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THE EVOLUTIONARY HISTORY AND ECOLOGY OF *TELMATHERINA* IN LAKE MATANO:
AN EXAMPLE OF ADAPTIVE RADIATION IN AN ANCIENT LAKE.

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A dissertation submitted to the Faculty of Graduate Studies and Research through the Department of Biological Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Windsor.

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ABSTRACT

Understanding factors generating biodiversity are critical in a world where continued anthropogenic disturbances threaten the survival of an increasing number of species. Ancient lakes provide ideal systems for the study of such factors as early invading species have a high potential for speciation and adaptive radiation. Lake Matano, a tropical island lake located on Sulawesi, Indonesia, is estimated to be 2-4 million years old. This isolated lake is characterized by very low productivity and has a high degree of endemism. The purpose of this research was to assess the potential for adaptive radiation within the fish community of this system and determine its causal root(s).

First, genetic data demonstrated that, of the endemic fish genera in this system, *Telmatherina* is monophyletic and demonstrates greater genetic diversity than any other genus in this system. *Telmatherina* are also characterized by more numerous colouration patterns and exhibits higher variation in feeding traits than the other genera in this lake as determined from standard morphometrics. These data support the radiation of *Telmatherina* in Lake Matano due to either sexual selection among the varied colouration types, or resource partitioning in this trophically limited system.

A second investigation used genetic, shape and trophic data to identify the number of *Telmatherina* species present in Lake Matano and determine if feeding trait differences among them were of an adaptive nature. Genetic data revealed that all *Telmatherina* previously described could be categorized into three distinct lineages. Skull and pharyngeal jaw features showed clear distinction among the three lineages and these data corresponded to lineage-specific diets determined from gut content and stable isotope analyses. Results separated the 3 *Telmatherina* lineages into a littoral specialist, a pelagic specialist and a generalist making use of both littoral and pelagic resources. The generalist was also found to be genetically basal to the two specialist lineages suggesting that the generalist is the most recent common ancestor to both specialists and that the radiation of *Telmatherina* is ongoing.

A third study used genetic, colouration and geometric shape analyses to assess whether or not the sympatric radiation of *Telmatherina* was primarily driven by sexual selection, specifically among colouration types, or if body shapes were more important to initial divergence. Results confirmed the *Telmatherina* genetic lineages and showed that colouration had little to do with initial divergence. Rather, body shape differences, most notably along the axis of body compression, but also in fin shape, caudal peduncle and head region, were key features segregating *Telmatherina* into three genetic lineages. Accrued data from this research provide strong support for the adaptive radiation of *Telmatherina* in Lake Matano, as a consequence of ecological selection within this trophically limited system and demonstrates one of the clearest examples of this phenomenon in a natural setting.

CO-AUTHORSHIP STATEMENT

This thesis includes materials reprinted from co-authored, submitted and published articles. In all cases the contribution of the co-authors was primarily in an advisory capacity or contributed intellectually by providing consultation, facilities and materials to complete the research. The primary contributions, experimental designs, data collection and interpretation and the preparation of all manuscripts were performed by the author except in the case of Appendix 1 where some data processing was done by CHJM Fransen.

Chapter 2, 3, 4, and the Appendix 1 are based in part on the articles listed below which were substantially modified to accommodate the requirements of this thesis.

1- Roy D, Docker MF, Hehanussa P, Heath DD, Haffner GD. (2004). Genetic and morphological data supporting the hypothesis of adaptive radiation in the endemic fish of Lake Matano. *Journal of Evolutionary Biology* **17**, 1268-1276.

Reprinted in amended form from the *Journal of Evolutionary Biology.*

2- Roy D, Paterson G, Hamilton PB, Heath DD, Haffner GD. Resource based adaptive radiation in *Telmatherina* from Lake Matano, Indonesia. (*Manuscript submitted to Molecular Ecology. Jan 2006*).

3- Roy D, Docker MF, Haffner GD, Heath DD. Body shape versus colour associated initial divergence in the *Telmatherina* from Lake Matano, Sulawesi, Indonesia. (*Manuscript submitted to Biological Journal of the Linnean Society. Jan 2006*)

4- Roy D, Kelly DW, Fransen CHJM, Heath DD, Haffner GD. Evidence of small scale vicariance in Caridina lanceolata (Decapoda: Atyidae) from the Malili Lakes, Sulawesi (*Manuscript submitted to Evolutionary Ecology Research. Aug 2005*).

For Franklin and Walace,

who love the most and expect the least.

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DEFINITIONS

Species: Although many definitions of a species exist (Schluter 2001; Turner 2001; Agapow et al. 2004; Coyne & Orr, 2004), for the purposes of this study I will use the neo-Darwinian definition which considers species as genetically distinct clusters of organisms with one to several distinctive autapomorphies, sharing a common ancestry and exhibiting reproductive isolation (Darwin 1859; Mallet 1995; Turner 1999).

Speciation: Divergence of a common lineage or species into two or more species. In this particular case, I also include the derivation of species due to branching from the ancestral line while the ancestral species remains extant, as well as the hybridization and introgression of two species forming a new intermediate species (i.e., in hybrid contact zones refs).

Allopatric speciation: Speciation occurring in response to geographical isolation between different taxa, usually populations of the same species. Can be due to drift, or divergent natural selection, founder effects and other mechanisms (Mayr, 1963; Coyne & Orr, 2004; Rundle & Nosil, 2005).

Sympatric speciation: Speciation among taxa which exhibit overlapping distributions. A good example is two overlapping populations diverging by means of sexual selection (but see Arnegard and Kondrashov 2004; Gourbiere 2004; Kirkpatrick and Nuismer 2004).

Endemism: Taxa native to, or originating from, a particular ecosystem.

Continental Island: An island whose tectonic plate(s) is independent and separated from other tectonic plates in its immediate vicinity. Examples include New Caledonia, Madagascar, and those of the Hawaiian archipelago (Wallace 1892).

Disruptive selection: Selection differentials in an environment that favours the individuals at the extremes of their phenotype or habitat or resource use (Coyne & Orr, 2004).

Reinforcement: Selection acting to increase reproductive isolation among already segregating groups (Schluter, 2000; Coyne & Orr, 2004; Rundle & Nosil, 2005).

Adaptive radiation: Rapid divergence among taxa from a common lineage in response to selective pressures leading to modification of traits allowing the adaptation along environmental adaptive peaks (Schluter 2000).

1.0 BACKGROUND

The unprecedented rate and extent of species loss, on a global scale, has led to a concerted effort among biologists and ecologists to stress the importance of biodiversity in natural systems. Though most would agree that conserving biodiversity is paramount, relatively few biologists and ecologists provide a clear rationale for such an argument. Perhaps the most well known reason for maintaining biodiversity stems from the 'diversity begets ecosystem stability' debate (McNaughton, 1977; Tilman & Downing, 1994; Johnson et al., 1996). According to reasoning therein, increased diversity of an ecosystem buffers it against perturbations such as habitat destruction, climate change and even pestilence (McNaughton, 1977; Tilman & Downing, 1994; Johnson et al., 1996). High biodiversity results in redundancy, with many species having similar roles or occupying similar niches (Elton, 1958; McNaughton, 1977; Ehrlich & Ehrlich, 1981). If some species are lost, other similar species can easily fill their vacated niches, causing minimal disruptions and repercussions to the function and maintenance of the ecosystem (Elton, 1958; McNaughton, 1977; Ehrlich & Ehrlich, 1981). Studies also suggest that ecosystems characterized by greater diversity are able to recover faster from extensive perturbation than less diverse ones (McNaughton, 1977; Tilman & Downing, 1994; Johnson et al., 1996).

Although the 'stability through diversity' concept is important and has greatly enhanced the push for biodiversity conservation throughout the world, an often forgotten or less acknowledged benefit of biodiversity is related to its evolutionary significance (Garner et al., 2005; Velland & Geber, 2005). Each extant genome represents the cumulative adaptation to a given set of environmental and ecological circumstances and is a direct consequence of ancestral evolution (modification by decent; Darwin, 1859; Wallace, 1892). Each genome has the potential to serve as a starting point for the evolution of new genomes through the action of selection, drift, reinforcement and character displacement (Garner et al., 2005; Velland & Geber, 2005). Conversely, the loss of genomes due to continued habitat destruction, and from hybridization and introgression among native and non-indigenous species (Rhymer & Simberloff, 1996), results in the loss of potentially new evolutionary pathways essential for continued adaptation in spatio-temporally heterogenous environments (Agapow et al., 2004; Garner et al., 2005). Thus, the conservation of biodiversity has very real evolutionary implications, and processes that generate biodiversity offer perhaps the only mechanisms countering species loss (Ehrlich, 1994). Processes such as speciation and adaptive radiation can generate new species allowing the increase in potential evolutionary pathways. Understanding under what circumstances these processes work can provide invaluable insight not only into the formation of new species and evolutionary mechanisms, but also into the wise application of limited conservation resources to areas where these processes have been identified (Ehrlich, 1994; Moritz & Faith, 1998; Myers et al., 2000; Agapow et al., 2004; Garner et al., 2005). Locations identified as centers of endemism where many unique species exist provide key ecosystems where such biodiversity generating mechanisms can be recognized and studied (Myers et al., 2000; Evans et al., 2004).

1.1 SULAWESI, LAKE MATANO

The continental island of Sulawesi, located in the Indonesian archipelago, is home to perhaps some of the most unique ecosystems in the world (Wallace, 1892; Evans et al., 1999; Whitten et al., 2002; Sodhi et al., 2004). It is from here that Wallace (1892) formulated his theories of sea level fluctuations, biogeographic distribution of species and evolution. Today, Sulawesi is recognized as an evolutionary zone of distinction separating the fauna and flora of South-East Asia from that of Australia, and is an integral part of what is commonly referred to as Wallace's line (Martens, 1997; Wilson & Moss, 1999; Myers et al., 2000).

Sulawesi Island has a unique geological history, and remains a highly active tectonic region (Hamilton, 1979; Wilson & Moss, 1999; Whitten et al., 2002; Rintelen & Glaubrecht, 2005). Formed approximately 8-12 million years ago (mya), Sulawesi is the result of a collision of three large tectonic plates, namely; the Gondwanan remnant (hereafter Australian), the Southern Ocean, and the Eurasian continental. The collision of these plates caused mantle uplifting, accretion and folding to occur along the marginal edges of both the Eurasian and the Australian plates, forming island arches (Hamilton, 1979; Wilson & Moss, 1999; Whitten et al., 2002). As the tectonic plates pressed further together, major imbrications caused two parallel arches from the leading edges of both the Australian and the Eurasian plates to collide. This collision caused the eastern part of Sulawesi (Australian arch) to begin subducting under the western (Eurasian) arc, but also caused considerable uplifting and accretion of marine sediment adding land mass between the two arches (Hamilton, 1979; Wilson & Moss, 1999; Whitten et al., 2002). Collision of the two island arches also caused substantial rifting and faulting on the newly formed island, while further tectonic action twisted the island into its characteristic 'K' shape (Katili, 1978; Hamilton, 1979; Wilson & Moss, 1999; Rintelen & Glaubrecht, 2005). Once formed, Sulawesi was pushed over major subduction zones causing substantial asthenospheric materials to be disgorged through the extensive faulting system within the island's land mass (Katili, 1978; Hamilton, 1979; Wilson & Moss, 1999). Asthenospheric materials forming Sulawesi have been estimated to be as old as 50 million years, and are rich in minerals and metal ores (Katili, 1978; Hamilton, 1979; Wilson & Moss, 1999; Hehanussa, LIPI, pers. communication).

Sulawesi has been isolated by deep oceanic trenches for millions of years and truly epitomizes the continental island concept (see definitions; Wallace, 1892; Wilson & Moss, 1999). The unique and aged origin of Sulawesi provides a rare opportunity to investigate key ecological and evolutionary concepts among its native species. Sulawesi has recently been recognized as a 'hotspot' for biodiversity and for conservation efforts largely because of its elevated endemism which is supported by rather unique ecosystems found nowhere else in the world (Myers et al., 2000; Evans et al., 1999; Wilson & Moss, 1999; Whitten et al., 2002; Sodhi et al., 2004).

One of the most unique ecosystem on Sulawesi is a series of five connected lakes forming the headwaters of the Larona River (see map Chapter 2). The Malili Lakes were likely formed at different times by complex faulting along the seismically active Matano and Palu Koro faults rendering them partially isolated from one another and joined by small tributaries (Brooks, 1950; Hamilton, 1979). Lake Matano, whose transverse axis follows the Matano fault (displacement = 12 cm year^{-1}), is the hydrological head of the watershed and is separated from downstream lakes by the Petea River consisting of highly flowing water over cascading rapids, negotiating a 72m elevation change (Brooks, 1950; Hamilton, 1979; Kottelat, 1990a; Haffner et al., 2001). It is estimated to be at 2-4 million years old, to have levels of endemism greater than 80%, and has been identified as the principal seeding source of endemic species within the Malili Lakes (Brooks, 1950; Haffner et al., 2001; Rintelen et al., 2004). This relatively small graben lake (surface area approximately 164 km^2) has very little littoral area surrounding a very deep basin (maximum depth 590m). Lake Matano contains very clear water (Secchi depths > 23m), low phytoplankton biomass consisting predominantly of cyanobacteria (Sabo, 2006) and supports rare top predators which are not purely piscivorus (Heath, pers. communication). These conditions indicate very low levels of primary productivity and support a highly oligotrophic status (Haffner et al., 2001; Rintelen et al., 2004).

Very little is known about the hydrological history or processes regulating Lake Matano, but the lake itself defies typical limnological and morphological classification (Lewis, 2000; Sabo, 2006). Lake Matano is characterized by unique water constructs, vertical circulation patterns, and water chemistry (Haffner et al., 2001). Much of the inflow to the lake is from small rivers and underground sources injecting cold water at various depths below the surface (Haffner et al., 2001). Regular mixing events are still conjectural but may take the form of large cold density currents along the extremely steep slopes and/or sedimentary warming (Haffner, unpublished data). Nevertheless, strong mixing processes prevent the formation of pronounced thermal stratification, and mix the water column to extraordinary depths; very abnormal characteristics for a deep, tropical lake (Lewis, 2000; Haffner et al., 2001).

As a consequence of its unique geological history, limnology and morphology, the endemism of the fauna adapted to Lake Matano is among the highest in the world (Haffner et al., 2001). The fish fauna in particular, are interesting as many are relatively new to science with little description as to their general biology, ecology and evolutionary histories (e.g., Kottelat, 1990a; 1990b; 1990c; Kottelat, 1991; Larson & Kottelat, 1992, Lovejoy, 2000). Others have yet to be described.

Ancient lakes, such as Matano, are increasingly recognized as potentially important models for the study of evolution and speciation (Rossiter & Kawanabee, 2000; McKinnon, 2002; Rintelen & Glaubrecht, 2003; Wilson et al., 2004; Schön & Martens, 2004). Characterized by complex geological histories (> 100 000 years old), most ancient lakes provide a high potential for evolutionary and speciation processes to occur and for the adaptation of their insular species to these ancient environments (Brooks, 1950; Martens, 1997; Rossiter & Kawanabee, 2000; Turner et al., 2001; Mckinnon, 2002; Rintelen & Glaubrecht, 2003; Schön & Martens, 2004). Accordingly, most ancient lakes are characterized by high levels of endemism and biodiversity, and have attracted special attention from their evolutionary and biodiversity perspectives (Rossiter & Kawanabee, 2000; McKinnon, 2002; Wilson et al., 2004; Schön & Martens, 2004). Ancient lakes provide rare opportunities to address, and empirically test, fundamental principals of ecology and evolution such as: What is a species (Kullander, 1999; Turner 1999)? How and why do so many closely related species coexist (e.g., Metz & Freitson, 1998; Sturmbauer, 1998; Albertson et al., 1999; Schön & Martens, 2004) and what are the mechanisms responsible for the colonization and radiation in these systems (Reinthal & Meyer, 1997; Markert et al., 1999; Seehausen et al., 1999; Kornfield & Smith, 2000; Wilson et al., 2000; Turner et al., 2001; Schön & Martens, 2004; Rintelen & Glaubrecht, 2005)?

Lake Matano definitely qualifies as aged (Haffner et al., 2001), and the fish fauna of the lake is clearly unique (Kottelat et al., 1993; Whitten et al., 2002). This is especially true of the endemic *Telmatherina* fishes characterized by a variety of coloured morphotypes. Currently, these fishes are described as seven species (Kottelat, 1991; Kottelat et al., 1993), but all are difficult to distinguish from one another based on traditional morphological characters alone. The high diversity of *Telmatherina* compared to other fish genera from Lake Matano (Kottelat, 1990a; 1990b; 1990c; Kottelat, 1991; Larson & Kottelat, 1992), hints to different mechanisms and processes regulating their divergence and speciation in this system. As such, the piscean community of this ancient lake provides a rare opportunity to investigate questions of intralacustrine speciation and adaptive radiation akin to that demonstrated for some cichlid lineages in the African Rift Valley Lakes (Fryer & Iles, 1972; McKaye et al., 1984; Reinthal & Meyer, 1997; McCune, 1997; Albertson et al., 1999; Salzburger & Meyer, 2004). More interestingly, however, Lake Matano is relatively pristine, quite small, has very low primary production and has rather simple fish species assemblages (Kottelat, 1990a; 1990b; 1990c; Kottelat, 1991;

Larson & Kottelat, 1992; Kottelat et al., 1993). Moreover, preliminary investigations of sedimentary cores taken from various areas around Sulawesi and in parts of Matano proper, suggest relatively constant water-levels since its formation (Haffner et al., 2001; Hope, 2001; Visser et al., 2004). Together, these attributes make Matano perhaps even more attractive in terms of tracking speciation events than any other ecosystem in the world.

1.2 SPECIATION

Speciation is generally accepted as the process by which species originate from the diverging forms of a common ancestor (Futuyma, 1986). Although this is one of the most fundamental of concepts in ecology and evolution, speciation remains one of the most complex and poorly understood (Coyne & Orr, 1994; Schiltzhuizen, 2000; Schluter, 2000; Schluter, 2001; Turelli et al., 2001; Coyne & Orr, 2004; Kocher, 2004; Rundle & Nosil, 2005). Speciation finds its roots in a fundamental understanding and accurate description of what is considered a species. Yet, even this most cardinal concept still eludes a clear definition (Moritz, 1994; Mallet, 1995; Moritz & Faith, 1998; Kullander, 1999; Turner, 1999; Hey, 2001; Agapow et al., 2004; Kocher, 2004). Part of the 'species concept' problem stems from a need to properly identify a practical definition of a species having a universal application (Moritz, 1994; Turner, 1999; Hey, 2001; Agapow et al., 2004; Coyne & Orr, 2004). This is largely because different perceptions as to what constitutes a species can result in inconsistent interpretations of the actual processes and causes of speciation (e.g., Funk, 1998; Kullander, 1999; Turner, 1999; Agapow et al., 2004). Most studies use the classic biological species concept, defining a species as a reproductively isolated group of individuals (Mayr, 1963; Turner, 1999; Coyne & Orr, 2004).

Traditional views of speciation characterize it in terms of the spatial extent over which reproductive isolation emerges among populations (Turelli et al., 2001; Schluter, 2001; Rundle & Nosil, 2005). Allopatric speciation (see definitions) involves the establishment of reproductive isolation between populations with non-overlapping distributions. This speciation mode has gained wide acceptance as the development of reproductive isolation in disjunct populations is intuitive and is not required to be strong in the presence of substantial physical barriers to gene flow (Turelli et al., 2001; Coyne & Orr, 2004). There have also been many clear examples reported in the literature (Mayr, 1963; Knowlton & Weigt, 1998; Turner, 1999; Hurt et al., 2005). In contrast, sympatric speciation (see definitions), whereby reproductive isolation is established among populations with overlapping distributions, continues to cause debate with few empirical confirmations (Turelli et al., 2001; Via, 2001; Arnegard & Kondrashov, 2004; Gourbiere, 2004; Kirkpatrick & Nuismer, 2004; Barluenga et al., 2006). Perhaps the best evidence in support of sympatric speciation was demonstrated by Barluenga et al. (2006), in the crater lake cichlids of Lake Apoyo in Nicaragua. There have also been a number of theoretically-based studies demonstrating how speciation in sympatry is possible (Higashi et al., 1999; Kondrashov & Kondrashov, 1999; Dieckmann & Doebeli, 1999). Yet, skepticism persists, for the evolution of species in sympatry entails the development of strong disruptive selection causing reproductive isolation among sometimes very closely related and sometimes morphologically very similar lineages (Dieckmann & Doebeli, 1999; Higashi et al., 1999; Kondrashov & Kondrashov, 1999; Turelli et al., 2001; Arnegard & Kondrashov, 2004; Kirkpatrick & Nuismer, 2004). Moreover, many researchers note that even the best examples of sympatric speciation (see definitions) have at least as parsimonious an explanation for the same speciation events in allopatry (i.e., microallopatry Seehausen, 2000; Turelli et al., 2001; Rico & Turner, 2002).

More recently, a shift away from considering speciation modes in a spatial context towards understanding the processes or mechanisms that establish reproductive isolation among closely related populations, has been taking place (Schluter, 2000; Schluter, 2001; Doebeli & Dieckmann, 2003; Arnegard & Kondrashov, 2004; Gourbiere, 2004; Kirkpatrick & Nuismer, 2004; Coyne & Orr, 2004; Rundle & Nosil, 2005). Ecological speciation is characterized by those speciation events in which ecological pressures such as inter- and/or intra- specific interactions, as well as environmental pressures, are responsible for the initiation of reproductive isolation among populations (Schluter, 2001; Doebeli & Dieckmann, 2003; Coyne & Orr, 2004; Rundle & Nosil, 2005). This typically involves the establishment of disruptive selection (see definitions) among varied phenotypes establishing reproductive isolation among groups (Higashi et al., 1999; Turelli et al., 2001; Coyne & Orr, 2004). In this light, sympatric speciation, especially when linked to both ecological and behavioural factors (such as in special cases of sexual selection) becomes increasingly more probable (Higashi et al., 1999; Doebeli & Dieckmann, 2003; Arnegard & Kondrashov, 2004; Gourbiere, 2004; Kirkpatrick & Nuismer, 2004; Kocher, 2004). Ecologically important diverging traits among populations that are linked to some recognizable feature(s) on which conspecifics and heterospecifics can cue, can quickly split populations along ecological and recognition axes regardless of their spatial proximity (Higashi et al., 1999; Arnegard & Kondrashov, 2004; Coyne & Orr, 2004; Gourbiere, 2004; Rundle & Nosil, 2005).

A number of studies have since demonstrated an ecological basis for many speciation events (Schluter, 1996; Lu & Bernatchez, 1999; Albertson et al., 2003; Coyne & Orr, 2004; Thorpe et al., 2005; Barluenga et al., 2006). However, even when speciation is recognized, difficulty remains in accurately assessing the underlying mechanisms responsible (Market et al., 1999; Kornfield & Smith, 2000; Seehaussen, 2000; Schluter, 2001; Rintelen & Glaubrecht, 2003; Thorpe et al., 2005). In most documented cases, speciation is attributable to a particular divergence agent which initiates divergence within taxa. Such agents can be ecological in nature and involve steep environmental gradients creating barriers to gene flow (Schluter, 2000; Doebeli & Dieckmann, 2003; Coyne & Orr, 2004). Non-ecological agents, such as the development of complete assortative mating and female preference for diverging male traits (Schluter, 2000; Streelman & Danley, 2003; Kocher, 2004; Rundle & Nosil, 2005) are also possible and can occur alone or in conjunction with ecological agents.

Once initiated, divergence among populations is pushed to completion by the action of diverging forces. These forces are processes such as drift, disruptive directional selection and reinforcement (see definitions), which widen divergence among populations, leading to complete reproductive isolation (Dieckmann & Doebeli, 1999; Schluter, 2000; Schluter, 2001; Turelli et al., 2001; Doebeli and Dieckmann, 2003; Coyne & Orr, 2004; Arnegard & Kondrashov, 2004). Many speciation studies offer particular causative agents and forces leading to the observed speciation (Seehausen et al., 1997; Markert et al., 1999; Kornfield and Smith, 2000; Losos and Miles, 2002). More likely, however, actual speciation is the result of several divergence agents and forces acting synergistically to create and complete species isolation. Teasing apart the relative contribution of various divergence agents and forces remains the fundamental challenge in numerous evolutionary investigations (Schluter, 2000; Schluter, 2001; Turelli et al., 2001; Rundle & Nosil, 2005; Thorpe et al., 2005).

A commonality to studies documenting speciation in nature is the adherence to a speciation framework which takes into account the four factors touched on above, namely; species concept, the development of reproductive isolation, divergence agents and forces (e.g., Meyer et al., 1990; Sturmbauer & Meyer, 1992; Seehaussen, 2000). How these latter are interrelated continues to be of great interest to students of evolution, challenging our understanding of evolutionary processes (Lu & Bernatchez, 1999; Schluter, 2000; Turelli et al., 2001; Thorpe et al., 2005).

1.3 ADAPTIVE RADIATION

One of the most interesting of processes associated with speciation is adaptive radiation. If speciation can be defined as diverging forms of a common ancestral species (Darwin, 1859; Futuyma, 1986) due to a variety of causative agents, and the process by which biodiversity increases, then adaptive radiation is essentially speciation occurring 'en mass' in an adaptive response to the environment (Schluter, 1996; Schluter, 2000; Schluter, 2001; Losos & Miles, 2002; Doebeli & Dieckmann, 2003; Schön & Martens, 2004). Adaptive radiation as a concept, just like many others in ecology and evolution, has suffered from a variety of definitions, each one context specific to the study describing it (Lack, 1947; Simpson, 1953; and reviewed by Schluter, 2000).

Originally formulated by Lack (1947), Dobzhansky (1951) and Simpson (1953), adaptive radiation has been described as phenotypic divergence and speciation driven by divergent natural selection pressures originating from the environment, resources, and resource competition among closely related taxa (McCune, 1997; Schluter, 2000). Since its original formulation, many studies have attempted to demonstrate the occurrence of adaptive radiation in natural systems (Fryer & Iles, 1972; Liem, 1974; Grant, 1986; Schluter, 1988; Nevo et al., 1994; Reinthal & Meyer, 1997). However, the identification of adaptive radiation has been complicated by the lack of a cohesive concept or distinguishing criteria that can be used to identify its occurrence among natural clades or taxa (Reinthal & Meyer, 1997; Schluter, 2000; Losos & Miles, 2002; Jordan et al., 2003). Even recent studies describing adaptive radiation events simply assume its occurrence without necessarily demonstrating the potential of the focal taxon to diverge in an adaptive manner (e.g., Rintelen & Glaubrecht, 2003).

Schluter (2000) greatly advanced the ecological basis of the adaptive radiation concept by suggesting that taxa exhibiting adaptive radiation should adhere to four distinct criteria, namely; 1) the focal taxon should have common ancestry, 2) should have a range of phenotypes correlated to some environmental gradient, 3) the traits causing the differentiation among groups should be utilitarian in nature, and finally, 4) the taxon should exhibit rapid divergence or speciation relative to that expected from random drift models. Schluter's framework provides a much more tangible set of criteria by which to evaluate adaptive radiation within and among taxa.

However, although these criteria seem reasonable and together demonstrate the occurrence of adaptive radiation within taxa in heterogenous landscapes, very few commonly regarded examples of adaptive radiation have ever been demonstrated to meet all of them. For example, Bouton et al., (1999) and Bouton et al., (2002) demonstrate the phenotype-environmental gradient correlation using body shape and habitat, and that the biting and suction forces are the modulated traits useful in food acquisition for haplochromine rock-dwelling cichlids in Victoria. These and other studies, however, fail to conclusively demonstrate common ancestry and consequently rapid speciation among haplochromine species in both Lakes Victoria, Malawi and Tanganyika (Meyer et al., 1996; Danley et al., 2000; Bouton et al., 2002). Although divergence among haplochromine cichlids in Lake Victoria is a well noted case of adaptive radiation, it nevertheless has not been completely demonstrated to fit all of Schluter's (2000) criteria. More objective evaluations of putative cases of adaptive radiation using established frameworks such as Schluter's can lead to a better understanding of the patterns and processes regulating adaptive radiations in natural systems (Schluter, 2000; Kocher, 2004).

Danley & Kocher (2001) and Streelman & Danley (2003) describe an emergent pattern observed in many of the most famous cases of species radiations and purport that such explosive speciations can often be traced back to distinct divergence events punctuating species' evolutionary histories. Using data gathered from published examples, these authors propose a model of adaptive radiation combining selective forces acting differently throughout this process (Danley & Kocher, 2001). The model is founded on cyclical bouts of high gene flow building variation within taxa followed by strong disruptive directional selection splitting taxa along varied lines ultimately causing punctuated diversification events. Under this model, divergence events typically occur in three stages and usually maintain their order through the radiation process. Initial divergence is related to body shape and its adaptation to microhabitat in a competitive environment for space (e.g., ground vs. tree finches; Sato et al., 1999; rock- vs. sand-dwelling cichlids in Lake Malawi; Danley & Kocher, 2001; various ecomorphs of the anolis lizards; Losos et al., 1998; Thorpe et al., 2005). The second involves divergence along the axis of trophic morphology giving rise to trophic differentiation capable of partitioning limited resources or allowing the exploitation of completely new ones (algae scappers, molluscivores, piscivores in cichlids; Bouton et al., 2002; coral crushers and algae excavators in parrotfishes; Streelman et al., 2002; various beak morphologies in finches; Grant, 1986). The final and typically most explosive stage of divergence is related to communication within newly formed divergent lineages, such as sexual selection based on male nuptial colouration and female preferences reinforcing divergence between closely related sister taxa. In this manner, communication allows divergence among closely related populations which would otherwise remain undifferentiated (repeated colouration patterns in African cichlid species flocks; Salzburger & Meyer, 2004; Kocher, 2004).

The 'radiation in stages' model of adaptive radiations (Danley & Kocher, 2001; Streelman and Danley, 2003; Kocher, 2004) is enticing for two reasons. First, as mentioned and although not universal, the order of divergence (habitat-trophic morphology-communication) is nearly always maintained through vertebrate radiations and especially when they occur within insular environments such as isolated lakes and islands (Streelman & Danley, 2003; Kocher, 2004). Second, this model proposes that although various divergence agents likely act throughout adaptive radiations, their predominant influence may be stage-specific (e.g., sexual selection is more disruptive and stronger at later stages). Consequently, identified putative radiations can be evaluated against this model to determine their relative progression, and the relative importance of different divergence agents.

1.4 THESIS OBJECTIVE

In the early 1990s, Kottelat described many fish species occurring in Lake Matano using traditional morphological descriptions. Many of the fish described were distinguished by colouration and overlapping morphological characteristics between closely related species. Since Kottelat, very few studies have attempted to describe Lake Matano or its fish fauna. Even less work has been done in attempts to determine the evolutionary relationships among the unique fish species occurring in this system. Yet, the fish species in Lake Matano are endemic, found nowhere else in the world, and provide one of the best opportunities to track the evolution of species within the rather confined spaces of this very old ecosystem.

The overall aim of this study is to use the Lake Matano ecosystem to assess the evolutionary history, and ecology of the more prominent members of the fish community. It is predicted that the unique physical characteristics of Lake Matano, coupled with intrinsic factors among certain endemic fish genera has led to the adaptive radiation of certain fishes within this system. The initial objective is to determine if there is evidence of adaptive radiation in the members of the fish community of this atypical tropical freshwater lake using the theoretical framework presented by Schluter (2000). If such a radiation is evident, its causal root(s) and the relative importance of divergence agents will be evaluated using the 'radiation in stages' model (Danley & Kocher, 2001; Streelman & Danley, 2003).

In this study I use the neo-Darwinian definition of species considering them as genetically distinct clusters of organisms sharing common ancestry and exhibiting reproductive isolation (Darwin, 1859; Mallet, 1995; Turner, 1999; Coyne & Orr, 2004). This study promises to challenge common speciation and radiation paradigms and will clarify some of the most fundamental principles regulating these important processes while at the same time describe one of the most unique aquatic ecosystems in the world.

1.5 CHAPTER 2 OBJECTIVES

Before an investigation of adaptive radiation can begin, it is vitally important to objectively discriminate if any taxa among those under study demonstrate qualities associated with adaptive radiation so as to establish a focal taxon (Losos & Miles, 2002). To determine if any of the fish genera from Lake Matano exhibit evidence of adaptive radiation, I use both morphological and mitochondrial DNA (mtDNA) sequence data to quantify and compare morphological and genetic divergence within each genus.

If all four genera colonized the lake at approximately the same time, there should be little differences in the level of variation estimated within them. If any of the genera, however, show substantially greater amounts of divergence, it may be related to differential colonization times, drift, or to directional selection having greater influence within a particular genus. If the predominant cause of divergence is drift, then I would expect substantially greater variation to occur evenly or randomly among morphological traits. If, however, divergence within the taxon is caused by directional selection, then I would expect greater amounts of variation in those morphological traits under selection. This directional selection acting on functional features could be the result of adaptive radiation acting to cause particular taxa to diverge in response to some sort of environmental gradient.

1.6 CHAPTER 3 OBJECTIVES

Many studies of adaptive radiation demonstrate a relationship between feeding morphology and diet among taxa exhibiting this process (e.g., Galapagos finches, Grant, 1986; cichlid fishes, Genner et al., 1999; Bouton et al., 2002; Lake Tana barbs, Sibbing & NagelKerke, 2001). Theory suggests that intense competition for food resources can often serve as a potent initiator of divergence among closely related taxa, whereby extreme feeding phenotypes within a population sharing less common resources diverge (Genner et al., 1999; Schluter, 2000; Turner et al., 2001; Bouton et al., 2002; Streelman & Danley, 2003). This divergence is even more prevalent in environments where common food resources are scarce, and where morphological divergent features allow transition of competing forms to make use of either under-utilized parts of the same resources or, a completely different one (Liem, 1974; Galis & Drucker, 1996; Genner et al., 1999; Schluter, 2000; Danley & Kocher, 2001).

The purpose of this chapter is to assess the hypothesis that there are no significant differences in the feeding morphology, diet composition and trophic status among the different *Telmatherina* species in Lake Matano. If all species within the lake have similar feeding morphologies, and share the same diet and trophic status, then food is not likely limiting, and competition for this resource is not likely a contributing factor of divergence among them. This study addresses the phenotype-environmental gradient correlation aspect of Schluter's (2000) criteria more closely, and initiates questions as to the progression of the *Telmatherina* radiation in this system.

1.7 CHAPTER 4 OBJECTIVES

Highly polymorphic colouration patterns, as observed in the *Telmatherina* genus of Lake Matano, are often associated with sexual selection and are though to be the initial cause of divergence among closely related taxa. Recent modeling and theoretical frameworks, however, describe that sexual selection, and more specifically that based on colouration segregation, is typically not strong enough to cause species divergence in singularity, but requires an ecological root (Arnegard & Kondrashov, 2004; Gourbiere, 2004; Rundle & Nosil, 2005). Studies based on other cases of adaptive radiations suggest that divergence based on communicatory cues, such as sexual selection based female preference for male nuptial colouration, typically occurs in the later stages of a radiation after initial ecological divergence (Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004).

In this study I map body shape features and colouration patterns onto derived *Telmatherina* phylogenies to assess whether or not the *Telmatherina* radiation follows the 'radiation in stages' model outlined by Danley & Kocher (2001), and Streelman & Danley (2003). The expectations are that if the *Telmatherina* radiation fits the radiation model described, then body shape ought to be a better predictor of species as can be resolved using mtDNA than will be colour patterns. In this case body shape adaptation to microhabitat would likely play a larger role in the initial divergence within this genus than would colour. Conversely, if colouration patterns fit the phylogenies better, then colouration patterns may be the initial root of divergence and the *Telmatherina* radiation does not fit the traditional radiation in stages model described (Danley & Kocher, 2001; Streelman & Danley, 2003). These data will also allow me to qualitatively estimate which selective pressures are most likely the causal root of the observed divergence within this genus.

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2.0 — GENETIC AND MORPHOLOGICAL DATA SUPPORTING THE HYPOTHESIS OF ADAPTIVE RADIATION IN THE ENDEMIC FISH OF LAKE MATANO*.

2.1 INTRODUCTION

An elevated rate of divergence (or level of variation) both within and among taxa is common to most examples of species radiation. Elevated divergence can either reflect drift acting in a non-directional manner, or be a response to selection acting directionally on particular traits. Radiation due to drift is characterised by the even, slow accumulation of variation within and among taxa. In contrast, radiation as a result of selection accumulates variation relatively quickly, is manifest in critical traits, and will be taxon biased. Rapid divergence among taxa in response to selection pressures is adaptive radiation and is an important mechanism driving biodiversity (Schluter, 1995).

The identification of adaptive radiation in natural systems has been complicated by the lack of a cohesive concept or distinguishing criteria used to detect its presence (Schluter, 2000; Losos & Miles, 2003). Even recent studies describing adaptive radiation events often assume its occurrence without demonstrating a potentially adaptive component within the focal taxon (e.g., Rintelen & Glaubrecht, 2003). Schluter (2000) recommends that taxa exhibiting adaptive radiation should adhere to four distinct criteria; the focal taxon should 1) have common ancestry, 2) exhibit varying phenotypes corresponding to their divergent environments, 3) be differentiated by traits that are functional (or utilitarian) in nature, and 4) exhibit rapid divergence, or 'burst' speciation (Schluter, 2000).

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Roy D, Docker MF, Hehanussa P, Heath DD, Haffner GD. (2004). Genetic and morphological data supporting the hypothesis of adaptive radiation in the endemic fish of Lake Matano. *Journal of Evolutionary Biology* **17**, 1268-1276.

Together, Schluter's criteria provide a framework by which taxa and phylogenies can be systematically evaluated for evidence of adaptive radiation. Presently, few published examples of adaptive radiation have been demonstrated to meet these criteria. There is a dearth of studies identifying natural systems where the influence of selection on taxa radiation can be easily recognized and investigated.

Ancient lakes are increasingly recognized as important study systems for evolution and speciation (Rossiter & Kawanabee, 2000; Rintelen & Glaubrecht, 2003). Such closed and aged environments provide a high potential for radiation events, especially among early invading fauna and flora. Consequently, ancient lakes typically support high levels of endemism such that divergence among taxa can be evaluated in terms of a response to drift or selection. The most famous examples of species radiation in ancient lakes are those described for the cichlids from the East African Rift valley lakes (e.g., Liem, 1974; Meyer et al., 1990; Danley & Kocher, 2001; Jordan et al., 2003). These complex assemblages of fish have made important contributions to our understanding of the theory of adaptive radiation and speciation (e.g., Sturmbauer, 1998; Seehausen, 2000). Yet, even these well known examples of adaptive radiation have not been shown to fulfill the requirements of Schluter's criteria. Furthermore, inferences of radiation and speciation in the East African Rift valley lakes are complicated by the tremendous number of species and species flocks still under scrutiny (e.g., Smith et al., 2003), and by the wide scope of factors purported to explain their divergences (Verheyen et al., 1996; Sturmbauer, 1998; Markert et al., 1999; Seehausen, 2000; Jordan et al., 2003; see Kornfield & Smith, 2000). The African Great Lakes have also been highly perturbed by continued anthropogenic stress, and are currently undergoing a rapid loss of biodiversity (Seehausen et al., 1997). To quantify the relative importance of adaptive radiation versus drift, there is a need to locate a system where the influence of selection on radiation among taxa can be readily identified. The ideal system would support a relatively simple assemblage of taxa to allow clarification of functional mechanisms driving phylogenetic radiation.

Lake Matano, located on the island of Sulawesi in the Indonesian archipelago (Fig. 2.1), is situated along an active fault line, forms the hydrological head of the Malili Lakes watershed, and is separated from downstream lakes by a 72m elevation change (Brooks, 1950; Kottelat, 1990b). It is estimated to be 2-4 million years old, to have levels of endemism greater than 80%, and has been identified as the principal seeding source of endemic species within the Malili Lakes watershed (Brooks, 1950; Haffner et al., 2001). This relatively small graben lake (surface area approximately 164 km^2) has very little littoral area surrounding a very deep basin (maximum depth = 590 m).

Lake Matano contains very clear water (Secchi depth > 23m), low abundances of phytoplankton and zooplankton and a lack of exclusively piscivorus top predators (Haffner et al., 2001; Sabo, 2006; Heath, pers. communication). These conditions indicate very low levels of primary productivity. Matano also possesses a comparatively simple fish community with few endemic genera (Kottelat, 1990a; 1990b; 1990c; Kottelat, 1991; Kottelat et al., 1993). Because of its isolation, Lake Matano has been subjected to few anthropogenic stressors and remains relatively pristine, and is therefore an excellent 'natural experiment' where the importance of drift versus adaptive radiation can be evaluated. Brooks (1950), concluded that intralacustrine speciation among native taxa is likely occurring, as the long isolation of Matano provided an extremely limited potential for multiple invasions. More recent investigations by Kottelat (Kottelat, 1990a; 1990b; 1990c; Kottelat, 1991; Kottelat et al., 1993) described multiple species endemic to Lake Matano belonging to 4 genera; all of which are of marine descent and thought to have colonized the lake at similar times (Brooks, 1950; Kottelat 1990a; 1990b; 1990c; Kottelat, 1991; Aarn et al., 1998).

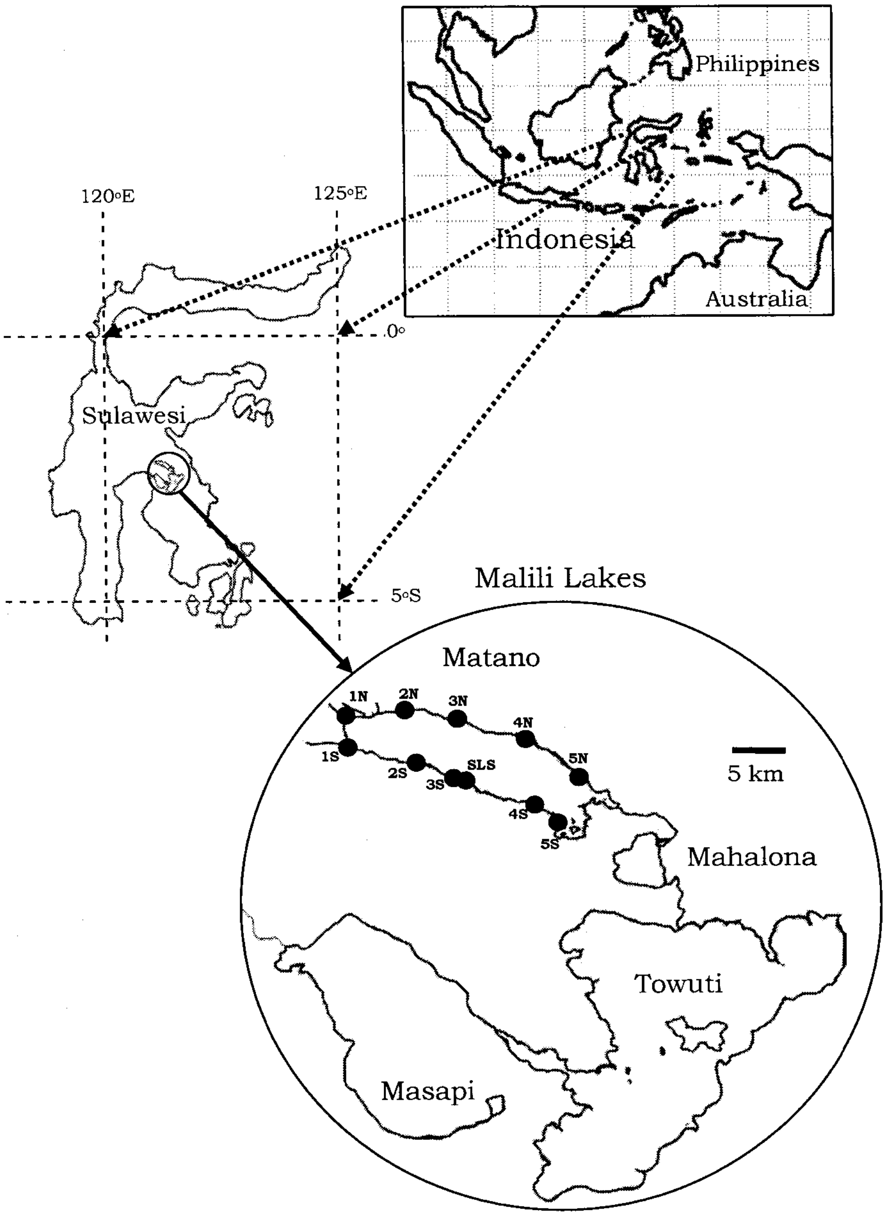

Figure 2.1 — Location of Lake Matano on the continental island of Sulawesi (inset) in the Indonesian archipelago. Beach seining locations where fish samples were collected around the lake are indicated with dark circles and alphanumeric values. *Glossogobius* collected from 1N, 1S, 3N, 3S, 4N, and 5S. *Oryzias* collected from 1N, 2N, 2S, 3S, 4N, and 5S. *Dermogenys* collected from 1S, 2N, 3N, and 4S. *Telmatherina* used for initial DNA analysis collected from 1S, 2N, 2S, 3S, 4N, and 5N. Additional *Telmatherina* were collected from all sampled beaches.

The purpose of the present study is to evaluate the evidence for adaptive radiation within the endemic fish community of this ancient tropical island lake using Schluter's (2000) criteria. We evaluate Schluter's first criteria using 16S rDNA gene fragment sequence to determine the ancestry of the four most common genera endemic to this lake. We then test for Schluter's fourth criteria by estimating relative rates of divergence among the 4 genera using genetic variability within each genus. Finally, Schluter's second and third criteria are evaluated using 3 categories of morphological traits to determine if potential radiations within this fish community have adaptive components. Results of these diverse approaches provide compelling data supporting the adaptive radiation within one endemic genus from this ancient lake system.

2.2 MATERIALS & METHODS

Approximately 50-100 fish were collected by beach seine from each of 11 locations around Lake Matano, Sulawesi Island, Indonesia (Fig. 2.1), and were identified to genus (*Glossogobius*, *Oryzias, Dermogenys*, and *Telmatherina*) according to Kottelat (1990a; 1990b; 1990c; and Kottelat, 1991; Kottelat et al., 1993). We observed no spatial bias in the abundance of fish from the four genera. Fish belonging to the genus *Telmatherina* were further separated into 5 morphotypes previously described as species by Kottelat (1991), and 4 new morphotypes based on colouration and other phenotypic characters used by Kottelat (1990c; 1991). Selected specimens from each genus were anaesthetized in clove oil, digitally photographed, fin clipped, and allowed to recover in oxygenated water before being released back into the lake. Collected fin tissue was stored in 95% ethanol. A smaller number of whole fish samples were also collected for morphometric analysis and were stored in 10% formalin and 95% ethanol (N=40).

Genetic analysis

Genomic DNA was extracted from fin tissue of 10 fish from each of the 4 genera (9 for *Glossogobius*), as well as 68 additional *Telmatherina* selected to include all identified colouration morphotypes (Kottelat 1991), using the Promega WIZARD® DNA extraction kit (Promega; Madison WI, USA) following the standard 'isolation from animal tissue' protocol. Polymerase chain reaction (PCR) amplification of approximately 600 bp of the mitochondrial 16S rRNA gene was accomplished using published primers (16Sar-L (5' CGCCTGTTTATCAAAAACAT 3' and 16Sbr- 5' CCGGTCTGAACTCAGATCACG 3'; Palumbi, 1996). The 16S rRNA gene was selected as most appropriate for the highly genetically diverse endemic genera of Lake Matano. Individual 25-μl reactions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer, 0.5 units of *Taq* DNA polymerase (Invitrogen, Burlington, Canada) and 1.0 μl of a 10:1 dilution of extracted genomic DNA. Reactions were run for 35 cycles consisting of denaturation at 96°C for 1 minute, primer annealing at 50°C for 1 minute, and extension at 72°C for 1.5 minute; the 35 cycles were followed by a final 5-minute 72°C extension. PCR products from a total of 107 fish were sequenced using the DTCS Quick Start cycle sequencing kit (Beckman Coulter, CA, USA) and CEQ8000 automated sequencer, following manufacturer's instructions.

Common ancestry

To determine ancestry of the genera sampled, derived sequences were aligned using the CLUSTAL W algorithm with a gap open penalty of 10, a gap extension of 5, a DNA transition weight of 0.5 (Omiga 1.2, Oxford Molecular Ltd., UK). Sequences were then analysed using the Molecular Evolutionary Genetics Analysis (MEGA) program version 2.1 (Kumar et al., 2001). Pairwise genetic distances were calculated and corrected for transition bias using the Kimura 2-parameter, Tamura-Nei, and the Jukes-Cantor distance models assuming a gamma distribution for base substitution (Kocher & Carleton, 1997). Insertions and deletions (indels) were treated equally as complete deletions, while tests for transition saturation were negative and therefore allowed both transitions and transversions to be used in distance estimates (Kocher & Carleton, 1997). A phylogenetic relationship based on corrected distances among the 107 fish was estimated using the Neighbour-joining algorithm supported by a confidence assessment of 10 000 bootstrap permutations (Saitou & Nei, 1987).

Lineage divergence rate

Because speciation rate is difficult to determine without complete phylogenies (Nee et al., 1994; Schluter, 2000), and because the phylogenies of the endemic genera from Lake Matano at the species level are not currently available, I used mean lineage divergence as a proxy for speciation rate. Lineage divergence within individual genera was estimated by calculating pairwise genetic distances among 10 (9 for *Glossogobius*) fish from each genus based on the 16S sequences (see above). We analysed the distribution of the pairwise genetic distance estimates among genera using a Kruskal-Wallis test and post-hoc tested for differences between pairs of genera using a Mann-Whitney U test. To verify anomalously high levels of divergence observed within *Telmatherina* relative to the other genera, mean pairwise genetic distance within this genus was re-calculated 50 times using 10 randomly chosen individuals from a pool of 78 individuals (with replacement). This was done to determine if the level of divergence observed within this genus was simply due to chance. Ultimately, I included all available sequence data (N=78) to increase the accuracy of the lineage divergence estimate for this genus.

Morphometric trait assessment

Although fish colouration patterns can be subjective and difficult to quantify, they have nevertheless been shown to be key morphological characters on which fish can cue and

Table 2.1 — Coefficient of variation calculated for several morphometric traits, separated into three trait categories (feeding, sexual display and neutral), in the four endemic genera of Lake Matano. The classification of these traits into feeding or sexual display traits is based on published descriptions (see text). Neutral traits are those typically used as diagnostic features delineating taxa, but on which selection has not been demonstrated.

Trait	*Glossogobius*	*Oryzias*	*Dermogenys*	*Telmatherina*
Feeding traits				
Snout length	9.63	3.77	9.80	14.94
Head depth	4.12	5.33	6.26	7.25
Mouth position	22.82	0.00	14.21	80.76
Mouth protruberosity	1.28	0.78	2.31	0.77
Gape width	7.80	7.45	11.20	14.95
Gape Height	8.16	10.17	16.54	13.07
Maxillary length	23.42	17.11	17.97	19.78
Number of gill rakers on 1st gill arch	9.39	3.42	5.21	18.18
Sexual display traits				
1st dorsal fin length	20.56	7.33	7.34	38.58
Caudal fin length	8.20	8.33	10.93	8.55
Caudal fin depth	7.88	3.91	1.94	4.19
Head length	5.98	5.03	6.27	3.03
Anal fin length	6.12	4.60	5.96	12.43
Body depth	9.08	3.50	4.79	10.18
Pre-anal length	9.00	3.26	1.95	3.79
Pelvic fin length	8.89	6.71	7.10	13.36
Neutral traits				
Number of lateral scales	6.31	3.99	6.35	3.10
Number of transverse scales	14.62	6.73	4.71	7.41
Number of caudal rays	0.03	0.04	0.03	0.09
Number of pectoral rays	1.87	4.52	2.66	8.37
Number of anal rays	0.00	2.85	1.96	11.48
Number dorsal rays	0.00	0.00	0.00	5.21
Inner orbital width	19.61	6.47	10.06	8.67
Number of pelvic rays	0.00	0.00	0.00	5.36

segregate (Seehausen, 2000). A chi-square (χ^2) analysis was performed to determine if any particular genus had a greater number of colour morphotypes than any other, based on in-field colour morphotype determination.

Ten fish from each of the four genera were also analyzed using morphometric parameters described in Kottelat (1990b; 1990c) along with five additional parameters (N = 32; Table 2.1). Morphometric traits were separated into three categories; feeding, sexual display, and neutral traits (see Table 2.1). Feeding traits were those commonly reported in the literature as associated with mouth morphology and food acquisition (Humphries, 1993; Hyndes et al., 1997; Gysels et al., 1997). Sexual display traits were those typically associated with attracting a mate or sequestering reproductive opportunities (Jennions et al., 2001; Karino & Haijima, 2001). Neutral traits consisted of traits not included in previous categories, and on which selection has not been demonstrated, but are conventionally used as taxonomically informative traits.

All measured traits were normalized using standard length to account for differences in body size, whereas data for meristic traits were kept as counts. Coefficients of variation for each trait, within a genus, were calculated and *z-score* corrected across traits within categories to fit a Gaussian distribution. *Z*-corrections applied to the coefficients of variation also standardized variances among traits within categories allowing parametric analyses (Sokal & Rohlf, 1998). Analyses of variance were then performed on corrected data to assess if there were significant differences in the amount of variation within categories among the four genera. *Post-hoc* Tukey tests identified specific genera which significantly differed from others (Sokal & Rohlf, 1998). All statistical analyses were completed using either SYSTAT statistical software package (SYSTAT v. 8.0 1998, SPSS Inc.) or specifically designed programs written in IDL (Interactive Data Language v. 5.3.1 2000, Research Systems Inc. CO, USA) and evaluated at the $p < 0.05$ level of significance.

2.3 RESULTS

Genetics

The 16S rDNA sequences generated in this study are available in GenBank under accession numbers AY62534-AY62538 and AY545822-AY545875. Aligned sequences are comprised of 560 bp; 170 sites (31%) were variable, 147 (26%) of which were phylogenetically informative. All distance models (listed above) yielded the same or very similar distance matrices. We report only the Kimura 2-parameter results, as this model is most commonly used for assessing fish mtDNA phylogenetics (Kocher & Carleton, 1997), and thus allows broad comparison to other work. Overall, sequence differences were characterised by 15 indels and an average of 15.5 and 14.9 transition and transversion substitutions, respectively.

Common Ancestry

The Neighbour-joining algorithm segregated sequences into four distinct monophyletic clusters corresponding to the four endemic genera (Fig. 2.2). We present all unique haplotypes as branch tips, despite low bootstrap support, to avoid misleading genus-level biases. Nodes separating genera were supported by > 90% of bootstrapping permutations. Equally shallow terminal branching exhibited within each genus indicates approximately equivalent divergence time from a common ancestor (Fig. 2.2). The *Telmatherina* genus showed high sequence variation, however the identified topology did not correspond to previously identified species. The *Glossogobius* genus was sub-divided into two groups with strong bootstrap support (98%). *Dermogenys* also demonstrated some topography with moderate bootstrap support, while *Oryzias* showed little intrageneric sequence divergence, with 9 identical 16S sequences.

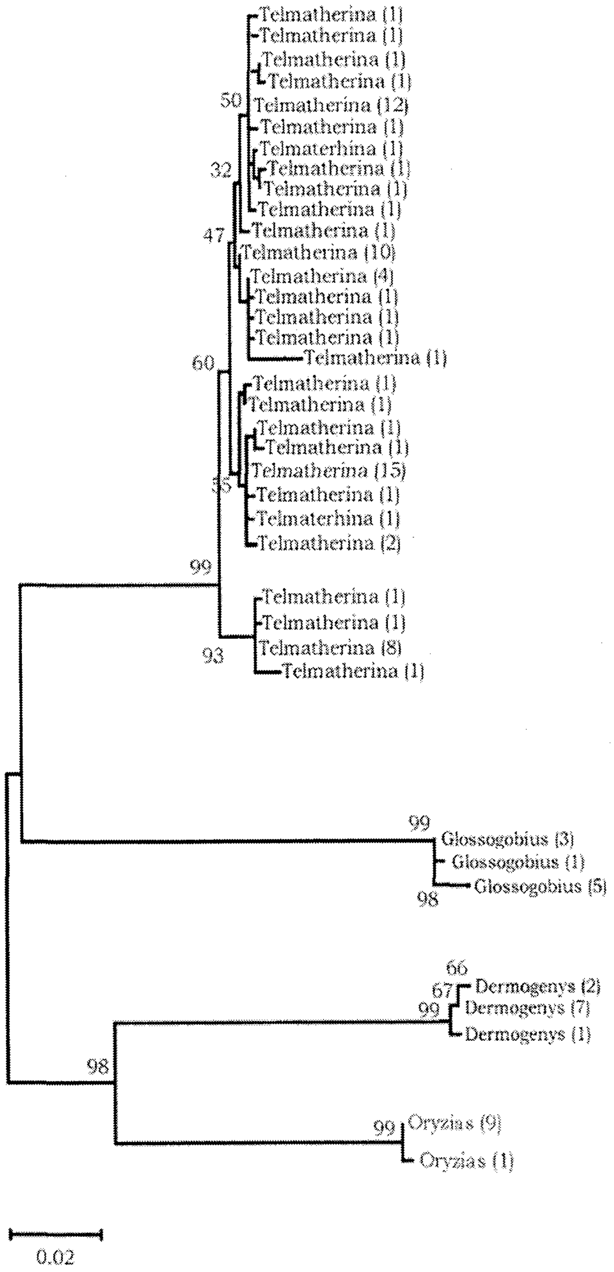

Figure 2.2 — Genetic relationship within and among the four endemic fish genera (*Glossogobius*, *Dermogenys*, *Oryzias*, and *Telmatherina*) of Lake Matano, constructed using Neighbor-joining analysis with the Kimura 2-parameter distance model assuming a gamma distribution for base substitution. Bootstrap support is indicated at all nodes. Numbers beside taxon name indicate the number of individuals sharing the same sequence, all labeled branch tips represent unique sequence data.

Lineage divergence rate

Frequency distributions of the pairwise genetic distances within each genus demonstrated that the *Telmatherina* genus had higher lineage divergence and genetic variation relative to the other genera endemic to the lake (Fig. 2.3). *Glossogobius* and *Dermogenys* showed intermediate levels of divergence and variation, while there was essentially no variation within the *Oryzias* genus (Fig. 2.3). The levels of genetic divergence among genera were highly significantly heterogeneous ($p < 0.0001$), and *post-hoc* Mann-Whitney U tests results confirmed that all comparisons among pairs of genera were significantly different ($p < 0.001$). All 50 bootstrap iterations used to re-calculate mean sequence divergence within *Telmatherina* were greater than the mean sequence divergence calculated within *Glossogobius*, the genus with the next highest mean sequence divergence (Fig. 2.4A). Including more individuals in the estimation of the *Telmatherina* mean sequence divergence marginally lowered the estimate and also reduced the standard error (Fig. 2.4B).

Morphometric trait assessment

A significant difference was observed in the number of colour morphotypes identified within each genus (Fig. 2.5A). The *Telmatherina* genus was characterised by 9 colour types, while the other genera were described by 3 or fewer. Significant differences were also observed in mean standardised, *z-score* corrected coefficients of variation calculated for 8 feeding traits among the different genera (Fig. 2.5B). Adjusted *post-hoc* analysis revealed *Telmatherina* to be more variable in feeding traits than either the *Glossogobius* or *Oryzias* genera, but not the *Dermogenys* genus. No significant differences were found in the amount of variation in sexual display traits estimated among the endemic genera sampled from this lake (Fig. 2.5C). The variation observed in neutral traits was greatest within the *Telmatherina* and lowest within the *Oryzias* genus, however, even differences using these extremes were not significant (Fig. 2.5D).

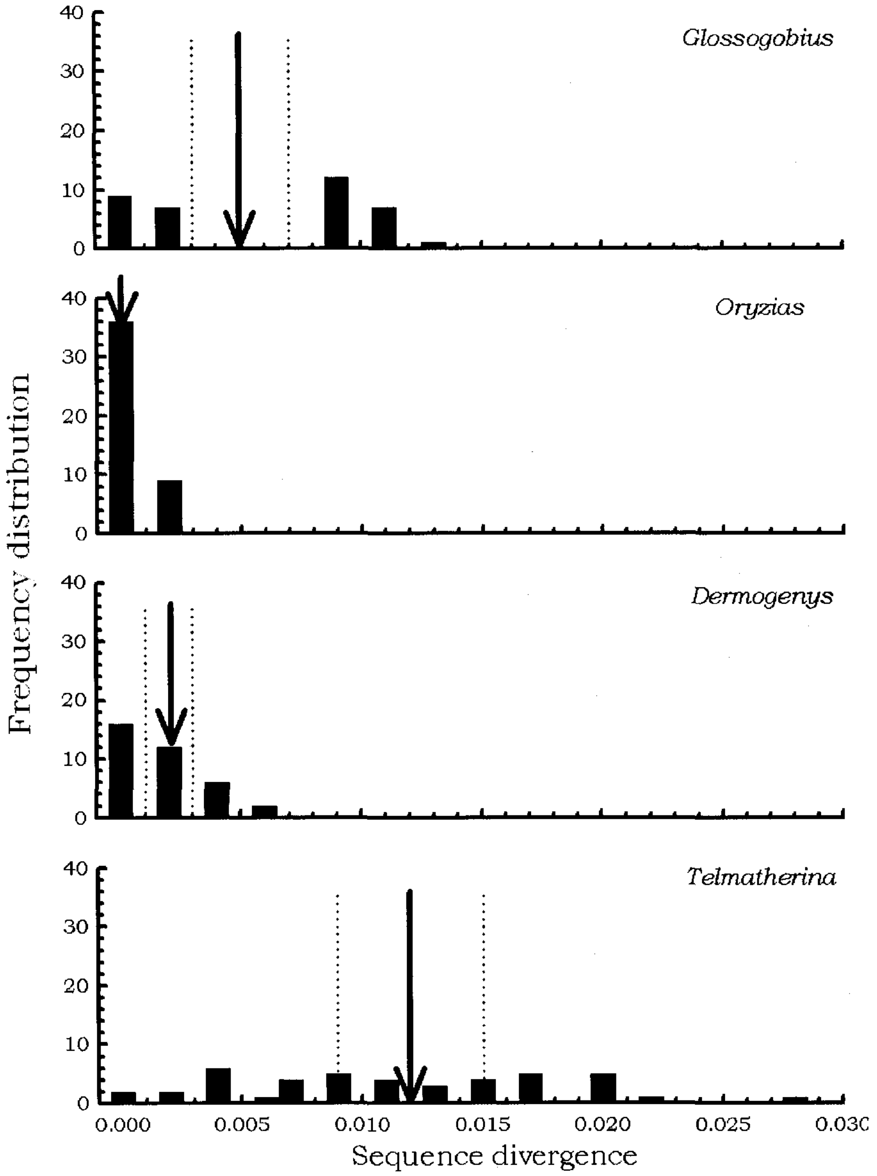

Figure 2.3 — Frequency distribution of pairwise genetic distances (a proxy for genetic divergence rate) calculated within each genus. Pairwise distances were corrected for transition bias using the Kimura 2-parameter distance model assuming a gamma distribution for base substitution. Arrows indicate mean sequence divergence within genus and dotted lines show ± 1.0 S.E (calculated using 10 000 bootstrap permutations). All four distributions differed significantly in mean genetic divergence (Mann-Whitney U test; $p < 0.001$).

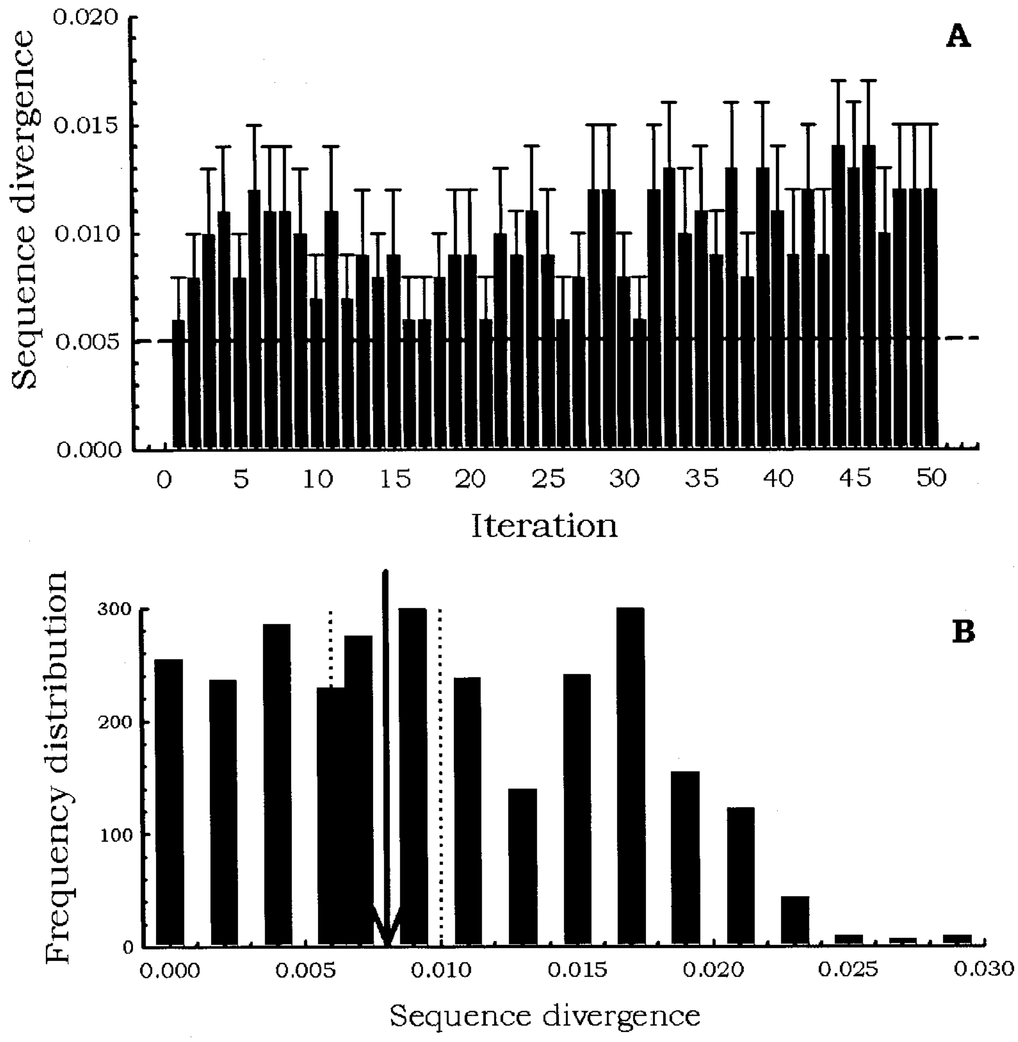

Figure 2.4 — A) Mean sequence divergence (± 1.0 S.E) calculated through 50 iterations within the *Telmatherina* genus using 10 randomly selected individuals from a pool of 78 (with replacement). All estimates of the mean were performed using 10 000 bootstrap permutations of the Kimura 2-parameter distance model assuming a gamma distribution for base substitution. All 50 iterations demonstrated greater sequence divergence within *Telmatherina* than that estimated within *Glossogobius*, the genus with the next highest divergence (indicated with dashed line). B) Frequency distribution of pairwise genetic distances using all 78 individual *Telmatherina*. Arrow indicates mean sequence divergence and dotted lines show ±1.0 S.E (calculated using 10 000 bootstrap permutations).

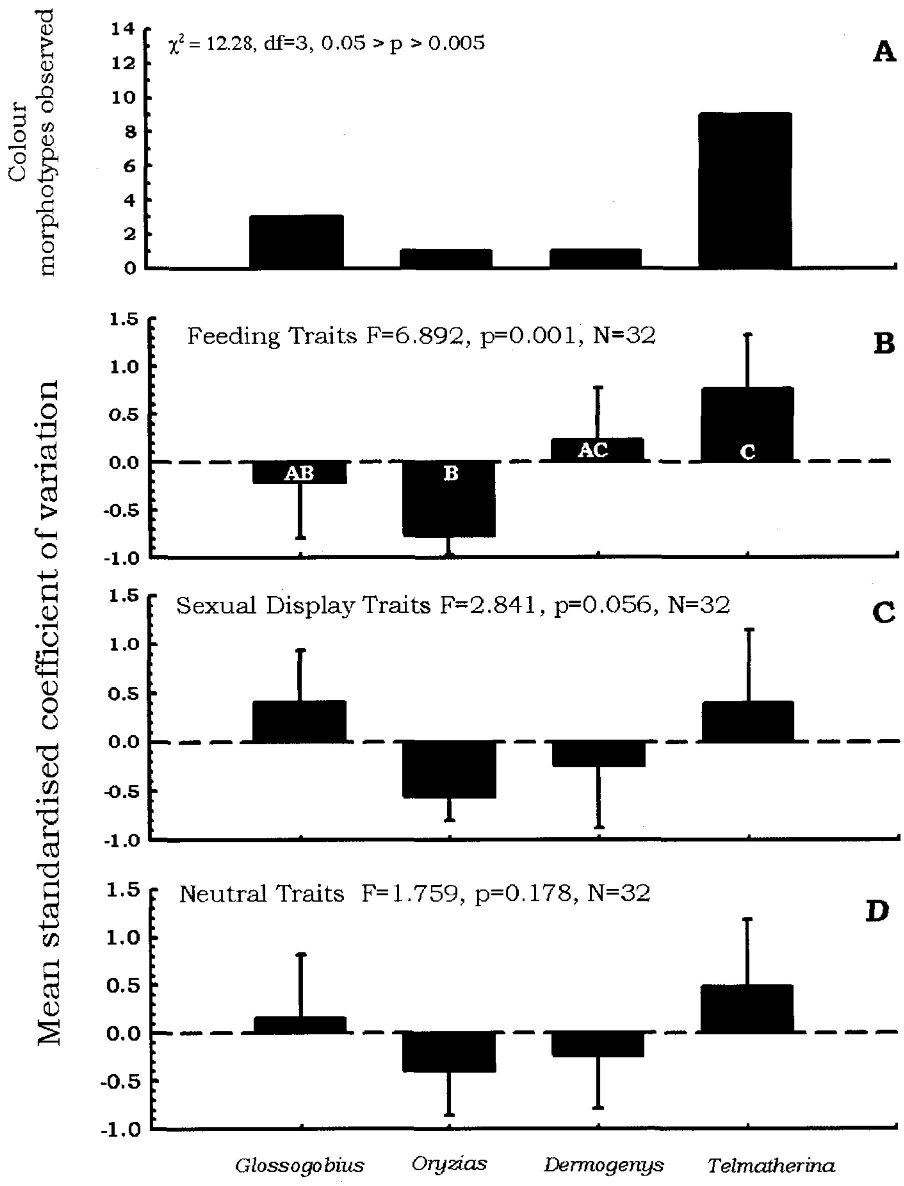

Figure 2.5 — Comparison of morphological variation in the four fish genera endemic to Lake Matano, Sulawesi Island, Indonesia: A) Number of colouration patterns observed within each genus, and mean z-score corrected coefficient of variation (± 2.0 S.E.) among: B) feeding traits, C) sexual traits, and D) neutral traits for each genus. Trait groups that showed significant variation among genera were tested for pairwise differences among genera using a Tukey's *post-hoc* test: shared letters indicate non-significant differences.

2.4 DISCUSSION

Adaptive radiations contribute to our understanding of the fundamental principles regulating speciation since it serves to rapidly increase variation, leading to speciation, in response to selective pressures. In this study, I provide data supporting the hypothesis of adaptive radiation in the *Telmatherina* genus in Lake Matano, based on the framework provided by Schluter (2000). The phylogenetic relationships generated using the 16S rDNA sequences demonstrate common ancestry for each of the endemic genera in Lake Matano, while elevated levels of sequence divergence within the *Telmatherina* and the *Glossogobius* genera were evident. These data satisfy the first and fourth criteria of Schluter's adaptive radiation framework, and indicate that both the *Telmatherina* and, to a lesser extent, *Glossogobius* are potentially radiating in this system.

Estimates of morphological variation in colouration patterns and feeding traits, meanwhile, imply adaptive components to the *Telmatherina* radiation that are not evident in *Glossogobius*. The larger number of colouration patterns exhibited, and the greater variation in feeding traits (Fig. 2.5A & B) demonstrate adaptive components to the *Telmatherina* radiation which address the second and third criteria of Schluter's (2000) framework. Although demonstrated no direct association between *Telmatherina* phenotypes and explicit environment or habitat differences, the evolution of variation in feeding morphology has been widely demonstrated for fish in low resource environments (Humphries, 1993; Schluter, 1995; Ruzzante et al., 1998; Rüber et al., 1999; Sibbing & NagelKerke, 2001; among others). In such cases, closely related species exhibit divergent phenotypic variation in feeding morphologies associated with variation in the food resource (Liem, 1974; Schluter & McPhail, 1993; Ruzzante et al., 1998; Rüber et al., 1999; Schluter, 2000; Sibbing & NagelKerke, 2001). The low level of primary productivity in Lake Matano is reflected throughout the food chain as indicated by the lack of pisciverous fish and the large secchi depth (Haffner et al., 2001). Greater variation in feeding traits observed in both

Telmatherina and *Dermogenys* suggests that both these genera are under selective pressure in response to a limited feeding resource. In particular, the feeding traits demonstrating the greatest variation in *Telmatherina* are those typically reported as associated with trophic specialization (i.e., snout length, mouth position, gape height, gill rakers - see Table 2.1; Sibbing & NagleKerke, 2001; among others). The *Dermogenys* have mouth morphology and spatial distribution consistent with obligate surface feeding (elongated lower jaws, and near surface distribution). The *Telmatherina* and *Oryzias* are both pelagic, however the *Oryzias* have the lowest variation in feeding morphology, indicative of feeding specialization.

Greater morphological variation in the *Telmatherina* also satisfies Schluter's third criterion. The larger number of colouration patterns and greater variation in feeding – but not sexual display or neutral traits supports the directed differential divergence in *Telmatherina* resulting from selection acting on functional (or utilitarian) features. Selection acting on colouration and/or feeding traits in the *Telmatherina* has the potential to create mating barriers that accelerate drift at neutral genetic markers (i.e. 16S rDNA) within reproductively isolated lines. This work thus establishes a link between genetic divergence and functional traits on which selection may be acting within the *Telmatherina*, and implicates trophic specialization as the most likely mechanism driving adaptive radiation.

Our data do not support assortative mating among colour morphotypes as a major contributing factor to the radiation observed in *Telmatherina*. Sexual selection, especially with strong assortative mating among colour types, will create barriers to gene flow and hence segregation among closely related taxa, even in sympatry (Seehausen et al., 1997; Seehausen, 2000). Hence, a consequence of speciation driven by sexual selection is high variability in sexual display traits among closely related taxa (Seehausen, 2000). Sexual selection of this nature has been proposed as a mechanism regulating the relatively recent explosive radiation of haplochromine and other cichlid fishes in the African rift lakes (Seehausen et al., 1997; Kornfield & Smith, 2000; Seehausen, 2000). Although I observed a significantly greater number of colouration patterns in *Telmatherina* relative to the other Lake Matano genera, I found no significant difference in the level of variation in other sexual traits (including dorsal and anal fins noted in the field to be used during displays).

An alternative explanation of the differences in genetic divergence observed among Lake Matano endemic genera is variation in colonization history. If the *Telmatherina* colonized the lake earlier than the other genera, or colonized the lake in multiple colonizing events, the *Telmatherina* would be expected to exhibit greater genetic variation, independent of selective effects. Pre-existing divergence expected from multiple colonists within a particular genus however, should manifest greater intra-generic genetic divergence at the 16S locus (deeper branching within genus phylogeny) than was observed here. Moreover, similar (but not identical) shallow terminal branch lengths among all genera suggests a common colonization time for all genera in this system. Common colonization of endemic genera in Lake Matano is consistent with the published reports available addressing the evolution of the fish community in this system (Brooks, 1950; Kottelat, 1990a; 1990b; 1990c; Kottelat, et al., 1993; Aarn et al., 1998). Brooks (1950) suggests that the tectonic origin of Lake Matano, its great depth, and location along an active fault are indicative of an ancient origin predating that of the other Malili lakes. Brooks (1950), Kottelat (1990b), and Aarn et al. (1998), all use local topology (Matano separated from downstream lakes by a 72m drop) to support the current isolated nature of this lake relative to others in this watershed. The isolated nature of Lake Matano and the paucity of shared species with downstream lakes are not consistent with multiple colonization of this system (Brooks, 1950; Kottelat, 1990c; Kottelat, 1991; Kottelat et al., 1993; Aarn et al., 1998). Therefore, I conclude that the endemic species of Lake Matano are the consequence of intralacustrine speciation from original, genus specific, common ancestors which colonized the lake at similar times and subsequently provided propagules for downstream varieties (Brooks, 1950; Aarn et al., 1998).

The most parsimonious explanation for the higher genetic diversity and the curious pattern of morphological variation of *Telmatherina* in Lake Matano, relative to the three other endemic fish genera, is differential selection acting on functional feeding traits. This study supports adaptive radiation in this genus as a consequence of divergence in resource acquisition in a resource poor environment. The adaptive radiation hypothesis for the *Telmatherina* in Lake Matano provides specific, testable predictions for studies of resource use specialization and phylogenetic relationships within the endemic genera of Lake Matano. Furthermore, the *Telmatherina* in this simple, isolated ancient lake represent a particularly valuable study system for the evaluation of the fundamental mechanisms driving the creation of biodiversity in tropical freshwater aquatic ecosystems.

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3.0 — RESOURCE BASED ADAPTIVE RADIATION IN *TELMATHERINA* FROM LAKE MATANO, INDONESIA*.

3.1 INTRODUCTION

Adaptive radiations are an important source of biodiversity, and understanding factors initiating these processes is critical in a world of ever increasing species loss (Myers 2000; Sodhi et al., 2004). Mounting evidence suggests that adaptive radiations are seeded by ecological selection among taxa which subsequently diverge via other selective forces related to inter- and/or intra-specific communication (Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004). Ecological pressures such as competition and predation have been predicted to play significant initiating roles in adaptive radiations and their implications in many notable systems supports this hypothesis (Schluter, 2000; Danley & Kocher, 2001; Doebeli & Dieckmann, 2003; McKinnon et al., 2004; Rundle & Nosil, 2005). Disentangling the relative importance of ecological pressure(s) responsible for initial divergence remains challenging largely because various pressures likely act synergistically to produce adaptive radiations (Schluter, 2000; Streelman & Danley, 2003; Kocher, 2004).

Some excellent studies have identified putative causal mechanisms in certain adaptive radiations and have demonstrated that resource polymorphism can play an initiating role in sympatric divergence. For example both Skúlason et al. (1993) and Schluter (1994) demonstrated that resource limitation in both Icelandic charr and in sticklebacks was likely the causative mechanism driving divergence leading to resource specialization among sympatric morphotypes. Such studies suggest that stable resource

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* Roy D, Paterson G, Hamilton PB, Heath DD, Haffner GD. Resource based adaptive radiation in *Telmatherina* from Lake Matano, Indonesia. (*Manuscript submitted to Molecular Ecology. Jan 2006*).

limited environments can often promote the evolution of resource specialists from polymorphic generalist ancestors (MacArthur and Pianka, 1966; Skúlason et al., 1993; Schluter, 1994). More recent theory on adaptive dynamics, however, proposes that the colonization of new environments by resource specialists can initiate niche expansion and subsequent contraction leading to the formation of many closely related resource-specific species (Waxman & Gavrilets, 2005). A general trend supporting either the generalists to specialist or the adaptive dynamic hypothesis in documented cases of adaptive radiations, however, remains elusive (Schluter et al., 1997; Schluter 2000).

The Indonesian island of Sulawesi has long been recognized as a centre of biodiversity due to the high degree of endemism among its native fauna (Wallace, 1892; Myers et al., 2000; Whitten et al., 2002). Most studies characterizing evolutionary relationships among Sulawesi biota come from terrestrial systems (Wallace, 1892; Whitten et al., 2002; Evans et al., 2003; Kingston & Rossiter, 2004), but equally intriguing results are emerging from investigations of Sulawesi's unique aquatic systems (Brooks, 1950; Kottelat, 1990a; 1990b; 1990c; Kottelat, 1991; Chapter 2; Rintelen et al., 2004). Lake Matano is an ancient fault lake forming the headwaters of the Malili lakes on Sulawesi (Brooks, 1950; Kottelat, 1990a; Haffner et al., 2001) and is isolated from downstream systems by physical barriers to aquatic dispersal (Brook, 1950; Kottelat, 1990a; Rintelen et al., 2004; Appendix I). This small (164 km^2) but ancient fault lake is very deep (~ 600m), has extremely steep sides and is surrounded by limited littoral areas. The fishes occurring in this system have been little studied and are found mainly within littoral areas (although reports are conflicting), with a marked absence of fish beyond the 30-40m lake contour (Roy, unpublished acoustic data). Lake Matano has very clear water (Secchi depths > 23 m) and low phytoplankton biomass (< 52 ug/L wet weight) composed predominantly of cyanobacteria. Such conditions do not maintain exclusively piscivorus top predators and indicate a highly oligotrophic state (Brooks, 1950; Chapter 2; Sabo, 2006; Heath, pers. communication; Appendix I).

Previous surveys of the fishes in this system reported several endemic species belonging to four principal genera, namely *Glossogobius*, *Oryzias*, *Dermogenys* and *Telmatherina* (Kottelat 1990a, 1990b, 1991, Larsen & Kottelat, 1992, Kottelat et al. 1993; Aarn et al., 1998). A more recent study (Chapter 2) used published criteria (Schluter, 2000) to demonstrate of the fish genera present in Matano, the *Telmatherina* are likely radiating as a consequence of either sexual selection among brightly coloured morphotypes, or adaptive feeding structures which may be important features (Liem, 1974; Galis & Drucker, 1996) allowing these fish to segregate along resource based adaptive peaks (Schluter, 2000; Doebeli & Dieckmann, 2003). Here I present evidence that resource partitioning is the critical ecological pressure likely responsible for the initial radiation of *Telmatherina* in this system.

3.2 MATERIAL & METHODS

Genetics

To determine the phylogenetic structure within *Telmatherina*, fish were collected by seine from eleven locations around the lake. Genomic DNA was extracted from fin tissue using the Promega WIZARD® DNA extraction kit following the standard 'isolation from animal tissue' protocol (Promega, Madison, WI, USA). Amplification of 490 bp of the 16S rDNA and 600 bp of the cytochrome *b* gene was performed using published and specifically designed primers [16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') & 16Sbr (5'-CCGGTCTGAACTCAGATCACG-3') (Palumbi, 1996)] and [GLUDG-5(5'-TGACTTGAARAACCACCGTTG-3') (Palumbi 1996) and (CBtelm–R (GTGGAGGAGGGGTACGACTA-3') developed here]. PCR reactions were performed as described in Chapter 2 using an annealing temperature of 50^{O}C and 60^{O}C for the 16S and cytochrome *b* reactions, respectively. PCR products of 82 *Telmatherina* and 5 *Marosatherina lagdesi*, a commonly available close relative of *Telmatherina* from South-west Sulawesi (Aarn et al. 1998) and used here as an outgroup, were cleaned and sequenced using the DTCS Quick Start cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) and CEQ8000 automated sequencer following manufacturer's instructions.

Resulting sequences from both genes were aligned separately using the clustal W algorithm (Omiga 1.2, Oxford Molecular Ltd, Oxford, UK), verified by eye and then concatenated to form a single sequence per individual. Generated sequences are available on GenBank (Accession # DQ023624-DQ023667, DQ002506-DQ002547 and DQ054795-DQ054796 and Chapter 2). The phylogenetic relationship among sequences was constructed by Neighbour-Joining using Kimura 3-parameter with unequal frequencies in base composition, a proportion of invariant sites (0.5517), and a gamma shape parameter for base substitution (0.6715). This distance model was determined by the Akaike information criterion survey of 56 different models implemented by Modeltest (Possada & Crandall, 1998) in PAUP version 4.0b10 (Swofford, 2001). A bootstrap 50 % majority consensus tree was generated from 10 000 permutations. As an additional test, phylogeny was also recovered from a Maximum parsimony heuristic search with close neighbour interchange of 1 and random addition trees of 10 replicates, supported by 1000 bootstrap permutations.

Three restriction endonucleases *FokI*, *SphI*, and *PleI* (New England Biolabs) provide diagnostic restriction fragment length polymorphisms in the cytochrome *b* fragment that identify *Telmatherina* to specific clades. DNA was subsequently extracted from more than 300 male individuals (anatomically sexed) and approximately 1000 bp of cytochrome *b* was amplified using PCR reaction and primers described above. Resulting

PCR products were digested with the three enzymes following manufacturer's instruction, to determine clade membership and 32 individuals belonging to each *Telmatherina* clade were selected with no spatial bias in regards to distribution around the lake. Subsequent to initial phylogenetic reconstruction, the same individual fish were used in all analyses, minimizing the number of specimens destroyed and providing strong support for relationships derived among various results. Samples of fish from the other three endemic genera were also collected in a similar manner (n=7).

Geometric Morphometrics

To explore putative morphological feeding specializations within *Telmatherina* lineages, the heads of 32 fish from each genetic clade were X-rayed using a SDS X-ray imaging system with PCCR 812 HS OREX digital scanner. The upper and lower pharyngeal jaw bones were also dissected and imaged using an FEI XL 30 ESEM scanning electron microscope using 60-65 KV. Images of skulls and upper and lower left pharyngeal jaw bones were analyzed using Geometric Morphometrics (GM), a landmark-based analysis of shape (Rohlf, 1999; Rüber & Adams, 2001; Zelditch et al., 2004).

Homologous landmarks on skull radiographs and electron scans were chosen based on standard landmark descriptions (Rohlf, 1999; Zelditch et al., 2004). Landmarks were chosen based on identifiable features that have important functions in feeding and food acquisition (Liem, 1974; Galis & Drucker, 1996; Rohlf, 1999; Zelditch et al., 2004). Landmarks from all specimens were superimposed using the generalized least squares Procrustes superimposition analysis (GPA; Rohlf, 1999) (TPSrelw; http://life.bio.sunysb.edu/; Rohlf, 2004). GPA generated an overall consensus configuration of landmarks for each analyzed structure and thin-plate splines were used to calculate interpolation functions among the landmarks of the consensus configuration (Rohlf, 1999; Rüber & Adams, 2001; Zelditch et al., 2004). The thin-plate spline superimposes each specimen's corrected landmark configuration onto that of the consensus. The deviations of each specimen from the consensus configuration (partial warps) were then used in standard multivariate analysis (Rüber & Adams, 2001; Zelditch et al., 2004). Canonical variates analysis (CVA) was used to discriminate among species along various ordination axes (Rüber & Adams, 2001; Zelditch et al., 2004). *Post-hoc* pairwise comparisons among the *Telmatherina* clades were performed using derived *F*-values from generalized Mahalanobis distances to determine which had significantly different shapes. Experiment-wise error was kept at 0.05 by Bonferroni correction of α-values to 0.0166.

Principal components analysis was performed on the partial warps of *Telmatherina* species, generating a series of relative warps outlining the biologically meaningful shape differences among the CVA identified groups (Rohlf, 1999; Zelditch et al., 2004). Deformation grids were constructed for each CVA defined group (TPSrelw; http://life.bio.sunysb.edu/; Rohlf, 2004) using their mean shapes defined along the most important relative warps (Zelditch et al., 2004). Each structure (skull, lower left pharyngeal jaw and upper left pharyngeal jaw) was analyzed separately and mean shapes defined using relative warp analysis were exaggerated to emphasize differences (skull 3X; lower jaw 2X; upper jaw 2X).

Diet Data

To determine whether feeding strategies of the *Telmatherina* clades matched those predicted from their morphology, I analyzed clade-specific gut content. Items in the gut of each selected *Telmatherina* was identified to general prey categories (Genner et al., 1999) and analyzed using the points method as described in Gysels et al. (1997) and Genner et al. (1999). Analysis was performed using a one-way ANOSIM in Primer v5 (Primer-E Ltd, Plymouth, 2001).

Stable isotopes

Each fish selected for diet analysis was also dried at 100^{O}C for 48 hrs after which whole specimens were macerated into a fine powder using mortar and pestle. A 1 mg subsample of the homogenate powder was weighed into a standardized tin capsule, sealed and processed for the stable isotopes of nitrogen (N) and carbon (C) (see methods in Paterson et al., 2006). Samples were analyzed using a continuous flow VG Micromass 903E isotope ratio mass spectrometer at the Environmental Isotope Laboratory (Department of Earth Sciences, University of Waterloo, Waterloo, Ontario, Canada). Stable isotope ratios were given as deviations from standard materials (Pee Bee belemnite limestone for C, or $\delta^{13}C$; and atmospheric nitrogen for N, or $\delta^{15}N$).

3.3 RESULTS & DISCUSSION

Genetics

Both distance and character based phylogenetic reconstructions separated *Telmatherina* into three distinct clades (Fig. 3.1). The resulting trifurcation of this genus, however, did not correspond to the seven *Telmatherina* species previously described based on standard morphological descriptions (Kottelat 1991). All three *Telmatherina* clades were highly divergent from each other at, or above, the species level (Table 3.1) (Avise & Walker, 1999). Clades I and II, however, appear more recently diverged relative to clade III. Thus, genetic phylogenies resolved three distinct *Telmatherina* lineages in Lake Matano.

Geometric Morphometrics

Significant differences were observed for all structures analyzed using GM (Skull: MANCOVA; Wilk's Λ=0.027, $F_{26,140}$=19.24, p<<0.0001 body size covariate; Lower pharyngeal jaw: MANCOVA; Wilk's Λ=0.037, $F_{44,130}$=12.28, p<<0.0001 body size

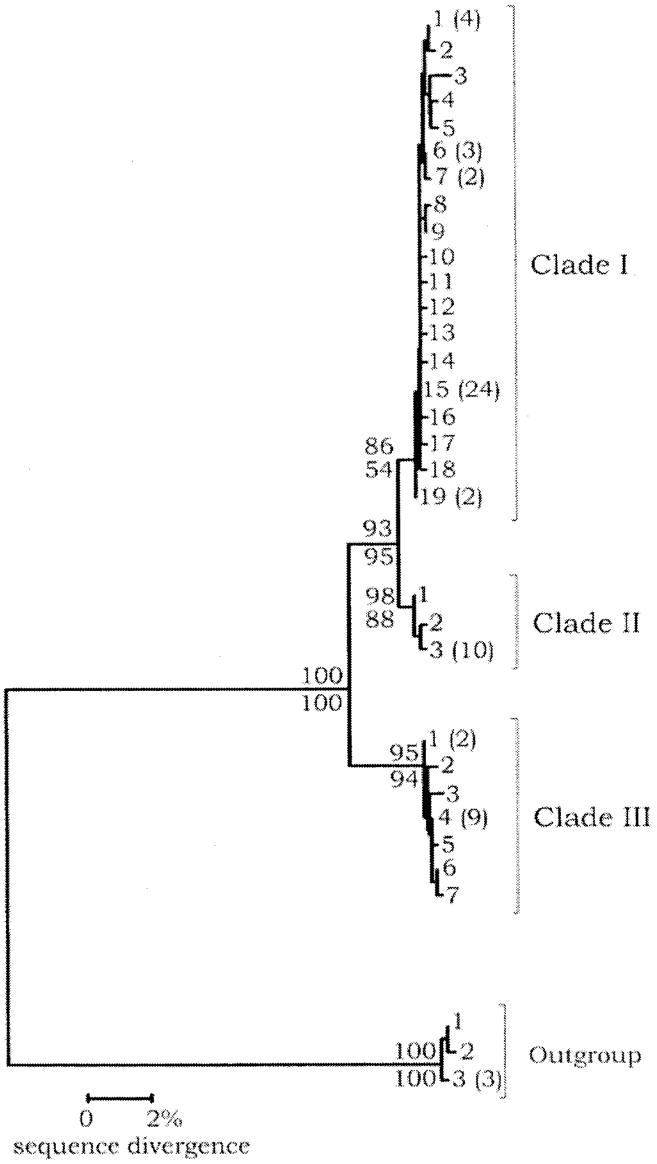

Figure 3.1 — Phylogenetic relationship among sampled *Telmatherina* (n=82) from Lake Matano, Sulawesi, Indonesia, using both 16S and cytochrome *b* mitochondrial sequence data. Numbers above and below internal nodes represent bootstrap support from both distance and Maximum parsimony based analyses, respectively. Number of haplotypes and individuals sharing them (in brackets) indicated at terminal nodes.

Table 3.1. Genetic distances within and among clades determined using Kimura 3-parameter with unequal frequencies in base composition, proportion of invariant sites (0.5517), and gamma shape parameter for base substitution (0.6715).

	Clade I	Clade II	Clade III
Clade I	0.32 %		
Clade II	1.9 %	0.12 %	
Clade III	3.9 %	3.5 %	0.41 %
Outgroup	18.0 %	18.2 %	17.7 %

covariate; 3rd pharyngobranchial (upper jaw): MANOVA; Wilk's $\Lambda=0.054$, $F_{44,132}=9.86$, $p<<0.0001$). *Post-hoc* comparisons among *Telmatherina* indicated that all genetic clades significantly differed in shape from each other except in the case of the upper jaw where clades II and III were not significantly different (Fig. 3.2). Clades II and III were more similar in shape to each other than either was to clade I (Fig. 3.2). Morphological data, however, nevertheless revealed significant differences among the three *Telmatherina* lineages in structures associated with the acquisition and processing of food.

Differences in skulls were attributable to the relative position and size of the eye, the ethmoid and the lower limb of the preopercle (Fig. 3.3: skull). The smaller and more elevated eye; the alignment of the ethmoid with the premaxilla tip; and the lengthened lower preopercle limb with its rostral tip dorsally rotated, in clades II and III relative to clade I, are features allowing the lateral expansion of the buccal cavity in these fish (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996; Grubich, 2001). The large lower preopercle limb also allows attachment of larger muscles which can produce powerful negative pressure required to dislodge substrate-associated prey in a vacuum-like manner (Galis & Drucker, 1996; Grubich, 2001). These features, more pronounced in clade III relative to II, indicate a greater ability for suction-based feeding. These same skull features dramatically differ in clade I where the eye is larger and more sunken into the head. The ethmoid is anteriorly extended and the lower limb of the preopercle is smaller, pushed more anteriorly with its rostral tip ventrally rotated (Fig. 3.3: skull). These characterize an elongated, cylindrical buccal cavity in clade I relative to the other clades. The larger eyes and a tube-like buccal cavity are consistent with tracking and ram feeding on smaller, more pelagic prey (Galis & Drucker, 1996; Grubich, 2001; Bouton et al., 2002).

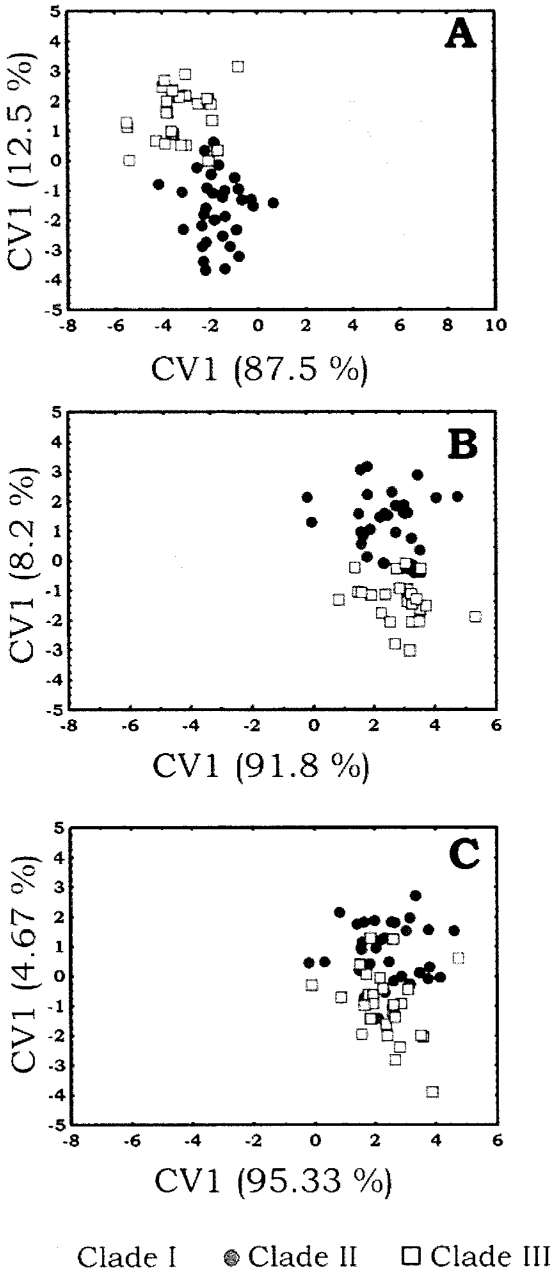

Figure 3.2 — Canonical variates plots based on partial warps analysis generated from independent geometric morphometric analysis of various head structures in *Telmatherina* clades sampled from Lake Matano. a) Skull: b) Lower pharyngeal jaw: c) 3rd pharyngobranchial (Upper jaw). Values inside brackets indicate proportion of total variation described along each axes. All three structures show statistically significant separation among clades except in the case of the left upper jaw (c) where no differences were observed between clades II and III.

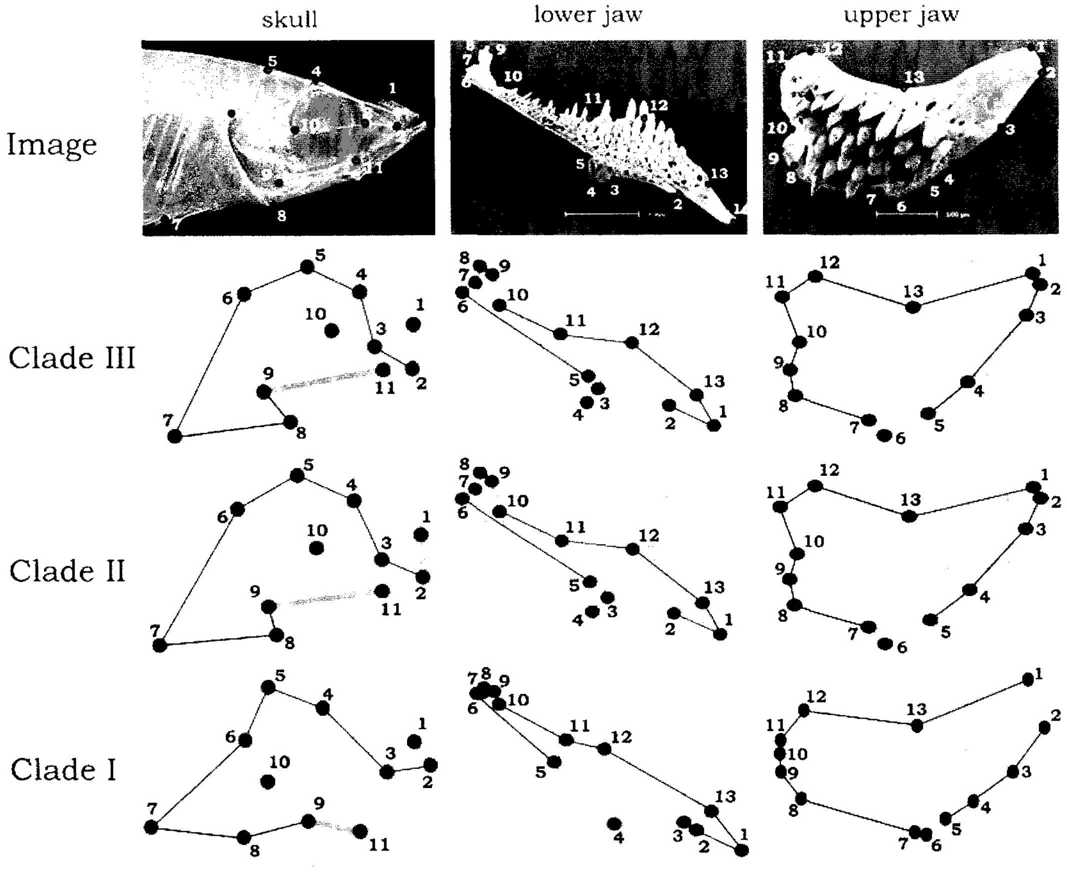

Figure 3.3 — Images and deformation grids outlining biologically meaningful shape differences in skull, lower left, and upper left pharyngeal jaws among the three *Telmatherina* clades in Lake Matano. Deformation grids show shape change using the first two relative warps accounting for 52.6%, 68.0% and 60.1% of the variation in skull, lower jaw, and upper jaw, respectively. Salient divergent features in structures outlined in colour. **Skull**: 3-10=eye diameter and position, 1-2=premaxilla tip to rostral tip of ethmoid, and 9-11=size and position of propercle lower limb. **Lower jaw**: 6-10=hinge, 3-5=keel. **Upper jaw**: 1-3=frayed zone, 5-7=ventral medial process.

The presence of large bony processes or 'hinges' on the dorso-caudal end of the lower pharyngeal jaw (Fig. 3.3: lower jaw) of *Telmatherina* allows the strong occlusion between upper and lower pharyngeal teeth (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996). The exaggerated hinge observed in both clades II and III (Fig. 3.3: lower jaw) suggests that these clades feed on hard shelled organisms where crushing is critical to process prey (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996). The relatively reduced hinge observed in clade I results in a substantial decrease in biting power. The extended keel of the lower jaw in clade I, however, provides an anchor point for larger muscles (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996) connecting it to the cleithrum, the hyoid and the 4th ceratobranchial (Fig. 3.3: lower jaw). Larger muscles can move the lower jaw more efficiently (Liem, 1974; Galis & Drucker, 1996) making it a more effective masticating apparatus for clade I, a feature consistent with pelagic feeding (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996; Grubich, 2001).

The crescent shaped 3rd pharygobranchial (Fig. 3.3: upper jaw) is the largest bone of the upper pharyngeal jaw (Barel et al., 1976). The frayed zone on this bone connects it to the 1st and 2nd pharyngobranchials (Barel et al., 1976). In clades II and III this region is smaller and anteriorly narrowed to a point, whereas in clade I it is broadened (Fig. 3.3: upper jaw). The larger and broadened frayed zone in clade I likely attaches larger 1st and 2nd pharyngobranchials providing larger chewing surfaces for softer, more malleable prey (Barel et al., 1976; Galis & Drucker, 1996). Both clades II and III are also characterized by a ventral medial process which is virtually absent in clade I (Fig. 3.3: upper jaw). This enlarged process permits larger muscles to attach to the upper jaw providing greater pressure during jaw occlusion (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996) allowing the crushing and breaking of harder prey. Thus, GM recovers significant morphological specialization within each *Telmatherina* genetic clade. If these morphological differences initiated adaptive radiation, then I expect predictably different feeding strategies among the three clades (Genner et al., 1999; Schluter, 2000; Bouton et al., 2002; Doebeli & Dieckmann, 2003).

Diet Analysis

The three clades differed significantly in their diets (Fig. 3.4A); clade I consumed mostly soft pelagic items and terrestrial insects, consistent with the types of prey predicted from the osteological data (Fig. 3.4A), while clades II and III fed mostly on littoral prey with items also matching predicted diets (Fig. 3.4A). Despite some overlap, the diets of clades II and III also significantly differed, primarily due to a substantial proportion of terrestrial insects in the clade III diet (Fig. 3.4A). This is consistent with skull differences between clade II and III, where greater suction in clade III would allow the capture of terrestrial insects from the surface. Terrestrial insects constituted 3, 16 and 34 % of the clade II, clade III, clade I diets, respectively, suggesting that clade III has a generalist feeding strategy combining items from both clades I and II diets.

Stable Isotopes

Diet analysis offers only a 'snapshot' of recent feeding activity (France, 1995; Genner et al., 1999; Post, 2002; Paterson et al., 2006). The stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$), however, provide time integrated measures of relative trophic position and energy sources (France, 1995; Genner et al., 1999; Post, 2002; Paterson et al., 2006). In aquatic systems $\delta^{15}N$ increases with trophic position while $\delta^{13}C$ remains conserved relative to pelagic or littoral pathways (France, 1995; Post, 2002; Paterson et al., 2006). The $\delta^{15}N$ signatures of all *Telmatherina* clades were significantly enriched relative to other genera in Lake Matano ($F_{3,24}$= 24.82, p<0.0001), but not relative to each

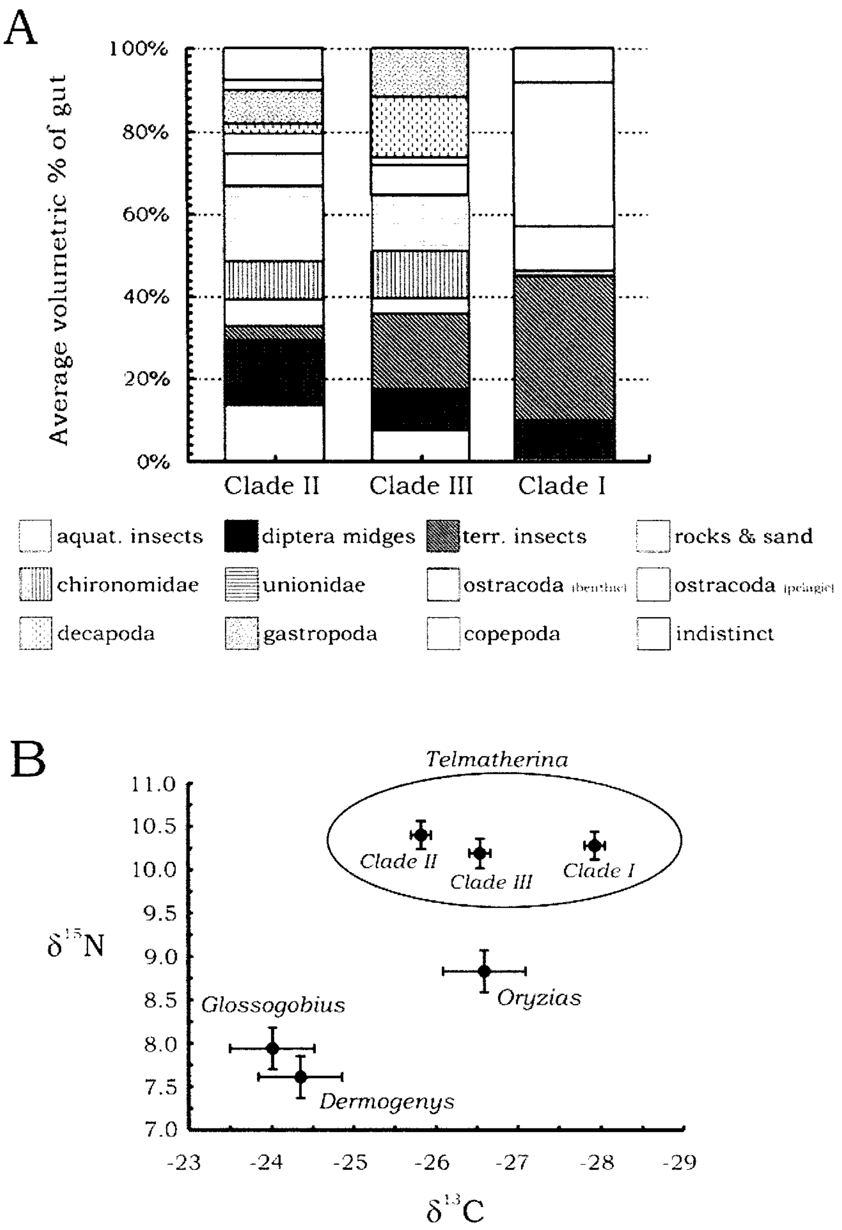

Figure 3.4 — *Telmatherina* diet analyses. a) Mean volumetric percent of various items in the gut of three *Telmatherina* clades in Lake Matano, Sulawesi, Indonesia (n=32). Clade I has a significantly different diet from both clade II and III (ANOSIM; R=0.504, p<<0.001 and R=0.444, p<<0.001, respectively), consisting of pelagic and surface prey such as copepods and terrestrial insects. Although clades II and III have many food items of a littoral nature in common, they nevertheless also have significantly different diets (ANOSIM; R=0.110, p<<0.001). b) Average carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope signature of the endemic fish from Lake Matano.

other ($F_{2,104}$=0.674, p=0.512) (Fig. 4B). δ^{13}C signatures, however, were all significantly different among clades ($F_{2,104}$=45.82, p<0.0001), with clade I and II representing the pelagic and littoral extremes of *Telmatherina* energy sources, respectively (Fig. 3.4B). The intermediate δ^{13}C signature for clade III, however, further supports a generalist feeding strategy resulting from an integration of both extremes of prey isotopic signatures (France, 1995; Post, 2002; Paterson et al., 2006). Both diet and stable isotope data confirm clear partitioning of resources within the *Telmatherina* trophic position. These diet differences associated with osteological specializations provide a definitive example of adaptive radiation initiated by resource partitioning in this trophically limited environment.

A departure of this radiation from that described in more notable examples, however, is the initial production of three divergent forms rather than two (Danley & Kocher, 2001; Streelman & Danley, 2003). We hypothesize that this trifurcation can be attributed to either of 2 possible scenarios. The first is that the *Telmatherina* are highly philopatric and their radiation is due to microallopatric speciation occurring over distances of only a few metres, as often fish from all three clades were caught in a single seine. Such highly developed philopatric behaviour driving species divergence has been demonstrated in other systems (Case & Taper, 2000; Rico & Turner, 2002), but seems unlikely here, considering the overlapping diets and morphological similarities of the generalist (clade III) and the littoral specialist (clade II). Morphologically similar sibling species relegated to small habitats in close proximity do not generally maintain strict assortative mating, a necessary condition for microallopatric speciation (Schluter, 2000; Doebeli & Deickmann, 2003; Rundle & Nosil, 2005). However, if the assortative mating among types is linked to other factors such as female preference for male colouration pattern, then such a condition may evolve as has been described in the African Lakes cichlids (Allender et al., 2003; Kocher, 2004). Such a condition would be consistent with the theory that many synergistic factors can contribute to the process of adaptive radiation, and that such interplaying factors can have varied degrees of influence over time (Schluter, 2000; Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004). Colouration pattern associations within and among the three *Telmatherina* lineages has not been tested and warrants further investigation.

An alternative explanation for this trifurcation, consistent with recent models of adaptive radiation (Streelman & Danley, 2003; Kocher, 2004), is that the generalist species (clade III) is the most recent common ancestor (Fig. 3.1) of the newly derived specialist species (clades I and II) and that the adaptive radiation of *Telmatherina* in this lake is ongoing, a consideration echoed in previous work (Chapter 2). This scenario supports the hypothesis that adaptive radiations initiated by resource limitation typically give rise to specialist species from more generalist ancestors (Pianka and McArthur, 1966). Although this scenario has been demonstrated in simulations and in manipulated conditions (Cooper & Lenski, 2000; Chow et al., 2004) few clear examples of this have been documented in natural systems. Schluter et al. (1997) reviewed several cases of adaptive radiation testing their statistical likelihood of either a generalist or specialist origin. Schluter et al. (1997) and subsequent investigations were inconclusive as to the general applicability of the generalist to specialist trend (Pagel, 1999; Nosil & Mooers, 2005). However, many investigations do show a preponderance toward generalist ancestry when such radiations were based on resource limitation (i.e., food and prey types; see Schluter et al., 1997; Schluter, 2000 p44-45). In our case, the littoral specialist in this study (clade II), shows slight yet distinct modification in specific features consistent with sharing many resources with the ancestral generalist. These features (e.g., eye size or pharyngeal keel size) are the very same that are most divergent in clade I (Fig. 3.3) allowing it to exploit completely different resources (pelagic specialist). The consistent and progressive differences among the same features in the three distinct genetic lineages indicate that morphological divergence is accomplished by simple modifications of pre-existing forms (Darwin, 1859; Goodwin, 2001; Lenski et al., 2003).

The relatively limited number of *Telmatherina* species in Lake Matano may be regarded as the product of both genetic and/or environmental constraints that may prevent the explosive divergence of this genus in this system (Streelman & Danley, 2003). It must be borne in mind, however, that the species described in this study reflect ancient divergences and lineages that may or may not continue to segregate. The morphologically distinct characters within each clade, however, testifies to the fact that segregation among them continues as otherwise clade-specific morphology would have quickly eroded through hybridization. Moreover, and as outlined in the 'radiation in stages' model (Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004), more recent divergence within *Telmatherina* clades cannot be ruled out and may be occurring based on communicatory features, such as colouration. This is especially likely considering the highly variable colouration patterns described in these fish (Kottelat, 1991; Chapter 2). Recent divergence (<20 000 years) are beyond the resolution of our genetic markers, and thus I may have underestimated the number of *Telmatherina* species present. Therefore, arguments pointing to the apparent constrained divergence of *Telmatherina* in Lake Matano ought to consider that more recently developed reproductive isolation among colouration groups within each clade may increase *Telmatherina* taxa number.

This study provides genetic, morphological diet and trophic data that are consistent with the ecological seeding of adaptive radiation among the *Telmatherina* in Lake Matano and provides an independent dataset, separate from more noted systems (i.e., Galapagos finches, African cichlids, Hawaiian drosophila), demonstrating the ecological basis of this process. Clearly, ecological pressures such as resource limitation are potent initiators of adaptive radiations in natural systems.

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4.0 — BODY SHAPE VERSUS COLOUR ASSOCIATED INITIAL DIVERGENCE IN THE TELMATHERINA RADIATION FROM LAKE MATANO, SULAWESI, INDONESIA*.

4.1 INTRODUCTION

Sexual selection is a powerful mechanism driving evolutionary divergence, and its ability to initiate and drive speciation has received considerable attention (Dieckmann & Doebeli, 1999; Panhuis et al., 2001; Arneguard & Kondrashov, 2004; Gourbiere, 2004). For example, investigations of sexual selection based on assortative mating among brightly coloured fishes have been conducted both *in situ* (Seehausen et al., 1997; van Oppen et al., 1998; Rico et al., 2003) and under laboratory settings (Seehausen & van Alphen, 1998; Couldridge & Alexander, 2002; Knight & Turner, 2004). Such studies usually attribute divergence among taxa to differentiating colouration patterns among closely related species. This process is thought to be due to female preference for specific male nuptial colouration patterns and is especially prevalent in polygynous systems (van Oppen et al., 1998; Seehausen & van Alphen, 1999; Allender et al., 2003; Knight & Turner, 2004). Sexual selection and assortative mating based on colouration patterns is an enticing hypothesis explaining sympatric speciation as the resulting selection is thought to be strong and disruptive even among closely related groups or populations (Lande, 1981; Mayr, 1963; Panhuis et al., 2001; Salzburger & Meyer, 2004). Sexual selection among brightly coloured fishes is especially prevalent in systems characterized by high clarity allowing mate recognition, and large amounts of predator-free space applying little cost to the development of conspicuousness (Lande, 1981; Seehausen et al., 1997; Higashi et al., 1999; Panhuis et al., 2001). Under such conditions, sexual selection can generate many

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* Roy D, Docker MF, Haffner GD, Heath DD. Body shape versus colour associated initial divergence in the *Telmatherina* from Lake Matano, Sulawesi, Indonesia. (*Manuscript submitted to Biological Journal of the Linnean Society. Jan 2006*).

polychromatic sibling species relatively quickly starting with few common ancestral lineages in a Fisherian runaway mode (Lande, 1981; Dieckmann & Doebeli, 1999; Panhuis et al., 2001; Rundle & Nosil, 2005).

Recent work, however, argues that sexual selection in general, and more specifically among brightly-coloured closely related species, is probably insufficient in itself to cause sympatric divergence (Arnegard & Kondrashov, 2004; Gourbiere, 2004). Rather, colouration segregation likely arises subsequent to differentiation initially brought on by ecological factors such as resource partitioning or niche specialization (Verheyen et al., 2003; Arneguard & Kondrashov, 2004; Salzburger & Meyer, 2004; Rundle & Nosil, 2005). Danley & Kocher (2001) Streelman & Danley (2003) and Kocher (2004) describe a pattern observed in many species radiations and propose that explosive speciation can often be traced back to distinct divergence events during species' evolutionary histories. Using data gathered from published examples, these authors present an integrative model of adaptive radiation whereby a combination of selective pressures act differentially throughout this process (Streelman & Danley, 2003; Kocher, 2004). Their model is predicated on cyclical bouts of high gene flow building variation within populations and strong disruptive selection splitting these populations along varied lines resulting in punctuated events of diversification. Initial divergences are related to body shape adaptations to microhabitat in a competitive environment limited for space (e.g., ground vs. tree finches, Sato et al., 1999; rock- vs. sand-dwelling cichlids, Danley & Kocher, 2001; various ecomorphs of the Anolis lizards, Losos et al., 1998). The second divergence involves trophic morphologies reducing competition in resource poor environments (e.g., piscivorous, benthivorous and planktivorous arctic charr, Skúlason et al. 1993, algae scrappers, molluscivores and piscivores in cichlids, Bouton et al. 2002; coral crushers and algae excavators in parrotfishes, Streelman et al., 2002). The final and typically most explosive divergence stage is often related to communication within newly formed divergent lines, such as that based on sexual selection (Streelman & Danley, 2003, Kocher, 2004). Sexual selection in many such cases often takes the form of female preferences for male nuptial colouration reinforcing divergence between already established closely related populations.

Lake Matano, located in the south central portion of Sulawesi Island, Indonesia (Fig. 4.1), is an ancient tropical freshwater lake estimated to be approximately 2-4 million years old (Brooks, 1950; Haffner et al., 2001). It occurs along an active fault, forms the hydrological head of the Malili lakes system, and is isolated from downstream lakes by a 72m elevation change which includes cascading rapids (Brooks, 1950; Hamilton, 1979; Kottelat, 1990a; Larson & Kottelat, 1992). Lake Matano is relatively small (~164 km^2), has two very deep basins (610 & 590 m) separated by a deep saddle (509 m), and the entire lake is surrounded by very small littoral areas (Fig. 4.1). The fish community in this lake is restricted to these shallower littoral areas with few or no fish below the 30-40 m contours (Roy, unpublished data). This graben lake is also highly oligotrophic, evidenced by its high Secchi depth (~23-25 m), low phytoplankton and zooplankton abundances, and by its rare top predators (Haffner et al., 2001; Sabo et al., 2006; Heath, pers. communication; Chapter 2). Four fish genera, all of which likely invaded the lake at similar times, are endemic to Lake Matano (Brooks, 1950; Kottelat, 1990a, 1991; Larson & Kottelat, 1992; Aarn et al., 1998; Chapter 2). Of these, the *Telmatherina* genus is the most genetically divergent and is characterized by a significantly greater number of colouration patterns and greater variation in feeding traits than the other genera present in this system (Chapter 2). In Chapter 2, I used published criteria (Schluter, 2000) to show that the *Telmatherina* are adaptively radiating in this system due to either sexual selection by assortative mating among the varied colourtypes, by trophic specialization in this resource limited environment, or a combination of both processes.

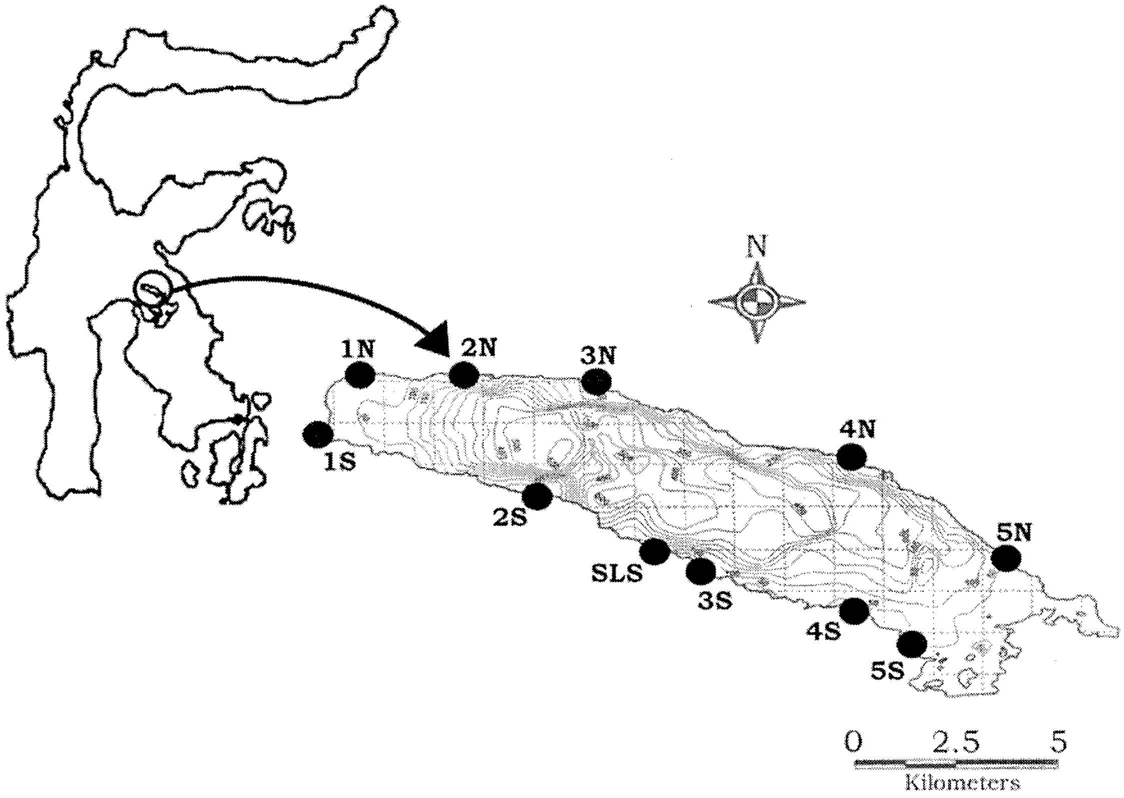

Figure 4.1 — Location of Lake Matano on the continental island of Sulawesi in the Indonesian Archipelago. Beach locations where fish were collected are indicated with dark circles and alphanumeric values (N=North, S=South).

In this study I address whether the radiation within the *Telmatherina* genus was initiated by strong sexual selection based specifically on colouration, or whether ecological or environmental factors driving microhabitat adaptation were more important factors. Lake Matano's clear water and brightly coloured *Telmatherina*, make it an ideal system to test for putative sexual selection among the sympatric colourtypes.

Here I characterize genetic variation within the *Telmatherina* genus using mitochondrial DNA and determine phylogenetic relationships among the various described types. We also determine if colouration or body shape traits better correspond to the genetic relationships by comparing both features with the derived genetic phylogenies. The expectations are that, if the genetic divergence within *Telmatherina* was initiated by assortative mating among colourtypes, then colouration ought to match genetic structure. However, if body shape was a more important factor driving the initial divergence, then body shapes should be a better match to the derived phylogenies. Determining the relative importance of different selective pressures seeding divergence in this system adds to our understanding of factors capable of inciting adaptive radiations and the creation of biodiversity in natural systems.

4.2 MATERIALS & METHODS

Approximately 50-100 *Telmatherina* were collected by beach seine from each of eleven beaches around the periphery of Lake Matano (Fig. 4.1). Collected fish were anatomically sexed and only male fish were used in subsequent analysis. Fish were separated into five morphotypes based on previous species descriptions (Kottelat, 1990b; 1991) and into four new morphotypes based on both colouration patterns and other phenotypic characters described by Kottelat (1990b; 1991). Standard morphometric procedures described by Kottelat, however, often led to ambiguous species determination

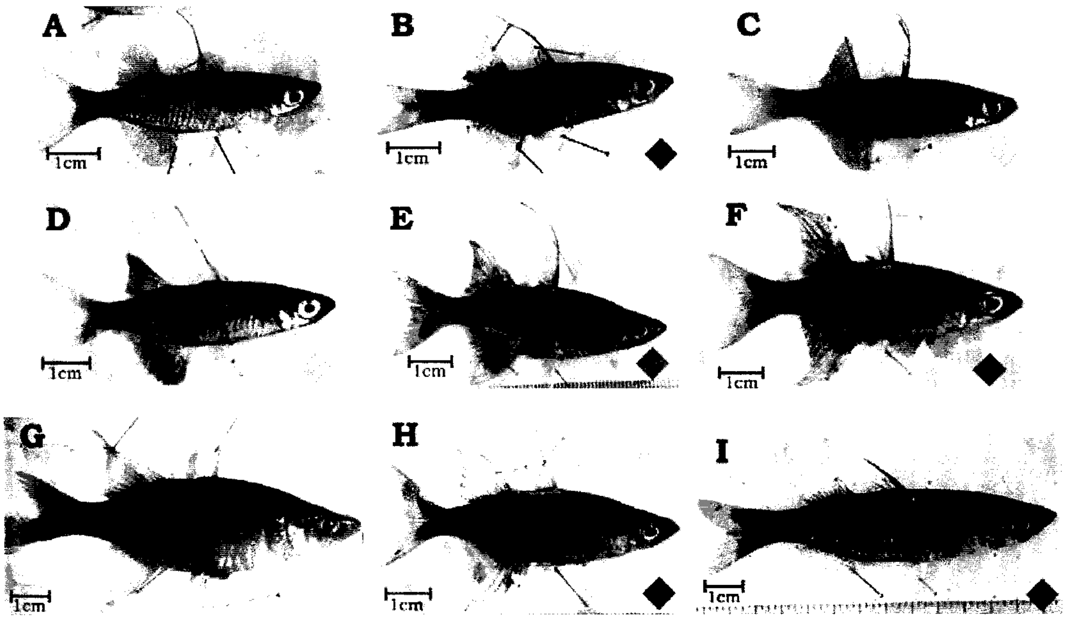

Figure 4.2 — Nine different *Telmatherina* morphotypes commonly encountered in Lake Matano. All types are endemic to Lake Matano and can be distinguished using colouration and shape. There are five distinct colouration types; yellow (A & G), blue (B, E & I), yellow blue (C) yellow green (D) and brown (F & H). Shapes were determined using geometric morphometrics (see text).

because of highly overlapping characters among described types (see Kottelat, 1991; Kottelat et al., 1993). Consequently, *Telmatherina* were identified by a combination of both body shapes and colourtypes (Fig. 4.2). No system-wide spatial bias was observed in the distribution of different morphotypes or colouration patterns collected from the different beaches.

In the field, collected specimens were anaesthetized using clove oil and pinned to a gridded cork board to carefully extend all fins in a standardized manner. Specimens were then photographed using a high resolution digital camera. Fin clips were taken from each individual and stored in 95% ethanol for subsequence DNA extraction. Specimens were then allowed to recover in oxygenated water before being released back to the lake. Fin clips were also collected from five *Marosatherina lagdesi*, a related, commonly available atheriniform fish from Sulawesi used as the outgroup (Aarn et al., 1998).

DNA was extracted from a random sub-sample of 118 fish while making certain to include individuals from each morphotype identified in Figure 4.2. Extractions were performed using the Promega WIZARD® DNA extraction kit following the standard 'isolation from animal tissue' protocol (Promega, Madison, WI, USA). Amplification of 490 bp of the 16S rDNA and 600 bp of the cytochrome *b* gene was performed using both published and specifically designed primers [16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') & 16Sbr (5'-CCGGTCTGAACTCAGATCACG-3'); Palumbi 1996], and [GLUDG-5(5'-TGACTTGAARAACCACCGTTG-3'); Palumbi, 1996 & (CBtelm–R (GTGGAGGAGGGGTACGACTA-3') developed here]. Individual 25-µl reactions contained 20 mM Tris-HCl (pH. 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer, 0.5 U of *Taq* DNA polymerase (Invitrogen, Burlington, Canada) and 1.0 µL of a 10:1 dilution of extracted DNA. Reactions were run for 35 cycles consisting of a denaturation at 96°C for 1 min, primer annealing at 50°C

(16S) or 60°C (cytochrome *b*) for 1 min, and extension at 72°C for 1.5 min; the 35 cycles were followed by a final 5 -min extension at 72°C. PCR products for a total of 118 fish were sequenced using the DTCS Quick Start cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) and CEQ8000 automated sequencer following manufacturer's instructions.

Derived sequences from both genes were aligned separately using the clustal W algorithm with a gap open penalty of 10, a gap extension of 5 and a DNA transition weight of 0.5 (Omiga 1.2, Oxford Molecular Ltd, Oxford, UK). Resulting alignments were verified by eye and analyzed for transition saturation using Molecular Evolutionary Genetics Analysis program version 3.1 (Kumar et al., 2001). Insertions and deletions (indels) were treated equally as complete deletions (Kocher & Carleton, 1997), and because both 16S and cytochrome *b* sequences exhibited no saturation but similar divergence rates, both fragments were combined into a single 1090 bp fragment for each individual. Phylogenetic reconstruction among derived sequences was done by Neighbour-Joining using the HKY85 model for base pair substitution characterized by equal base frequencies, a proportion of invariant sites (0.6005) and a gamma shape parameter (0.7095). This substitution model was specified by the Akaike Information Criterion resulting from a maximum likelihood survey of 56 different base substitution models implemented by Modeltest (Possada & Crandall, 1998) performed in PAUP version 4.0b10 (Swafford, 2001). As an additional test of phylogenetic structure and topology, Maximum Parsimony (MP) heuristic searches were performed with a Close Neighbour Interchange of 1 and an initial tree chosen by Random Addition Trees of 10 replicates. A 50 % majority consensus tree was constructed from pre-calculated distances by 10000 bootstrap iterations assessing nodal confidence, while MP was reiterated 1000 times.

Digital pictures from all genetically analyzed specimens were used for geometric morphometrics (hereafter GM), a landmark based analysis assessing body shape variation among specimen groups (Caldecutt & Adams, 1998; Rüber & Adams, 2001; Adams et al., 2004; Zelditch et al., 2004; Chapter 3). GM allows the decoupling of shape from non-shape variation in landmark configurations by essentially removing the effects of size, orientation and scale (Rohlf & Slice, 1990; Adams et al., 2004; Zelditch et al., 2004). The two-dimensional coordinates of 18 biologically homologous landmarks, selected based on standard landmark descriptions (Bookstein, 1991; Zelditch et al., 2004), were identified on digitized *Telmatherina* images using the TPSDig program (Rolhf, 2004). Landmarks included fin shapes as these features can be diagnostic in this genus (Kottelat, 1990b; 1991). Fin positions were standardized by holding the fins in full extension using entomological pins. The first dorsal fin, however, was standardized using methods described by Adams (1999) because of its length and centre of articulation at its base.

Digitized landmarks from all specimens were superimposed using a Generalized least squared Procrustes superimposition Analysis (GPA). GPA calculates the average X- and Y- coordinates of the landmarks for each specimen (centroid). Specimen landmark configurations were re-scaled to unit size and their centroids translated to a common position. Re-scaled landmark configurations were then superimposed and fit to minimize the least squared Procrustes distance between homologous landmarks for all specimens (Rohlf, 1999; Zelditch et al., 2004). The GPA produced a consensus configuration of landmarks based on all superimposed specimens (Rohlf, 1999; Adams et al., 2004; Zelditch et al., 2004).

A thin-plate spline was used to generate an interpolation function between the landmarks of the consensus configuration, describing each landmark by geometrically orthogonal components termed 'principal warps'. Principal warps represent multidimensional shape axes through each landmark. As the thin-plate spline superimposes each specimen's corrected landmark configuration onto the consensus configuration, it calculates the amount of deviation needed along each principal warp to fit each landmark in the specimen to that in the consensus configuration. This procedure generates a set of orthogonal shape variables, or partial warp scores that can be used in standard multivariate analyses (Caldecutt & Adams, 1998; Rüber & Adams, 2001; Zelditch et al., 2004). Thus, after the thin-plate spline procedure, each fish is characterized by its unique set of partial warps.

A discriminant function analysis (DFA) was performed on the partial warps from all fish to determine if there were significant differences in body shape variables among the groupings derived from the genetic relationships. A canonical variates analysis (CVA) was performed to discriminate among specimens along various ordination axes minimizing variation within, but maximizing differences among, groups (Rüber & Adams, 2001; Klingenberg et al., 2003; Zelditch et al., 2004). *Post-hoc* pairwise comparisons among the genetic groups were performed using derived *F*-values from the generalized Mahalanobis distances to determine which ones were characterized by significantly different shapes. We adjusted α-values to 0.0166 using Bonferroni correction to keep experiment-wise error at 0.05 (Kassam et al., 2004).

Deformation grids were constructed for each CVA defined group using their mean shapes defined along the most important relative warps (Rohlf et al., 1996; Caldecutt & Adams, 1998; Rüber & Adams, 2001; Klingenberg et al., 2003; Zelditch et al., 2004). Deformation grids allow the visualization of biologically relevant shape differences among the pre-determined groups. Consensus configuration, GPA, the thin-plate spline generation of partial warps, relative warps and deformation grids were all accomplished using the TPSRelw program (Rohlf, 2004).

Colouration for each specimen was scored from the digital pictures (Fig. 4.2) and subsequently compared to the derived genetic phylogeny. We assigned 3 dominant colours (blue, yellow and brown), plus two intermediate colourtypes (yellow-blue and yellow-green; Fig. 4.2). Significant relationships between colourtypes and clade membership were assessed using an Exact test for population differentiation. Significance of Exact tests, including all pairwise comparisons among clade membership and colourtypes were assessed using an unbiased Markov chain approach considering colouration as an allele inherited in a haplotypic manner and clade number as a population. The Exact tests were permutated 10000 times using 10000 de-memorization steps per batch of 100 using the algorithm developed by Raymond & Rousset (1995), implemented in Tools For Population Genetics Analysis program version 1.3 (Miller, 1997). Colouration among fish were assigned discrete values (1-5; see Fig. 4.2) and added to the list of shape variable (i.e., partial warps) for discriminant function analysis. DFA and CVA were repeated with the colour code included to determine whether colouration provided additional discriminating information among the *Telmatherina* genetic clades. To determine whether coloration or shape best predicted genetic structure individual clade assignments based on colour and shape were compared. Possible colouration specific segregation among haplotypes was also assessed using TCS (Clement et al., 2000) generating a minimum spanning network while keeping track of individual haplotype colouration. The maximum parsimony default settings in TCS were used to produce network linkages. To assess whether body shape and colouration patterns were related independent of genetic data, an additional DFA and CVA was performed using colouration as the grouping variable. All DFA and CVA were performed using STATISTICA (StatSoft, Inc. 2001. data analysis software system, version 6).

4.3 RESULTS

Sequences used in this study are available on GenBank under the accession numbers DQ023624-DQ023667, DQ002506-DQ002547, DQ054795-DQ054796, and AY545822-AY545875. Aligned sequences were characterized by 204 variable sites (19%), 182 (17%) of which were parsimoniously informative. Overall sequence differences were characterized by 3 indels, and a transition to transversion ratio of 3.36. The Neighbour-Joining analysis based on HKY85 distances and the MP algorithm recovered very similar trees with identical general topology and support for major nodes (Fig 4.3).

Generated phylogenetic relationships separated sampled *Telmatherina* specimens into three distinct and well supported clades (Fig. 4.3). All clades, with the exception of clade II, were supported by greater than 85% of bootstrap permutations. The observed trifurcation of the *Telmatherina* specimens into three distinct clades did not correspond to the seven different species previously reported (Kottelat, 1991), but did match previous phylogenies (Chapter 2; Chapter 3). Genetic variation within clades was highest amongst clade I followed by clade III and then clade II (Table 4.1). All *Telmatherina* clades were approximately equidistant from the outgroup, but showed variable interclade divergence (Fig. 4.3; Table 4.1). Clades I and II were more closely related but were clearly divergent at or above the species level (Table 4.1; Avise & Walker, 1999; Hebert et al., 2003). Both clades I and II exhibited large and similar distances from clade III, indicating that, apart from the distances to the outgroup, the distance between clades I and II vs. clade III was the most pronounced within the *Telmatherina* specimens sampled (Fig. 4.3, Table 4.1). The distances exhibited between each *Telmatherina* clade are consistent with the presence of three *Telmatherina* species in Lake Matano with possible internal structures indicating subspecies or races.

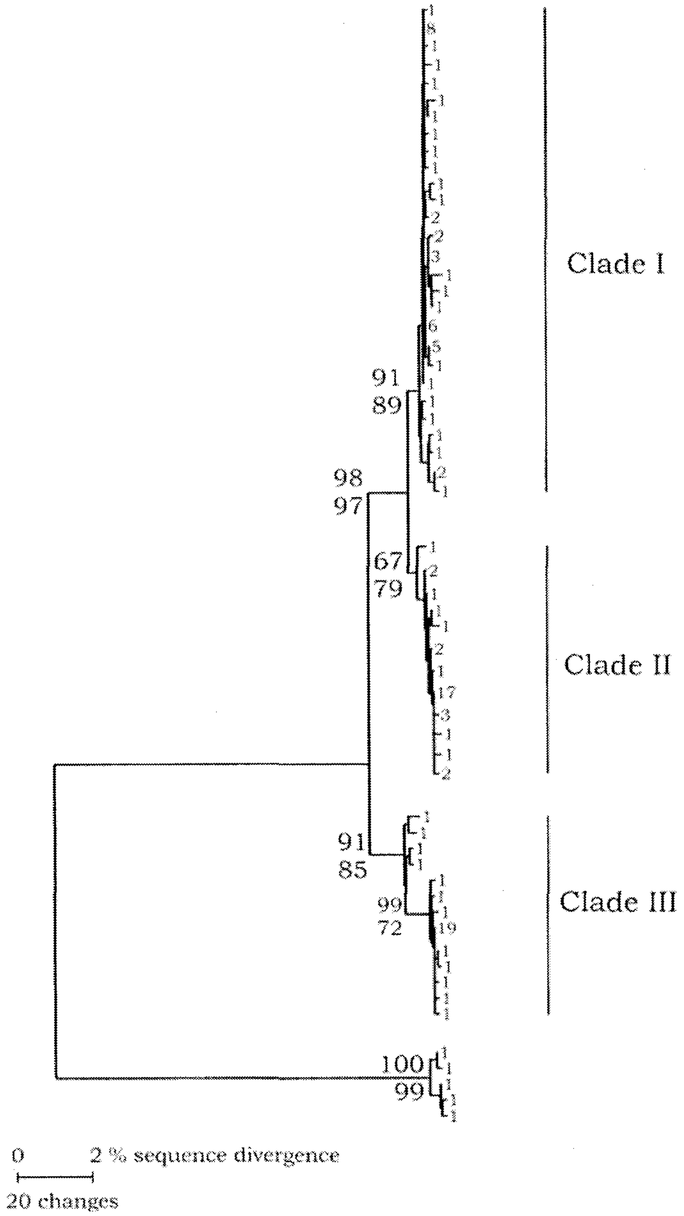

Figure 4.3 — Phylogenetic relationships derived from 1086 bp of mitochondrial genome from 118 *Telmatherina* from Lake Matano, Sulawesi, Indonesia using a 50 % majority-rule consensus tree determined from Neighbour-Joining algorithm with genetic distances calculated using HKY+I+G (I=0.6055; G=0.7095) model of base substitution implemented in PAUP version 4.0b10. Maximum parsimony (MP) recovered similar topology using Closest Neighbour Interchange of 1 with the addition of 10 replicates. Bootstrap nodal support for both distance based (above) and MP (below) topology indicated at root nodes (10000 for NJ and 1000 for MP). Numbers at terminal nodes indicate number of individuals sharing the same sequence.

Table 4.1– Genetic distances calculated within (on diagonal) and among *Telmatherina* clades identified from the Neighbour-Joining tree. Genetic distances estimated using the Hasegawa-Kishino-Yano (1985) base substitution model with equal base frequencies, a proportion of invariable sites (I=0.6005) and a gamma shape parameter (G=0.7095). Distance model chosen by Akaike Information Criterion result from maximum likelihood survey of 56 different substitution models (Modeltest, Possada & Crandall, 1998).

	Clade I	**Clade II**	**Clade III**
Clade I	0.32 %		
Clade II	1.34 %	0.15 %	
Clade III	3.87 %	3.46 %	0.28 %
Outgroup	43.49 %	44.68 %	40.22 %

No significant differences were found in overall morphometric-based centroid sizes among the three genetically determined *Telmatherina* clades (Krukal-Wallis$_{(116)}$=3.84, P>0.05) indicating that size had no significant influence on shape differences among them (Calvacanti, 2004). Significant differences, however, were observed among the body shapes of the three different clades (Wilk's $\Lambda = 0.11219$, $P \ll 0.00001$). Pairwise, *post-hoc* comparison among the three clades determined that all significantly differed in body shape from each other (clades I vs. II $F_{(32,82)}$=8.48, P<<0.00001; clades I vs. III $F_{(32,82)}$=7.29, P<<0.00001; clades II vs. III $F_{(32,82)}$=1.93, P=0.009). CVA revealed that nearly all the variation in body shape could be explained using the first two canonical variate (CV) axes (Fig. 4.4A). Although CVA clearly segregated all three clades from one another, some overlap was evident from the 95% equal frequency ellipses. Clades II and III were much more similar in body shape to each other than either was to clade I (Fig. 4.4A). Adding colouration as an additional variable in the DFA and CVA gave almost identical results (Wilk's $\Lambda = 0.11159$, $P \ll 0.00001$) and did not enhance the segregation among clades any further (Fig. 4.4B).

Relative warp analysis of shape variables demonstrated biologically meaningful shape differences among clades and allowed visualization of shape change using deformation grids (Rohlf et al., 1996; Rüber & Adams, 2001; Adams et al., 2004; Calvalcanti, 2004; Zelditch et al., 2004). Deformation of the consensus configuration along the relative warps 1 and 2 was determined for the average of all specimens belonging to each clade and exaggerated five times to emphasize differences (Zelditch et al., 2004). Relative warps 1 and 2 accounted for > 75 % of the variation in shape differences among the three clades and deformation proved to be clade-specific (Fig. 4.5). Individuals in clade I were characteristically more fusiform in body shape, had rounded anal and second dorsal fins, an enlarged caudal peduncle and a terminal mouth. Based on these features I classified this fusiform body shape as 'Torpedo' (Fig. 4.5). Specimens in clades II and III

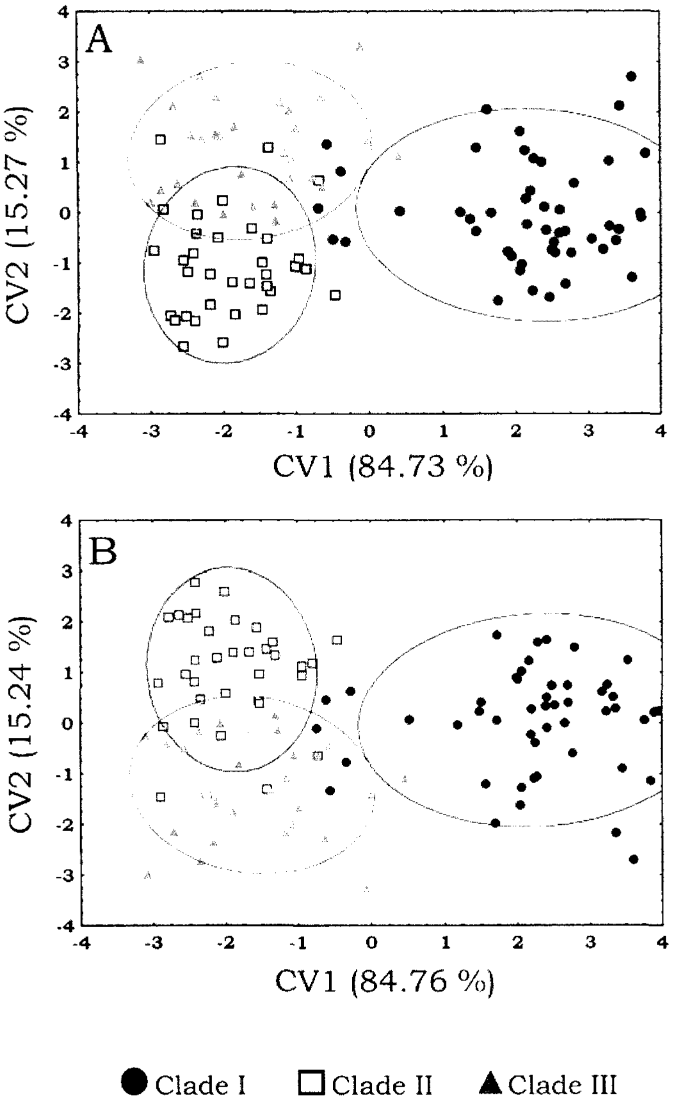

Figure 4.4 — Canonical variates ordination plots based on partial warps analysis of body shape segregating the three genetic clades identified from the phylogenetic relationships. Plots show individual *Telmatherina* scores along CV axes and 95 % frequency ellipses surround each clade. In both cases CV1 and CV2 account for near 100% of the total variation among clades. Values in brackets indicate the proportion of total variation described along each axis. A) Shape variables only. B) Shape variables with an additional variable representing colour scored from digital images as in Figure 2.

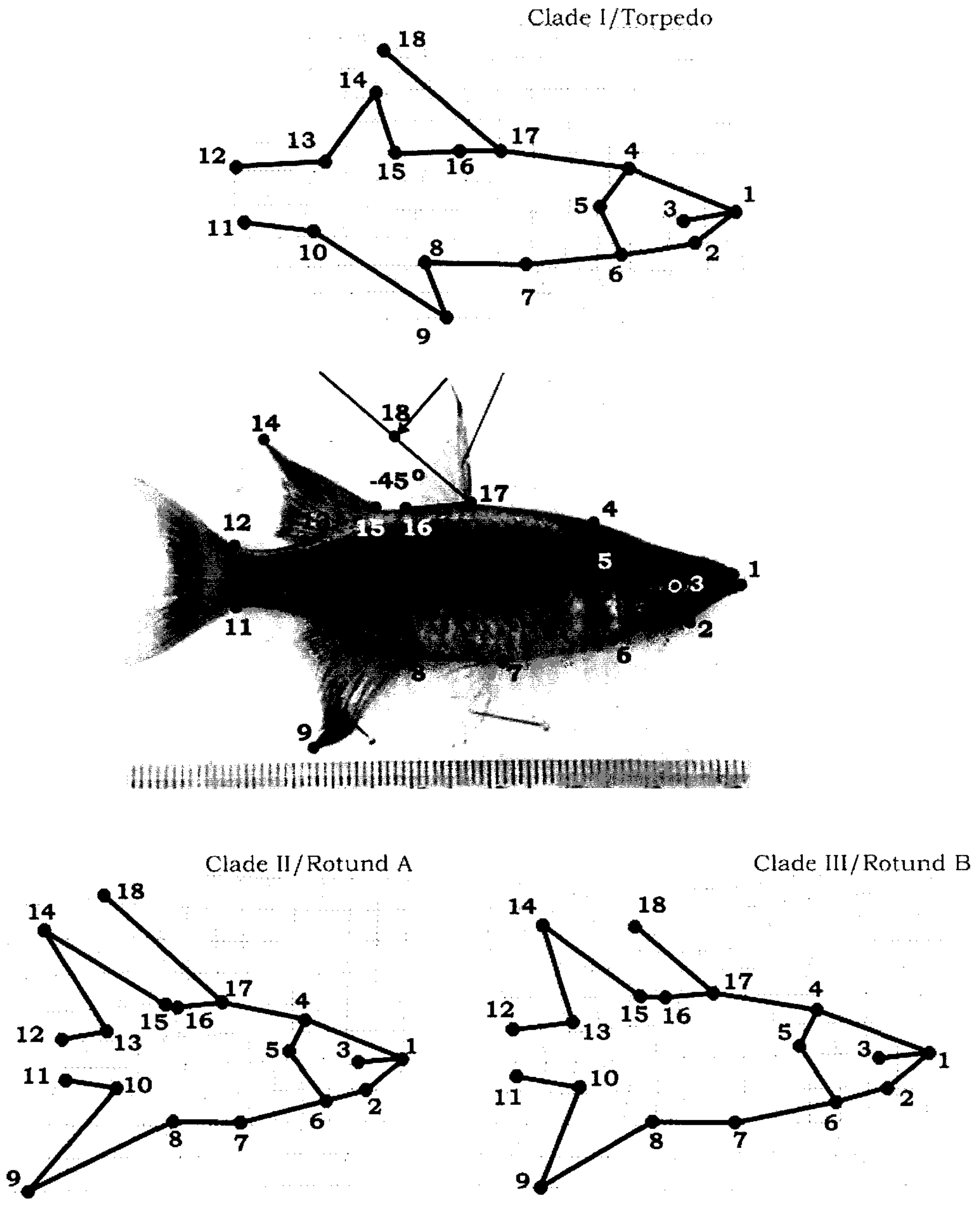

Figure 4.5 — Image of typical *Telmatherina* showing overlaid landmarks used in Generalized Procrustes superimposition analysis determining overall consensus configuration. Deformation grids generated from thin-plate spline analysis of partial warps (shape variables) demonstrate how consensus configuration must be warped to fit average shapes determined for each clade and outline biologically meaningful shape differences among clades. Generated deformation grids show shape change using first two relative warps which account for > 75 % of the shape differences among clades. Mean shapes for each clade was exaggerated five times to emphasize differences.

were more compressiform in body shape with their bodies compressed along the posterior anterior axis (Fig. 4.5). Both clades were also distinguished from the 'Torpedo' type by longer and more pointed anal and second dorsal fins, a deeper body and a protruding mouth. Based on this distinction from Torpedo I classified these latter clades as 'Rotund'.

Distinguishing features between clades II and III were more subtle but nonetheless apparent. Individuals in clade II were even more longitudinally compressed, had longer and more pointed fins reaching further back along the body, had a smaller and more tapered caudal peduncle and a smaller head than individuals belonging to clade III (Fig. 4.5). Individuals in clade III generally displayed morphological features intermediate between those in the other two clades (Fig. 4.5). Clade II and III specimens were subsequently classified as 'Rotund A' and 'Rotund B', respectively. Shape differences were more pronounced between both Rotund clades and the Torpedo clade, while the greatest genetic divergence occurred between Rotund A and B (clades II and III), separating Rotund A and Torpedo shaped fish from those of Rotund B (Figs. 4.3, 4.4A & B, 4.5 and Table 4.1).

Exact tests demonstrated a significant difference in colourtypes assigned to the different clades ($P<<0.0001$). Pairwise comparisons between clades showed that colourtype assignments to clade I were significantly different from those assigned to clades II and III ($P < 0.001$ and $P < 0.001$, respectively). No significant differences, however, were found between clades II and III ($P = 0.97$). All three clades shared similarities in member colourtypes except that yellow blue individuals were predominantly present in clade I with the exception of a single individual in clade III. All yellow green individuals were found in clade I (Fig. 4.6 & 4.7). Blue and yellow individuals were present in all clades including clade I in similar proportions, while brown individuals were present in both clades II and III in similar proportions, but were underrepresented in clade I (Fig. 4.6

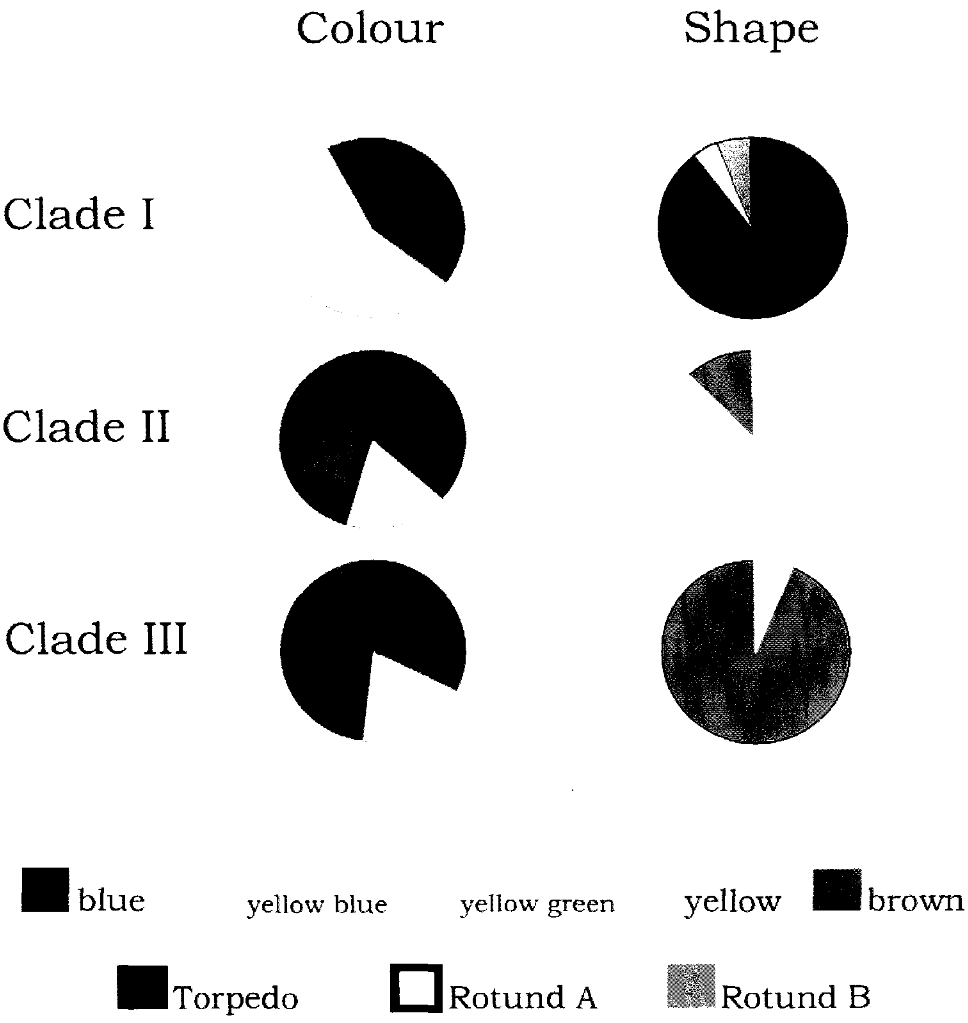

Figure 4.6 — Frequency of the different colouration types and shapes associated with individuals within each clade. Colouration types for each individual determined from digital images as in Figure 4.2, while shape was determined from geometric morphometric analysis of shape differences among clades (Figure 4.4). Individuals occurring inside their genetic clade 95 % frequency ellipse were assigned that clade's respective shape. Those falling outside their respective clade 95 % frequency ellipses were assigned the shape of the clade in closest proximity.

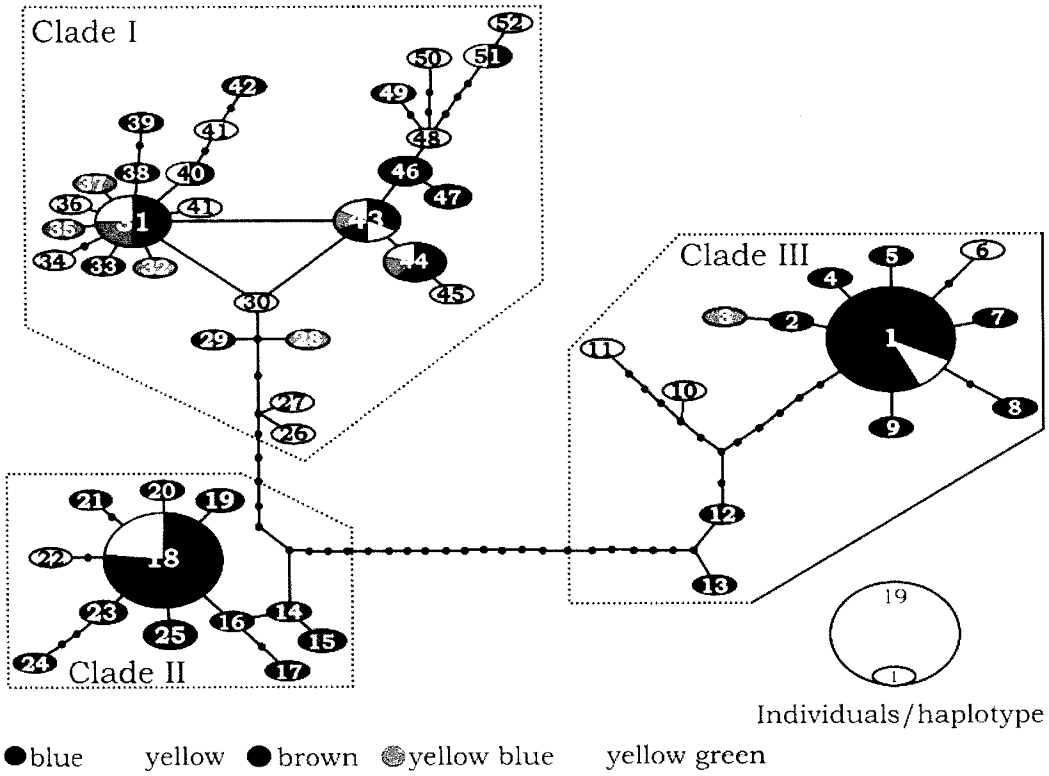

Figure 4.7 — Unrooted minimum spanning haplotype network determined (using Maximum Parsimony to generate network linkages) among individual *Telmatherina* sampled in this study. Network derived from 1080 bp sequences of mtDNA (cytochrome *b* & 16S). Haplotype sizes and colour reflect number of individuals sharing a particular haplotype (scale in lower right corner) and their respective colouration as determined from digital images (see Figure 4.2). Haplotype # indicated inside haplotype ovals.

& 4.7). Predicting clade membership using only colourtype proved to be ineffective except in the case of yellow blue and yellow green *Telmatherina*. Conversely, predicting clade membership based on shape, although not perfect, was generally more successful (Fig. 4.6).

The minimum spanning haplotype network determined for *Telmatherina* revealed results consistent with both the distance based phylogeny (Figure 4.3) and with the colouration assignment tests (see above). In particular, the network demonstrated three genetic clades separated by several mutations each, with clades I & II more closely related than either is to clade III (Figure 4.7). The network also confirmed that most colouration types are spread about all clades with the exception of yellow blue and yellow green which are predominantly in clade I. In addition, brown is underrepresented in clade I and blue and yellow have similar proportions in all three clades (Fig. 4.7). Thus, although some colouration types proved to be clade specific (yellow blue and yellow green specific to clade I) the network showed that colouration types within each clade were not haplotype-specific as many haplotypes exhibited various colouration types (e.g., haplotypes 1,18, & 43; Fig. 4.7).

Using colourtype as a grouping variable in a DFA for shape revealed a significant difference in the shapes assigned to colouration groups (Wilk's $\Lambda = 0.13341$, $P << 0.002$). *Post-hoc* comparisons in this case did not show significant shape differences among yellow, blue or brown *Telmatherina* ($P >> 0.05$), but did show significant differences between yellow blue vs. blue ($F_{(32,80)}=2.46$, $P<0.00001$), yellow blue vs. brown ($F_{(32,80)}=2.52$, $P<0.00001$), yellow green vs. blue ($F_{(32,80)}=2.83$, $P<0.00001$) and yellow green vs. brown ($F_{(32,80)}=2.28$, $P<0.00001$). Yellow blue and yellow green *Telmatherina*, however, were not significantly different in shape from each other ($P = 0.1163$). CVA segregated both yellow blue and yellow green coloured fish from all other colourtypes but a high degree of overlap was observed among blue, brown and yellow colourtypes (Fig 4.8). The first two CV axes accounted for just over 75 % of the shape variation among colourtypes, substantially less than what was accounted for by clade membership (compare Fig. 4.4 & 4.8).

4.4 DISCUSSION

Understanding the mechanisms regulating speciation in natural systems is critical for clarifying the evolutionary processes generating biodiversity. This is especially important in systems involved in species radiations where the number and rate of species formation is high (Schluter, 2000). A common paradigm among studies of radiation in brightly coloured, polychromatic species is that sexual selection based on male nuptial colouration patterns and female preferences is the cause of rapid divergence (Lande, 1981; van Oppen et al., 1998; Panhuis et al., 2001; Knight and Turner, 2004). In this study I show that this is likely not the reason for initial divergence in the radiation of *Telmatherina* in Lake Matano. Rather, the development of specific body shape adaptations to particular microenvironments is key to initial *Telmatherina* divergence within the confines of this highly oligotrophic system, whereas colouration appears to have had a minimal influence, at least initially. Our results are consistent with the 'radiation in stages' pattern demonstrated in many other adaptive radiations, whereby species initially diverge by body shape specialization to environmental adaptive peaks, followed by communicatory cues such as that initiated by sexual selection among colouration types (e.g., Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004).

In this study, I identified three distinct *Telmatherina* mitochondrial lineages within the Lake Matano system separated by levels of divergence consistent with that of well-established species (Avise & Walker, 1999; Hebert et al., 2003). The three genetic

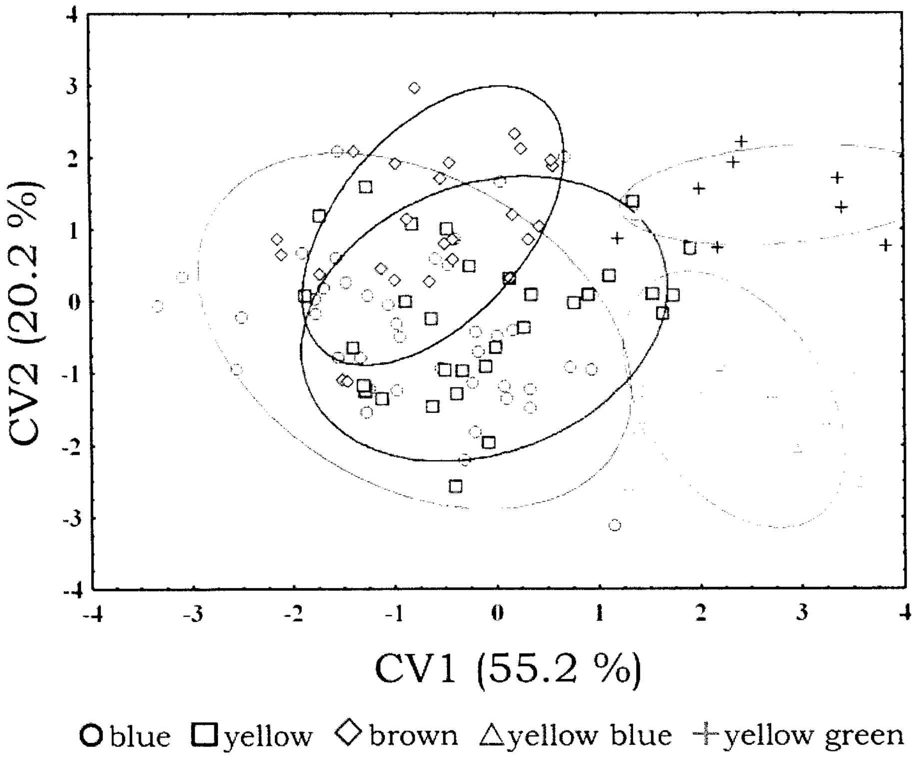

Figure 4.8 — Canonical variates ordination plot based on partial warps analysis of body shape segregating the *Telmatherina* based on colouration types defined in Figure 2. Plot shows individual *Telmatherin*a scores along CV axes and 95 % frequency ellipses surround each colouration type. CV1 and CV2 accounting for just over 75 % of the total variation among colouration types and values in brackets beside CV axes indicate the proportion of total variation described along each axis.

Telmatherina clades were not consistent with previous work describing seven different *Telmatherina* species in this system based on traditional morphometric techniques (Kottelat, 1990b; 1991; Kottelat et al., 1993). It must be recognized, however, that morphometric techniques take into consideration morphological adaptations (whether generated by phenotypic plasticity or genetically based) which may be a better representation of overall and more recent character evolution reflecting both nuclear and mitochondrial divergence among species. As such, morphological species estimates may often be elevated in comparison with those estimated using strictly mitochondrial DNA sequence data. Whether or not recent (<20 000 yrs) reproductive isolation within *Telmatherina* clades has occurred is impossible to determine using our data alone. The constructed phylogeny presented here, nevertheless represents the evolutionary history of *Telmatherina*, and it is useful in determining deep evolutionary relationships among the *Telmatherina* in Lake Matano, particularly with reference to their initial divergence.

All evidence suggests an intralacustrine origin of these three clades from a common colonizing ancestor. Lake Matano is isolated from the remaining Malili lakes by an elevation change of more than 70 m (Brooks, 1950; Kottelat, 1990a; Aarn et al., 1998). Although such geological discontinuities can form rather quickly, evidence suggests that this topographical feature is ancient (Brooks, 1950; Hamilton, 1979; Hope, 2001). The formation of Lake Matano predates that of other Malili systems based on its tectonic origins, its great depth and elevation, deviations along the active fault, and its level of endemism (Brooks, 1950; Hamilton, 1979). From this, Brooks (1950) postulated that species from Matano have served as propagules for downstream colonisations. More recent studies support this hypothesis by demonstrating a paucity of downstream species present in Matano (Kottelat, 1990b; 1991; Kottelat et al., 1993; Aarn et al., 1998; Rintelen et al., 2004). Furthermore, multiple colonization events would also be expected to be associated with much higher levels of divergence within most genera than observed

(Chapter 2). Sympatric speciation of *Telmatherina* is also suggested by the apparent lack of any spatial segregation among the three mitochondrial lineages. Fish used in this study were taken from various beaches where all clades were often caught together in a single seine. Smaller scale differences in habitat preference (e.g., rock formations or the extension of sandy reefs) were not quantified in this study and may be informative in linking different *Telmatherina* types to specific microhabitats, especially since body shape differences among the three clades suggest ecological specializations. Nevertheless, considerable debate continues as to whether or not microallopatry in the form of behavioural or habitat preference differences constitutes sympatry (see Fillatre et al., 2003; Coyne & Orr, 2004).

The intralacustrine divergence of *Telmatherina* in Matano may have resulted from fluctuating lake levels as has been purported for many of the African Rift Valley cichlids (Salzburger & Meyer, 2004). This scenario seems questionable, however, considering Matano's depth and bathymetry. Matano water levels would need to drop several hundred metres (509 m) below current levels before isolated basins could be established. Moreover, although small scale spatial variation in climate conditions and temperature are possible, sedimentary cores taken in this region suggests that conditions on Sulawesi have been relatively constant over the last two glaciations (~1.6 million years) (Hope, 2001; Visser et al., 2004). Therefore, the drastic lake level fluctuations necessary to postulate historic vicariance events in Matano due to changing climate patterns or temperatures are unlikely.

An increasing number of empirical studies document sympatric speciation in various taxa attributable to ecological adaptation among various body shapes (Skúlason et al., 1993; Schluter, 1994; Stern & Grant, 1996; Losos & Miles, 2003; Verheyen et al., 2003; Chapter 3). In most reported cases, the divergence due to microhabitat adaptation separates taxa into distinctively divergent forms (Streelman & Danley, 2003). In this study I demonstrate the occurrence of three clade-specific shapes with two extremes and an intermediate recovered from the GM analysis (Fig. 4.4 & 4.5). Clade I is characterized by a fusiform body, short rounded fins, broadened caudal peduncle and a terminal mouth making it well suited to an open-water pelagic existence where food items are chased. In particular, the slender body and broad caudal peduncle enhances swimming speed and a terminal mouth is consistent with pelagic feeding on organisms residing within the mid-water domain (Moyle & Cech, 1996; Kassam et al., 2004). The extreme compressiform shape, larger and longer fins and protruding mouth of individuals in clade II (Rotund A) make these latter better suited to more littoral/benthic areas where manoeuvrability among mangrove roots, submerged rocky outcrops, or reefs is essential (Moyle & Cech, 1996; Kassam et al., 2004). A protruding mouth is also better suited for plucking invertebrates from different substrates than is a terminally set one (Sibbing & NagelKerke, 2001; Bouton et al., 2002; Kassam et al., 2004). Clade III is also characterized by a distinct shape (Rotund B), but is intermediate between the other two clades (Fig. 4.5). Clade III is moderately compressifom, has longer fins and a shorter caudal peduncle than Torpedo shaped fish, but these same features vary in the opposite direction when compared to Rotund A (Fig. 4.5). These differences suggest that members of clade III may also be intermediate in habitat choice, perhaps using a combination of littoral/benthic and pelagic environments within the lake. Microenvironment adaptation of each clade predicted from their shapes as described above is consistent with a previous analysis revealing clade-specific diet and isotopic signatures reflecting items within these respective microenvironments (Chapter 3). Thus, body shape does match initial genetic divergence within *Telmatherina* and is likely related to microenvironment adaptation.

Assortative mating and sexual selection among colouration patterns has been proposed as a driving mechanism responsible for the creation of many sibling cichlid species in the African Great Lakes (Seehausen & van Alphen, 1999; Allender et al., 2003; Knight & Turner, 2004). It is tempting to propose that this same mechanism may be operating in Lake Matano, especially considering the high water clarity and negligible number top predators which do not hinder the development of conspicuous colouration patterns. Results from this study, however, reveal that colouration was generally not found to be clade specific, except in the case of yellow blue and yellow green (Fig. 4.6 & 4.7). In fact, the significant differences among *Telmatherina* clade colour assignments were largely attributable to the high frequencies of both yellow blue and yellow green individuals reported in clade I. The other colourtypes were more abundant than yellow blue and yellow green overall, and were present in substantial numbers in all clades (Fig. 4.6 & 4.7). The recurring pattern of similar colouration types in each of the three genetic clades may be the result of parallel evolution of colourtype within each clade, and demonstrates that colouration patterns may be important to intra-clade segregation and speciation (Rundle et al., 2000; McKinnon & Rundle, 2002). The minimum spanning haplotype network, however tends to discount this possibility, at least in as much as it is related to mtDNA lineages and thus to initial divergence. Colouration types related to intra-clade divergence ought to show clear segregation of haplotypes within clades based on colouration, a condition not observed here. The fact that many haplotypes, in all clades, have various colouration patterns (e.g., Fig. 4.7 haplotypes 1, 18, & 43) indicates a lack of haplotype specific colouration types. Whether or not colouration types are related to more recently evolved segregating patterns within each clade is beyond the resolution of our genetic markers. Certainly, the presence of yellow blue and yellow green colouration types in clade I suggest some colouration type segregation breakdown occurring in this clade and may be related to possible hybridization among colouration types in at least clade I. The lack of haplotype-specific colouration types within each clade and the possibility of more recently evolved parallel evolution of colour in the three *Telmatherina* clades demonstrate that colouration is not related to initial divergence in these fish. These data are consistent with the ‘radiation in stages’ model of adaptive radiation which describes colour as a potent initiator of divergence in more derived stages of radiations, subsequent, however, to body shape divergence (Danley & Kocher, 2001; Streelman & Danley, 2003; Kocher, 2004).

Alternatively, colouration may be linked to body shape and both traits acted together to cause initial divergence among the *Telmatherina* clades. Several lines of evidence tend to discount this hypothesis. First, if colouration and body shape were linked, either trait should be equally diagnostic in terms of assigning clade membership for any random individual within the population. This, however, is clearly not the case (Fig. 4.6). Second, adding colouration as a variable in the clade specific shape analysis should have substantially enhanced segregation among clades by shape. This, however, was not observed and adding colour produced an identical shape segregation pattern and enhanced the explanatory power of the first CV axis only marginally (Fig. 4.4B). Finally, if colour was linked to shape, colouration should predict *Telmatherina* shape (Fig. 4.8). Apart from yellow blue and yellow green showing segregation, blue, yellow and brown coloured individuals showed substantial overlap and were not significantly different in shape (Fig. 4.7). Thus the prediction of body shape from colour alone would be highly inaccurate.

An alternative to both scenarios is that the repeated colouration types observed in each genetic clade may have been inherited as a polymorphism from a common ancestor to all clades and is maintained by mechanisms unrelated to the establishment of reproductive isolation. Colouration-based mate choice experiments could provide valuable insight into the reason why various colourations types have developed in these fish.

This study provides genetic, morphological and body colour data demonstrating that the *Telmatherina* radiation in Lake Matano was most likely initiated by body shape adaptation to microenvironments and that colouration had little to with this initial process. Our results further suggest, however, that colour may be an important factor associated with more recent divergence and the development of reproductive isolation within individual clades. Interestingly, the presence of apparent intermediate colourtypes in clade I suggest hybridization events. This study represents robust evidence supporting the 'radiation in stages' model of vertebrate radiation and describes the evolutionary history of the unique Lake Matano *Telmatherina.* Continued study of speciation processes in *Telmatherina* in this relatively simple and pristine ecosystem will provide valuable insight towards refining theories of speciation and adaptive radiation in natural systems.

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5.0 — GENERAL DISCUSSION

The data presented in this thesis characterizes the evolutionary history and ecology of the fish community of Lake Matano, an ancient tropical freshwater lake with little productivity. Genetic, morphological, dietary, isotopic and colouration data were determined and integrated to demonstrate that adaptive radiation has occurred within the fish community of this lake. This research concludes that this radiation was primarily ecologically based and fit recognized patterns of ecological adaptive radiation as described by both Schluter (2000) and Danley & Kocker (2001). In highlighting the adaptive radiation of *Telmatherina*, key ecological pressures likely shaping the evolutionary history of this genus were identified. This chapter briefly summarizes the major findings of this thesis and discusses their overall implications for the Lake Matano system and for the theory of adaptive radiation in general. This chapter also identifies pathways along which future research can be directed in order to clarify our understanding of the evolution and ecology of the unique species in this ancient ecosystem.

5.1 CONTEXT

Chapter 1 provided an introduction to some of the major concepts in this thesis and gave a brief outline of how these concepts are related to the fundamental theory of evolution. Concepts such as speciation and adaptive radiation were discussed and how the characterization of these processes in natural system relied heavily on the species concept one chooses to adhere to. A brief outline of how both speciation and adaptive radiation were related to the generation of biodiversity was described. Two emerging theories of adaptive radiation were also presented. The first, which has since developed into a framework, allows the objective identification of adaptive radiation in natural systems. The second describes how many such radiations involving vertebrates typically progress through various characteristic stages over time. A brief description of the study system was given, emphasizing our lack of knowledge with regards to the evolutionary history of its organisms and in particular its native fish community, and their potential to exhibit adaptive radiation in this ancient system.

5.2 ADAPTIVE RADIATION IN LAKE MATANO

The objective of Chapter 2 was to assess the fish community of Lake Matano using the framework developed by Schluter (2000), which outlines criteria for the evaluation of adaptive radiations. Establishing whether or not focal taxa fit the criteria for adaptive radiations is the first step toward unambiguously identifying this process in natural systems. Comparative analyses of genetic and traditional morphometric data revealed a common ancestry for each genus (criterion 1) endemic to the lake and a rapid divergence within both *Telmatherina* and *Glossogobius* (criterion 4). *Telmatherina*, however, was also characterized by greater colouration patterns and a higher variation in feeding traits than the other endemic genus in this lake. A high degree of feeding trait variation in a resource limited environment was taken as indirect but compelling evidence supporting a phenotype-environment correlation (criterion 2) within *Telmatherina* traits that are most likely utilitarian in nature (criterion 3). It was shown, using Schluter's criteria (Schluter 2000), that *Telmatherina* have indeed radiated in Lake Matano, and that the radiation was either a consequence of assortative mating among colouration types or partitioning of resources in a trophically limited system. This study demonstrated the usefulness of Schluter's framework, not only in identifying putative cases of adaptive radiations, but also in focusing future research directions.

The application of Schluter's framework in a comparative manner among the different genera in Lake Matano also drew attention to differences in their abilities to undergo adaptive radiation. For example, *Oryzias* was found to have limited morphological and genetic variation to the point where the entire genus is likely represented by a single cosmopolitan species (Chapter 2). Although microhabitat preference and general ecology of *Oryzias* in this system remains poorly characterized, this genus was collected in the same environments as was *Telmatherina*. Such limited genetic and phenotypic variation within *Oryzias* compared to that within *Telmatherina* points to very different genetic and/or ecological constraints imposed on these two co-occurring genera (Kassen et al., 2004). Understanding why *Telmatherina* have radiated in this system while *Oryzias* have not, may have important implications in identifying key intrinsic factors of taxanomic groups promoting rapid speciation (i.e., key innovations ; Liem, 1974; Hunter, 1998). Thus, the comparative evaluation of Schluter's (2000) framework also demonstrated natural controls or constraints within the Matano fish community and further emphasizes the framework's effectiveness in clarifying important principles related to adaptive radiation. Future studies in Lake Matano should investigate potential ecological and genetic constraints on the other members of the fish community.

In order to solidify support for the intralacustrine radiation of *Telmatherina* and the isolation of Matano endemic species, a separate study outside the general topic of this thesis was initiated to investigate the possibility of gene flow among lakes (Appendix 1). In this work, populations of *Caridina lanceolata*, a taxon known for its great dispersal ability (Woolshot et al., 1999; Baker et al., 2004) were sampled both above and below the 72 m elevation change separating Matano from downstream lakes. The within-lake genetic and morphological variation in these populations was small, but that expressed among lakes was sufficient to consider different populations incipient cryptic species. The conclusions derived in this study were consistent with more recent investigations among the highly dispersing gastropods in the Malili Lakes (Rintelen et al., 2004; Rintelen & Glaubrecht, 2005). These studies revealed that while many Matano species are shared with the lower Malili Lakes, few lower lake species were present in Matano. These findings together with the results obtained form the *Caridina* study provide strong evidence supporting the isolation of Matano species for a long period of time (Appendix 1).

5.3 ECOLOGICAL SPECIATION

Although the radiation of *Telmatherina* in Lake Matano had been identified, its causal factors remained ambiguous. Results from Chapter 2 suggested that feeding trait selection (perhaps resulting from limited resources) was the most likely mechanism by which the radiation within *Telmatherina* was initiated. Such a notion, however, was derived predominantly from indirect evidence of high feeding trait variation in a trophically limited environment and from reports of such scenarios in other systems (Skúlason et al., 1993; Rüber et al., 1999; Sibbing & NagelKerke, 2001). The purpose of Chapter 3 was to determine whether or not feeding trait variation represented functional features adapted to environmental fitness peaks (Schluter's 2000 criteria 2 and 3) and to further characterize the radiation of *Telmatherina* in this system. More fine-scale phylogenetic analyses revealed that all *Telmatherina* in Lake Matano belonged to one of three genetically distinct clades. The shape of specific head features such as the skull and the pharyngeal jaw apparatus showed clear distinction among the three clades, wherein distinctive features corresponded to clade-specific diets determined from both gut content and stable isotopes analyses. These data separated the three *Telmatherina* clades into a littoral specialist, a pelagic specialist and a generalist that makes use of both littoral and pelagic resources. The generalist was also found to be the most genetically divergent and basal to the two specialist species, suggesting that the generalist was the most recent common ancestor of both specialist species and that the radiation of *Telmatherina* is incomplete. Results from this chapter, along with those of Chapter 2 provided further support of the adaptive radiation of *Telmatherina* in Lake Matano and provided strong evidence for its ecological root. The more highly resolved phylogeny, with its associated feeding trait variation matched to diet, provided three independent datasets suggesting that the adaptive radiation was initiated by the partitioning of limited resources in this system.

Geometric morphometrics used in this study (Chapter 3 & 4) proved to be a much better tool to demarcate morphological differences among *Telmatherina* than were traditional morphometric techniques (Kottelat, 1990a ; 1990b; 1991; Kottelat et al., 1993). GM techniques, especially when combined with genetic information as described in this and other studies (Chapter 3 & 4, Rüber & Adams, 2001; Barluenga et al., 2004; Albertson et al., 2005; Harmon et al., 2005) can clarify important morphological changes describing adaptive divergence of various species within adaptive landscapes (Schluter 2000; Adams et al. 2004). The use of GM techniques coupled with the derived phylogenies in this study, demonstrated key divergent ecological features allowing the exploitation of different resources in Lake Matano by the different *Telmatherina* clades. Studies on African cichlids show how many of these same features also vary substantially in those fish as well (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996; Bouton et al., 2002). Future studies could repeat the GM evaluation of pharyngeal jaws and skull elements across many different fish lineages in efforts to determine if divergence patterns are associated with particular arrangements of these (or other) features within the piscean body plan. Such studies would make important contributions toward clarifying key morphological features associated with increased speciation in fish (and perhaps other taxa).

Although this study revealed associations among clade-specific morphologies, both diet and trophic status, it did not demonstrate clear selective advantages among the different feeding strategies associated with each clade. Such selective advantages are essential to determine differential selection acting on feeding traits based on the different resources available within the identified microhabitats (littoral and/or pelagic) (Schluter, 2000; Rundle & Nosil, 2005). For example, do littoral feeding traits offer littoral specialists an advantage in littoral areas relative to pelagic specialists in terms of resource acquisition and use? Although the presence of such selective advantages among the three clades seems likely, especially between the littoral and pelagic specialists, this was not specifically tested. Future research needs to establish whether such selective advantages exist among different *Telmatherina* clades and to determine the effectiveness of the generalist relative to the two specialists. Ideally, such investigations would entail rearing experiments under standard food treatment conditions. Such data would conclusively determine if differential selection acting on diverging feeding traits within the different microhabitats was ultimately responsible for initiating adaptive radiation in these fish.

5.4 *TELMATHERINA* RADIATION IN STAGES

The previous chapters (2 and 3) identified the adaptive radiation of *Telmatherina* in Lake Matano and demonstrated its ecological base. The next logical step was to compare this adaptive radiation with other identified cases in other systems. Rather than compare and contrast radiations in singular form, comparing the radiation in *Telmatherina* to a composite of many radiations was performed to better describe both its commonalities and its unique aspects. The 'radiation in stages' model of vertebrate radiations comes from a synthesis of the most notable cases of adaptive radiations demonstrated (Streelman & Danley, 2003; Kocher, 2004). Consequently, comparing the *Telmatherina* radiation to this model provides an evaluation of the model using an independent example of adaptive radiation, and highlights the unique and important aspects of this particular radiation relative to other more familiar ones.

The 'radiation in stages' model is characterized by two main principles, first, radiations occur in stages, the sequence of which (microhabitat, trophic status & communication) is often conserved (Streelman & Danley, 2003; Kocher, 2004), and second, different selective pressures, although active throughout radiations, exert their principal influence at different stages of the radiation (Danley & Kocher, 2001; Kocher, 2004). Previous work demonstrated that although the radiation of *Telmatherina* was ecologically based, bright and diverse colouration patterns were also observed in these fish, a fact that had thus far received little attention in this thesis. The purpose of Chapter 4 was two fold: First, it compared the *Telmatherina* radiation in Lake Matano to the 'radiation in stages' model, and second it demonstrated the role of colour in this adaptive radiation. This work revealed that although the various colouration patterns found within the *Telmatherina* genus suggests assortative mating among colourtypes driven by sexual selection, colouration within these fish did not match their derived genetic phylogenies and was thus not likely a significant contributor to initial divergence. Body shape adaptation to microhabitats, however, did match genetic phylogenies and did appear to be linked to initial divergence which was supported by results obtained in Chapter 3. Results suggested, however, that colouration may be important to more recent (< 20 000 yr) divergence within the defined mtDNA clades. Thus, the *Telmatherina* radiation did fit the 'radiation in stages' model quite well and supports the generality of this pattern in vertebrate adaptive radiations.

The argument can be raised that the divergence of a lineage into three extant forms does not necessarily constitute a radiation event. This is especially true considering that more famous examples of this process occurring in other systems lead to a much larger number of closely related species, such as in the African Great Lake cichlids (Salzburger et al., 2005) and the Galapagos finches (Harrel et al., 2005). Recent studies offer possible reasons why certain radiations are more speciose than others (Streelman & Danley, 2001; Lenski et al., 2003; Kassen et al., 2004; Kocher, 2004). Radiations resulting in limited numbers of species are related to intrinsic (genetic and morphological potential) features of the ancestral taxon and/or to extrinsic environmental variables (e.g., resource limitation, niche availability, predation pressures) limiting the success of varied forms (Lenski et al., 2003; Kassen et al., 2004).

The data presented here tend to suggests that the divergence of *Telmatherina* in Matano is constrained more by extrinsic than by intrinsic features. *Telmatherina* in Lake Matano show similar levels of genetic divergence as reported within many cichlid fish lineages and genera of Lake Tanganyika (Rüber et al., 1999; Baric et al., 2003). *Telmatherina* also share many of the same 'key innovations' in terms of morphological features as the generalized cichlid forms both in body plan (Danley & Kocher, 2001; Kassam et al., 2004) and feeding structures (Liem, 1974; Barel et al., 1976; Galis & Drucker, 1996). Thus, *Telmatherina* possess sufficient genetic and morphological variation necessary for explosive divergence to occur.

The environment in which the *Telmatherina* radiation has occurred, on the other hand, may be the source of substantial ecological constraining factors. Many studies, including those in this thesis, underscore the limited productivity of this system (Haffner et al., 2001; Sabo, 2006). Factors such as very clear water, low primary production dominated by cyanophytes, a derth of exclusively piscivorus top predators and exogenous material constituting major portions of *Telmatherina* diets (Chapter 3) support the oligotrophic nature of Lake Matano. The lack of sufficient autochthonous production in this system may indicate that most trophic positions in the lake have been filled, leaving little ecological opportunity for the development of novel ecological niches among the fish fauna (Schluter, 2000; Rundle and Nosil, 2005). Evidence also suggests that the climate conditions and lake levels have been relatively consistent since the last two glaciations (*c.* 1.6 mya) (Hope, 2001; Visser et al., 2004). The aged and stable nature of this system compared to the dramatic fluctuations in lake levels and climate patterns reported in East African lakes may have limited the number of oscillations between population bottlenecks and expansions purported to explain the explosive speciation in many cichlid lineages (Kornfiled & Smith, 2000; Fryer, 2004).

Another potential constraining factor may be stress from the natural leaching of ultrabasic rocks which constitutes the geological foundations of Lake Matano and its surroundings (Hamilton, 1979). Recent investigations have demonstrated that high metal concentrations dissolved in Matano water, such as cadmium, chromium species, selenium and copper, are substantial constraining factors limiting production of both phytoplankton and zooplankton in this system (Sabo et al., 2006; Bramburger et al., submitted). These same processes may influence, both directly in terms of metal toxicity and indirectly in terms of food-web limitation, the potential for further radiation of *Telmatherina* in Lake Matano. Further investigations of the Matano fish community ought to investigate such metal concentrations and their toxicokinetics. If the fish endemic to Matano are all characterized by 1) high concentrations of such metals and metal complexes in their tissues, and 2) have a high tolerance and/or elimination rates for such metals, then these factors may play important roles in fish species survivability and evolution. Perhaps many of these described constraints along with others not mentioned are acting synergistically to limit the potential for explosive adaptation and speciation in this system.

Alternatively, the adaptive radiation identified among *Telmatherina* in Matano may not be constrained at all and there may be a great deal of diversity in this system, but it has been presented here in a conservative manner. This thesis reports only three genetically identified clades of *Telmatherina* in Matano using mitochondrial DNA phylogenies. These three clades are separated by species level distances (Avise & Walker, 1999) and can be considered three distinct species. Such a classification, however, follows a more phylogenetic species description (Avise & Wollenberg, 1997) than a biological one (Mayr, 1963). Whether or not reproductive isolation both among and within the clades exists was not specifically tested and remains an area in need of clarification. Certainly, the data presented in Chapters 3 and 4 suggest that each clade is indeed distinct and exhibits reproductive isolation. Distinct body shapes and trophic specialization among clades, along with discrete stable isotope and diet data suggests strong segregation among clades. Hybridization among clades would quickly erode established differences in such patterns and no significant differences among clades in these features would be expected (Klingenberg et al., 2003; Zelditch et al., 2004). The colouration types examined in Chapter 4, however, are fascinating as most strict coloration types (i.e., blue, yellow, & brown) occurred in all clades, and mostly in equal proportions. The repeated colouration types occurring in each clade suggests the parallel evolution of such colouration within each clade and that colouration is indeed important to within-clade segregation. Reproductive isolation and assortative mating may be occurring by colour type in each clade. Further evidence of this comes from the apparent breakdown of such isolation in clade I, wherein intermediate colouration types are apparent. Future studies on colouration patterns and whether or not these fish exhibit reproductive isolation along colouration lines in Lake Matano is would be a promising approach to further characterizing the evolutionary history of *Telmatherina*. These data emphasize the importance of species concepts, as was described in the first chapter, and clearly demonstrate that species concept and definitions can have major implications in characterizing evolutionary histories in any system. This thesis has demonstrated the need to clarify and standardize evolutionary significant units in evolutionary studies (Moritz, 1994; Carvalho & Hauser, 1999; Hey, 2001; Barraclough & Nee, 2001; Agapow et al., 2004).

5.5 FINAL NOTE

Previous research on the fish of Lake Matano has done little to characterize the general history or ecology of these unique fishes, and thus not much was known in terms of their resource needs, habitat preferences and behaviours. Yet, such important ecological parameters have clearly contributed to their survival and evolution. Many such important parameters are the result of substantial environmental selective pressures that have formed these species and species groups. Understanding the phylogenetic relationships among the fish populations and species within a lake and supplementing this information with ecological and physical observation can serve to identify many important selective pressures that isolate populations and species over time and provide key evidence of speciation and radiations in natural systems. The molecular, morphological and ecological data presented in this thesis demonstrate the uniqueness of this aquatic ecosystem providing motivation for its conservation. This work also provides a better understanding of a key process generating biodiversity. A greater understanding of Lake Matano and its unique limnology, hydrology and faunal ecology from studies such as this will help to maintain its responsible usage and establish baseline studies to initiate its conservation as a world-wide heritage.

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APPENDIX 1.0 — EVIDENCE OF SMALL SCALE VICARIANCE IN *CARIDINA LANCEOLATA* (DECAPODA: ATYIDAE) FROM THE MALILI LAKES, SULAWESI*.

A.2 INTRODUCTION

Speciation is dependent on variation among individuals and divergence between populations (Darwin 1859; Futuyma 1986). While divergence may result from selection on functional traits such as behaviour or physiology, the effects of selection are most apparent in morphological differences that are consequently used in species identification. Alternatively, vicariance, or limitations to dispersal, may produce allopatric populations that diverge through genetic drift but where balancing selection may limit changes in morphology (Slatkin 1985; Futuyma 1986). This leads to the formation of cryptic species and indicates that drift plays an important role in the speciation process.

Cryptic species often confound traditional species definitions in that, if they differ genetically but are morphologically very similar, do they represent different species (Mayr 1963 ; Coyne & Orr 2004)? This is dependent on the choice of species concept, which can be viewed from phylogenetic or morphological perspectives, among many others (Hey 2001; Schluter 2001; Agapow et al. 2004; Coyne & Orr 2004). Nevertheless, clarifying mechanisms building variation among populations is critical to understanding processes

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* Roy D, Kelly DW, Fransen CHJM, Heath DD, Haffner GD. Evidence of small scale vicariance in *Caridina lanceolata* (Decapoda: Atyidae) from the Malili Lakes, Sulawesi (*Manuscript submitted to Evolutionary Ecology Research. Aug 2005*).

leading to species formation, but is often clouded by an inability to discriminate effects of drift from those of selection (Moritz and Faith 1998; Agapow et al. 2004). Studies of allopatric cryptic sibling species may address this problem and allow an assessment of dispersal limitations and the time span over which neutral processes shape divergence free of the confounding effects of selection (Knowlton et al. 1993; Knowlton & Weigt 1998; Cristescu et al. 2003; Page et al. 2005).

Sulawesi Island, Indonesia, has been isolated from other land masses since its formation 12-14 million years ago (mya) and exhibits elevated patterns of species diversity and endemism (Brooks 1950; Whitten et al. 2000; Evans et al. 2003). Such patterns may be explained by its isolation, unusual geological history and variety of unique ecosystems (Brooks 1950; Myers et al. 2000; Whitten et al. 2000). The Malili lakes are a series of interconnected tropical lakes centrally located on Sulawesi with Lake Matano constituting the hydrological head (Brooks 1950; Kottelat 1990a, b, 1991; Haffner et al. 2001). Although the age of these lakes is uncertain, Brooks (1950) estimated an origin 2-4 mya on the basis of local geology and Matano's substantial depth (maximum depth = 598 m). In addition, Hamilton (1979) estimated a similar age (2-4 my old) based on displacement along both the Matano and the Palu Koro faults purported to have been the formative agents of these aquatic systems. Investigations of sedimentary cores taken from various areas around Sulawesi and in parts of Matano proper, suggest relatively constant water levels since its formation (Haffner et al. 2001; Hope 2001; Visser et al. 2004). Lake Matano, located along the active Matano fault and at the head of the Malili Lakes, has likely provided source propagules for colonization of downstream lakes (Brooks 1950).

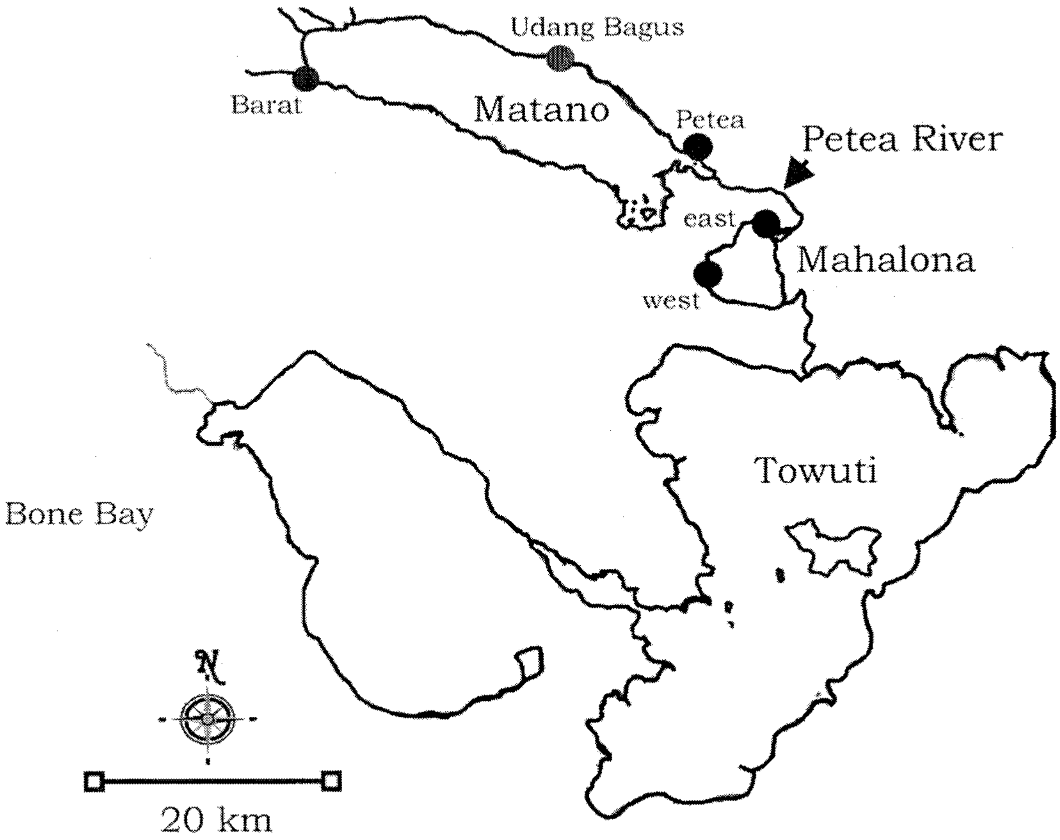

Figure A.1. Map of the Malili Lakes located in the south central portion of Sulawesi Island, Indonesia. Dark circles indicate the geographic location of each beach from where *Caridina lanceolata* specimens were collected. Populations in Matano are separated from those in Mahalona by the Petea River which flows down a 72 m elevation change between the lakes (see Brooks 1950) (B = Barat, UB = Udang Bagus, P = Petea, Me = Mahalona East and MW = Mahalona West).

However, it remains effectively isolated by a 72m elevation change, consisting of cascading rapids within the connecting Petea River (Brooks 1950; Kottelat 1990a; see Fig. A.1). This barrier has likely limited the dispersal of aquatic species between Matano and the other Malili Lakes. The Malili Lakes thus provides an opportunity to assess the influence of physical barriers on population divergence and evolutionary processes in tropical freshwater systems.

Using molecular and morphological data, I assess small scale phylogeographic structure of the endemic crustacean, *Caridina lanceolata* (Decapoda: Atyidae) by comparing populations above and below the Petea River. Given that other Atyid species exhibit extensive population structure among different river drainages separated by inhospitable environments (Woolschot et al. 1999; Hurwood & Hughes 2001; Baker et al. 2004), I expect that populations will be divergent across the Petea River.

A.3 MATERIALS AND METHODS

Approximately 53 *C. lanceolata* were collected by both seine and dip nets from five beaches in the Malili Lakes. Three beaches, located in Matano, consisted of Barat and Udang Bagus separated by 19.3 km, and Petea separated from Udang Bagus and Barat by 12.1 and 30.3 km, respectively. Two beaches were selected below the Petea River in Lake Mahalona and included Mahalona East located at the Petea River inflow and Mahalona West, located 7.2 km southwest of Mahalona East (Fig. A.1). Mahalona East is located 8.5 km, 20.6 km and 36.9 km away from Petea, Udang Bagus and Barat, respectively, while Mahalona West is located 9.6 km, 19.8 km and 34 km, respectively from these same beaches. Sub-samples from each beach were registered and deposited at the National Museum of Natural History Naturalis, Leiden, The Netherlands, under the specimen voucher numbers RMNH D 51165-51178.

DNA was extracted from 10 individuals per beach, using the WIZARD® DNA kit following the standard protocol (Promega, Madison, WI, USA). Universal primers (Folmer et al. 1994) amplified COI only in individuals from Mahalona but not those from any of the Matano beaches. Consequently, Mahalona sequences were aligned with other Atyid sequences from Genbank, and new primers were designed within highly conserved regions. Newly developed primers (CLW-F 5'– TYCGAGCAGAACTAGGHCAAC –3', and CLW-R 5'– CGATCTGTTARTAGTATAGTA –3') were used with 1 ul raw genomic DNA in standard PCR reactions, described elsewhere (Chapter 2) amplifying a 480 bp region of the COI mitochondrial genome. Reactions were run for 5 cycles with a denaturation of $96^{O}C$ for 1min, primer annealing at $45^{O}C$ for 1 min, and an extension at $72^{O}C$ for 1.5 min. This was followed by 30 cycles with an annealing temperature of $50^{O}C$ and a final termination by extension for 5 min at $72^{O}C$. PCR products were then sequenced as in Chapter 2 and aligned using the clustal W algorithm with a gap open penalty of 10, gap extension of 5 and a DNA transition weight of 0.5 (Omiga, 1.2, Oxford Molecular Ltd, Oxford, UK). Aligned sequences were entered into PAUP version 4.0 (Swofford 1998) for phylogenetic analyses. Both transition and transversion substitutions were used in the analyses since transition saturation tests were negative (Kocher & Carleton 1997). Insertions and deletions (indels) were treated as complete deletions, and pair-wise distances were calculated using the GTR + G substitution model determined from an Akaike information criterion (AIC) survey of 56 substitution models implemented by MODELTEST in PAUP (Posada and Crandall 1998). Patterns of phylogeographic divergence in the distance matrix were assessed using Neighbour-Joining with a gamma parameter of 0.4149, and nodal support estimated using 10 000 bootstrap permutations (Kocher & Carleton 1997). An additional test of phylogenetic structure was also conducted by Maximum Parsimony (MP) heuristic searches with Close Neighbour Interchange of 1 and a Random Addition Trees of 10 replicates supported by 1000 permutations. Sequences of *Paratya australiensis* Kemp 1917, an Atyid species from the Indo-Pacific region, were obtained from GenBank for use as an outgroup (Baker at al. 2004).

Patterns of population structure both among and within lakes were assessed using an analysis of molecular variance using populations within lakes as a nested factor (AMOVA, Excoffier et al. 1992) and pair-wise F_{ST} based on derived distances, implemented in ARLEQUIN version 3.0 (Excoffier et al. 2005). Global significance was determined at the $\alpha = 0.05$ level using 10 000 permutations and pair-wise differences were evaluated with an α value corrected for multiple comparisons ($\alpha = 0.005$). To determine if patterns of genetic variation were attributable to neutral mutations (drift) or exhibited signs of directional selection, population expansion, or recent bottlenecks at the COI locus, Tajima's (1989) *D* statistic was calculated across all populations.

Morphometric measurements were taken (following Woltereck 1937a,b) from remaining individuals collected from each population to determine morphological population differences. Morphological differences were analysed using a multivariate analysis of covariance (MANCOVA) across all traits using pre-orbital carapace length as a covariate and populations within lakes as a nested factor. The multivariate test was followed by a series of univariate ANCOVAs (corrected for type I errors using sequential Bonferroni adjustment) using populations within lakes as a nested factor to determine if populations among and within lakes differed in specific traits. *Post-hoc* comparisons (also corrected using sequential Bonferroni) were performed to determine which populations within lakes were different (STATISTICA v. 6.0 Statsoft Inc. 2001, Tulsa, OK).

A.4 RESULTS

COI mtDNA sequences generated in this study are available on GenBank under the accession numbers DQ155568-DQ155590. Of the 480bp fragment of the COI gene, 103 sites were polymorphic, 91 of which were phylogenetically informative. There were no indels and I found an average transition: transversion ratio of 3.376. Similar topologies were recovered using both distance and parsimony based analyses, and both demonstrated substantial distances to the outgroup. As such, distances to the outgroup were truncated for display purposes (Fig. A.2). *C. lanceolata* sampled from the Malili lakes were strongly spatially structured, revealing two distinct clades with strong bootstrap support (Fig. A.2). The first, designated 'Matano', encompassed 20 different haplotypes of individuals from all three Matano beaches, but without any structure with respect to beach (Fig. A.2). Two Matano haplotypes were present at both Barat and Udang Bagus, while another was present at all three sampled Matano beaches. The second clade, designated 'Mahalona' consisted of 14 haplotypes, two of which were shared among Mahalona beaches (Fig. A.2).

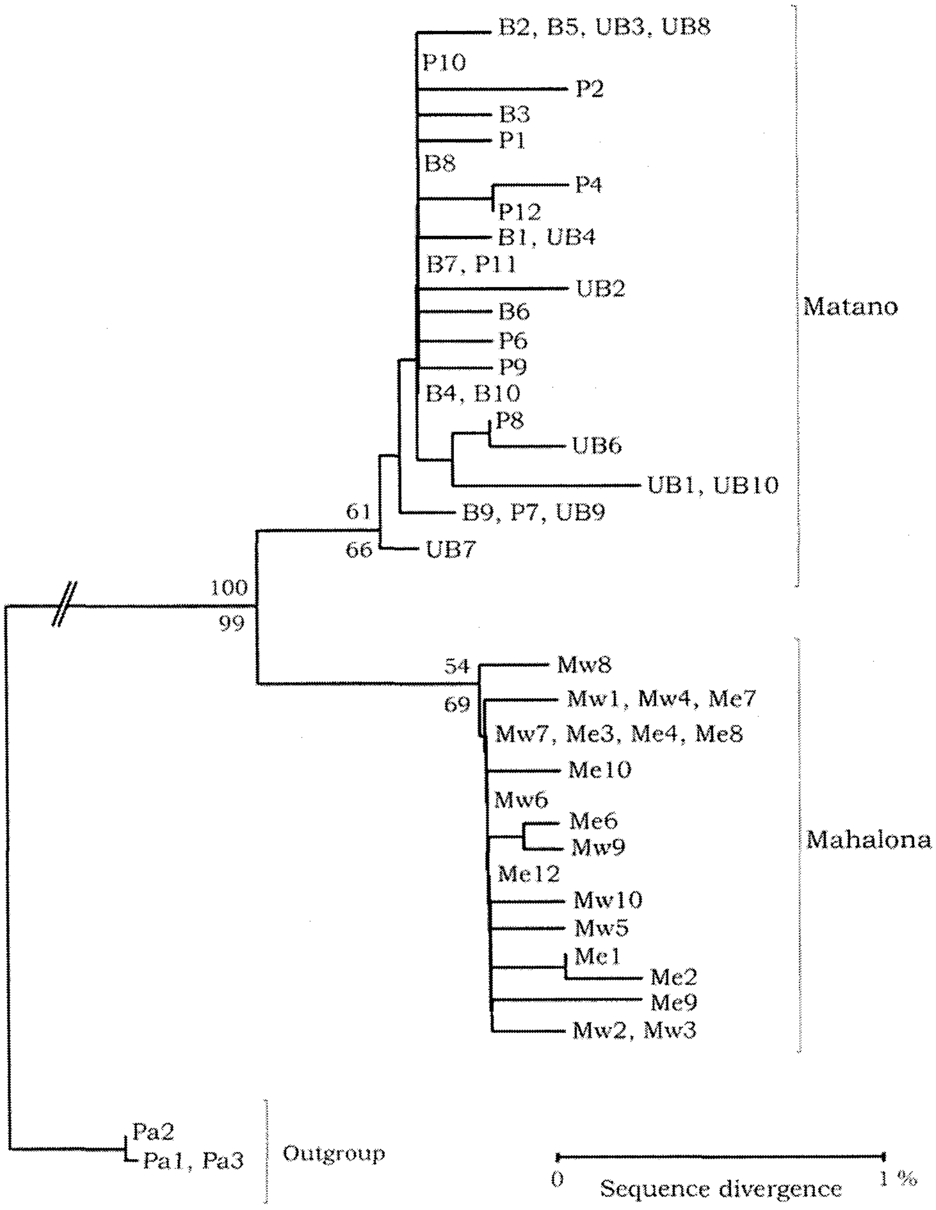

Figure A.2. Phylogenetic relationship determined among five populations of *Caridina lanceolata* (Decapoda: Atyidae) endemic to the Malili Lakes, Sulawesi Island, Indonesia. Bootstrap 50 % majority rule consensus tree constructed by Neighbour-Joining using GTR + G distance model with a gamma distribution for base substitution ($\gamma = 0.4149$). Phylogeny recovered using Maximum Parsimony heuristic searches with Close Neighbour Interchange of 1 and a Random Addition Trees of 10 replicates recovered identical general topology. Bootstrap support for distances (from 10 000 permutation) and MP (1000 permutations) indicated above and below the nodes, respectively. Each specimen is identified in the phylogeny by an alphanumeric code using beach designation (see Fig. A.1) and specimen number. Lake of origin is given to the right of the tree.

The sharing of haplotypes among various beaches within individual lakes indicates no impediment to within-lake dispersal (Fig. A.1 & A.2). Matano and Mahalona clades however did not share any haplotypes indicating a lack of gene flow between the two lakes.

Limited gene flow between lakes was also indicated by similar mean sequence divergence within the Matano and Mahalona clades (0.403 % 0.0013 S.E. and 0.31 % 0.0013 S.E., respectively) whereas inter-clade divergence was high (1.5 % 0.0048 S.E.). AMOVA revealed that most of the genetic variation among sampled beaches was attributable to differences between lakes (Table A.1), with pair-wise population comparisons showing that the Mahalona populations were significantly different from all those in Matano (Table A.2). No population structure, however, was observed either within the Matano or Mahalona populations (Table A.2). Tajima's D was not significant across populations suggesting neutral mutations rather than selection was responsible for the observed variation among populations at the COI locus ($D = -0.1131$, $P > 0.05$).

MANCOVA revealed significant differences both between lakes and among populations within lakes in morphometric measurements (Wilk's $\Lambda = 0.098$, $F = 14.25_{18,28}$, $P < 0.0001$; Wilk's $\Lambda = 0.049$, $F = 2.73_{54,84.25}$, $P < 0.0001$, respectively). Univariate ANCOVAs between lakes demonstrated that six traits related to the rostrum and the chelae differed between individuals in each lake (Table A.3). Individuals in Mahalona were characterized by a longer rostrum with fewer ventral but more dorsal teeth. Matano individuals also had significantly fewer rostral postorbital dorsal teeth and shorter first and

Table A.1 — Results of an analysis of molecular variance (Excoffier et al. 1992) performed on five populations of *Caridina lanceolata* collected from the Malili Lakes, Sulawesi Island, Indonesia. Analysis performed using COI sequence data and calculated pairwise differences using the GTR+G ($\gamma = 0.4149$) base pair substitution model, and algorithm permutated 1023 times. *P* values estimated from permutations provided as footnote.

Source of variation	Degrees of freedom	Sum of squares	Variance component	Percentage of variation
Among lakes	1	60.817	2.490ct	73.19
Among populations within lakes	3	3.183	0.017sc	0.49
Within populations	45	40.300	0.896st	26.32

F_{ST} : *P*(rand. values < obs. values) = 0.0000; *F*sc : *P*(rand. values < obs. values)=0.1740; *F*ct : *P*(rand. values < obs. values) = 0.0000;

Table A.2 — Pairwise F_{ST} values and associated P-values (in brackets) determined among five populations of *Caridina lanceolata* (Decapoda: Atyidae) sampled from the Malili Lakes. P-values generated from 1023 permutations of AMOVA algorithm performed in ARLEQUIN version 3.0 (Excoffier 2005). Bold entries indicate significant genetic differentiation among compared populations with α-value corrected to 0.005 for multiple comparisons.

	Barat		Udang Bagus		Petea		Mahalona east	
Barat	*	*						
Udang Bagus	0.007	(0.473)	*	*				
Petea	0.016	(0.490)	0.030	(0.265)	*	*		
Mahalona east	**0.788**	**(0.0001)**	**0.698**	**(0.0001)**	**0.741**	**(0.0001)**	*	*
Mahalona west	**0.791**	**(0.0001)**	**0.699**	**(0.0001)**	**0.744**	**(0.0001)**	0.011	(0.376)

Table A.3 —Results of univariate nested ANCOVAs performed on morphological features measured (as per Woltereck 1937a, b) in 5 populations of *Caridina lanceolata* (Decapoda: Atyidae) collected from the Malili Lakes. Significance for univariate tests were assessed by sequential Bonferroni correction (starting at $\alpha = 0.0027$ between lakes and *post hoc* tests adjusted to $\alpha = 0.00027$ among population comparisons). Bold entries and superscripts indicate significant differences both among lakes and population for particular traits (UB = Udang Bagus, B = Barat, P = Petea, Mw = Mahalona west, and Me = Mahalona east).

Trait		UB	B	P	Mw	Me
Rostrum						
	length	**5.43±2.57^{A}**	**5.02±1.38^{A}**	**5.52±1.49^{A}**	**7.03±3.16^{B}**	**7.43±1.52^{B}**
	# dorsal teeth	**14.00±3.43^{A}**	**14.22±1.33^{A}**	**15.50±3.92^{A}**	**11.90±2.20^{B}**	**12.82±2.50^{B}**
	# postorbital dorsal teeth	**2.00±0.63^{A}**	**2.44±1.05^{A}**	**2.40±1.03^{A}**	**1.70±0.97^{B}**	**1.55±1.04^{B}**
	dorsal subdistal	1.00±0.84^{A}	1.89±0.67^{A}	1.60±1.03^{A}	1.70±1.35^{A}	2.18±0.81^{A}
	# ventral teeth	**6.00±1.35^{A}**	**5.44±1.76^{A}**	**6.80±3.24^{A}**	**8.10±2.57^{B}**	**8.18±2.34^{B}**
Diaeresis						
	left	8.00±1.05^{A}	8.25±2.18^{A}	8.20±1.26^{A}	6.80±3.24^{A}	8.09±2.09^{A}
	right	8.00±1.75^{A}	7.13±1.20^{A}	8.10±1.48^{A}	6.80±1.58^{A}	8.00±1.55^{A}
Telson						
	# dorsal spines left	4.00±1.03^{A}	3.44±1.05^{A}	3.00±0.00^{A}	3.50±1.41^{A}	3.82±0.81^{A}
	# dorsal spines right	4.00±1.05^{A}	3.22±0.88^{A}	3.20±0.84^{A}	3.60±1.03^{A}	3.82±1.21^{A}
6th Abdominal segment		2.95±1.13^{A}	2.82±0.59^{A}	2.95±0.50^{A}	2.96±0.87^{A}	3.33±0.61^{A}

P1 carpus						
	length	0.77 ± 0.26^{A}	0.73 ± 0.18^{A}	0.83 ± 0.17^{A}A	0.73 ± 0.23^{A}	0.86 ± 0.23^{A}
	height	0.37 ± 0.05^{A}	0.20 ± 0.09^{A}	0.19 ± 0.06^{A}	0.21 ± 0.04^{A}	0.25 ± 0.06^{A}
P1 chela						
	Length	**0.82 ± 0.25^{A}**	**0.77 ± 0.12^{A}**	**0.80 ± 0.15^{A}**	**0.84 ± 0.15^{B}**	**0.94 ± 0.18^{B}**
	Height	0.37 ± 0.09^{A}	0.33 ± 0.07^{A}	0.29 ± 0.08^{A}	0.33 ± 0.05^{A}	0.39 ± 0.10^{A}
P2 carpus						
	length	1.43 ± 0.42^{A}	1.40 ± 0.23^{A}	1.45 ± 0.35^{A}	1.44 ± 0.54^{A}	1.60 ± 0.35^{A}
	height	0.16 ± 0.04^{A}	0.15 ± 0.03^{A}	0.15 ± 0.02^{A}	0.15 ± 0.03^{A}	0.18 ± 0.04^{A}
P2 chela						
	length	**0.86 ± 0.26^{A}**	**0.83 ± 0.17^{A}**	**0.89 ± 0.18^{A}**	**0.91 ± 0.25^{B}**	**1.02 ± 0.22^{B}**
	height	0.28 ± 0.06^{A}	0.26 ± 0.04^{A}	0.25 ± 0.06^{A}	0.29 ± 0.04^{A}	0.31 ± 0.07^{A}

second chelae lengths than individuals in Mahalona (Table A.3). Once univariate tests were corrected for sample sizes (N = 10/population) and for multiple comparisons, significant differences among populations were only observed in those traits that differed between lakes (Table A.3). Within these traits, Mahalona populations were not significantly different from each other, but were significantly different from all Matano populations.

A.5 DISCUSSION

Assessing biological diversity often depends on accurate identification of species which are usually recognised by clear morphological criteria (Barton 1988). A lack of obvious morphological differences among groups, however, may overlook genetic population structure and possibly speciation due to vicariance barriers to dispersal (Slatkin 1985; Palumbi et al. 1997; Collin 2005). The data presented here provide an example of this phenomenon, whereby dispersal appears restricted across the Petea River, leading to divergence between populations of *C. lanceolata* in Lakes Matano and Mahalona. Lake Matano populations showed substantial dispersal ability over at least 30 km as evidenced by haplotype sharing between populations, and intermixing of individuals from both populations within the Matano clade. This is consistent with the lack of morphological differentiation between the three Matano and the two Mahalona populations, and the relatively low level of genetic divergence within both clades (Table A.1 & A.2; Table A.3; Fig. A.2). Low genetic divergences observed within each lake, and the small F_{ST} among both Matano and Mahalona populations are consistent with studies describing high dispersal and gene flow among other Atyids, including *Caridina* species, within contiguous systems such as river catchments and drainage basins (Woolschot et al. 1999; Hurwood & Hughes 2001; Baker et al. 2004; Page et al. 2005).

Matano and Mahalona populations, however, are separated by physical distances less than that separating the two most distant Matano populations (Fig. A.1). Thus, physical distances alone are not a significant hindrance to dispersal of this species, but rather the Petea River is likely an unsuitable environment, preventing dispersal. Whether the river itself or some specific feature within the river (i.e., cascading rapids) is responsible for the discontinuity in genetic structure is difficult to determine. However, *C. lanceolata* specimens recovered from both the inflow and outflow of the Petea River suggests that the river alone is not the dispersal barrier (Fig. A.1 & A.2). This is consistent with studies describing dispersal of other Atyid and *Caridina* species across a variety of habitats ranging from lakes and riverine systems to estuaries, and small terrestrial divides (Woolschot et al. 1999; Hurwood & Hughes 2001; Baker et al. 2004; Page et al. 2005). Those studies also demonstrate significant population structure across substantial barriers, such as divided watersheds and large marine expanses (Woolschot et al. 1999; Hurwood & Hughes 2001; Baker et al. 2004). Yet, the cascading rapids and fast flowing water of the Petea River are likely an obstruction to *C. lanceolata* dispersal in the Malili Lakes system. Kottelat (1990a, b; 1991) described a similar pattern in the fish species from Lake Matano which are endemic and restricted to it. More recently, Rintelen et al. (2004) demonstrated a segregation of gastropod genetic clades wherein the Matano clade was present in all lakes but lower lake clades were absent from Matano. Together, these data suggest that the

Petea River is a substantial barrier to dispersal for *C. lanceolata* and likely for many other species in the Malili Lakes.

Although the formation of such geological discontinuities can occur rather quickly, the high levels of divergence observed in *C. lanceolata* between the two lakes suggests that this barrier is not recent. Invoking established rates of divergence (1.4-2.6 % per million years) among other Caridean lineages separated by the uplift of the Isthmus of Panama set by Knowlton et al. (1993) and Knowlton & Weigt (1998), and assuming a constant rate of evolution, the data indicate a separation dating back to the Pleistocene. Hence, the approximate age of the Petea River obstruction is 0.6 – 1 mya. The divergence reported here is comparable to that observed in other invertebrates occurring throughout the world, but is manifest over a much smaller scale of less than 10 km (Knowlton et al. 1993; Knowlton & Weigt 1998; Woolschot et al. 1999; Critescu et al. 2003).

In this study, I report differences in both rostral and chelae features (see Table A.3; Woltereck 1937b). These morphological differences may be due to either differential selective pressures acting in the two different lakes, and is a response to selection, or may be neutral traits allowed to drift unchecked by balancing selection. Indeed, Woltereck (1937b) suggested that these traits do not provide adaptive advantages to particular environments and thus are likely not under selection. Morphological differences reported here are consistent with other studies of allopatric cryptic species demonstrating subtle yet distinct differences in various phenotypic traits among closely related sibling species (reviewed by both Mayr 1963, see also Fernandez et al. 2006; Sanders et al. 2006).

In fact, the genetic divergence observed in this study is also accompanied by relatively few morphological differentiations between the *C. lanceolata* populations in the two lakes. The overall morphological similarities between these populations despite the absence of gene flow may be maintained by balancing selection. Such balancing selection has been invoked to explain deep population and species level divergence without accompanied similar levels of morphological divergence in other cryptic species (e.g., Knowlton et al. 1993; Palumbi et al. 1997; Collin 2005).

Nevertheless, the few obvious morphological differences coupled with insignificant levels of selection on the COI locus (Tajima's *D* statistic) indicates that the divergence observed in the *C. lanceolata* clades is predominantly an effect of drift, and provides one of the clearest examples of drift building variation among closely related populations. Elevated levels of divergence between the two clades further suggests the presence of cryptic, or at the very least, incipient species using the phylogenetic species concept, resulting from the separation of the populations by the Petea River.

The formation of such cryptic species highlights the need to identify species from a variety of perspectives which can provide a more reliable and tangible consensus of an ecological/evolutionarily significant unit. Such consensus units would be far more practical and useful in conservation and mechanistic-based studies (Moritz and Faith 1998; Hey 2001; Agapow et al. 2004).

This study demonstrates the importance of physical topography and resulting vicariance for gene flow patterns in *C. lanceolata*, and perhaps other species in the Malili Lakes. Local and broader scale geography has had a significant impact on the generation of faunal and floral endemism in the Malili Lakes region, and Sulawesi Island in general (Whitten et al. 2000; Evans et al. 2003). These data add to a growing body of evidence demonstrating the unique evolutionary history of this isolated continental island.

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