955 in SubGroup 4 are also from Oriental region.

4.4.5.2 Dactylogyridae in MP and ML trees

Similar trends can also be observed in MP and ML trees (Figs. 4.17 & 4.18) where SubGroup 1 consists of *Dactylogyrus* spp. from Oriental origin and Europe, SubGroups 2, 3 & 4 consist of *Dactylogyrus* spp. from Peninsular Malaysia (except for *D. quanfami* Ky, 1971, *D. inversus* Goto & Kikuchi, 1917 and *D. gotoi* Gusev, 1965) while SubGroup 5 consists of *Dactylogyrus* spp. from Oriental region (Figs. 4.17 & 4.18).

4.4.5.3 Summation for Dactylogyridae group

These trends show that *Dactylogyrus* spp. from the same biogeographical region are more related to each other. This could be due to the fact that the lineages of *Dactylogyrus* spp. from different biogeographical regions must have diverged from each other very early in their evolutionary history and subsequently evolve and speciate separately. Ġimková *et al.* (2007) had also shown that the different lineages of *Dactylogyrus* spp. had separated from each other in a very short period of time in their evolutionary history which might also have contributed to the current observed trend in the different groups of *Dactylogyrus* spp. from different biogeographical regions.

4.4.6 Heteronchocleididae – Pseudodactylogyridae group (Figs. 4.20, 4.21 & 4.22)

This group consists of 13 members (from three genera, i.e. *Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus*) and five members (from two genera, i.e. *Pseudodactylogyrus* and *Pseudodactylogyroides*) which correspond to the family Heteronchocleididae and Pseudodactylogyridae, respectively. Three subGroups can be observed in Heteronchocleididae ï Pseudodactylogyridae group (Figs. 4.20, 4.21 & 4.22).

4.4.6.1 *Trianchoratus* (SubGroup 1)

Within SubGroup 1, it can be observed that species with relatively shorter and less sharply recurved anchor points (*T. pahangensis* Lim, 1986, *T. longianchoratus* Tan & Lim, 2009, *T. ophicephali* Lim, 1986 and *T. malayensis* Lim, 1986) are clustered together while species with longer and more sharply recurved anchor points (*T. acleithrium* Lim, 1986, *T. gussevi* Lim, 1986, *T. leerium* Lim, 1986, *T. trichogasterium* Lim, 1986, *T. parvulus* Lim, 1986 and *T. grandis* Lim, 1986) are in another separate cluster (Figs. 4.20, 4.21 & 4.22). Thus this results show the *Trianchoratus* spp. are grouped according to the general shape of their three developed anchors. Similar observations were also noted in previous studies by Lim (1986) and Tan & Lim (2009) based on morphological characteristic of *Trianchoratus* spp. Therefore, the interrelationships of the heteronchocleidids obtained from molecular data (28S rDNA) in this study are congruent with the classification of the heteronchocleidids based on morphological characteristics by Lim (1986), Lim (1989) and Tan & Lim (2009).

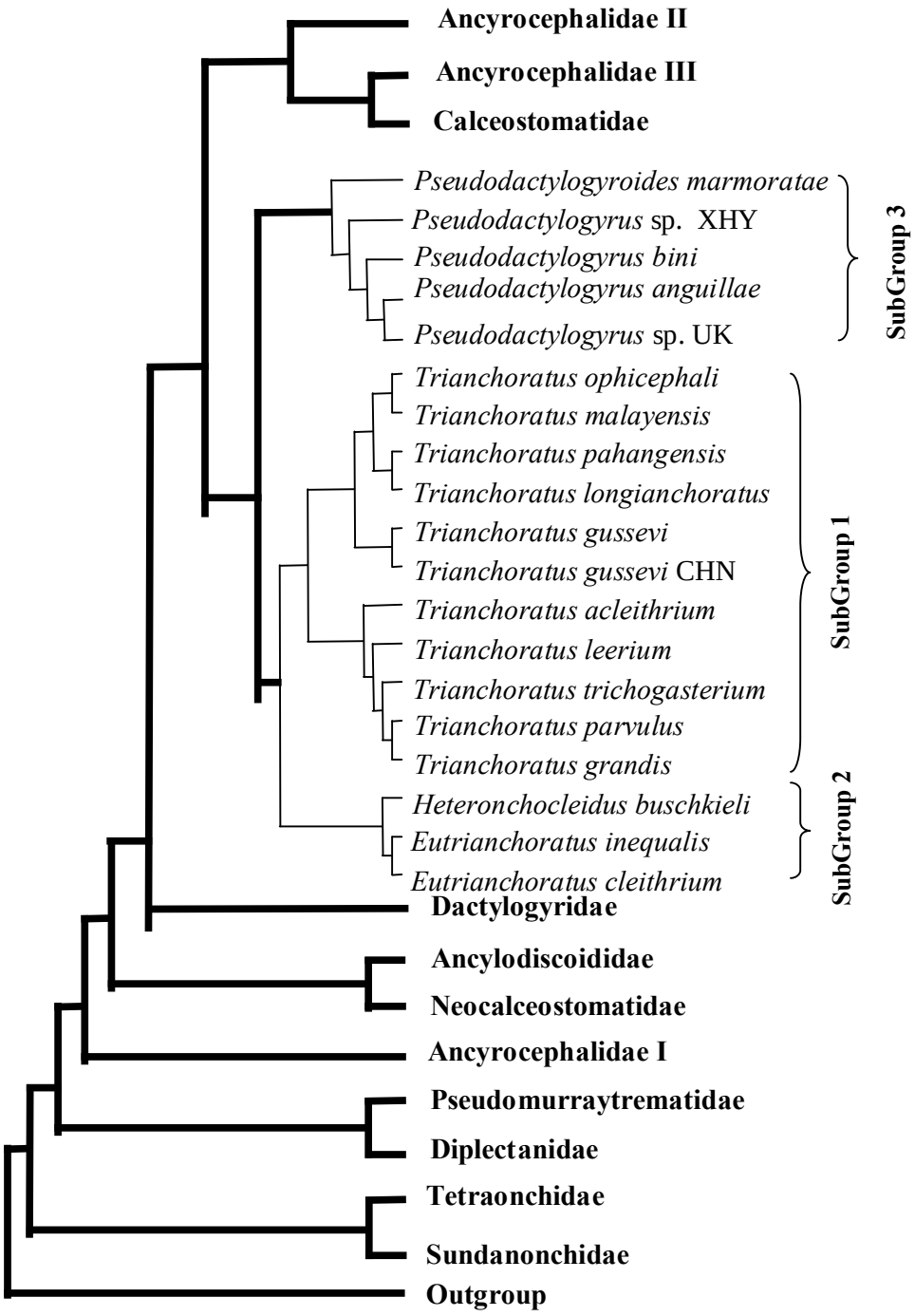


Figure 4.20 Maximum parsimony (MP) tree depicting the interrelationships within the Heteronchocleididae-Pseudodactylogyridae group.

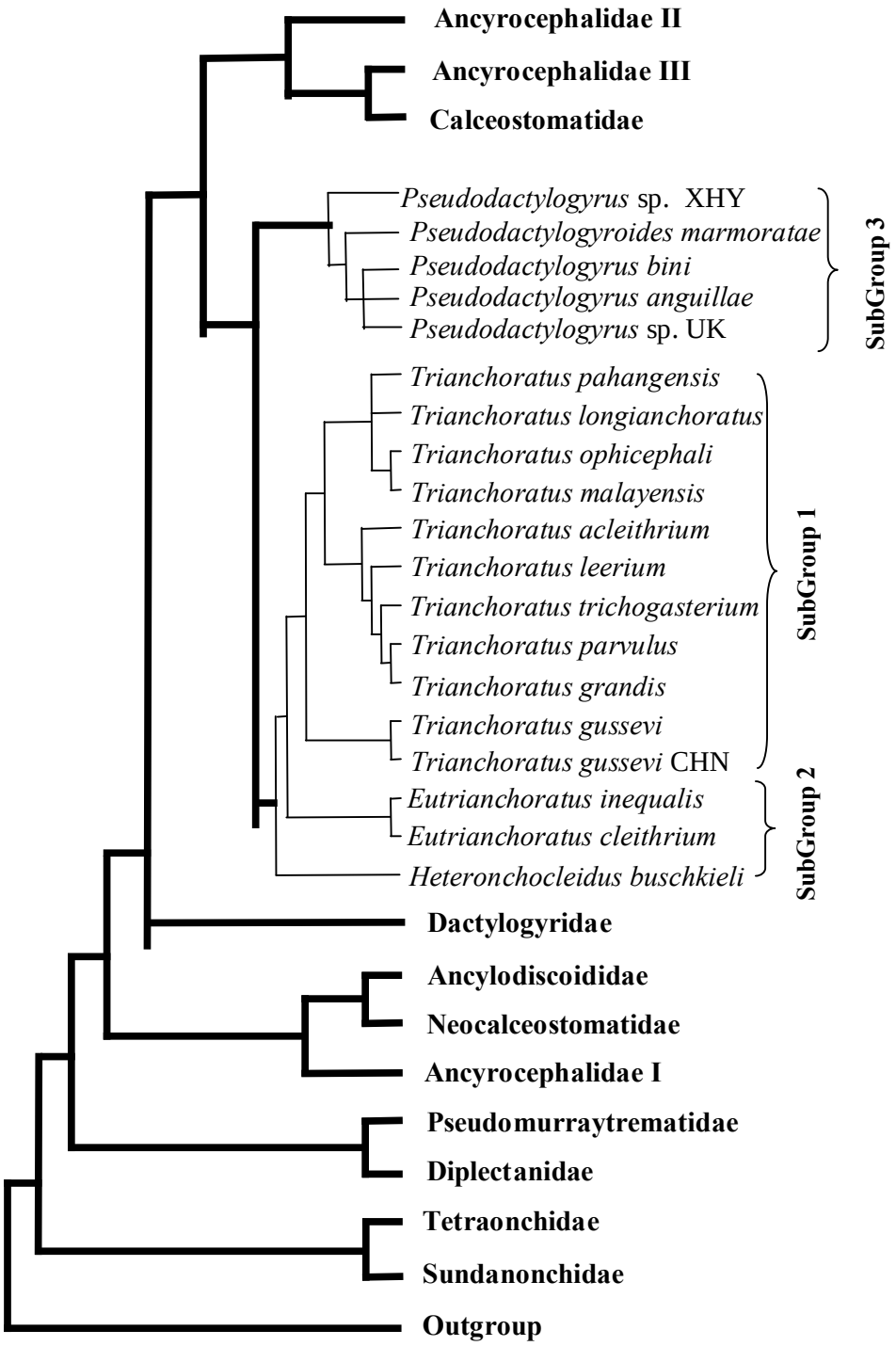


Figure 4.21 Maximum likelihood (ML) tree depicting the interrelationships within the Heteronchocleididae (*sensu* Tan, Fong & Lim, 2011).

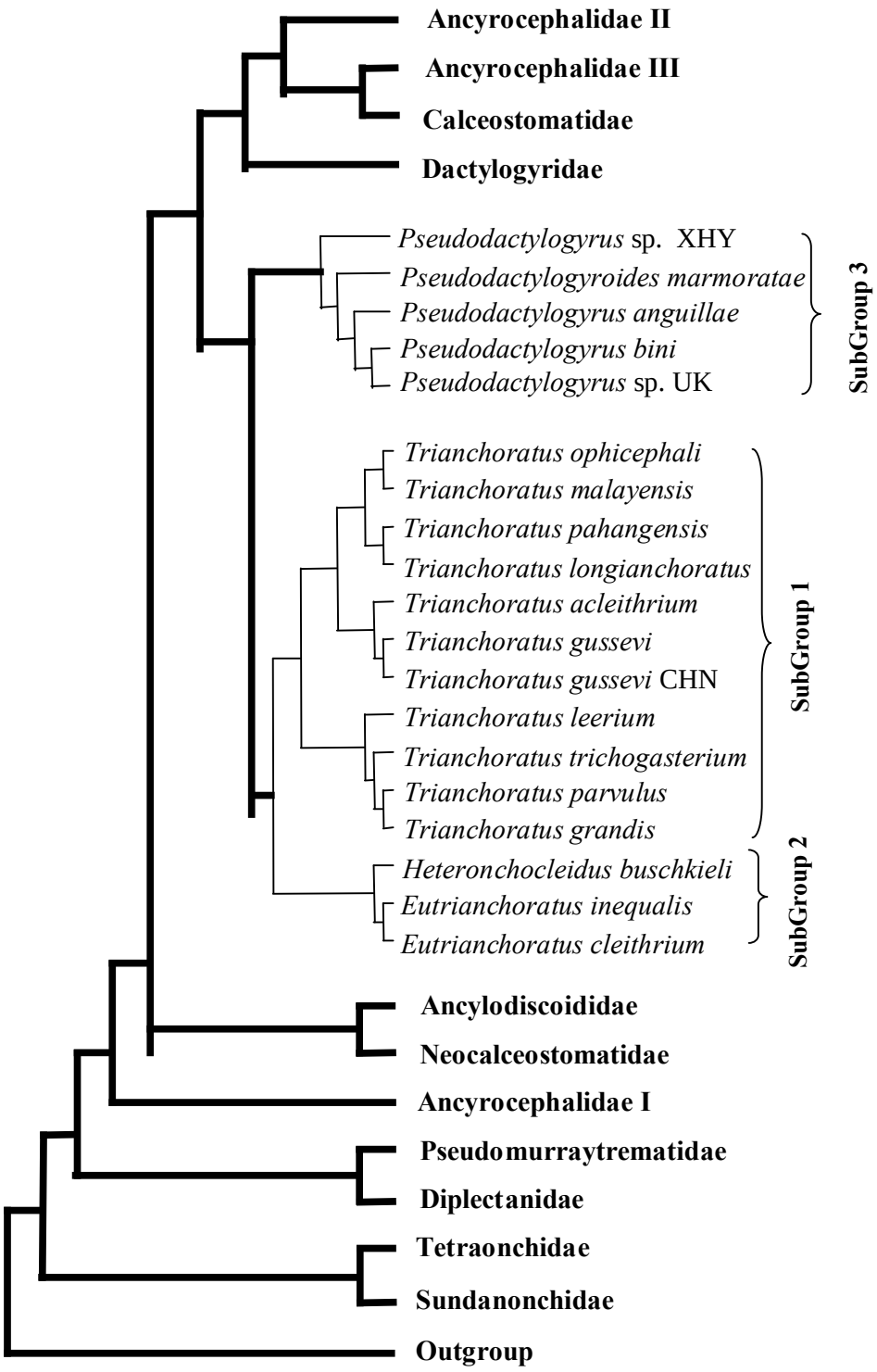


Figure 4.22 Neighbour-joining (NJ) tree depicting the interrelationships within the Heteronchocleididae (*sensu* Tan, Fong & Lim, 2011).

4.4.6.2 *Heteronchocleidus* & *Eutrianchoratus* (SubGroup 2)

SubGroup 2 consists of *Heteronchocleidus buschkieli* Bychowsky, 1957, *Eutrianchoratus inequalis* Lim, 1989 and *E. cleithrium* Lim, 1989 where *Heteronchocleidus buschkieli* is shown to form sister group with *E. inequalis* and *E. cleithrium* (Figs. 4.20, 4.21 & 4.22). All these members within SubGroup 2 possess connective bar i.e. *Heteronchocleidus buschkieli* possess two connective bars, *E. inequalis* and *E. cleithrium* possess one connective bar while the *Trianchoratus* group (see above) do not possess any connective bar (Fig. 4.23). This result shows that the heteronchocleidids which possess one to two connective bars (*Heteronchocleidus* and *Eutrianchoratus*) are more related to each other.

4.4.6.3 *Pseudodactylogyrus* & *Pseudodactylogyroides* (SubGroup 3)

Within SubGroup 3, there are species from the genera *Pseudodactylogyrus* i.e. *Pseudodactylogyrus* sp. XHY, *Pseudodactylogyrus* sp. UK, *P. bini* Kikuchi, 1929 and *P. anguillae* Yin & Sproston, 1948 and *Pseudodactylogyroides* i.e. *Pseudodactylogyroides marmoratae* Lim, 1995. In MP tree (Fig. 4.20), the four species of the *Pseudodactylogyrus* used in this study form a monophyletic group which is related to its sister group the *Pseudodactylogyroides*. Similar finding was reported in previous studies using molecular data by Ġimková *et al.* (2003), Ġimková *et al.* (2004) and Ding & Liao (2005) where *Pseudodactylogyrus* and *Pseudodactylogyroides* were shown to form sister group.

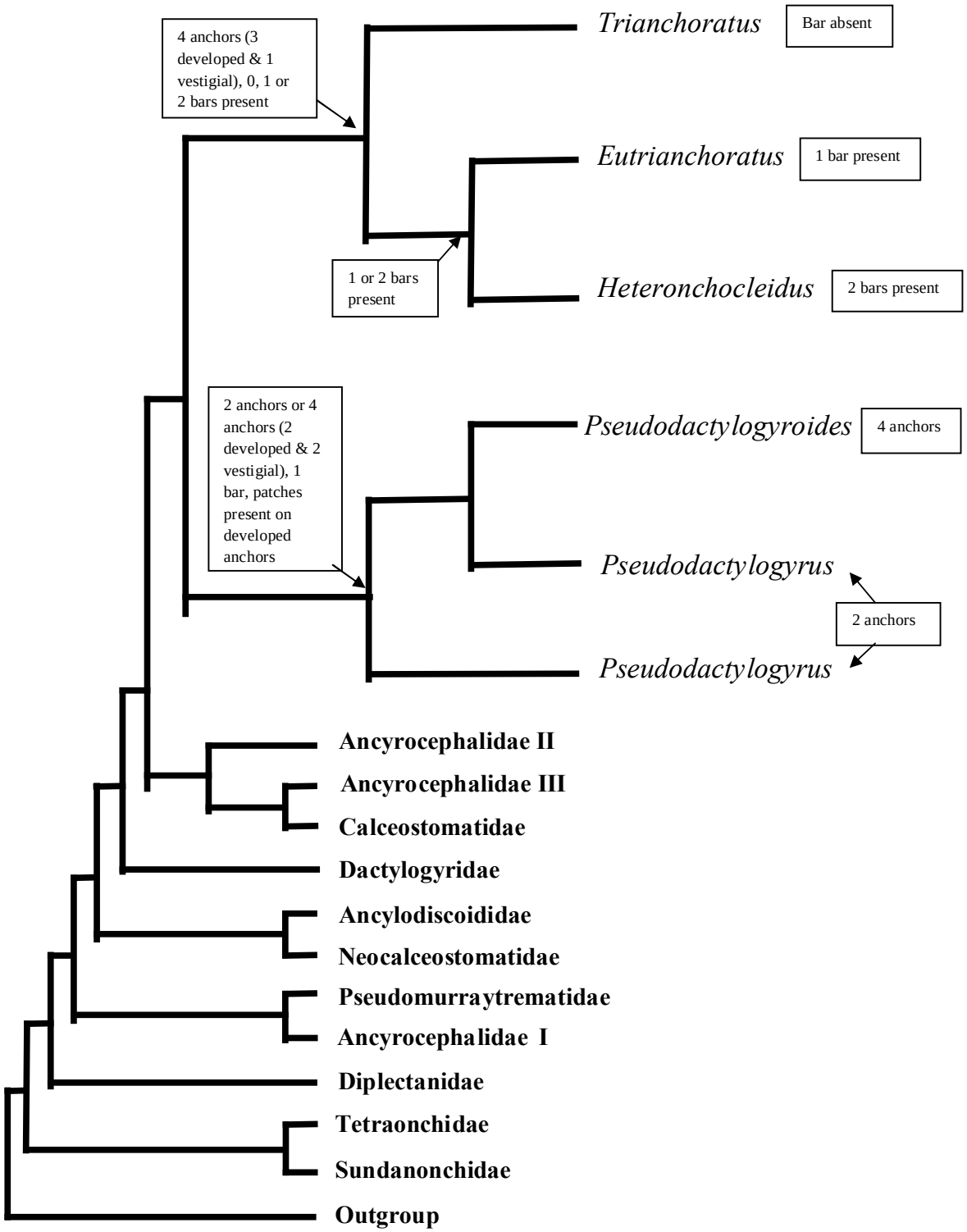


Figure 4.23 Neighbour-joining (NJ) tree depicting the interrelationships within the Heteronchocleididae and Pseudodactylogyridae with their generic morphological characteristics.

4.4.6.4 Summation for Heteronchocleididae–Pseudodactylogyridae group

The heteronchocleidids and pseudodactylogyrids form sister group (Figs. 4.20, 4.21 & 4.22). The sister group relationship of Heteronchocleididae and Pseudodactylogyridae has never been shown in any previous studies. It is reasonable to accept that Pseudodactylogyridae and Heteronchocleididae are closely related as members of these two families are characterised by having ventral anchors in different stages of development. For example the heteronchocleididids have 3 well developed and one reduced anchor (see Lim, 1986), whereas in the pseudodactylogyrids the ventral anchors are poorly developed in *Pseudodactylogyroides* and completely absent in *Pseudodactylogyrus* (only two dorsal anchors are present) (see Lim, 1995a).

4.5 Discussion

In the following sections, interrelationships of the different dactylogyridean families depicted in the relationships trees generated in this study (Section 4.3.1) are discussed with reference to the morphological characteristics of the dactylogyrideans to see if there are any interesting trends (Section 4.5.1). The status and validity of the various dactylogyridean families are also discussed using information obtained from molecular data (Section 4.5.2).

4.5.1 Relationship of dactylogyridean families

Sundanonchidaeï Tetraonchidae group and Diplectanidaeï Pseudomurraytrematidae group are morphologically very different from the other dactylogyridean families. These two groups are also shown to be the first two groups separated in MP, ML and NJ relationships trees indicating that they are distantly related to the other dactylogyridean families (Figs. 4.1, 4.2, 4.3 & 4.4). The Sundanonchidaeï

Tetraonchidae group are characterised by having 16 marginal hooks, cross-vitellaria and a single sac-like intestine (Lim & Furtado, 1985) while the Diplectanidaeï Pseudomurraytrematidae group possess ovary that overlaps the intestinal caecum, morphological characteristics which are not present in the other dactylogyridean families.

After the separaration of Sundanonchidaeï Tetraonchidae group and Diplectanidaeï Pseudomurraytrematidae group, the third and fourth groups which are formed in the relationships trees are the Ancyrocephalidae I group (see later) and Ancylodiscoididaeï Neocalceostomatidae group (Figs. 4.1, 4.2, 4.3 & 4.4). The separation of Ancylodiscoididaeï Neocalceostomatidae group at this point indicates that it is least related to the remaining groups in the relationships trees, i.e. Dactylogyridae group, Heteronchocleididaeï Pseudodactylogyridae group, Ancyrocephalidae II group and Ancyrocephalidaeï Calceostomatidae group. This could be due to the unique characteristics of ancylodiscoidids in having variable types of seminal vesicle, i.e. dactylogyrid type seminal vesicle, blind-sac like seminal vesicle or both while all the other dactylogyridean families included in this study only possess dactylogyrid type seminal vesicle.

It can be observed that the families from Node 4 (in ML tree) and Node 5 (in MP and NJ trees) onwards are families where the characteristics of anchors are highly plastic and heterogeneous. There are Dactylogyridae group with 2 anchors + 2 needle-like structures, Heteronchocleididaeï Pseudodactylogyridae group with variable types of anchors, i.e. heteronchocleidids and *Pseudodactylogyroides* (Pseudodactylogyridae) with 4 anchors (2 developed anchors + 2 reduced anchors) and *Pseudodactylogyrus*

(Pseudodactylogyridae) with 2 anchors while the Ancyrocephalidae II group and Ancyrocephalidaeï Calceostomatidae group possess 4 developed anchors.

Within the group of families which are having anchors with high plasticity mentioned above, the Acyrocephalidae II group and Ancyrocephalidaeï Calceostomatidae group are shown to be more related to the Heteronchocleididaeï Pseudodactylogyridae group compare to the Dactylogyridae group as they form sister group in MP and ML trees (Figs. 4.1, 4.2 & 4.4). It is reasonable to accept that the Heteronchocleididaeï Pseudodactylogyridae group is closely related to the four anchors Acyrocephalidae as all the members of Heteronchocleididaeï Pseudodactylogyridae group possess 4 anchors (three developed and one reduced anchors in Heteronchocelididae and two developed and two reduced anchors in *Pseudodactylogyroides*) except the *Pseudodactylogyrus* with 2 anchors. The Dactylogyridae group is shown to be less related to the Ancyrocephalidae, Heteronchocleididae and Pseudodactylogyridae as it possesses the unique pair of ñneedle-likeò structures which is not present in other dactylogyridean families (Figs. 4.1, 4.2 & 4.4).

It is also noted that Ancyrocephalidae is the only family with its members present in three separate groups (Ancyrocephalidae I group, Ancyrocephalidae II group & Ancyrocephalidaeï Calceostomatidae group) within the MP, NJ and ML trees generated. This indicates that ancyrocephalids are not a monophyletic group and highly heterogeneous. The heterogeneity of the Ancyrocephalidae has also been reported in previous studies based on morphology (Gusev, 1978; Kritsky & Boeger, 1989; 1993; Lim, 1998; Lim *et al.*, 2001) and molecular data (Plaisance *et al.*, 2005; Ġimková *et al.*,

2003; 2006b; Wu *et al.*, 2006). The status of Ancyrocephalidae and the other dactylogyridean families are discussed in the next section.

4.5.2 Status of dactylogyridean families

4.5.2.1 Status of Heteronchocleididae

The heteronchocleidids have been assigned to the subfamily Heteronchocleidinae in the family Dactylogyridae by Price (1968), subfamily Ancyrocephalinae in the family Ancyrocephalidae by Gusev (1978) and subfamily Heteronchocleidinae in the family Ancyrocephalidae by Lim (1989). However, results from this study show that the heteronchocleidids form a monophyletic group (Figs. 4.20, 4.21 & 4.22). This confirms that the heteronchocleidids which possess three well-developed anchors and one reduced anchor are unique and this finding is in agreement with the relationships proposed by Lim (1986, 1987a & 1989). Lim (1987a, 1989) noted the relative homogeneity between the three heteronchocleidid genera and the possibility of raising Heteronchocleidinae to family status. The relationships trees (Figs. 4.20, 4.21 & 4.22) from the present study support the move and Heteronchocleidinae is herein raised to family status, Heteronchocleididae. This result is already published in Tan *et al.* (2011) (see also Appendix B).

4.5.2.2 Status of Pseudodactylogyridae

Although *Pseudodactylogyrus* and *Pseudodactylogyroides* are shown to cluster in the same subGroup and thus closely related, Lim (1995a) suggested that these two genera should not belong to the same family as *Pseudodactylogyrus* has two anchors while *Pseudodactylogyroides* has four anchors. The arrangement of anchors in these two genera is also different as the anchors of *Pseudodactylogyrus* exemplified by *P. anguillae*, are medial peduncular anchor (Le Brun *et al.*, 1986), whereas the large

anchors of *Pseudodactylogyroides marmoratae* are lateral peduncular anchors (see Lim, 1995a). Lim (1995a) predicted that a new family may eventually be erected to accommodate *Pseudodactylogyroides* and proposed that *Pseudodactylogyroides* should be assigned to family Ancyrocephalidae.

However, it should be noted that *Pseudodactylogyrus* and *Pseudodactylogyroides* are both genera with low numbers of species where only three species have been described for each of this genus (see Lim, 1995a; Ogawa, 1984; Ogawa, 1986). In fact, molecular data from only two *Pseudodactylogyrus* spp. and one *Pseudodactylogyroides* species are analysed in this study. The possibility of re-assigning these two genera into two separate families as suggested by Lim (1995a) based on their morphological differences can only be determined until more species of these two genera are described in the future. Despite their morphological differences, results from this study show that the *Pseudodactylogyrus* and *Pseudodactylogyroides* are closely related (Figs. 4.20, 4.21 & 4.22) and support the current placement of these two genera under the family Pseudodactylogyridae (see Ogawa, 1986; Le Brun *et al.*, 1986).

4.5.2.3 Status of Ancylodiscoididae

The subfamily Ancylodiscoidinae was erected by Gusev (1961) within the family Dactylogyridae to accommodate monogeneans from the siluriforms. In 1978, Bychowsky & Nagibina transferred Ancylodiscoidinae into the family Ancyrocephalidae. Lim (1998) re-assigned the ancylodiscoidid genera with a dactylogyrid-type seminal vesicle e.g. *Bychowskyella*, *Quadriancanthus*, *Chauhanellus*, *Hamatopeduncularia*, into the Ancyrocephalinae, leaving the genera with blind sac-like seminal vesicle e.g. *Anchylodiscus*, *Bifurcohaptor*, *Cornudiscoides*, *Malayanodiscoides*,

Pseudancylodiscoides, *Thaparocleidus* in the Ancylodiscoidinae (see also Lim, 1991, 1992, 1994, 1996; Lim & Lerssutthichawal, 1996). Later Lim *et al.* (2001) raised the Ancylodiscoidinae to family status and grouped within this family the monogeneans from siluriforms and notopterids (two species of *Thaprocleidus*, one species of *Malayanodiscoides* and two species of *Notopterodiscoides*; see Lim & Furtado, 1986).

The current result indicates that the genera previously re-assigned to the Ancyrocephalinae by Lim (1998) (*Bychowskyella*, *Quadriancanthus*, *Chauhanellus* and *Hamatopeduncularia*) do not cluster with any of the ancyrocephalids (Ancyrocephalidae I group, Ancyrocephalidae II group & Ancyrocephalidaeï Calceostomatidae group) but are clustered in the same group with the other ancylodiscoidids (Figs. 4.13, 4.14 & 4.15). These results unambiguously support the move by Lim *et al.* (2001) and further confirmed the validity of familial status of Ancylodiscoididae.

4.5.2.4 Status of Neocalceostomatidae and Calceostomatidae

Neocalceostoma and *Neocalceostomoides* have been placed in subfamily Calceostomatinae under family Dactylogyridae (Kritsky *et al.*, 1978) and in family Calceostomatidae Parona & Perugia, 1890 under suborder Calceostomatinea (Boeger & Kritsky, 1993). However, Lim (1995c) separated *Neocalceostoma* and *Neocalceostomoides* from Calceostomatidae into Neocalceostomatidae based on the fact that *Neocalceostoma* and *Neocalceostomoides* are morphologically different from the calceostomatids (see Lim, 1995c). Although Lim (1995c) separated *Neocalceostoma* and *Neocalceostomoides* from Calceostomatidae into Neocalceostomatidae, Kearn *et al.* (1995) decided to include the Australian *Neocalceostomoides* into Calceostomatidae. Result from this study show Neocalceostomatidae and Calceostomatidae are not related

therefore supporting the separation of Neocalceostomatidae from Calceostomatidae by Lim (1995c) (Figs. 4.1, 4.2, 4.3 & 4.4).

4.5.2.5 Status of Ancyrocephalidae

The three ancyrocephalid groups (Ancyrocephalidae I group, Ancyrocephalidae II group & Ancyrocephalidae IIIï Calceostomatidae group) formed in this current study show members from these groups are highly heterogeneous. The heterogeneity of the Ancyrocephalidae has caused a lot of controversies especially in the way researchers had tried to resolve its artificial grouping within the Dactylogyridea. Kritsky & Boeger (1989) and Boeger & Kritsky (1993, 1997) had also shown similar relationships for the members of the Ancyrocephalidae based on analysis of morphological data. Based on the analysis of morphological data, it is confusing what Kritsky & Boeger (1989) are trying to say. Kritsky & Boeger (1989) had suggested a major revision of the Ancyrocephalidae. They provided two options of either reducing Ancyrocephalidae to subfamily status or raising all the subfamilies within the Ancyrocephalidae to family status. They chose to reduce the Ancytrocephalidae and reassigned Ancyrocephalinae, Linguadactylinae, Linguadactyloidinae, Heterotesiinae, Pseudodactylogyrinae, Hareocephalinae, Ancylodiscoidinae and Anacanthorinae into the family Dactylogyridae in order to make the groups monophyletic (see Section 1.4). Lim (1998) and Lim *et al.* (2001) however did not agree with this move given that *Dactylogyrus*, the type genus of Dactylogyridae, possess a pair of unique needle-like structure which is not present in any ancyrocephalids.

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Results from this study indicate that the Ancyrocephalidae needs revision but not by reducing Ancyrocephalidae but by creating a new family to accommodate

members of Ancyrocephalidae II group & Ancyrocephalidae IIIï Calceostomatidae group and leaving the Ancyrocephalidae for members of Ancyrocephalidae I group which include *Ancyrocephalus*, the type genus of Ancyrocephalidae as it is common in Linnean taxonomy that as the numbers of a taxon increase, the status of the taxon would be altered accordingly (see Section 6.6; General Discussion).

4.5.2.6 Status of Dactylogyridae

The current result supports the suggestion by Lim (1998), Timofeeva *et al.* (1997) and Lim *et al.* (2001) that the Dactylogyridae should include monogeneans with two anchors, two unique óneedle-likeô structure (considered to be hooks by some and anchors by others), 14 marginal hooks and one to two bars. The revison by Kritsky & Boeger (1989) served only to cause the family Dactylogyridae to become more heterogeneous and artificial by including the four anchors ancyrocephalids into Dactylogyridae. Lim *et al.* (2001) also suggested that the Ancyrocephalidae (*sensu* Bychowsky & Nagibina, 1978) should be left intact within the Dactylogyridea until further studies are done on more members of the Ancyrocephalidae. A much better option suggested by Kritsky & Boeger (1989) would have been their other suggestion of raising the status of the subfamilies of the Ancyrocephalidae.

It should also be noted that the relationship trees based on molecular data obtained from previous studies (e.g. Ġimková *et al.*, 2003, 2006b) for the dactylogyridean families are almost similar to that obtained in the current study despite these previous studies using few representatives in their analysis (cf. Figs. 4.1, 4.2 & 4.3). In these previous analyses, members from major dactylogyridean groups of Dactylogyrinae, Pseudodatcylogyrinae, Ancylodiscoidinae, Pseudomurraytrematidae and Diplectanidae formed distinct yet related groups and the Ancyrocephalinae are

clustered in two groups (Ġimková *et al.*, 2003; 2006b). Despite the clustering of dactylogyrids and ancyrocephalines as distinctly different groups, Ġimková *et al.* (2003, 2006b) had interpreted their relationships trees according to Kritsky & Boeger (1989) by considering the polyphyletic ancyrocephalines as Dactylogryidae. As already noted above (Section 4.5.2.5), Kritsky & Boeger (1989) and Boeger & Kritsky (1993) tried to resolve what they considered to the paraphyly of the Ancyrocephalidae by including all the subfamilies within the Ancyrocephalidae into the Dactylogyridae. Could the approach of these cladists to introduce strict monophyly into the current monogenean classification based on ranked Linnean system causes more conflict than resolving paraphyly and polyphyly? (see Section 6.6; General Discussion).

4.6 Summary of chapter

Eight major groups can be observed in the MP, ML and NJ relationship trees generated in this study using partial 28S rDNA sequences from 191 dactylogyridean monogeneans. The memberships for each of the eight groups are the same and correspond to the different dactylogyridean families and genera (Table 4.1). These groupings of different individuals of dactylogyrideans corresponding to their respective family as well as some at generic level show the partial 28S rDNA can be used to group the dactylogyrideans and indicate there are molecular diversities within partial 28S rDNA sequences among the dactylogyrideans (see General Discussion).

The groupings formed in the MP, ML and NJ trees have also provided information to examine the relationships and status of the different dactylogyrideans especially at family level (Sections 4.4 & 4.5.1). For example, based on information derived from the relationship trees generated in this study, the subfamily Heteronchocleidinae Price, 1968 was raised to family status, Heteronchocleididae (see

Tan *et al.*, 2011) (Section 4.5.2.1). The validity of the family Ancylodiscoididae, Neocalceostomatidae and Pseudodactylogyridae are also confirmed based on information from partial 28S rDNA (Sections 4.5.2.2, 4.5.2.3 & 4.5.2.4).

CHAPTER 5

RESULTS & DISCUSSION

FISH HOST RELATIONSHIP BASED ON PARTIAL CYTOCHROME *b* SEQUENCES AND FISH-MONOGENEAN RELATIONSHIP

5.1 Introduction

This chapter deals with the fish hosts of the dactylogyridean. In this study the relationship of the fish host species are inferred using partial Cytochrome *b* sequences (see Section 2.4.2). The relationship of these fish hosts (Section 5.2.1) are used to assist in discussing the correlation with the distribution pattern of the dactylogyridean monogeneans (Section 5.3). The main objective of doing so is to determine if there is any association between the dactylogyridean monogeneans and their fish hosts.

5.2 Partial Cytochrome *b* sequences of fish hosts

In this study, 176 partial Cytochrome *b* sequences from fish species belonging to 34 families and the partial Cytochrome *b* sequences of three fish species from Salmonidae, viz. *Salmo salar* (Linnaeus), *Oncorhynchus mykiss* (Walbaum) and *O. kisutch* (Walbaum) (as outgroup) (Table 2.4) are analysed by PAUP*4.0b10 in High Performance Computer (HPC) (see Sections 2.5 & 2.6.2.2). Three clustering methods of neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) are used to generate the three relationship trees (Figs. 5.1, 5.2 & 5.3). In the sequence alignment of the partial Cytochrome *b* of these 179 fish species, 692 alignable positions containing 451 variable sites were obtained and 377 of these variable sites were

considered to be parsimony informative. Based on Modeltest 3.7, the current dataset of aligned sequences resulted in the best likelihood score for the general time reversible model with invariable sites and rate heterogeneity (GTR+I+G). Base frequencies are unequal where A=0.3465, C =0.3908, G=0.0559, T=0.2068 and the estimated proportion of invariable sites (pinvar) is 0.3227. The rate matrix (rmat) for the selected substitution model is [A-C]=0.1059, [A-G]=3.5164, [A-T]=0.2532, [C-G]=0.3328, [C-T]=3.1356, [G-T]=1.0000. The shape parameter of the gamma distribution is Ŭ = 0.4168. This model and parameters are used in NJ and ML analyses.

5.3 Relationship of the fish hosts (Figs. 5.1, 5.2 & 5.3)

The NJ, MP and ML relationship trees generated using partial Cytochrome *b* sequences show that the 176 species of fish hosts form distinct separate groups which correspond to their respective family (Figs. 5.1, 5.2 & 5.3). It can also be observed that there are consistently two major groups formed in the NJ, MP and ML relationship trees. The first group consists of 26 families from the order Perciformes and the second group correspond to the non-Perciformes group which consist of members from eight families.

5.3.1 Perciformes group

Within the Perciformes group, different number of subGroups can be observed in the NJ, MP and ML trees, i.e., seven subGroups in MP tree (Fig. 5.1), nine subGroups in ML tree (Fig. 5.2) and five subGroups in NJ tree (Fig. 5.3).

5.3.1.2 MP tree (Fig. 5.1)

In MP tree, the subGroups formed within the Perciformes group are almost similar with the NJ tree except for the two extra subGroups formed by Malacanthidae and Chaetodontidae (SubGroup 4 in MP tree) and Cichlidae and Mugilidae (SubGroup 6 in MP tree) (Fig. 5.1). All the members in the other subGroups are similar to the subGroups formed in NJ trees except for position of the family Haemulidae, Scatophagidae and Sciaenidae. Thus, the membership of the Perciformes families within the seven subGroups observed in MP tree is as follow: SubGroup 1 = Helostomatidae, Anabantidae, Osphronemidae, Nandidae and Channidae; SubGroup 2 = Nemipteridae, Scatophagidae, Sciaenidae, Sparidae, Lutjanidae and Siganidae; SubGroup 3 = Serranidae, Latidae and Haemulidae; SubGroup 4 = Malacanthidae and Chaetodontidae; SubGroup 5 = Terapontidae, Mullidae, Centrarchidae, Lateolabracidae and Percidae; SubGroup 6 = Cichlidae and Mugilidae; SubGroup 7 = Gobiidae, Eleotridae and Apogonidae (Fig. 5.1).

5.3.1.3 ML tree (Fig. 5.2)

In ML tree, there are nine subGroups formed within the Perciformes group (Fig. 5.2). The first subGroup consists of fish species from families which are similar to those in the NJ tree, viz., Helostomatidae, Anabantidae, Osphronemidae, Channidae and Malacanthidae. The second subGroup contains members from the family Scatophagidae and Mugilidae. The third subGroup possesses similar groupings which can also be observed in NJ and MP trees, where members of Nemipteridae, Sparidae, Siganidae and Lutjanidae are present. The fourth subGroup contains the sister group of Serranidae and Latidae (also observed in NJ and MP trees) as well as members of the Nandidae which are shown to form sister group with the Osphronemidae in NJ and MP trees. The fifth subGroup is made up of fish species from Haemulidae, Mullidae and Cichlidae while

the sixth subGroup consists of members from Terapontidae, Percidae, Lateolabracidae and Centrarchidae where these families can also be observed to be grouped together in NJ and MP trees. The seventh and eighth subGroups are the Sciaenidae and Chaetodontidae, respectively. Lastly, the ninth subGroup contains members from Gobiidae, Eleotriidae and Apogonidae, where these three families are also grouped together in NJ and MP trees.

5.3.1.1 NJ tree (Fig. 5.3)

In NJ tree, there are 5 subGroups within the Perciformes group (Fig. 5.3). SubGroup 1 consists of fish species from the family Helostomatidae, Anabantidae, Osphronemidae, Nandidae, Channidae and Malacanthidae. All of these families in SubGroup 1 are primarily freshwater families except Malacanthidae. SubGroup 2 includes members from the family Nemipteridae, Sparidae, Chaetodontidae, Lutjanidae and Siganidae which are all marine species while SubGroup 3 consists of families with mostly freshwater fish species (Latidae and Cichlidae) or with some freshwater and mostly marine species (Serranidae). SubGroup 4 includes both marine fish species from Haemulidae, Terapontidae, Mullidae, Lateolabracidae, Mugilidae and freshwater fish species from Centrarchidae and Percidae. Lastly, SubGroup 5 consists of members from Scatophagidae, Sciaenidae, Gobiidae, Eleotridae and Apogonidae which are all primarily marine species. Despite currently being assigned in the order Mugiliformes, the Mugilidae is consistently grouped with the perciforms families in this analysis (Fig. 5.3) (Figs. 5.1 & 5.2; see above). It should also be noted that the Mugilidae was previously a perciform family (Nelson, 1994; Kottelat *et al.*, 1993). Thus, based on current results from molecular data, Mugilidae is referred to as a perciform family in this study.

5.3.2 Non-Perciformes group

Within the non-Perciformes group, there are members from Anguilliformes (Anguillidae), Osteoglossiformes (Notopteridae), Siluriformes (Ariidae, Plotosidae, Bagriidae, Clariidae & Siluridae) and Cypriniformes (Cyprinidae). The Siluriformes are either forming sister group with Cyprinidae in MP tree (Fig. 5.1) or with Anguillidae + Notopteridae in NJ tree (Fig. 5.3). The Anguillidae and Notopteridae are shown to be closely related where they consistently form sister group in MP, ML and NJ trees (Figs. 5.1, 5.2 & 5.3).

5.4 Fish – monogenean distribution patterns

Table 5.1 shows the dactylogyridean monogeneans analysed in this study (see Chapter 4) are from 115 species of marine and freshwater fish hosts. It should be noted that this table is compiled based on the dactylogyrideans analysed in Chapter 4 and it is not a complete list of the dactylogyrideans and their hosts. A review on the dactylogyridean monogeneans found on marine and freshwater fish of Peninsular Malaysia and oriental biogeography region which include Indo China and Southeast Asia has been done by Lim (1998).

5.4.1 Perciformes

Perciformes is one of the largest fish order with over 10,000 species (Nelson, 1994; Froese & Pauly, 2012). Members of perciformes from 26 families (see Table 2.5) with information regarding the partial 28S rDNA sequences of their dactylogyridean monogeneans (see Chapter 4) are included in this study. The dactylogyridean monogeneans found in these 26 perciforms families range from members of Ancyrocephalidae, Diplectanidae, Heteronchocleididae, Pseudodactylogyridae and Dactylogyridae (Table 5.1).

Table 5.1 Fish host of the monogeneans analysed in this study with information of the host order, family and species as well as the monogenean family (*names used in original paper/GenBank).

Host order/family	Host species	Monogenean	Monogenean family
Order			
Perciformes			
Family			
Channidae	*Channa lucius*	*Trianchoratus malayensis*	Heteronchocleididae
		T. pahangensis	Heteronchocleididae
		T. longiancchoratus	Heteronchocleididae
	Channa striata	*T. ophicephali*	Heteronchocleididae
	Channa micropeltes	*Sundanonchus foliaceus*	Sundanonchidae
		S. tomanorum	Sundanonchidae
		S. micropeltis	Sundanonchidae
Anabantidae	*Anabas testudineus*	*T. gussevi*	Heteronchocleididae
		T. parvulus	Heteronchocleididae
		T. grandis	Heteronchocleididae
Helostomatidae	*Helostoma temminkii (Helostoma temminckii*)*	*T. acleithrium*	Heteronchocleididae
Osphronemidae	*Trichogaster leerii*	*T. leerium*	Heteronchocleididae
	Trichogaster trichopterus	*T. trichogasterium*	Heteronchocleididae
	Macropodus opercularis	*Heteronchocleidus buschkieli*	Heteronchocleididae
	Belontia hasselti	*Eutrianchoratus inequalis*	Heteronchocleididae
		E. cleithrium	Heteronchocleididae
Serranidae	*Epinephelus coioides*	*Diplectanum grouperi*	Diplectanidae
		Pseudorhabdosynochus coioidesis	Diplectanidae

		Pseudorhabdosynochus shenzhenensis	Diplectanidae
	Epinephelus bruneus	*Pseudorhabdosynochus lantauensis*	Diplectanidae
		P. epinepheli	Diplectanidae
	Epinephelus sexfasciatus	*Haliotrema epinepheli*	Ancyrocephalidae
	Cromileptes altivelis	*Haliotrema cromileptis*	Ancyrocephalidae
Latidae	*Lates calcarifer*	*Diplectanum penangi*	Diplectanidae
		Lamellodiscus spari	Diplectanidae
		Pseudorhabdosynochus latesi (Pseudorhabdosynochus latesis)*	Diplectanidae
		Laticola seabassi (Pseudorhabdosynochus seabassi)*	Diplectanidae
		L. paralatesi	Diplectanidae
		L. lingaoensis	Diplectanidae
Nemipteridae	*Nemipterus japonicus*	*Calydiscoides indianus*	Diplectanidae
	Nemipterus bathybius	*Calydiscoides* sp.	Diplectanidae
Terapontidae	*Terapon jarbua*	*Lepidotrema longipenis*	Diplectanidae
Sciaenidae	*Johnius amblycephalus*	*Diplectanum umbrinum*	Diplectanidae
	Johnius sp.	*Lobotrema* sp.	Diplectanidae
	Nibea albiflora	*Lobotrema sciaenae*	Diplectanidae
		Murraytrema bychowskyi (*M. pricei**)	Diplectanidae
		Sinodiplectanotrema sp. HGY	Diplectanidae
	Pennahia anea	*Sinodiplectanotrema malayanum*	Diplectanidae
Sparidae	*Sparus macrocephalus*	*Haliotrema kurodai*	Ancyrocephalidae
		H. grossecurvitubus	Ancyrocephalidae

		Lamellodiscus japonicus	Diplectanidae
	Pagrosomus major	*Lamellodiscus pagrosomi*	Diplectanidae
	Acanthopagrus schlegelii	*Haliotrema eukurodai*	Ancyrocephalidae
	Acanthopagrus australis	*Lamellodiscus acanthopagri*	Diplectanidae
Haemulidae	*Pomadasys hasta*	*Bravohollisia gussevi*	Ancyrocephalidae
		B. reticulata	Ancyrocephalidae
		B. kritskyi	Ancyrocephalidae
		Bravohollisia sp.	Ancyrocephalidae
		Caballeria liewi	Ancyrocephalidae
		C. pedunculata	Ancyrocephalidae
		C. intermedius	Ancyrocephalidae
	Pomadasys maculatus	*B. rosetta*	Ancyrocephalidae
		B. maculatus	Ancyrocephalidae
		B. parvianchoratus	Ancyrocephalidae
Lutjanidae	*Lutjanus russellii*	*Euryhaliotrema* sp. HBDD	Ancyrocephalidae
	Lutjanus stellatus	*Haliotrema spirotubiforum*	Ancyrocephalidae
	Lutjanus monostigma	*Haliotrema anguiformis*	Ancyrocephalidae
	Lutjanus argentimaculatus	*Haliotrema shenzhenensis*	Ancyrocephalidae
		H. nanaoensis	Ancyrocephalidae
	Lutjanus guttatus	*Euryhaliotrema perezponcei*	Ancyrocephalidae
		Haliotrematoides plectridium	Ancyrocephalidae
		H. spinatus	Ancyrocephalidae
		H. guttati	Ancyrocephalidae
	Lutjanus rhodopterus	*Euryhaliotrema johnii*	Ancyrocephalidae
		Euryhaliotrematoides sp. HQDD	Ancyrocephalidae
Scatophagidae	*Scatophagus argus*	*Metahaliotrema mizellei*	Ancyrocephalidae

		M. geminatohamula	Ancyrocephalidae
Mullidae	*Upeneus quadrilineatus*	*Haliotrema bihamulatum*	Ancyrocephalidae
	Upeneus luzonius	*H. johnstoni*	Ancyrocephalidae
Siganidae	*Siganus* sp.	*Pseudohaliotrema* sp.	Ancyrocephalidae
	Siganus doliatus	*P. sphincteroporus*	Ancyrocephalidae
	Siganus fuscescens	*Tetrancistrum* sp.	Ancyrocephalidae
Cichlidae	*Tylochromis intermedius*	*Cichlidogyrus pouyaudi*	Ancyrocephalidae
	Hemichromis fasciatus	*C. falcifer*	Ancyrocephalidae
		C. tilapiae	Ancyrocephalidae
		Onchobdella aframae	Ancyrocephalidae
		O. bopeleti	Ancyrocephalidae
	Sarotherodon galilaeus	*C. acerbus*	Ancyrocephalidae
	Oreochromis niloticus	*Scutogyrus longicornis*	Ancyrocephalidae
Centrarchidae	*Lepomis gibbosus*	*Actinocleidus recurvatus*	Ancyrocephalidae
		Urocleidus similis	Ancyrocephalidae
	Lepomis macrochirus	*Onchocleidus* sp.	Ancyrocephalidae
Percidae	*Sander lucioperca (Stizostedion lucioperca*)*	*Ancyrocephalus paradoxus*	Ancyrocephalidae
	Gymnocephalus cernua (Gymnocephalus cernuus)*	*Dactylogyrus hemiamphibothrium*	Dactylogyridae
Eleotridae	*Oxyeleotris marmorata*	*Pseudodactylogyroides marmoratae*	Pseudodactylogyridae
Lateolabracidae	*Lateolabrax japonicus*	*Dactylogyrus inversus*	
		D. gotoi	
		D. kikuchii	
Malacanthidae	*Branchiostegus auratus*	*Haliotrema chenhsintaoi*	Ancyrocephalidae

Chaetodontidae	*Chaetodon auriga*	*Haliotrema aurigae*	Ancyrocephalidae
	Chaetodon kleinii	*Haliotrema angelopterum*	Ancyrocephalidae
		Euryhaliotrematoides triangulovagina	Ancyrocephalidae
	Forcipiger flavissimus	*Haliotrema scyphovagina*	Ancyrocephalidae
	Chaetodon citrinellus	*Euryhaliotrematoides berenguelae*	Ancyrocephalidae
		Aliatrema cribbi	Ancyrocephalidae
	Chaetodon vagabundus	*Euryhaliotrematoides annulocirrus*	Ancyrocephalidae
		E. grandis	Ancyrocephalidae
		E. aspistis	Ancyrocephalidae
	Chaetodon lunula	*Euryhaliotrematoides pirulum*	Ancyrocephalidae
	Heniochus chrysostomus	*Euryhaliotrematoides microphallus*	Ancyrocephalidae
Nandidae	*Pristolepis fasciatus*	*Sundanonchus triradiacatus*	Sundanonchidae
Gerreidae	*Gerres filamentosus*	*Haliotrema subancistroides*	Ancyrocephalidae
	Gerres macrosoma	*Haliotrema digyroides*	Ancyrocephalidae
	Eugerres axillaris	Calceostomatidae sp.	
Leiognathidae	*Leiognathus brevirostris*	*Haliotrema geminatohamula*	Ancyrocephalidae
Lethrinidae	*Lethrinus nebulosus*	*Haliotrema fleti*	Ancyrocephalidae
Acanthuridae	*Acanthurus nigrofuscus*	*Haliotrema leporinus*	Ancyrocephalidae
	Ctenochaetus strigosus	*Haliotrema ctenochaeti*	Ancyrocephalidae
	Acanthurus nigroris	*Haliotrema macracantha*	Ancyrocephalidae
	Acanthurus olivaceus	*Haliotrema pratasensis*	Ancyrocephalidae
Sillaginidae	*Sillago sihama*	*Diplectanum blairense (Paradiplectanum blairense*)*	Diplectanidae
		Diplectanum sillagonum (Paradiplectanum sillagonum)*	Diplectanidae

Polynemidae	*Polydactylus sextarius (Polynemus sextarius*)*	*Diplectanum veropolynemi*	Diplectanidae
Kyphosidae	*Kyphosus vaigiensis*	*Acelotrema* sp.	Diplectanidae
Order			
Osteoglossiformes			
Family			
Notopteridae	*Notopterus notopterus*	*Malayanodiscoides bihamuli*	Ancylodiscoididae
		Thaparocleidus notopteri	Ancylodiscoididae
Order			
Siluriformes			
Family			
Plotosidae	*Plotosus canius*	*Ancylodiscus malayensis*	Ancylodiscoididae
Ariidae	*Osteogeneiosus militaris*	*Hamatopeduncularia simplex*	Ancylodiscoididae
		Chauhanellus osteogenosus	Ancylodiscoididae
	Arius maculatus	*Hamatopeduncularia* sp.	Ancylodiscoididae
		H. papernai	Ancylodiscoididae
		C. poculus	Ancylodiscoididae
		C. pulutanus	Ancylodiscoididae
	Hexanematichthys sagor (Arius sagor)*	*H. isosimplex*	Ancylodiscoididae
		C. digitalis	Ancylodiscoididae
		Neocalceostomoides hamatum	Neocalceostomatidae
	Arius venosus	*H. venosus*	Ancylodiscoididae
		Neocalceostoma sp.	Neocalceostomatidae
	Arius caelatus	*H. malayanus*	Ancylodiscoididae

Bagridae	*Mystus nigriceps*	*Cornudiscoides facicirrus*	Ancylodiscoididae
	Mystus sp.	*Bifurcohaptor lanchangensis*	Ancylodiscoididae
		Cornudiscoides sp.	Ancylodiscoididae
	Mystus vittatus	*Cornudiscoides proximus*	Ancylodiscoididae
	Pseudobagrus fulvidraco	*Bychowskyella pseudobagri*	Ancylodiscoididae
		Pseudancylodiscoides sp. HSY1	Ancylodiscoididae
		Pseudancylodiscoides sp. HSY3	Ancylodiscoididae
		Pseudancylodiscoides sp. HSY4	Ancylodiscoididae
Siluridae	*Silurus astus*	*Thaparocleidus magnicirrus*	Ancylodiscoididae
		T. obscura	Ancylodiscoididae
		T. mutabilis	Ancylodiscoididae
		T. omegavagina	Ancylodiscoididae
		T. infundibulovagina	Ancylodiscoididae
		T. asoti	Ancylodiscoididae
		T. varicus	Ancylodiscoididae
		T. cochleavagina	Ancylodiscoididae
	Silurus glanis	*T. vistulensis*	Ancylodiscoididae
		T. siluri	Ancylodiscoididae
Pangasiidae	*Pangasianodon hypophthalmus (Pangasius sutchi*)*	*T. campylopterocirrus*	Ancylodiscoididae
Clariidae	*Clarias batrachus*	*Quadriacanthus kobiensis*	Ancylodiscoididae
Ictaluridae	*Ameiurus nebulosus (Ictalurus nebulosus*)*	*Cleidodiscus pricei*	Ancyrocephalidae

Order			
Cypriniformes			
Family			
Cyprinidae	*Cyclocheilichthys apogon*	*Dactylogyrus apogonae*	Dactylogyridae
		D. temperasi	Dactylogyridae
	Hypophthalmichthys nobilis (Aristichthys nobilis)*	*D. aristichthys*	Dactylogyridae
	Hypophthalmichthys molitrix	*D. hypophalmichthys*	Dactylogyridae
	Hampala macrolepidota	*D. hampalai*	Dactylogyridae
		D. quadribrachiatus	Dactylogyridae
	Osteochilus hasselti	*D. cheligenitalis*	Dactylogyridae
	Rasbora elegans	*D. elegani*	Dactylogyridae
	Rasbora sp.	*Dactylogyrus* sp.	Dactylogyridae
	Systomus sophore (Puntius sophore)*	*Dactylogyroides longicirrus*	Dactylogyridae
	Ctenopharyngodon idella	*D. lamellatus*	Dactylogyridae
	Labiobarbus sp.	*Dactylogyrus* sp. LAB	Dactylogyridae
	Gobio gobio	*D. cryptomeres*	Dactylogyridae
	Carassius auratus	*D. inexpectatus*	Dactylogyridae
	Cyprinus carpio	*D. extensus*	Dactylogyridae
	Rutilus rutilus	*D. nanus*	Dactylogyridae
		D. sphyrna	Dactylogyridae
	Megalobrama amblycephala	*D. petruschewskyi*	Dactylogyridae
		D. pekinensis	Dactylogyridae
	Megalobrama terminalis	*D. parabramis*	Dactylogyridae
	Cirrhinus molitorella	*D. quanfami*	Dactylogyridae
	Systomus binotatus (Puntius binotatus)*	*D. damansari*	Dactylogyridae

		D. spirocopulatrium	Dactylogyridae
		D. sclerovaginalis	Dactylogyridae
	Systomus laterstriga (Puntius laterstriga)*	*D. laterstriga*	Dactylogyridae
	Systomus gonionotus (Puntius gonionotus)*	*D. puntii*	Dactylogyridae
Cobitidae	*Misgurnus anguillicaudatus*	*Gyrodactylus macracanthus*	Gyrodactylidae
Catostomidae	*Catostomus ardens*	*Pseudomurraytrema* sp. USA	Pseudomurraytrematidae
Order			
Anguilliformes			
Family			
Anguillidae	*Anguilla anguilla*	*Pseudodactylogyrus* sp. UK	Pseudodactylogyridae
		Pseudodactylogyrus sp. XHY	Pseudodactylogyridae
		P. bini	Pseudodactylogyridae
		P. anguillae	Pseudodactylogyridae
Order			
Mugiliformes			
Family			
Mugilidae	*Mugil cephalus*	*Ligophorus vanbenedenii*	Ancyrocephalidae
		L. leporinus	Ancyrocephalidae
Order			
Beloniformes			
Family			
Hemiramphidae	*Hemiramphodon pogonognothus*	*Dactylogyrus hemiramphodonus*	Dactylogyridae

Order			
Scorpaeniformes			
Family			
Platycephalidae	*Platycephalus indicus*	*Haliotrema platycephali*	Ancyrocephalidae
		H. macasarensis	Ancyrocephalidae
Order			
Esociformes			
Family			
Esocidae	*Esox lucius*	*Tetraonchus monenteron*	Tetraonchidae
Order			
Salmoniformes			
Family			
Salmonidae	*Salmo salar*	*Gyrodactylus salaris*	Gyrodactylidae
	Oncorhynchus mykiss	*G. derjavini*	Gyrodactylidae

5.4.1.1 Chaetodontidae, Lutjanidae, Sparidae, Scatophagidae & Mugilidae

From the host relationship trees (Figs. 5.1, 5.2 & 5.3), the perciformes hosts of the closely related ancyrocephalid genera (*Euryhaliotrematoides*, *Aliatrema*, *Haliotrema*, *Haliotrematoides*, *Euryhaliotrema*, *Ligophorus* and *Metahaliotrema* in Group 8; see Section 4.2.3) are shown to be closely related as well. In NJ tree, fish species from the family Chaetodontidae (hosts to *Euryhaliotrematoides*, *Aliatrema*, *Haliotrema*), Lutjanidae (hosts to *Haliotrematoides*, *Euryhaliotrema*, *Euryhaliotrematoides*, *Haliotrema*) and Sparidae (hosts to *Haliotrema*) are shown to be related where they are found in the same subGroup (SubGroup 2) (Fig. 5.3). In MP tree, fish hosts from Lutjanidae, Sparidae and Scatophagidae (hosts to *Metahaliotrema*) are shown to be related and present in the same subGroup (SubGroup 2) (Fig. 5.1). Also in ML tree, the closely related grouping of Lutjanidae + Sparidae (SubGroup 3) and Scatophagidae + Mugilidae (hosts to *Ligophorus*) (SubGroup 2) can be observed (Fig. 5.2).

5.4.1.2 Serranidae, Latidae, Sparidae & Nemipteridae

For fish species from the family Serranidae and Latidae, they are shown to be closely related where they consistently form sister group in MP and NJ tree (Figs. 5.1 & 5.3). The Serranidae and Latidae are hosts to the diplectanids analysed in this study, i.e. *Pseudorhabdosynochus* spp. and *Diplectanum* spp. which are shown to be closely related (see Section 4.2.2). The Sparidae and Nemipteridae, hosts of closely related diplectanids, i.e. *Lamellodiscus* spp. and *Calydiscoides* spp. (see Section 4.2.2), are shown to form sister group in the NJ tree (Fig. 5.3) and present in the same subGroup in the MP and ML trees (Figs. 5.1 & 5.2).

5.4.1.3 Gobiidae, Apogonidae & Eleotriidae

Perciforms species from Gobiidae, Apogonidae and Eleotriidae are host to the pseudodactylogyrids, i.e. *Pseudodactylogyrus* spp. (Gobiidae) and *Pseudodactylogyroides* spp. (Apogonidae and Eleotriidae) (Gussev, 1965; Ogawa, 1984; 1986; Lim, 1995) (Table 5.1). Although one species of *Pseudodactylogyroides* from *Oxyeleotris* sp. (Eleotriidae) and four species of *Pseudodactylogyrus* from an *Anguilla* sp. (Anguilliformes; see later; Section 5.4.2) are included in this study, the fish species from Apogonidae and Gobiidae are included in the host relationship tree to provide a better understanding of the host-pseudodactylogyrid relationship. From the host relationship trees of NJ, MP and ML, it can be observed that Apogonidae, Gobiidae and Eleotridae are consistently grouped in the same subGroup (Figs. 5.1, 5.2 & 5.3). This indicates that these three families are closely related as similar to their closely related pseudodactylogyrid monogeneans (see Section 4.4.6.3).

5.4.1.4 Lateolabracidae, Percidae & Hemiramphidae

Fish species from Lateolabracidae (*Lateolabrax* sp.), Percidae (*Gymnocephalus* sp.) and Hemiramphidae (*Hemirhamphodon* sp.) are host to *Dactylogyrus* spp., viz. *D. inversus* Goto & Kikuchi, 1917, *D. gotoi* Gusev, 1965 and *D. kikuchii* Gusev, 1965 from *Lateolabrax* sp. (Lateolabracidae), *D. hemiamphibothrium* Ergens, 1956 from *Gymnocephalus* sp. (Percidae) and. *D. hemiramphodonus* Lim, pers. com. from *Hemirhamphodon* sp. (Hemiramphidae) (Lim, pers. com.) (Figs. 5.1, 5.2 & 5.3) (see Table 2.5). Current results from the host relationship trees show that the *Lateolabrax* sp. (Lateolabracidae) and *Gymnocephalus* sp. (Percidae) are grouped together with the other Perciformes families. Although harbouring similar dactylogyrids monogenean (*Dactylogyrus* spp.) as the cyprinid hosts (see later; Section 5.4.4), the *Lateolabrax* sp.

(Lateolabracidae) and *Gymnocephalus* sp. (Percidae) are shown to be not related to the Cypriniformes. Hemiramphidae is not represented in the current host relationship trees (Figs. 5.1, 5.2 & 5.3).

5.4.1.5 Osphronemidae, Helostomatidae, Anabantidae & Channidae

The NJ, MP and ML host relationship trees generated using partial Cytochrome *b* sequences show that the anabantoids (Osphronemidae, Helostomatidae, Anabantidae) and channids are consistently present in the same subGroup, indicating that they are closely related (Figs. 5.1, 5.2 & 5.3). This supports the relatedness of the anabantoids and channids postulated by various ichthyologists based on the similar morphological characteristics such as possessing accessory breathing organs and ecological habitats (Nelson, 1994; Lim, 1997). Thus, current results show the closely related heteronchocleidid species (see Section 4.2.6) are found on closely related anabantoid and channid hosts species.

5.4.1.6 Channidae & Nandidae

It should be noted that instead of harbouring heteronchocleidids, *Channa micropeltes* (Cuvier) from Channidae are host to members of Sundanonchidae, i.e. *Sundanonchus micropeltis* Lim & Furtado, 1985, *S. foliaceus* Krtisky & Lim, 1995 and *S. tomanorum* Krtisky & Lim, 1995 (see Lim & Furtado, 1985; Krtisky & Lim, 1995). The *Sundanonchus* spp. are also found previously on nandid hosts, as exemplified by *S. triradicatus* Lim & Furtado, 1985 on *Pristolepis fasciatus* (Bleeker) (Nandidae) (Lim & Furtado, 1985). Current host relationship trees generated using NJ and MP analyses show that the Nandidae and Channidae are closely related where they are found in the

same subGroup (Figs. 5.1 & 5.2). Thus, *Sundanonchus* spp. are shown to be present on the related channid and nandid hosts.

5.4.1.7 Centrarchidae & Percidae

The freshwater perciformes hosts of the closely related ancyrocephalid genera, i.e. *Actinocleidus*, *Urocleidus*, *Ancyrocephalus* and *Onchocleidus* (see Section 4.2.3) are shown to be closely related in the host relationship trees (Figs. 5.1, 5.2 & 5.3). From the NJ, MP and ML host relationship trees, fish species from the family Centrarchidae (hosts to *Actinocleidus*, *Urocleidus, Onchocleidus*) and Percidae (hosts to *Ancyrocephalus*) are consistently present in the same subGroup (Figs. 5.1, 5.2 & 5.3).

5.4.2 Anguilliformes

Fish species from Anguillidae, i.e. *Anguilla* sp. possess *Pseudodactylogyrus* spp. (Pseudodactylogyridae). Despite harbouring closely related pseudodactylogyrids, current host relationship trees (Figs. 5.1, 5.2 & 5.3) show Anguillidae is distantly related to other fish hosts of pseudodactylogyrids from Apogonidae, Gobiidae and Eleotridae (Section 5.4.1.3).

5.4.3 Siluriformes and Osteoglossiformes

The distribution patterns of the host-ancylodiscoidid included in this study show that all the ancylodiscoidid monogeneans are found on Siluriformes hosts (Siluridae, Bagridae, Clariidae, Plotosidae, Ariidae) except two ancylodiscoidid species which are found on an Osteoglossiformes host (Notopteridae). From the host relationship trees (Figs. 5.1, 5.2 & 5.3), it is shown that all the fish host families from the Siluriformes are

clustered in the same group. This indicates that the Siluriformes hosts which harbour the related ancylodiscoidids are closely related as well. Within this Siluriformes group, fish species from the family Ariidae and Plotosidae can be observed to be closely related where these two families form sister group in NJ and MP trees (Fig. 5.1 & 5.2). Similarly, the ancylodiscoidid monogeneans, *Chauhanellus* and *Hamatopeduncularia* spp. from fish species of Ariidae and *Ancylodiscus* sp. from fish species of Plotosidae are shown to be closely related in the monogenean relationships trees where they are present in the same subGroup (see Section 4.4.4).

It should be noted that ancylodiscoidids are also found on osteoglossiformes host from Notopteridae. This is exemplified in the current study by *Thaparocleidus notopteri* Lim & Furtado, 1986 and *Malayanodiscoides bihamuli* Lim & Furtado, 1986 from *Notopterus notopterus* (another ancylodiscoidid genus from notopterids, *Notopterodiscoides* is not analysed in this study; see Lim & Furtado, 1986a).

5.4.4 Cypriniformes

From the host relationship trees (Figs. 5.1, 5.2 & 5.3), all the cypriniform hosts included in this study are shown to be closely related where they consistently form a monophyletic group. The dactylogyrids (*Dactylogyrus* and *Dactylogyroides*) found on these cyprinid hosts are shown to be closely related as well (see Section 4.4.5). It should also be noted that there are *Dactylogyrus* spp. which are present on non-cyprinid hosts (see above; Section 5.4.1.4).

5.5 Discussion

Current analysis of the fish hosts relationships and distribution patterns of the dactylogyridean monogeneans included in this study indicate that these dactylogyrideans are host-specific (see Gusev, 1978; Lim, 1987a; Lim, 1998 and Lim *et al.*, 2001). This is exemplified by the presence of dactylogyrids on cyprinid hosts (Section 5.4.4), ancylodiscoidids on the siluriforms (Section 5.4.3), heteronchocleidids on the related anabantoids and channids (Section 5.4.1.5) (see also Lim, 1986; Lim, 1989; Tan *et al.*, 2011) and pseudodactylogyrids on the related gobiids, eleotridids and apogonids (Section 5.4.1.3). The presence of diplectanids and marine ancyrocephalids on their respective perciforms families (Sections 5.4.1.1 & 5.4.1.2) as well as the freshwater ancyrocephalids on their related freshwater fish hosts (Percidae & Centrarchidae) (Section 5.4.1.7) also supports the fact that related host species harbour related monogeneans.

Current results also show the unusual presences of dactylogyrideans on apparently unrelated hosts. There are *Dactylogyrus* spp. on non-cyprinid hosts (Section 5.4.1.4), ancylodiscoidids on non-siluriform hosts (Section 5.4.3) and pseudodactylogyrids on the distantly related anguilliform and perciform (gobiids + eleotriidids + apogonids) hosts (Sections 5.4.1.3 & 5.4.2). The non-cyprinids, i.e. *Gymnocephalus cernuus* (Percidae) and *Hemiramphodon pogonognothus* (Hemiramphidae) (which might have shared similar freshwater habitats with the Cypriniformes) could have acquired the *Dactylogyrus* from cyprinid hosts. For the marine non-cyprinid host of *Dactylogyrus*, i.e. *Lateolabrax japonicus* (Lateolabracidae), this anadromous fish host might have acquired the *Dactylogyrus* spp. from cyprinid hosts during their migration into freshwater habitat (Lim, 2005).

As for the presence of ancylodiscoidids (*Thaparocleidus*, *Malayanodiscoides* and *Nototperodiscoides*) on Notopteridae (Osteoglossiformes), it suggests that the notopterid hosts have most probably acquired their ancylodiscoidids from siluriform hosts since some siluriformes hosts possess similar characteristics such as sharing similar habitats (swampy areas and rivers) with the notopterids and both are air-breathing fish (Nelson, 1974). The presence of *Pseudodactylogyrus* spp. on distantly related Anguilidae (Anguilliformes) (Section 5.4.2) and Gobiidae (Perciformes) (Section 5.4.1.3) also indicates *Pseudodactylogyrus* spp. could be acquired by Anguillidae from Gobiidae or vice versa as these two families are catadromous fishes with overlapping habitat during their migration between marine to freshwater habitats.

It can be noted that there are absences of dactylogyrideans on their related hosts. This is exemplified by the absence of heteronchocleidids from *Channa micropeltes* (Channidae), indicating that the heteronchocleidids could be lost (species loss) during the dispersion of *C. micropeltes* which later enable it to capture a new group of monogenean species, *Sundanonchus* spp. probably from a *Pristolepis* sp. (Section 5.4.1.6) (see also Lim & Furtado, 1985). It is also interesting to note that only one heteronchocleidid genus is present on each anabantoid and channid host species with no specificity of any of the three heteronchocleidid genera (*Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus*) to any anabantoid and channid species (Section 5.4.1.5).

Therefore, current results indicate that there are associations between the fish host relationships and distribution patterns of the dactylogyridean monogeneans as shown in the host specificity of dactylogyrideans where related hosts harbour related monogeneans, the unusual presences of dactylogyrideans on unrelated fish host species

and the absences of dactylogyrideans on their related hosts (species loss). These associations between the dactylogyridean monogeneans and their fish hosts mentioned above suggest that ecological processes such as co-evolution (related hosts harbour related monogeneans), host transfer (unusual presences of dactylogyrideans on unrelated fish host species) and failure to speciate (absences of dactylogyrideans on their related hosts) could have taken place during the diversification and dispersion of the dactylogyrideans (see General Discussion).

CHAPTER 6
GENERAL DISCUSSION
DIVERSIFICATIONS & STATUS OF DACTYLOGYRIDEA

6.1 Introduction

In this Chapter, a synthesis of the salient findings discussed in Chapters 3 to 5 will be done to encapsulate what is known to date about the diversification and status of the monogeneans in particular the Dactylogyridea. The significance of the morphological and morphometrical variations of species at the population level will be discussed in relation to speciation processes. In this chapter, an attempt is made to determine if the phylogenetic relationships of the monogeneans based on 28S rDNA sequences will be able to explain the morphological diversifications of the monogeneans or is the role of 28S rDNA in phylogenetic reconstruction overstated. The associations of the dactylogyrideans with their fish hosts might help to elucidate the evolutionary history of the diversifications of monogenean and might explain the unusual distribution patterns of some of the monogeneans. Issues encountered in the present different classification systems proposed for the dactylogyrideans will be discussed in the light of the current results particularly from Chapter 4. Finally aspects arising from this study which requires more investigation will be included and discussed under Future Studies.

It should also be noted that some results of this study had already been published in partial requirement for Ph.D. from this university (Tan & Lim, 2009; Tan, Khang & Lim, 2010; Tan, Fong & Lim, 2011). These 3 papers dealt with the analyses of the morphological, morphometrical and molecular characteristics of heteronchocleidines.

Two other papers published in connection with this Ph.D., viz., Wong, Tan & Lim (2006) & Lim, Tan & Gibson (2010) are from my supervisorôs related studies.

6.2 Diversifications of dactylogyrideans

Morphological and morphometrical differences are observed amongst different congeneric species and different individuals of a species (Chapter 3) (Lim, pers. com.). These differences, in particular, the morphometrical differences provide the necessary variations for the diversification of the monogeneans (Section 6.3). This study has shown that metric measurements of morphological features can be used to distinguish between morphologically similar species (PCA, Section 3.2.1) and hence could be used for differential diagnoses of species. The morphometric data of the different monogenean individuals supports that morphometric variations do exist in the monogenean species at population level (Section 3.2.2). These morphovariants or morphometic variants seem to be affected by host locations or/and host individuals and that different species are affected differently by these two environmental factors (Sections 3.2.2.1 & 3.2.2.2).

The number of morphovariants within each species population not constant and vary according to species: 3 morphovariants in *Trianchoratus malayensis* and *T. pahangensis*, 2 morphovariants in *T. ophicephali*, 2 morphvariants in *Bravohollisia rosetta*, *B. reticulata*, *B. gussevi*, *Bravohollisia* n. sp., *Caballeria liewi*, *C. intermedius* and *C. pedunculata* (see Section 3.2; Tables 3.1 & 3.2). The fact that different species have different number of morphovariants suggests that the amount of variability (genetic variations) is different in different species.

Lim (pers. com.) noted the diversity of certain morphologies in some group of monogeneans and the lack of diversity of the same morphologies in other monogeneans (cf stable male copulatory organ features in sundanonchids and tetraonchids and the diverse male copulatory organs in *Dactylogyrus* spp. (Lim, pers. com.). It will be interesting to see if the numbers of morphovariants do reflect genetic variations at generic and familial levels (see Future studies).

In this investigation we have tried to determine if all the different morphovariants are present in all host individuals (Section 3.4) and although there are some indication that this is the case, current investigation is limited by the way the data was collected for analysis (see Section 3.6) and more data are necessary for a more definite conclusion (see Future studies). As already noted morphovariants are highly likely to be due to genetic differences as monogeneans are known to prefer cross-fertilisation (Lim, pers. com.; Lim, 2002) which provides the variations observed in the different morphovariants within the populations (see later).

The amount of variations or differences found amongst the different morphovariants within a species population are low as indicated by their low differentiation indices, ū (an index used to detect amount of variations) as compared to the ū for species (ū =1 ï 7.3 for morphovariants cf ū =14 ï 192 for congeneric species) (Section 3.3; Tables 3.3ï 3.6). The indices show that although the variations within the population are real, the amount of differences are almost 50% less than that for species and hence not large enough to consider them as species (Section 3.3).

At the moment we know very little about the molecular diversity of monogeneans at population level although molecular data are available for different monogenean species. An attempt was made to use 28S rDNA sequences to determine whether two morphologically similar monogenean species are the same species or different species. The conserved 28S rDNA of 3 different individuals of the same species (*Sinodiplectanotrema malayanum*) are 100% similar (see Lim, Tan & Gibson, 2010; Appendix E) supporting the argument 28S rDNA of different individuals of the same species should be 100% similar. The 28S rDNA of different individuals of a species are not readily available and hence the molecular diversifications of monogeneans cannot be discussed herein.

6.3 Significance of variations in speciation process of monogeneans

Speciation is the evolutionary process by which new biological species arise (DeQueiroz, 1998). For speciation process to occur the speciating populations must be isolated from one another and possess enough variations (Lim, pers. com.). There are four modes of speciation in nature, i.e. allopatric (a population splits into two geographically isolated populations which undergone different selective pressure, independent genetic drift and mutations), peripatric (a subform of allopatric speciation where new species are formed in isolated, smaller peripheral populations that are prevented from exchanging genes with the main population), parapatric (two diverging populations are only partially separated where individuals of each species may come into contact from time to time, but with mechanisms that prevent inter-breeding) and sympatric (formation of two or more descendent species from a single ancestral species while inhabiting the same geographic region) (Templeton, 1981; Barraclough & Volger, 2000).

As already noted above (Section 6.2), monogeneans cross-fertilisation (Lim, 2002; Lim, pers. com.) provides the necessary genetic differences in morphovariants. Hypothetically it is possible for the morphovariants to eventually become new species if there are ecological barriers to isolate them. This study shows that probable isolating mechanisms are already present within the ecosystem of the parasites. The presence of the locality dependent morphovariants and host factor dependent morphovariants (Section 3.2.2) suggest these factors might served as ecological barriers or isolating mechanisms for eventual speciation of these morphovariants into new species. However, it is not possible in this study to determine which types of speciation has monogeneans undergone and how these speciation processes can take place amongst the monogeneans.

6.4 Can relationships trees generated from molecular data explain the evolutionary diversification of morphological characteristics among dactylogyrideans?

In this study a total of 190 species belonging to 53 genera and 11 families are used to generate the 3 relationship trees (Section 4.3). These dactylogyridean monogeneans (Lim, pers. com.) are diverse in terms of the number of anchors (2 to 4), bars (1, 2 or 0), marginal hooks (14 to 16), the presence of needles, squamodiscs or lamellodiscs as well as in the anatomical structures (e.g. bifurcating intestinal caeca which end blindly or confluent to single sac-like intestine of the sundanonchid-tetronchid). The relationship trees generated indicate in general that the ancyrocephalids are heterogeneous and this is supported by the splitting of the ancyrocephalids into 3 main groups (Section 4.5.2.5); that Dactylogyridae is unique (Section 4.5.2.6); the validity of Ancylosidcoididae (Section 4.5.2.3) and the grouping of heteronchocleidids (Section 4.5.2.1; see also Tan *et al*., 2011). However as already noted the current

relationship trees support the validity of the different family groups except for the ancyrocephalids which in this study we suggest should be split into two groups with one group, the freshwater forms retaining under the Ancyrocephalidae while the other groups which are more associated with the marine 4 anchor forms should be assigned to a new family (see later; Section 6.6).

When the morphological characteristics of the dactylogyridean monogeneans are superimposed onto the relationship tree generated from molecular data, the sundanonchid-tetronchid with 16 marginal hooks and single intestinal track and diplectanids with squamodiscs or lamellodics (this can be secondarily lost in some groups) and ovary overlapping the caeca are shown to diverged early from the other monogeneans on the relationship tree (Fig. 6.1). These groups are indeed different with features not found in the other dactylogyrideans. The other dactylogyridean groups observed in the relationship tree are highly heterogeneous especially in the anchors where family with 2 anchors, 2 needle-like structures (Dactylogyridae) and family with 2 anchors (Neocalceostomatidae) are grouped among the families with 4 anchors (Ancylodiscoididae, Ancyrocephalidae I, Ancyrocephalidae II, Ancyrocephalidae IIIï Calceostomatidae and Heteronchocleididae) as well as a family with members with 2 and 4 anchors (Pseudodactylogyridae) (Fig. 6.1).

Therefore, current relationship trees generated from molecular data are not able to explain how the present morphological characteristics or structures evolved and what the ancestral form of dactylogyridean is like. For example based on current relationship tree and examining the diversity of the number of anchors, marginal hooks and needles it is not possible to pinpoint when the 14 marginal hooks and 16 marginal hooks split and when the 4 anchors and 2 anchors split: current relationship tree suggest that the 2

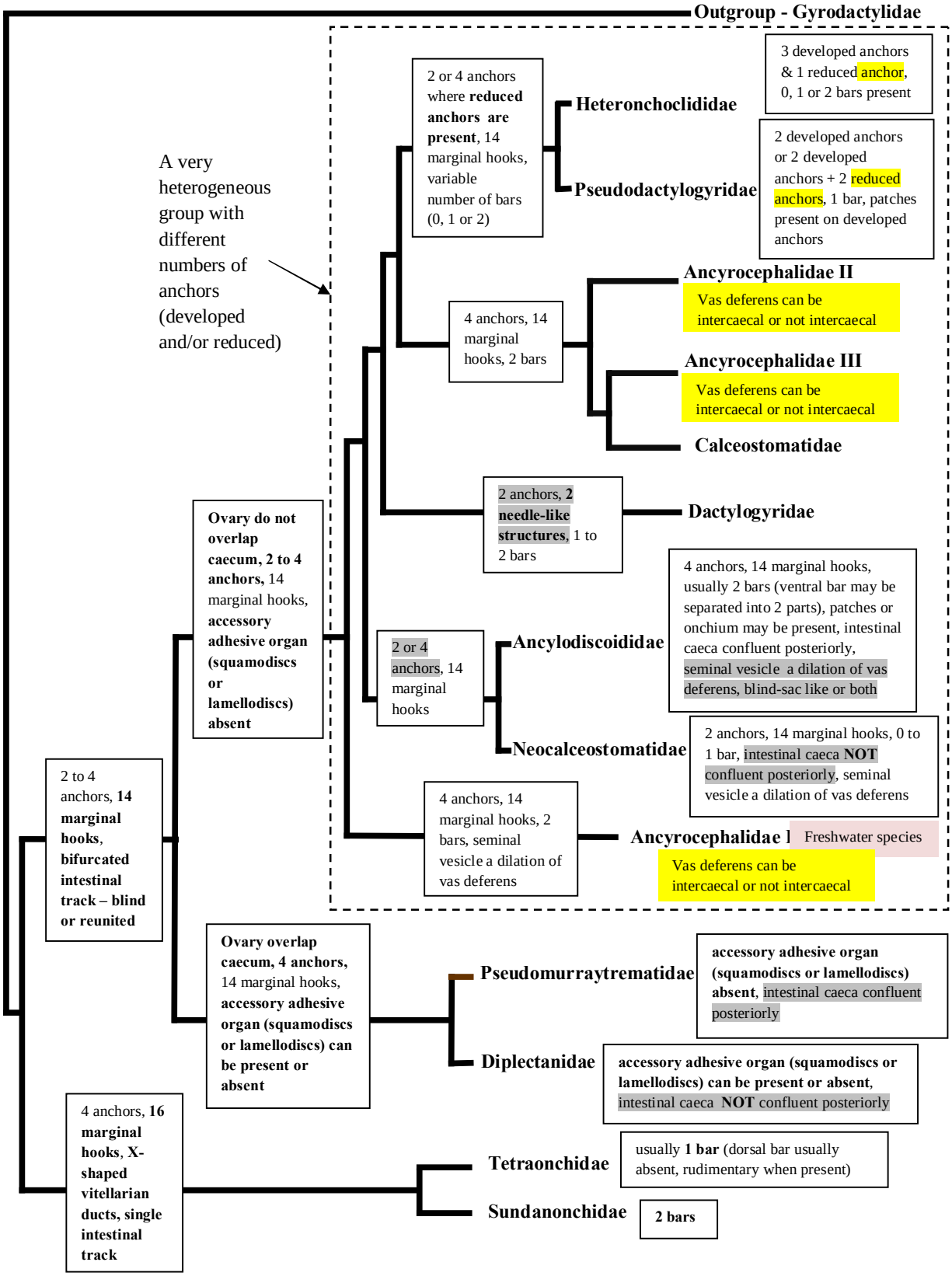

Figure 6.1 Interrelationships among different families within the order Dactylogyridea Bychowsky, 1937 with their morphological characteristics (*using MP tree as example).

anchors form evolved 3 times in the evolution of the dactylogyrideans (Fig. 6.1; see also Section 4.5.1) if one assumes that the 2 anchors are derived from ancestral dactylogyrideans with 4 anchors. The relationship tree is also not able to explain how the two types of seminal vesicles (dactylogyrid-type and blind sac ancylodiscoidid type) evolved. The presence of the needles in Dactylogyridae is also controversial ï some argued that they are anchors, and some that they are marginal hooks or a feature of unknown origin ï the relationship trees generated are not able to suggest much. They could be anchors and this seems to be supported by the presence of dactylogyridae within the 4 anchor dactylogyrideans such as the heteronchochoclidis and ancyrocephalids with ôlabileô anchors. But structurally the needles are different from the vestigial anchor of the heteronchocleidines (Lim pers comm., Lim).

The relationship trees generated from molecular data are not able to explain the relationships of the different morphological features. Therefore are we giving the 28S rDNA too much weight as a marker of relationships? Or is our analysis flawed because not all the monogeneans are represented in this analysis due to the lack of DNA information and also there might be species out there still undescribed as suggested by Lim that only about 10% of the monogeneans have been described in Malaysia for instance (Lim., 1998). Or is this analysis showing us that more sequences from other parts of the genome should also be incorporated? Using more morphological data did give us more information about the relationships. Perhaps more DNA data might give us a better picture of the molecular diversity which might mirror the morphological diversity. There are now currently other DNA sequences in the Genbank (Table 1.2) but only a few such sequences are available and most data are from the 28S rDNA (Table 1.2).

In conclusion we need more sequences from more monogenans before a proper relationship tree can be generated. This could be one reason why there are so much controversies and contradictory results from molecular analyses. The use of morphological information is still valid and has been statistically shown to be reliable diagnostic tool and therefore morphological information should thus not be pushed aside in favour of DNA results. The inability of the DNA results to explain morphological diversity shows that tree generated by DNA data should be used with great caution and more DNA data from more monogenean species are needed.

6.5 Dactylogyridean-host relationships

Several ecological processes such as co-evolution, host transfer and failure to speciate have been used to explain parasite-host relationships (Lim, pers. com.; Lim, 1997; 2005; Huyse & Volckaert, 2005; Dick & Patterson, 2007). These ecological processes are also shown in this study to play an important role in affecting the diversification and distribution patterns of dactylogyrideans (see below).

6.5.1 Co-evolution

It is well established that the majority of monogeneans are highly host specific (Gusev, 1976; Poulin, 1992; 2002; Lim, 1998; 2005; Sasal *et al*., 1999). Host specificity can be defined as the phenomenon produced by parasite-host couplings and indicates the degree to which a parasite occurs in association with a single host species (Dick & Patterson, 2007). The host specificity of monogeneans indicates the long term evolutionary association of the monogeneans and their hosts where co-evolution (the process of reciprocal, adaptive genetic change in two or more species) (see Woolhouse *et al*., 2002) could have occurred (Lim, 2005). The dactylogyrideans are host specific

and related host species can be observed to harbour related dactylogyrideans as exemplified in this study by the presence of dactylogyrids on cyprinid hosts, ancylodiscoidids on the siluriforms (see also Lim *et al*., 2001), heteronchocleidids on anabantoids and channids (see also Lim, 1986; Lim, 1989; Tan *et al*., 2011) as well as diplectanids and marine ancyrocephalids on the perciforms (Section 5.5). Therefore the dactylogyridean-host relationships support the Fahrenholzôs rule (parasito-phyletic rules) that related host species harbour related parasite species (Farenholz, 1913) and indicate possible evolutionary link between the fish hosts and their dactylogyrideans. Thus, co-evolution could be used to explain why related dactylogyrideans are found on related group of host species observed in this study.

6.5.2 Host transfer or host switching

The presence of *Dactylogyrus* spp. on non-cyprinids (Lim, pers. com.), ancylodiscoidids on non-siluriforms (Lim & Furtado, 1986a) and *Pseudodactylogyrus* spp. on the distantly related Anguillidae (Anguilliformes) and Gobiidae (Perciformes) (Sections 5.4.1.4 & 5.5) could be the result of host transfer, a process where parasites are acquired through close ecological (environmental) association between host and potential host species (Lim, 2005; Lim, pers. com.). For example, *Dactylogyrus* spp. and ancylodiscoidids could be acquired by non-cyprinids and non-siluriforms, respectively, whilst *Pseudodactylogyrus* spp. could be acquired by Anguillidae from Gobiidae or vice versa, through host transfer due to sharing of similar habitat or ecosystem (Section 5.5).

6.5.3 Failure to speciate

Parasite species could be lost due to failure to speciate (Johnson *et al*., 2003) leading to their extinction in certain host species as indicated by the distribution patterns of the 3 genera of heteronchocleidids (Section 5.5) where *Heteronchocleidus* are found on Chinese *Macropodus* (anabantoid), Malaysian *Trichopsis* (anabantoid) and African *Ctenopoma* (anabantoid), *Eutrianchoratus* on Malaysian *Belontia* (anabantoid) and African *Parachanna* (channid) and *Trianchoratus* on Malaysian and Indian anabantoids, Malaysian and Thai *Helostoma* and Malaysian channids (see also Tan *et al*., 2011; Lim, pers. com.). These distribution patterns suggest that the ancestral form of the heteronchocleidids could be present on both the ancestral anabantoids and channids. During subsequent speciation process, failure to speciate and extinction of the heteronchocleidids within their hosts could result in the survival of only one group of heteronchocleidids on each of the anabantoid and channid species (see also Tan *et al*, 2011).

6.6 Dactylogyridean systematics

6.6.1 Status of Dactylogyridea – present controversies

From Figure 6.1 (Section 6.3.1) it can be seen that the molecular data are not able to explain the morphological diversities of the different dactylogyridean families. In fact, Kritsky & Boeger (1989) assumption of monophyly of the dactylogyrideans (especially the ancyrocephalids) by putting them all (except Diplectanidae, Sundanonchidae and Tetraonchidae) back into the Dactylogyridae cause more problems and this move is not supported by the relationship trees generated in this study (Section 4.3). The inclusion of all the 4 anchor monogeneans into the Dactylogyridae did not resolve the paraphyly issue of ancyrocephalids (Sections 4.5.2.5 & 4.5.2.6). The

relationship trees obtained show that Dactylogyridae is unique and include only the monogeneans with 2 anchors, 1-2 bars, 14 marginal hooks and 2 needles (Sections 4.3 & 4.5). The relationship trees also show that ancyrocephalids are heterogeneous and should be divided into two groups (Section 6.4.1.1).

As already noted it is not easy to determine how the 16 marginal hooks can completely disappeared during the evolution of the major groups of dactylogyridean separated from the Sundanonchidae-Tetraonchidae group (Lim, pers. com.) (Section 6.3.1). The possible explanation is that it is still being retained as needle-like structures within the Dactylogyridae but again how do we explain the number of anchors present in the Dactylogyridae (2 anchors) and the other dactylogyrideans (4 anchors) and also explain why the other 2 anchor dactylogyrideans (Neocalceostomatidae and *Pseudodactylogyrus* spp.) are not closely related to the Dactylogyridae (Section 6.3.1)? Is the current interpretation too premature? This could be the case since the analysis itself lacks representations from all members of the Dactylogyridea as exemplified by the missing of other genera, e.g. *Dogielus* and *Thaprogyrus* from Dactylogyridae, Neocalceostomatidae is represented by 2 members, *Pseudodactylogyrus* and *Pseudodactylogyroides* are represented by 4 and 1 member, respectively (see Table 2.4; Section 4.4).

6.6.1.1 Ancyrocephalidae – paraphyly and separation into Ancyrocephalidae and Haliotrematidae n. fam.

The Ancyrocephalidae is not a cohesive monophyletic group and clustered in three separate groups based on current results from molecular data (Section 4.4.3). The non-monophyly of the Ancyrocephalidae has also been shown previously in relationship

trees based on morphological characters from various studies by Kritsky & Boeger (1989), Boeger & Kritsky (1993) and Boeger & Kritsky (1997). The non-monophyly of Ancyrocephalidae has led Kritsky & Boeger (1989) to reduce the family status of Ancyrocephalidae and include all its members (subfamilies) into the family Dactylogyridae. This move has caused the Dactylogyridae to be heterogeneous (Section 1.4).

The non-monophyly of Ancyrocephalidae could be a result from incorporating the Linnean classification system based on morphological characteristics into the relationships tree generated based on cladistic system which is not compatible to each other. The Linnean classification system and the cladistic system have several conceptual differences (Mayr & Bock, 2002). For instance, in cladistic system the taxon are not classified into ranks while there are clear hierarchies of ranks in the Linnean system. The cladistic methods are also based solely on dichotomous branching pattern where ancestral taxon splits up and ancestral species cannot exist as terminal species. Thus, cladistic system recognizes only monophyletic taxon which contains all of its descendants.

It has been shown by various authors that the strict adherence of monophyly for grouping of taxa in cladistic system is problematic (Horandl, 2006; Brummit, 2002; 2003; Brummitt & Sosef, 1998). In fact, it has been shown that most evolutionary processes result in descendants without extinction of the parental group (Horandl, 2006). The coexistence of the ancestral species and descendant species results automatically in paraphyly of the parental group. Thus, paraphyly has been recognized

as natural and inevitable in an evolutionary process (Horandl & Stuessy, 2010; Horandl, 2006; Brummit, 2002; 2003; Brummitt & Sosef, 1998).

Despite these facts, to date the relationship trees generated using cladistic methods in the study of systematic of dactylogyridean monogenean based on morphological and molecular data (Lim, pers. com.; Kritsky & Boeger, 1989; Boeger & Kritsky, 1993; 1997; Simkova *et al.*, 2003; 2006b; Wu *et al*., 2006; 2007a; 2007b) are interpreted according to cladistic system which place the search for strict monophyly as their ultimate aim. All the taxon which is found to be non-monophyletic are considered unnatural and revisions are needed. There are no other alternative and to date there has not been any suggestion to accept paraphyly in monogenean systematic.

Based on morphological data, Kritsky & Boeger (1989) has attempted to push for monophyly of the Ancyrocephalidae by changing the composition of Dactylogyridae to return it to its former composition of the pre-1978 when the data on monogeneans are only growing and poorly known (Lim, pers. com.). The relationship trees from current study based on molecular sequences of 191 species have shown that the authors are wrong in trying to ópushô their cladistic view into a Linnean system. When they do this, Kritsky & Boeger (1989) has caused confusion in the ranked Linnean system which has well defined characteristics to define the various groupings. Not only that they have not achieved monophyly for the Ancyrocephalidae, but at the same time their revision makes the family of Dactylogyridae (under the Linnean system) unnatural and heterogeneous as it has to accommodate the 4 anchors monogeneans, the Ancyrocehalidae and the two anchor monogeneans, the Dactylogyridae. Although when Dactylogyridae was first erected, it did include both 2 and 4 anchor forms, by 1978 the

Dactylogyridae was limited to accommodate monogeneans with 2 anchors and 2 needle-like structures with *Dactylogyrus* as the type species and the 4 anchor forms were all included under the Ancyrocephalidae. Current relationship trees also indicate the monophyly of the Ancylodiscoididae (see Lim *et al*., 2001).

Base on the results from current study, 2 possible options are proposed to resolve the paraphyly issue and heterogeneity of Ancyrocephalidae. The 2 possible options are elaborated as below:

1) Establishing 2 families – Ancyrocephalidae and Haliotrematidae n. fam.

In the first option, revision is proposed to resolve the heterogeneity of Ancyrocephalidae (Lim, pers. com.). Despite the revision done by Kritsky & Boeger (1989) (Section 1.4), the Ancyrocephalidae remains to be paraphyletic. Although the members of the ancyrocephalid genera included in this study are shown to be clustered in three separate groups based on partial 28S rDNA sequences, it should be noted that the type genus of the Ancyrocephalidae, the genus *Ancyrocephalus* is present in Ancyrocephalidae I group (Section 4.4.3.1). Thus in order to resolve the heterogeneity of Ancyrocephalidae, it is proposed that the family Ancyrocephalidae be amended to only include the genera in Ancyrocephalidae I group, i.e. *Ancyrocephalus*, *Actinocleidus*, *Cleidodiscus*, *Urocleidus* and *Onchocleidus*. At the same time, a new family should be erected to accommodate genera in Ancyrocephalidae II and Ancyrocephalidae III group (which essentially is one group without considering the only one member of Calceostomatidae; see Sections 4.4.3.2 & 4.4.3.3). A new family, Haliotrematidae n. fam., is hereby tentatively proposed for Ancyrocephalidae II and Ancyrocephalidae III group. By doing so, monophyly can be achieved for the Ancyrocephalidae and the newly erected family, the Haliotrematidae n. fam.

2) Acceptance of the paraphyly of Ancyrocephalidae

In the second option, the paraphyly of Ancyrocephalidae can be explained as evidence which have shown that the parental taxon does not experience extinction but co-exist with the descendent species as also shown by previous authors (Horandl & Stuessy, 2010; Horandl, 2006; Brummitt, 2002; 2003; Brummitt & Sosef, 1998). In the case of the 4 anchors Ancyrocephalidae, it is most probably the ancestral form which still exists after giving rise to its descendent, the other 4 anchors and 2 anchors form (e.g. Heteronchocleididae, Pseudodactylogyridae, Ancylodiscoididae, Dactylogyridae & Neocalceostomatidae). This has caused the family Ancyrocephalidae (the ancestral taxon) to be paraphyletic (Sections 4.5.1 & 6.2.3.1). Thus it is reasonable to accept the co-existence of parental and descendent taxon as terminal species and paraphyly is an inevitable phenomenon in the evolutionary process of the ancyrocephalid monogeneans (Lim, pers. com.).

6.7 Future studies

This study has shown several limitations due to constraints in obtaining morphometric and molecular data from more monogenean specimens and need to be rectified. It is shown in this study that large amount of morphometric data are needed for PCA to accurately detect the presence of morphovariants (Section 3.2.2). In fact, there is a need to collect morphometric data from every individual within a monogenean species population to determine the total number of morphovariants. Although this was done for *Trianchoratus* species population from different localities, larger sample size is needed especially for *T. longianchoratus* which has low abundance (Section 3.2.2.2; see also Tan *et al*., 2010). Despite current results showing the presence of morphovariatnts within *Bravohollisia* and *Caballeria* species (Section 3.2.2.1), not all the individuals

within the *Bravohollisia* and *Caballeria* species population are measured for morphometric data due to their large population size (high abundance). Thus, to determine the total numbers of morphovariant in future studies, morphometric data has to be collected from all the individuals within *Bravohollisia* and *Caballeria* species population.

Similarly, morphometric data of monogenean population from a single host is needed to determine if morphovariants are present within a single host. This information is important as it can be used to indicate that the morphovariants present in the same host (with similar macro- and micro-environment) could possess genetic basis. Due to limited morphometric data of monogeneans from single host population, current results can only show some indications that morphovariants could be caused by genetic variations in two monogenean species (*Trianhcoratus pahangensis* and *Bravohollisia* n. sp.; Section 3.4). Thus, morphometric data of monogeneans from single host population should be collected from more monogenean species in future studies to determine the role of genetic variations in affecting the presence of morphovariants.

This study shows variations within monogenean species population can be detected based on morphometric data analysed by PCA and differentiation indices (Section 3.3). In contrast, molecular data of monogeneans is currently inadequate to be used to detect variations at species population level (Section 6.2). Comparison of the 28S rDNA of 3 different individuals of the same species (*Sinodiplectanotrema malayanum*) are also shown to be 100% similar (see Lim *et al*., 2010), indicating that 28S rDNA being conserved are not suitable to detect variations at population level. Therefore, DNA sequences from other parts of the genome should be examined in future studies to search for suitable molecular sequences which can detect variations at

population level and until then, morphometric data are more readily available for the detection of variations within monogenean species population.

Current results show relationship trees generated from molecular data are unable to fully explain the evolutionary diversification of morphological characteristics among dactylogyrideans (Section 6.4). This could be due to not all the dactylogyridean groups are well represented in this study. For example, Neocalceostomatidae is represented by 2 members, Pseudodactylogyridae with 5 members while Pseudomurraytrematidae and Calceostomatidae with 1 member each (Table 2.4). It should also be noted that Dactylogyridae is only represented by 2 genera (*Dactylogyrus* and *Dactylogyroides*) where other dactylogyrid genera such as *Dogielus* and *Thaprogyrus* are not represented (Section 4.3.1.5). All these could have resulted in generating relationship trees which do not reflect the actual relationships of the dactylogyrideans. Thus, DNA sequence data from more species are needed to represent the different dactylogyridean groups in future studies so that the interrelationships of the dactylogyrideans can be properly assessed. The need for more taxa data versus more characters have been well debated by Hillis *et al*. (2003).

Molecular diversity of dactylogyrideans shown in this study strongly suggests the possible existence of unique segments within the DNA sequences of the dactylogyrideans. In fact, an analysis using INVERTER (a tandem repeats finder software) (Wirawan *et al*., 2010) reveals the presence of genus specific short DNA segments (5-6 base pairs) within the partial 28S rDNA sequences of the dactylogyridean monogeneans (preliminary results not shown). These short DNA segments could most probably be part of the tandem repeat in DNA sequences. A tandem repeat in DNA is a

sequence of two or more contiguous, approximate copies of a pattern of nucleotides. These tandem repeats evolve very rapidly where the type of repeats present and their number of repeats are highly diverse and variable. These characteristics make tandem repeats the ideal candidates as molecular markers or species diagnostic tool in various studies (e.g. Nathues *et al*., 2011; Hilty *et al*., 2006).

In fact there is very little information regarding the tandem repeats of monogenenas. To date, only two studies have been done on the tandem repeats of monogeneans where only a few gyrodactylids species were studied (see Matejusova *et al*., 2001; Collins & Cunningham, 2000). Thus more studies on tandem repeats should be done for the dactylogyrideans as well as other monogeneans in the future. The genus specific short DNA segments observed in partial 28S rDNA sequences from current study most probably indicates the presence of molecular markers. These molecular markers can be potentially used as diagnostic tools in the future study of monogenean taxonomy in view of the high number of estimated monogenean species which are yet to be described (Section 1.1).

SUMMARY

The main objectives of this thesis are (1) to evaluate the relevance of morphological and morphometric characters in diagnosis and the significance of the observed intraspecific variations using statistical methods and (2) to appraise the current use of molecular data in reconstructing phylogenetic relationships. Morphological and morphometric data are most commonly used diagnostic characters in the characterisation of monogeneans. Wide ranges in morphometric data are often observed in species descriptions raising question on its significance in the species population. The use of morphological and morphometric data in description has been deemed subjective by many and the validity and reliability of morphologies as diagnostic features are an on-going debate. A possible solution is to use statistical tool to analyse morphometric data to see whether species and intraspecific morphovariants can be delimited using morphometric data. Recently more molecular data (especially 28S rDNA) are becoming available for inferring phylogenetic relationships. However despite the increase in molecular data there are still discrepancies in the phylogenetic relationships reconstructed based on molecular data. In this study an attempt is made to determine relationships of the monogeneans based on molecular data from as many species and genera as possible. The molecular data used (partial 28S rDNA) are data sequenced in this study (64 sequences) as well as data from the GenBank (127 sequences). There are also only a few studies where molecular data are used for differentiating species and a related study provided data for the use of molecular data to support the separation of morphologically similar species (Lim, Tan & Gibson, 2010).

Current results from Principal Component Analysis (PCA) show that morphometries of the sclerotised hard parts of the monogenans can be used to differentiate morphologically similar species as indicated by the differentiation of the 744 *Bravohollisia* into 5 spp., 295 *Caballeira* into 3 spp. and 448 *Trianchoratus* spp. into 4 spp. (see Section 3.2.1 Chapter 3). The important diagnostic features can also be detected in the biplots produced from the PCA results (see Section 3.2.1.3) and these are congruent with the features used in description of the species. Thus, statistical analytical tools such as PCA can remove subjectivity in the use of morphologies in differentiating species and this result indicates that morphologies and morphometries are still relevant diagnostic characters. The subplots within the scatterplots for each species indicate that variations do occur within the species population and these variations can be group into variant groups or morphovariant group. Morphometric variations can be detected in four species of *Bravohollisia*, three species of *Caballeria* and three species of *Trianchoratus*. The numbers of morphovariant groups vary between species: for example there are two morphovariants in *B. rosetta*, *B. reticulata*, *B. gussevi*, *Bravohollisia* n. sp, *C. liewi*, *C. intermedius, C. pedunculata*, *T. ophicephali* and three morphovariants each in *T. malayensis* and *T. pahangensis*. The varying numbers of morphovariants present within each of these monogenean species are possibly due the amount of genetic diversity within each species population (Lim, 2002; Lim, pers. com.; Chapter 6). The analysis of morphometric data for the *Trianchoratus* has been published (Tan, Khang & Lim, 2010).

Differentiation indices, $\bar{u}$, are calculated to provide an estimate of the amount of variations existing amongst the different species and amongst different morphovariants within species population. The indices show that although the variations within the population are real, the amount of variations are almost 50% less than that for species

(ū =1 ï 7.3 for morphovariants cf ū =14 ï 192 for congeneric species). The PCA results and the Differentiation indices, ū, indicate that within a species populations genetic differences are present although not enough to differentiate them as species. These morphovariants are the results of sexual reproduction (cross-fertilisation) within a species population and these diversities in variations probably form the basic DNA materials for future speciation (Lim, pers. com.; Lim, 2002; Chapter 6).

Previous molecular studies on relationships of dactylogyrideans focus only on certain groups and some groups were represented by only very few species as shown in the review done (Chapter 1). In this study, the most available molecular data (partial 28S rDNA) from 190 dactylogyridean species (cf to 51 sequences in Ġimková *et al.*, 2004 and 47 sequences in Wu *et al.*, 2007a) are used to reconstruct the relationship trees of the dactylogyrideans to determine how the different members of the dactylogyrideans are grouped based on partial 28S rDNA. Eight major groups can be observed in the MP, ML and NJ relationship trees and the memberships for each of the eight groups correspond to the different dactylogyridean families and genera. The present analysis indicates the need to change the status of some of the families within the Dactylogyridea: for example subfamily Heteronchocleidinae should be raised to family status and the ancyrocephalids should be split into two groups (see later). This analysis also supports the validity of the family Ancylodiscoididae, Neocalceostomatidae and Pseudodactylogyridae. The uniqueness of the Dactylogyridae is also supported in this analysis which contradicts the postulation and interpretation of Kritsky and Boeger (1989). The results for the heteronchocleidids are already published in Tan, Fong and Lim (2011).

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The host relationships are done to show the relatedness of the fish host of the dactylogyrideans for inference to the host-dactylogyridean relationship (Chapter 5). The analysis shows that the fish species are congruent with the current knowledge based on morphologies (Section 5.3). The current analysis of host-monogeneans relationships (Chapter 5) also supports the accepted associations between the fish host relationships and distribution patterns of the dactylogyridean monogeneans especially that of their host-specificity and in most cases the host-monogenean relationships are in agreement with the parasitophyletic rule that related hosts harbour related parasites as exemplified by the presence of *Dactylogyrus* only on cyprinids and ancylodisocidids on the siluriforms. The host-monogenean relationships in particular host specificity indicate ancient relationships and suggest that co-evolution had occurred between the host-specific monogneans and their host species (Section 6.5.1). The unusual presence of *Dactylogyrus* spp. on non-cyprinid hosts (in the monogenean relationships trees these *Dactylogyrus* are related to the *Dactylogyrus* of the cyprinids) and the absences of some heteronchocleidids on the related anabantoid and channid fish groups (the anabantoid and channid are shown to be related in the host relationships trees) suggest that host transfer (from freshwater cyprinids to the migrating marine *Lateolabrax* sp. into the freshwater system) and failure to speciate might have occurred in evolutionary history giving rise to the present day diversification and distribution patterns of dactylogyrideans (Lim, 2005; Tan *et al.*, 2011) (Chapter 5 and 6).

The results from this study which are presented and discussed in the various chapters are synthesised and their significance discussed in Chapter 6. Morphological and morphometric information are still valid characters in differential diagnosis as indicated by the PCA scatterplot results. However such analyses require a substantial amount of data (Section 2.4.1; Tables 2.2 & 2.3). The morphometric variants could be

due to genetic variabilities resulting from cross-fertilisation amongst the hermaphrodite monogenean species (Lim, pers. com & unpublished data; Lim, 2002). The morphological and morphometric variations indicate that similar variations in the past evolutionary history could give rise to the the species diversification and speciation process for the dactylogyrideans and these current variations could form the basis for future diversifications of the monogeneans if the right isolating mechanisms are present (Section 6.3).

However the reconstructed tree from molecular data is limited in its ability to explain the possible evolutionary relationships of the different diagnostic characters and the possible evolutionary diversification of the morphological characters amongst the dactylogyrideans (Section 6.4). This is probably due to incomplete data since some groups are not represented or poorly represented for example Pseudomurraytrematidae is only presented by one unknown species (Table 2.4) and some groups such as *Dogielus* and *Thaparogyrus* are not represented at all in the analysis and probably also due to the use of only one molecular sequence 28S rDNA. Although basically the dactylogyrideans are related based on the present reconstructed tree, the present reconstructions did not take into consideration the other monogenean groups such as the polystomatideans which might show a different relationships. This molecular analysis is not possible without the use of PAUP in HPC (High Performance Computer) because of the large amount of data. The limitation in the reconstructed tree could be due to the use of the tree-building software PAUP. Other softwares might provide some insights into the relationships not revealed using PAUP.

Kritsky and Boeger (1989) in an attempt to make ancyrocephalids monophyletic had proposed that all the ancyrocephalids be grouped under the family Dactylogyridae which Lim vehemently disagree noting that Dactylogyridae as defined by Bychowsky & Nagibina (1978) and Gusev (1978) are unique in having 2 needles, 2 anchors and 14 marginal hooks (Section 1.4). Furthermore Lim (pers. com.) suggested that they are wrong in using a Linnean system (ranked system) of naming and incorporate it into the cladistic classification which is basically rankles (Section 6.6.1.1). It is suggested in this study that the heterogeneity of the ancyrocephalidae could be resolved by separating the group into two with the Ancyrocephalidae housing the freshwater ancyrocephalids (*Ancyrocephalus*, *Actinocleidus*, *Cleidodiscus*, *Urocleidus* and *Onchocleidus*) and a new family to house the marine ancyrocpehalids viz. *Haliotrema*, *Bravohollisia, Caballeria, Pseudohaliotrema, Metahaliotrema, Euryhaliotrema, Euryhaliotrematoides, Tetrancistrum, Haliotrematoides, Ligophorus* and *Aliatrema* as well as freshwater members from the cichlids, i.e. *Cichlidogyrus*, *Scutogyrus* and *Onchobdella* (Section 6.6.1.1). The ancestors of cichlid hosts have been postulated to have a marine origin since some of the most primitive species of Cichlidae have high salinity tolerance and prefer to live in estuarine environments (Murray, 2001).

In this present study statistical analytical methods are used on the morphometric data from 12 monogenean species with individuals ranging from 59 ï 180 individuals per species (none of the previously reported studies have used this large amount of morphometric data) (Section 2.4.1). This is also the first time that differentiation index is used to estimate variations amongst species and morphovariants (Section 3.3). However shapes of the different sclerotised parts have not been taken into consideration and the shape might provide more information on the relatedness of the different individuals and species (Lim, pers. com). In this study, a total of 191 sequences (62

from present study and 129 from GenBank) belonging to 190 species representing 53 genera and 12 families are used to reconstruct phylogenetic trees using PAUP and HPC has to be used (for example analysis using PC can take more than 5 weeks to go through). The present study indicates that there are limitations and delimitations in this study which should be looked into and these include the need to collect large amount of morphometric data which is needed for PCA to accurately detect the presence of morphovariants within species popuplation, more DNA sequences from other parts of the genome should be examined for suitable molecular sequences which can detect variations at population level and DNA sequence data from more species are needed to represent the different dactylogyridean groups (Section 6.7).

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