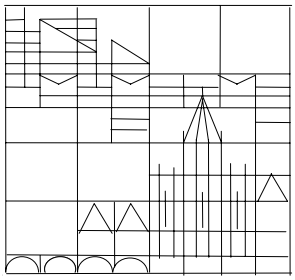

Molecular Investigations of Speciation in the Sea: Comparing Patterns of Diversification in Freshwater And Marine Organisms

Dissertation zur Erlangung eines

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ZUSAMMENFASSUNG

Die neue Entwicklungen der Molekularbiologie innerhalb der letzten Zeit haben das Studium der Evolutionsbiologie revolutioniert und Forscher mit leistungsfähigen neuen Hilfsmitteln versehen, um Muster und Prozesse der Evolution in der Natur zu erforschen. Die Einblicke, die durch die molekulare Populationsgenetik und die Phylogenie gewonnen wurden, haben eine wichtige Rolle gespielt, indem sie umstrittene Fragestellungen in der Evolutionsbiologie erhellt haben. In vielen Fällen haben diese Einblicke kategorisch die Weise geändert, wie wir über Evolution denken. In miener Doktorarbeit stelle ich drei Fallstudien vor, in denen ich aquatische Organismen benutze, um die Anwendung molekularer Methoden zur Beantwortung wesentlicher Fragen bezüglich der Rolle von sympatrischer Artbildung, sexueller Selektion und adaptiver Radiation in der Evolution zu demonstrieren.

Detailierte ökologische Studien haben erhebliche morphologische Veränderungen des Midas Cichlid, *Amphilophus citrinellus*, in Süsswassersee-Populationen aufgedeckt. Diese Untersuchungen hat zu verschiedenen Hypothesen hinsichtlich des Ursprungs dieser Variation geführt, doch das Fehlen molekularer Daten hat die rigorose Überprüfung dieser Hypothesen bisher verhindert. Im ersten Kapitel dieser Doktorarbeit stelle ich eine Reihe von neuen Microsatelliten vor und nutze diese neutralen Marker, um die heutige Populationsstruktur von *A. citrinellus* in vier Zentralamerikanischen Süsswasserseen zu untersuchen. Die erhaltenen Daten legen nahe, daß jeder der vier untersuchten Seen unabhängig

voneinander besiedelt wurde. Weiterhin hat assortatives Paarunsverhalten, gekoppelt mit der Diversifikation in der Färbung, zu reproduktiver Isolation zwischen den Cichlid Populationen in mindestens zwei von diesen Seen geführt. Die molekularen Resultate bestätigen die aufspaltende Stärke der sexuellen Selektion und liefern einen Hinweis, um theoretische Modelle der sympatrischen Artbildung zu unterstützen.

Im zweiten Kapitel meiner Doktorarbeit verwende ich molekular-phylogenetische Techniken, um die Evolution der väterlichen Fürsorge in syngnathiden Fischen (Seenadeln und Seepferdchen) zu untersuchen. Väterliche Fürsorge ist weit verbreitet unter Fischen, jedoch ist dieses Verhalten besonders ausgeprägt bei Arten der Familie Syngnathidae, wo die Männchen eine speziell angepasste "Brut-Struktur" entwickelt haben, die entweder am Schwanz oder am Abdomen zu finden ist. Syngnathide Fische zeigen auch häufig einen Tausch der Geschlechtsrollen; Weibchen konkurrieren stärker um Partner als Männchen. Dies macht diese Arten ideal für die Untersuchung des Einflusses von elterlichen Investitionen auf den Wettkampf um Paarungspartner. Mit Hilfe einer molekularen Phylogenie, die auf drei mitochondrialen DNA Markern basiert, habe ich die Evolution von Brut-Strukturen rekonstruiert. Diese Phylogenie liefert den Beweis, daß die Komplexität der Brut-Taschen unabhängig voneinander in den Schwanz-brütenden und den abdominal-brütenden Gruppen zugenommen hat. In der Gruppe der syngnathiden Fische scheint die schnelle Artbildung mit der Diversifikation der Brut-Strukturen zusammen zu hängen, was nahe legt, daß diese Form von hochentwickelter väterlicher Fürsorge eng assoziiert ist mit ihrer Radiation.

Obwohl man erwartet, daß Geschlechtsrollen Umkehr stark mit der väterlichen Investition korreliert, findet man mehrfache Ursprünge der Geschlechtsrollen Umkehr, sowie keinen Zusammenhang mit der väterlichen energetischen Investition, die durch die Brut-Struktur Komplexität gemessen wurde. Statt dessen scheinen die Geschlechtsrollen sehr stark vom Paarungssystem abzuhängen; Arten mit Geschlechtsrollen Umkehr haben fast immer ein polygames Paarungssystem. Diese Resultate zeigen an, daß das Paarungssystem möglicherweise die Intensität der sexuellen Selektion stark beeinflussen kann, indem es die Konkurrenz um Paarungspartner und/oder die Möglichkeit für Fortpflanzung verändert.

Im abschließenden Kapitel meiner Doktorarbeit nutze ich molekular-phylogenetische Techniken, um die endemischen Gastropoden des Tanganyika-Sees in Afrika zu studieren. Während die Schnecken des Tanganyika-Sees schon lange das wissenschaftliche Interesse wegen ihrer einzigartigen Süsswassermorphologie und der konchologischen Ähnlichkeit mit Meeresschnecken erregt haben, sind die Debatten hinsichtlich des Ursprungs dieser Gastropoden lange durch den Mangel an phylogenetischen Daten behindert worden. Eine mit molekularer Uhr kalibrierte Phylogenie schlägt vor, daß die endemischen Gastropoden des Tanganyika-Sees aus mindestens fünf Hauptlinien, die bereits vor der Enstehung des Sees existierten, entstanden sind. Eine weit verbreitete Gattung von afrikanischen paludomiden Gastropoden, die in Flüßen und Süsswasserseen vorkommt, scheint sich aus Arten entwickelt zu haben, die heute nur in Tanganyika-See vorkommen und sie hat ihr aktuelle Verbreitungsgebiet ausgehend von dem See möglicherweise in nur 8

Millionen Jahren erreicht. Dies zeigt, daß der Tanganyika-See eine wichtige Rolle als evolutionäres Reservoir gespielt hat für Gastropoden Arten, die ausserhalb des Sees ausgestorben sind. Morphologische Unterschiede zwischen diesen Schnecken scheinen nicht zu einer grossen adaptiven Radiation der Gastropoden im Tanganyika-See geführt zu haben. Diese Resultate haben wichtige Auswirkungen auf das Vertständnis von "Punctuated Equilibria", eine Theorie, die fossile Nachweise verwendet, anhand derer auf die Rate der morphologischen Diversifikation und der Artbildung während der evolutionärer Zeiträume geschlossen wird.

Die Untersuchenen, die in dieser Doktorarbeit dargestellt werden, zeigen sehr unterschiedliche Einsichten bei den Mustern der Evolutionsbiologie an, aber alle unterstützen konsistent die Tatsache, daß mikroevolutive Prozesse häufig zum Erklären der ausgedehnten makroevolutiver Muster fähig sind. Die populationsgenetischen und phylogenetischen Methoden, die hier dargestellt wurden, haben geholfen, Fragen bezüglich der sympatrischen Artenbildung, der adaptiven Radiation und der sexuellen Selektion bei Wasserorganismen zu lösen. Zusammengenommen helfen diese Studien, die oft sehr aussagekräftige Art und Weise zu veranschaulichen, in der die molekularen Techniken zu den grundlegenden Debatten der Evolutionsbiologie beitragen.

SUMMARY

Recent major developments in molecular biology have revolutionized the study of evolution, providing researchers with powerful new tools to investigate patterns and processes of evolution in nature. Insights gained from molecular population genetics and phylogenetics have played important roles in resolving major debates in evolutionary biology and, in many cases, have categorically changed the way we think about evolution. In this thesis, I present a series of three case studies, using aquatic organisms to demonstrate applications of molecular-based approaches to major questions relating to the role of sympatric speciation, sexual selection, and adaptive radiation in evolution.

Detailed ecological study has revealed substantial morphological variation in lacustrine populations of the Midas cichlid, *Amphilophus citrinellus*, and while this research has led to hypotheses concerning the origins of this variation, the absence of molecular data has hindered rigorous testing of these hypotheses. In the first section of this thesis, I present a suite of novel microsatellite markers and use these neutral markers to investigate present-day population structuring in four Central American lake populations of *A. citrinellus*. These data suggest that each of the four study lakes was colonized independently and subsequent assortative mating coupled with diversification in coloration has led to divergence between populations of cichlids in at least two of these lakes. Molecular results highlight the diversifying power of sexual selection and provide evidence to support theoretical models of sympatric speciation.

In the second section of my thesis, I use molecular phylogenetic techniques to investigate the evolution of male parental care in syngnathid fishes (pipefishes and seahorses). While male parental care is common amongst fishes, this pattern has been carried to its extreme in species of the family Syngnathidae, where males have developed specially-adapted brooding structures that are located under their tail or abdomen. Syngnathid fishes are also often sex-role reversed, with females competing more strongly for mates than males, and are ideally suited for the investigation of the impact of parental investment on competition for mates. Using a molecular phylogeny based on a suite of three mitochondrial DNA markers, I reconstruct the evolution of brooding structures and provide evidence to suggest that brood pouch complexity has increased independently in tail-brooding and abdominal-brooding groups. Rapid speciation in the group appears to be correlated with diversification of brooding structures, suggesting that this form of highly developed male parental care has been closely associated with the evolution of syngnathid fishes.

Although sex-role reversal is expected to be highly correlated with paternal investment, multiple origins of sex-role reversal appear to be unrelated to paternal energetic investment as measured by brood pouch complexity. Instead, sex-roles appear to be highly sensitive to mating systems, and sex-role reversed species almost always have polygamous systems of mating. These results indicate that mating systems may strongly mediate the intensity of sexual selection by influencing mating competition and/or opportunities for reproduction.

In the final section of my thesis, I apply molecular phylogenetic techniques to the study of the endemic gastropods of Lake Tanganyika, Africa. While the gastropod snails of Lake Tanganyika have long attracted scientific interest due to unique freshwater morphologies and conchological similarity with marine shells, debates concerning the origins of these gastropods have long been hindered from a lack of phylogenetic perspective. A molecular clock-calibrated phylogeny suggests that the endemic gastropods of Tanganyika have originated from at least five major lineages of snails that predated the lake. A widespread genus of African riverine and lacustrine paludomid gastropods appears to have evolved from species currently restricted to Tanganyika and reached its current range of distribution in as little as 8 million years, indicating that Lake Tanganyika has played an important role as an evolutionary reservoir of gastropod species extirpated outside the lake. Morphological divergence in these snails does not appear to have led to a major adaptive radiation of gastropod species in Lake Tanganyika. These results have important implications for the study of punctuated equilibria, a theory that has used fossil evidence to infer changes in rates of morphological diversification and speciation through evolutionary time.

The studies presented in this thesis offer very different perspectives into patterns of biological evolution, but all consistently highlight the fact that micro-evolutionary processes are often capable of explaining broad macro-evolutionary patterns. Population genetic and phylogenetic methods presented here have helped resolve questions related to sympatric speciation, adaptive radiation and sexual selection in aquatic organisms. Together, these

studies help to illustrate the often powerful ways that molecular techniques can contribute to fundamental debates in evolutionary biology.

CHAPTER 1

Introduction – Modern Molecular Methods in Zoology

INTRODUCTION

Darwin's (1859) theory of evolution by natural selection is arguably the singularly most significant scientific and cultural contribution of the past two hundred years. The concept of biological evolution has influenced social, political and philosophical thought since its introduction and has long formed the cornerstone of the biological sciences, offering a wider frame of reference for the intricate complexities of modern biology.

Darwin's publication of the *Origin of Species* immediately attracted considerable interest, but the lack of a clear theory of genetic inheritance hindered detailed investigations of organismic evolution and the wide scope of the theory was not immediately evident. Theoretical work by Fisher (1930), Wright (1931) and Haldane (1932) contributed to the Modern Synthesis (Huxley, 1942; Mayr, 1942; Simpson, 1944), which saw the fusion of the disparate fields of experimental genetics, population biology and paleontology into the broadly-based modern discipline of evolutionary biology (Mayr, 1980).

While the past sixty years of research in evolutionary biology have remained surprisingly close to Darwin and largely validated the theory, a novel interpretation of patterns in the fossil record (Eldredge & Gould, 1972) has led to debates concerning general rates and patterns of evolutionary diversification, questioning the gradualistic concept of evolutionary change. The problem of integrating data from broad spatial and temporal scales in the inference of biological phenomena has become one of the principal challenges in ecology and evolution (Levin, 1992). Insights gained from

molecular methods have been key in these debates and largely define the field of evolutionary biology today.

Molecular methodologies are essential for the study of evolution

Genetic data have always been an important resource for the formulation and assessment of evolutionary theories, whether in the form of morphological information or, more recently, as molecular data that have enabled even more intimate insights into the genealogical history of organisms, populations and species. The development of allozyme electrophoresis and its use in population genetics (Lewontin & Hubby, 1966; Lewontin, 1991) provided researchers with a relatively inexpensive way to look beyond the model organisms of *Drosophila* and *Mus* and broadly investigate the theory of evolution in a diversity of plants and animals (Lewontin, 1991). Allozymes provided a generation of researchers with a new tool to answer a broad array of evolutionary questions.

Molecular biology became even more intimately associated with evolutionary biology with the discovery of restriction enzymes (Smith & Wilcox, 1970), an innovation that enabled researchers to isolate discrete fragments of DNA (Avise *et al.*, 1979; Brown, 1980) and, following the development of molecular cloning, amplify them in large quantities (Cohen *et al.*, 1973). While methods of manual sequencing (Sanger *et al.*, 1977), finally allowed scientists to directly access the genotype of any organism, it was not until the advent of the polymerase chain reaction (Mullis *et al.*, 1986) that such techniques became readily accessible to evolutionary biologists. These

relatively recent advances have helped change the way we think about evolution.

Given its relative cellular abundance in relation to nuclear DNA, mitochondrial DNA was one of the first sources of genetic data for the study of evolutionary relationships (Avise *et al.*, 1979; Brown *et al.*, 1982) and has continued to be one of the most important sources of data for phylogenetic investigations. The majority of molecular phylogenetic data collected over the past thirty years have been mitochondrial (Anderson *et al.*, 1981; Avise *et al.*, 1987; Moritz *et al.*, 1987; Kocher *et al.*, 1989; Meyer, 1993a) and insights gained from these data have led to some of the first broad-scale comparative studies of human origins (Hasegawa *et al.*, 1985; Di Rienzo & Wilson, 1991; Ingman *et al.*, 2000), helped to identify the ancestors of tetrapods (Meyer & Wilson, 1990; Zardoya & Meyer, 1997) and illuminated relationships among teleost fishes (Lee *et al.*, 1995; Miya *et al.*, 2001). At the same time, analyses of mitochondrial data have illuminated evolutionary concepts such as the molecular clock (Ayala, 1986; Strauss, 1999), biparental organelle inheritance (Zouros *et al.*, 1992; Lunt & Hyman, 1997), and gene rearrangements (Boore *et al.*, 1995; Boore & Brown, 1998). Much of our present view of phylogenetic relationships among species is based on mitochondrial DNA.

Hypervariable microsatellites have had a similarly significant impact on the field of population genetics, largely replacing allozyme studies due to their high level of variability, near ubiquitous distribution in the genome and selective neutrality (Tautz, 1989; Bruford & Wayne, 1993; Schlotterer & Pemberton, 1994). These repetitive stretches of short (2-6 bp) sequences have proven to be extremely valuable for genome mapping (Routman &

Cheverud, 1994), kinship studies (Queller *et al.*, 1993) and in investigations of population structure (Bruford & Wayne, 1993). Details on the birth (Messier *et al.*, 1996), expansion and contraction (Rubinsztein *et al.*, 1995; Goldstein & Pollock, 1997) and death (Taylor *et al.*, 1999) of microsatellites have expanded our understanding of genome evolution.

In this thesis, I present a series of three case studies, using aquatic taxa to demonstrate the effectiveness of molecular methodologies in addressing questions previously inaccessible to evolutionary biologists. Aquatic organisms are particularly well suited to investigate questions related to sympatric speciation, sexual selection and adaptive radiation. By comparing and contrasting patterns of diversification and speciation in the sea, I provide empirical insights into Darwin's (1859) theory of evolution and investigate modern formulations of his theory in a phylogenetic context.

Case Study I: Nicaraguan crater lake cichlid fishes: A model system for sympatric speciation?

While allopatric speciation has long been recognized as a major force in evolution (Darwin, 1859; Mayr, 1988), the significance of sympatric diversification in the speciation process has long been debated (Bush, 1994). Although several examples of sympatric speciation have been presented, these have often been discounted due to lack of genetic data and/or possible alternative explanations involving historical allopatry (reviewed in Via, 2001). Recent molecular studies have been pivotal in this debate and have strengthened the idea that sympatric diversification plays an important role in the speciation process.

Given their clearly delineated borders, freshwater lakes, particularly crater lakes with gradually sloping bottoms (Schliewen *et al.*, 1994), are especially important for the study of sympatric speciation. Studies of diversification and speciation in freshwater species flocks, monophyletic groups of species endemic to a geographically circumscribed area (Greenwood, 1984), have provided some of the strongest empirical evidence in support of sympatric speciation (Meyer *et al.*, 1990; Schliewen *et al.*, 1994; Pigeon *et al.*, 1997b; Schluter, 2000).

While African cichlid fishes are well known as examples of rapid speciation in freshwater (Meyer *et al.*, 1990; Sturmbauer *et al.*, 1994; Sturmbauer *et al.*, 2001), far less is known about the diverse cichlids of Central and South America. Although both sexual selection (Seehausen *et al.*, 1997; Knight *et al.*, 1998) and ecological partitioning (Witte, 1984; Schliewen *et al.*, 1994) have been implicated in the rapid speciation of African cichlids, the absence of molecular data for neotropical cichlids has hindered the inference of rates and patterns of speciation in these animals (but see Roe *et al.*, 1997; Farias *et al.*, 1999; Farias *et al.*, 2000).

The Neotropical cichlid, *Amphilophus citrinellus*, has been the subject of detailed ecological study for over twenty years (Barlow & Munsey, 1976; McKaye, 1980; Meyer, 1987; Meyer, 1990a; Barlow *et al.*, 1990). This species is highly variable in both coloration (Barlow *et al.*, 1977) and pharyngeal jaw morphology associated with diet (Meyer, 1987; Meyer, 1990a) and appears to mate assortatively with respect to color, offering unique opportunities to investigate the relative importance of reproductive isolating mechanisms in wild populations. While various hypotheses have been

proposed for the diversification of populations of *A. citrinellus* in sympatry (Barlow *et al.*, 1990; Meyer, 1993b), the absence of molecular data has hindered rigorous testing of these scenarios. Chapter 2 outlines the development of a unique set of highly polymorphic microsatellite markers in *A. citrinellus*. Chapter 3 uses these markers to investigate of the factors responsible for sympatric diversification in this species.

Case Study II: Syngnathid fishes as a model system for the study of sexual selection

Darwin recognized that natural selection and sexual selection are often antagonistic processes that operate in conflict in the wild (Darwin, 1871). Natural selection results in the differential survival and reproduction of organisms that vary in some heritable characteristic. Sexual selection is a special case of natural selection, where differences in heritable sexual characteristics of individuals impact on their reproductive success. As characters which may enhance mating success may concurrently be detrimental for survival, sexual selection theory has often been invoked to explain unusually extreme secondary sexual characteristics (i.e. Peacock feather train) (Darwin, 1871). The calculation of organismic fitness must balance the tradeoffs between natural and sexual selection.

Through the mechanisms of sexual selection, mating behaviour and reproduction strongly influence the dynamics of wild populations. Parental investment is defined as:

> "Any investment by the parent in an individual offspring that increases the offspring's chance of surviving (and hence reproductive

> success) at the cost of the parent's ability to invest in other offspring"
>
> (Trivers, 1972, p. 139)

Parental investment Includes both energetic investment in the primary sex cells and any additional parental care that benefits the young. Trivers (1972) recognized that in the vast majority of cases, female parental investment exceeds that of males. As a result, female mate choice tends to be a driving force behind selection on secondary sexual characteristics in males. The bulk of sexual selection theory has been derived from empirical examples such as these, where female reproductive investment exceeds that of males and male competition for mates results in strong sexual selection (Trivers, 1972; Andersson, 1994)i.

In contrast to the majority of vertebrate animals, fishes of the family Syngnathidae are characterized by dramatic adaptations for paternal care. Male pipefishes and seahorses provide all post-fertilization parental care through specially-adapted brooding structures or pouches located on their trunk (Box 1). Syngnathid fishes are ideal as a model system for the study of sexual selection, due to their novel reversal of sex roles, with males providing sole parental care and females competing most strongly for mates. Chapter 4 chronicles a molecular phylogenetic investigation of the evolution of male brooding structures in the Syngnathidae. Chapter 5 uses the comparative method to study the relationship between the evolution of mating systems and sex-roles in the family.

Case Study III: **Ancient Lakes as natural laboratories for the study of evolution**

Darwin's (1859) concept of natural selection stressed the importance of micro-evolutionary processes on the origin of new species. While paleontologists have long recognized that periods of stasis in the fossil record are frequently interrupted by short bursts of rapid morphological change (Agassiz, 1866; Olson, 1952), Darwin (1859) believed that this apparent deviation from strict phyletic gradualism results from the incomplete preservation of the fossil record and does not reflect intrinsic biological factors. Eldredge and Gould (1972) suggested that the pattern of rapid punctuated change followed by long periods of stasis was too widespread to be due solely to limitations in the fossil record. They proposed that fossils were telling us something new, that evolution was not gradual, but instead often follows a model of punctuated equilibria. Recognizing that coordinated stasis across multiple taxonomic lineages is only possible when evolutionary stability is favored at the level of the ecosystem, Morris et al. (1995) refined the theory of punctuated equilibria, proposing a model of ecological locking, whereby complex ecological interactions generally favor a stable adaptive landscape. Following a significant disruption that overcomes this stable state, ecological constraints are removed, opening the system to invasion and presenting a new adaptive landscape, simultaneously driving evolutionary change in multiple lineages.

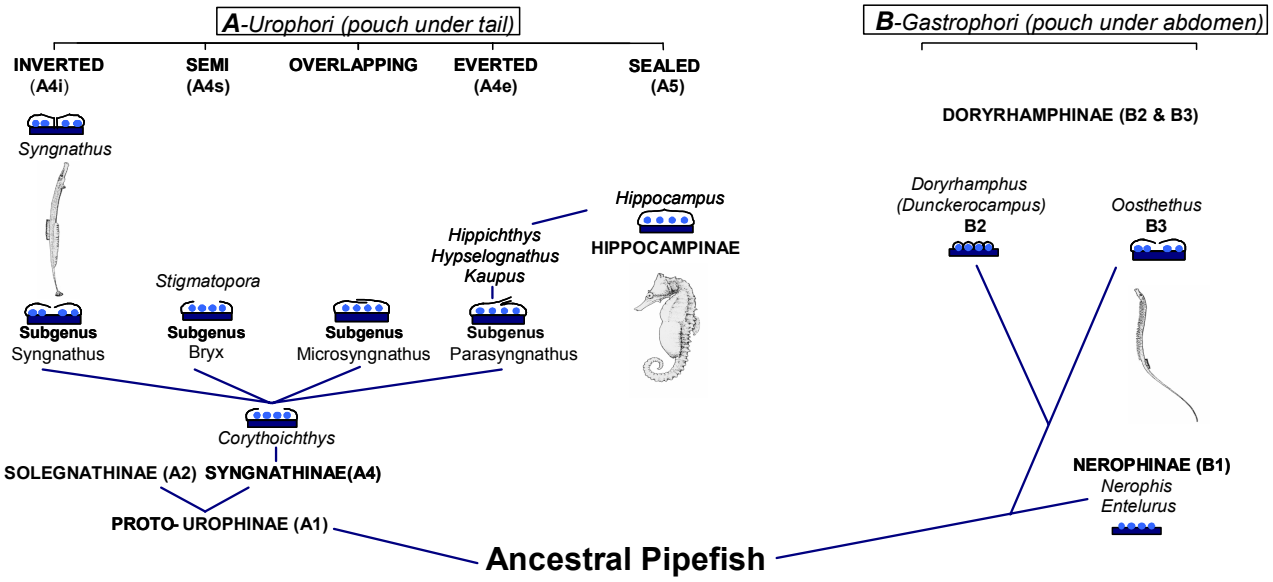
While most freshwater lakes are transitory and susceptible to subtle environmental fluctuations, a small number of lakes of extreme depth harbor the majority of the biodiversity of freshwater organisms. As some of the

largest and oldest lakes in the world, the East African Great Lakes Tanganyika, Malawi and Victoria have long attracted scientific interest due to the unusually high diversity and endemism of their flora and fauna (Box 2).

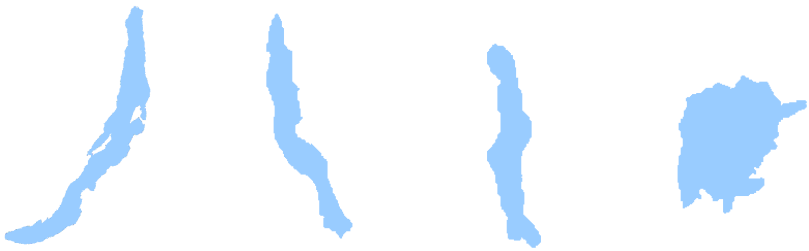
The gastropod snails of Lake Tanganyika are a group that is particularly notable for dramatic shell morphologies that appear much more similar to those of marine species than to other gastropods occurring in freshwater. These unique morphologies led Moore (1903) to suggest that Lake Tanganyika was a relict marine sea that was once directly connected to the ocean. As geological studies have recently confirmed the freshwater origin of Lake Tanganyika (Tiercelin & Mondeguer, 1991; Cohen *et al.*, 1993), various alternative hypotheses have been proposed to explain the startling diversity of life in Tanganyika (Coulter, 1991; West & Cohen, 1994). For the investigation of these hypotheses, a molecular phylogenetic framework is clearly necessary.

In chapter 6, I present a multi-gene phylogenetic study of the gastropods of Lake Tanganyika, identifying factors responsible for diversification over a geological time scale using Lake Tanganyika as a natural laboratory for the study of evolutionary processes.

Box 1. Seahorse male brood pouch types. Hypothesized evolution of male brooding in the Syngnathidae (Herald, 1959).



Box 2. Endemicity in Ancient Lakes. Species diversity and endemism in four major ancient lakes (data from Coulter, 1994).



	Lake Baikal	Lake Tanganyika	Lake Malawi	Lake Victoria
Max. Depth	1637m	1470m	704m	93m
Age (MY)	25-30	9-12	1-2	0.25-0.75
Cichlid Fishes (endemism)	-	180/185 (97%)	595/600 (99%)	248/250 (99%)
Other Fishes (endemism)	27/56 (48%)	61/145 (42%)	25/46 (54%)	17/40 (43%)
Gastropoda(endemism)	130/170 (76%)	37/60 (62%)	Low	Low
Amphipoda (endemism)	254/259(98%)	-	Low	Low
Decapoda (endemism)	-	22/25 (88%)	Low	Low
Ostracoda(endemism)	150/174(86%)	60/84 (71%)	Low	Low
Overall (endemism)	*982/1828 (54%)*	*477/819 (55%)*	*<40%*	*<40%*

CHAPTER 2

Broad taxonomic applicability of microsatellites developed for the highly polymorphic neotropical cichlid, *Amphilophus citrinellum*

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Broad taxonomic applicability of microsatellites developed for the highly polymorphic neotropical cichlid, *Amphilophus citrinellum*

2.1 INTRODUCTION

Neotropical cichlids are the some of the most important food fishes of Central America (Barlow, 1976). In addition to its important economic role as part of the freshwater fishery, the Midas cichlid, *Amphilophus citrinellum*, exhibits a high level of intraspecific variation in both coloration (Barlow 1976) and pharyngeal jaw morphology (Meyer, 1990a), which has made it a model species for the study of sympatric speciation (McKaye, 1980; Meyer, 1990a). While behavioural and ecological study of *A. citrinellum* has been extensive, low levels of variation in the mitochondrial control region and cytochrome *b* gene suggest a recent origin of the species (Meyer et al., unpublished data).

While a suite of microsatellite loci have been developed for African cichlids, these microsatellite primer sets have proven largely ineffective in amplifying Neotropical species (Kellogg *et al.*, 1995; Zardoya *et al.*, 1996; Van Oppen *et al.*, 1997). In this study, we describe the identification of six di- and tri-nucleotide microsatellite loci in *A. citrinellum* that can also be amplified in both Neotropical and Old World cichlids.

2.2 MATERIALS & METHODS

Genomic DNA was extracted from a single *A. citrinellum* specimen collected from Lake Nicaragua using a previously published extraction protocol (Karl & Avise, 1993). *Eco*R1-digested DNA fragments were ligated to

pUC18 (Gibco BRL) and transformed into SURE cells (Stratagene). The resulting library was screened with a [γ-^{32}P]-ATP end-labelled $(GT)_{10}$ oligonucleotide using standard hybridization techniques. Twenty-five positive clones of 300-1000 bp were sequenced using a Taq Dye-Deoxy Termination Cycle Kit (ABI - following manufacturer's recommendations) and analyzed with an ABI 373 Stretch DNA automated sequencer. Primer sets were developed for six of the 25 clones containing GT-microsatellites and adequate single-copy flanking DNA for primer design.

Amplification of the six microsatellite loci was carried out in a Geneamp 9700 Thermocycler (ABI) using 25 ul reaction volumes (Tris 67 mM, pH. 8.8; 1.5 mM $MgCl_2$; 0.4 mM of each dNTP; 75 ng of each primer and one unit of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus)). Forward primers were labelled with tetrachloro-6-carboxyfluorescin (TET). Amplification reaction conditions consisted of an initial denaturing step of 3 min at 94° C followed by 30 cycles of 94° C for 1 min, an optimized annealing temperature (see Table 1) for 30 seconds, and 72° C for 1 min. PCR products were visualized on agarose gels stained with ethidium bromide and diluted according to their strength. One microlitre of each sample was then mixed with 2 uL formamide and 0.5 uL each of size standard (GeneScan TAMRA-500, Applied Biosystems) and TAMRA buffer. The samples were denatured at 98° C for two minutes, loaded on a 5% denaturing 19:1 acrylamide:bisacrylamide gel and analyzed using an ABI 373A Stretch Automated Sequencer. Allele sizes were determined by the GeneScan software (Perkin Elmer) based on comparison of migration distances with the TAMRA ladder fragments of known size within each lane.

2.3 RESULTS & DISCUSSION

The six microsatellite loci were amplified in 140 *A. citrinellum* individuals from four lacustrine populations in Nicaragua. Levels of intraspecific variation varied considerably amongst loci, with allele numbers at each locus ranging from 1 to 26 and observed heterozygosity ranging between 0.000 and 0.664.

In addition to a high overall level of intraspecific variation, these six microsatellite loci have also proven useful in a broad taxonomic array of cichlid species (Table 2). In contrast to the majority of microsatellites identified in African species which fail to amplify Neotropical cichlids (Zardoya *et al.*, 1996), the present six microsatellite loci amplify both Neotropical and Old World species. These markers may prove effective in a further taxonomic clarification of relationships between New World and Old World cichlids.

The high intraspecific variation of these microsatellites makes them ideally suited to a detailed molecular investigation of observed anatomical and behavioural polymorphism in *A. citrinellum* and to molecular characterization of wild stocks of the species. At the same time, the broad taxonomic applicability of these markers offers an opportunity to further examine questions related to the rapid evolution of the Cichlidae.

Table 1 Primer sequences and core repeat structure for *Amphilophus cichlasoma* microsatellites. All loci were tested on 140 individuals from four lacustrine populations in Nicaragua.

Locus	No. of Alleles	H_O	H_E	Primer Sequence (5'-3')	Cloned Repeat Motif	Size of sequenced product (bp)	Annealing temperature (°C)
***Acit*1**	6	0.101	0.487	**F** AAA TGA GTT CAG CGA TGG CTG AG **R** TGC ACA TCA TGT CCG CCG AAC A	$(AG)_{11}$	168-174	49
***Acit*2**	26	0.593	0.926	**F** GGC ACT GAG GAT TTA TAT TAC AGG **R** GAG GTC CAG CTG AGA ACA GGG	$(GT)_{34}$	184-232	52
Acit3	18	0.664	0.905	**F** CTT AAG GTG TAC CTG CTT AGC R GAG TGG GAA GAC AGA TGT TGA GG	$(GT)_{32}$	161-195	**51**
Acit4	15	0.593	0.829	**F** CCT TCC TAC TAG TTA GTC TTT CAC **R** CAC ATA GCA CAG TGC ATT CAC CC	$(GT)_{22}(GCACGT)_9$	347-375	49
Acit5	1	0.000	0.000	**F** GCC GCA CCC TCA TTA TCC TCA C **R** GTG ACT CCA ACG TGT AGC TTC C	$(AGC)_8$	157	52
Acit6	1	0.000	0.000	**F** GAA TTC ACA AAG GCC AAT CCT AC **R** GGA TAC TGA GCA TGA CAA TAA GC	$(CA)_3(CGCA)_5(TATGTA)_{14}(TG)_9(CGTG)_8(TTA)_3$	268	50

*GenBank Accession Numbers: AF237713-AF237718
H_O = observed heterozygosity; H_E = expected heterozygosity.

Table 2 Success of cross-species amplification of *Amphilophus citrinellus* microsatellite loci.

	Acit1	***Acit2***	***Acit3***	***Acit4***	***Acit5***	***Acit6***
Neotropics:						
Amphilophus citrinellum	+	+	+	+	+	+
Cichla cichla	+	+	+	+	+	+
Crenicichla saxatilis	+	+	+	+	?	+
East Africa:						
Astatoreochromis alluaudi	+	-	+	+	+	+
West Africa:						
Hemichromis bimaculatus	+	-	+	-	-	+
Madagascar and India:						
Etroplus maculatus	-	+	-	+	?	-

(+) product; (-) no obvious product, (?) product of questionable size

CHAPTER 3

Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: sexual selection versus ecological diversification

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Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: Sexual selection versus ecological diversification

3.1 ABSTRACT

A growing body of empirical evidence for sympatric speciation has been complemented by recent theoretical treatments that have identified evolutionary conditions conducive to speciation in sympatry. The Neotropical Midas Cichlid (Amphilophus citrinellum) fits both of the key characteristics of these models, with strong assortative mating on the basis of a color polymorphism coupled with trophic and ecological differentiation derived from a polymorphism in their pharyngeal jaws. In an investigation of putative incipient sympatric speciation in this species, we used four microsatellite markers and a 480bp segment of the mtDNA control region to study four polymorphic populations of the Midas cichlid from three crater lakes and one large lake in Nicaragua. All populations are strongly genetically differentiated on the basis of geography. We identify strong genetic separation based on the color polymorphism for populations from Lake Nicaragua and one crater lake (Lake Apoyo), but fail to find significant genetic structuring based on trophic differences and ecological niche separation in any of the four populations studied. These data support the notion that sexual selection through assortative mating contributes more strongly or earlier during speciation in sympatry than ecological separation in these crater lakes. The long persistence of divergent cichlid ecotypes (as measured by % sequence divergence between populations) despite lack

of fixed genetic differentiation in Central American crater lakes differs strikingly from patterns of extremely rapid speciation in the cichlids in Africa, including its crater lakes. It is unclear whether extrinsic environmental factors or intrinsic biological differences, e.g. in the degree of phenotypic plasticity, promote different mechanisms and thereby rates of speciation of cichlid fishes from the Old and the New World.

3.2 INTRODUCTION

In natural populations of most species much morphological and behavioural variation exists, yet, empirical evidence for the role of this variation in establishing genetic differentiation, and ultimately speciation, remains limited (Lynch, 1989; Coyne, 1992; Ricklefs & Schluter, 1993). It is clear that allopatric speciation through vicariant events and colonizations has played a key role in the diversification of terrestrial (Bleiweiss, 1998) and aquatic animals (Brooks, 1950) as well as plants (Ricklefs & Renner, 1994). However, even in the absence of geographical barriers, it is thought that variation can lead to partial or complete reproductive isolation between sympatric populations through the reduction of gene flow (Bush, 1994).

The challenges inherent in identifying instances of sympatric speciation have contributed to ongoing debates concerning its importance (Mayr, 1988; Bush, 1994). Research into morphological diversification and speciation in aquatic systems (McKaye, 1980; Meyer, 1990a; Meyer, 1990b; Meyer, 1993c; Schliewen *et al.*, 1994; Pigeon *et al.*, 1997a; Nagel & Schluter, 1998) and islands (Grant, 1998) highlights the potential for the establishment of reproductive isolation and possible speciation in sympatry. In addition to these empirical studies, a series of recent theoretical treatments have attempted to identify evolutionary conditions that promote sympatric

speciation (Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999). Although the methodologies underlying these studies differ in several important respects, the models are consistent with classical studies (Maynard Smith, 1966) in identifying two key characteristics that are conducive to sympatric speciation. Variation in an ecological trait (such as differences in resource use) is necessary for disruptive selection against intermediates to take place (Losos, 2000). Assortative mating drives this disruptive selection and leads the divergent subpopulations on separate evolutionary trajectories, resulting in speciation in as few as 300 generations (Dieckmann & Doebeli, 1999).

The adaptive radiations of East African cichlid fishes are well known for their explosive rates of speciation and spectacular diversity (Meyer, 1993c; Stiassny & Meyer, 1999). The cichlid species flock of Lake Victoria is possibly as young as 12,400 years old, suggesting that these species not only formed extremely rapidly, but also within one continuous body of water (Meyer *et al.*, 1990; Johnson *et al.*, 1996). Recent evidence suggests that both niche diversification (Schliewen *et al.*, 1994) and sexual selection (Seehausen *et al.*, 1999) have played significant roles in the speciation of cichlid fishes (Meyer *et al.*, 1990; Meyer, 1993c). Unfortunately, determining underlying patterns of diversification in cichlids is complicated by a meagre fossil record, which confounds the elucidation of the historical distribution patterns and the dynamics of species formation. Even when historical distributions are known, documenting genetic effects of historical barriers to gene flow in initiating the speciation process is only rarely possible (Sturmbauer & Meyer, 1992; Ruber *et al.*, 1999).

While cichlids from African lakes are highly specialized (Fryer & Iles, 1969; Witte, 1984), Neotropical cichlids are characteristically generalists with respect to

habitat and diet (Kornfield *et al.*, 1982; Meyer, 1987; Meyer, 1990a), possibly reflecting the relative stability of their Old and New World habitats (Barlow, 1976). Long term field and laboratory studies of the highly polymorphic Neotropical cichlid *Amphilophus citrinellum* (Midas cichlid) (Barlow, 1976; Barlow & Munsey, 1976; Barlow & Rogers, 1978; Meyer, 1989; Meyer, 1990b; Meyer, 1990a; Barlow, 1998) has yielded extensive insights into its ecology and behaviour, making it an excellent model species for the study of non-geographical isolating factors in tropical lakes. This species lives in the large lakes of Nicaragua (Lakes Managua and Nicaragua) as well as in several small crater lakes (Lakes Masaya, Jiloa, and Apoyo) (figure 1).

All *A. citrinellum* individuals start out as normal protectively coloured, mostly vertically black-and-white striped, young. During their ontogeny, some of the normal morphs, irrespective of sex, will lose their melanophores and will become gold-coloured (figure 2). The age at which the gold morphs undergo this metamorphosis is variable and in some individuals it may not occur until after they have reached sexual maturity (Barlow 1976). It has been demonstrated in the field (McKaye, 1980; McKaye, 1986) and in the laboratory (Barlow & Rogers, 1978; Barlow *et al.*, 1977) that this species shows a strong tendency to mate assortatively with respect to coloration. This assortative mating may be enhanced by the fact that while territories of gold morph individuals dominate the benthic zone (where >50% are gold: (McKaye, 1980)), normal morphs tend to exclude gold morphs from the limnetic zone during the breeding season (>90% normal; (McKaye, 1980)), a pattern of segregation that is commonly observed between divergent ecotypes of fish such as limnetic and benthic morphs of sticklebacks, sunfish and salmonids (Smith & Skulason, 1996).

The functional decoupling of the upper and lower pharyngeal jaw in cichlid fishes led to a fundamental shift in function from food transport to food manipulation and preparation, and is believed to be partly responsible for their subsequent explosive diversification (Liem, 1973). Distinct pharyngeal jaw polymorphisms have been identified in several species of cichlids from both the Old and the New World (Greenwood, 1965; Kornfield *et al.*, 1982; Meyer, 1990a; Meyer, 1993b), and two distinct pharyngeal jaw morphologies have been found in *A. citrinellum* (Meyer, 1990b). In *A.citrinellum*, papilliform jaw morphs are characterized by slender, pointed teeth, while in molariform morphs, the pharyngeal jaws are heavier and the teeth are stouter and sturdier (figure 2; (Meyer, 1990a). While all fish are initially papilliform, the frequency of molariform adult Midas cichlids found in wild populations is highly correlated with the abundance of hard-shelled prey (table 1; (Meyer, 1990a)). Although molariform fish are able to crack larger and harder snails than papilliform morphs, their handling time for soft prey is significantly longer (Meyer, 1989). This ecological trade-off may help to maintain divergent ecotypes in natural populations and explains their highly different resource usage in nature (Meyer, 1990a).

The formation of discrete adaptive fitness peaks has been shown to be a key intermediate step during the process of sympatric speciation (Dieckmann & Doebeli, 1999; Doebeli, 1996; Kondrashov & Kondrashov, 1999). Both assortative mating (Kondrashov & Mina, 1986) and resource polymorphisms (Meyer, 1993b; Smith & Skulason, 1996) have been implicated as potentially significant factors in sympatric speciation. *A. citrinellum* exhibits both assortative mating on the basis of color and a trophic polymorphism that is correlated with prey availability. Moreover, these two polymorphisms may be coupled, as study by Meyer (Meyer, 1990b) identified that

within a single population, 76% of all normal morphs have molariform pharyngeal teeth and 57% of all gold morphs have papilliform jaws (see figure 2). We, therefore, hypothesized that if assortative mating is strong and trophic polymorphisms generally enhance reproductive isolation through ecological differentiation, this should result in decreased gene flow, possibly leading to speciation in sympatry.

In an effort to measure and describe population structuring that might be congruent with possible sympatric speciation in lacustrine populations of the Midas cichlid, we sequenced a 480 bp fragment of the most variable segment of mitochondrial DNA (mtDNA), the control region, and conducted a microsatellite analysis based on four hypervariable microsatellite markers. Previous analyses of these fishes identified only a single *cytochrome b* mtDNA haplotype in populations from several Central American lakes (Meyer, Biermann & Pålsson, unpubl. data). The higher evolutionary rate of the mtDNA control region makes it particularly sensitive to subtle changes in population structuring (Avise *et al.*, 1987). Microsatellites are generally also highly variable and have proven useful in previous studies of cichlid populations (Van Oppen *et al.*, 1998; Agnese *et al.*, 1999). Our combined microsatellite and mtDNA sequence data analyses are used in an effort to clarify the relative role of geographical and ecological factors in promoting reproductive isolation within and among four lacustrine populations of *A. citrinellum* in Nicaragua.

3.3. MATERIALS & METHODS

Sample Collection / Analysis of Microsatellite Loci & mtDNA Control Region

Fresh specimens of *Amphilophus citrinellum* (figure 2) were collected in 1987 from Lake Nicaragua, and from three crater lakes, Apoyo, Jiloa and Masaya (figure 1) which vary in their surface area, depth, and water transparency (table 1). Lake Nicaragua samples were purchased at two fish markets in Granada, while samples from crater lake populations were gill-netted from the shoreline of the lakes. Tissues of heart, liver, and muscle were stored frozen at -80ºC prior to DNA extraction that was performed following a previously published extraction protocol (Kocher *et al.*, 1989). Identification and characterization of the four microsatellite loci (*Acit*1-4) was performed as outlined in (Noack *et al.*, 2000). A 480 bp portion of the mtDNA control region was amplified with primers L15995 (Meyer *et al.*, 1994b) and H00651 (Kocher *et al.*, 1989) under previously published reaction conditions (Kocher *et al.*, 1989). Approximately 0.2 μg of Qiagen-column purified product from this PCR reaction was cycle-sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit following manufacturer's instructions (Applied Biosystems), with 5 pmol primer L15926 and 2μL Terminator Ready Reaction Mix. The cycling profile for the sequencing reaction consisted of 25 cycles of 96°C for 10 sec, 40°C for 5 sec, and 60°C for 4 min. Ethanol/sodium acetate purified cycle sequencing products were analyzed on an ABI 377 Automated Sequencer (Applied Biosystems).

Statistical Analyses

(i) Microsatellites

To ensure independent assortment of microsatellite markers, exact tests of linkage disequilibrium between microsatellite loci were performed using GENEPOP V3.1d (Raymond & Rousset 1995a). The significance of tests was estimated using a permutation procedure (Raymond & Rousset, 1995) . Genetic distances between populations and morphs were measured by calculating both F_{ST} and R_{ST} as implemented by ARLEQUIN V2.0 (Schneider *et al*. 2000). The significance of these estimates was tested under the null hypothesis H_O = "No difference between populations" by permuting genotypes between populations (10,000 iterations).

Exact tests of both global and pairwise tests of genotypic equilibrium were performed using GENEPOP V3.1d (Raymond & Rousset 1995a), in an effort to independently clarify the pattern of inter- and intra-population differentiation. An unbiased estimate of the significance of these tests was calculated through a 10,000 step, 1,000 iteration Markov chain series of permutations (10,000 dememorization steps) of a R X C contingency table of allelic distribution for each population. The significance of the P-values across the six loci was determined using Fisher's probability combination test (Raymond & Rousset 1995b).

Finally, to test for deviations from random mating, observed heterozygosities within populations were tested for departure from Hardy-Weinberg expectations using GENEPOP V3.1d (Raymond & Rousset 1995a). A 10,000 step, 1,000 iteration Markov chain method (10,000 dememorization steps) was used to calculate an unbiased estimate of the P-value.

(ii) Control Region

DNA sequences were aligned by eye and a minimum spanning haplotype network was constructed following a star decomposition search using the parsimony method as implemented by PAUP*V4.b3a (Swofford, 2000). Geographical and morphological population subdivision was measured using F_{ST} estimates as calculated by ARLEQUIN V2.0 (Schneider *et al*. 2000) from a Kimura 2-parameter distance matrix based on sequence data. Significance of these estimates was determined by a 10,000 step, 1,000 iteration Markov chain method (10,000 dememorization steps).

The relative significance of within- and between-lake variation of color morphs was clarified with an analysis of molecular variation (AMOVA). Kimura 2-parameter distance estimates were calculated from sequence data and an AMOVA was conducted with ARLEQUIN V2.0 (Schneider *et al*. 2000).

3.4 RESULTS

Microsatellite data

(i) Descriptive statistics / linkage disequilibrium

The four microsatellite loci detected consistently high levels of intraspecific variation (between four and 26 alleles) for the four populations examined (n=141) with intra-locus heterozygosity averaging 0.488 (± 0.260) (Noack *et al.*, 2000). Although exact tests for genotypic linkage disequilibrium between microsatellite loci within populations indicated a single significant P-value (χ^2 test; $P<0.05$) out of 24 pairs of loci tested (4.17%) (data not shown), this value was rendered insignificant following Bonferroni correction. Global tests of linkage disequilibrium calculated from within-population data were not significant at the 5% level (χ^2 test), indicating that all loci segregate independently.

(ii) Differentiation among lake populations

Global exact tests of genotypic differentiation indicated significant heterogeneity in gene frequencies among the four lacustrine populations (P<0.0001). In an effort to further partition these data, pairwise estimates of genotypic differentiation were calculated. All six pair-wise combinations of lake populations also showed highly significant differences (P< 0.001).

Differences in allele frequency distribution translated into highly significant fixation indices among the four lake populations investigated. To assess overall genetic differences between pairs of populations, we calculated both F_{ST} and R_{ST}, to take into account uncertainty over the mode of mutation in microsatellites (Slatkin, 1995). When all loci were combined, overall estimates of genetic differentiation among the four populations were highly significant for both F_{ST} (P<0.0001) and R_{ST} (P<0.0001). Pairwise population comparisons indicated significant differentiation between all population pairs except for a single R_{ST} estimate calculated between Lake Nicaragua and Lake Masaya (table 2). Overall these results indicate that highly significant genetic differentiation exists among the four lacustrine populations of the Midas cichlid.

(iii) Departures from HW proportions

Comparison of expected heterozygosities with observed values indicate a substantial heterozygote deficit in all four populations (figure 3). In a total of fourteen population-locus comparisons thirteen showed highly significant departures from Hardy-Weinberg proportions (P<0.0001). Pooling across all four loci, a significant heterozygote deficit was found for each population (P < 0.0001),

indicating that observed deviations from Hardy-Weinberg equilibrium are due to consistent heterozygote deficits at all loci, and not solely the result of deviations at a single locus. Taken together, these results indicate non-random associations of allele frequencies within each lake population, which may be explained by non-random mating in these populations (see Discussion).

(iv) Differentiation between pharyngeal morphotypes and color morphs

To examine the genetic structure within each lake population, we tested within each lake, as far as the abundance of morphs (see table 1) and our sampling allowed, for genetic differentiation between color morphs and pharyngeal jaw morphs.

Comparison of the two color morphs was possible for Lake Masaya, Lake Apoyo and Lake Nicaragua populations. Comparison of color morphs in the two crater lakes failed to identify significant genetic differentiation on the basis of microsatellite data, but the mitochondrial data found significant support for a genetic separation based on color for the Lake Apoyo population (table 2). For Lake Nicaragua we had a larger sample size for both of the color morphs (25 golds vs. 16 normals). Consistent differences between the two color-subpopulations in mtDNA haplotype frequency distributions ($P<0.05$) and overall F_{ST} (0.033) and R_{ST} (0.065) estimates ($P<0.05$) supported the separation of the Lake Nicaragua A. citrinellum population on the basis of the color polymorphism.

Tests for differentiation on the basis of jaw morphology were possible for populations from Lakes Nicaragua and Jiloa. For both comparisons, estimates of

F_{ST}, R_{ST}, and genotypic differentiation were non-significant (table 2), indicating lack ofigenetic differentiation on the basis of jaw morphology in these populations.

mtDNA control region data

To further explore genetic differentiation, we sequenced a 480 bp fragment of the mtDNA control region from a random subset (N = 76) of cichlid individuals, identifying a total of 36 haplotypes (figure 4). While the dominant mtDNA haplotype can be found in individuals from all four lakes, clustering of secondary haplotypes largely reflects geographical structuring of populations (figure 4), suggesting independent post-colonization molecular diversification of this mtDNA region.

Quantitative estimates of population structuring based on the mtDNA sequences support this qualitative interpretation of the data (table 2). F_{ST} estimates based on mtDNA indicate strong partitioning of populations on the basis of geography. In addition color morphs within Lake Apoyo and Lake Nicaragua populations are also significantly differentiated (table 2). In agreement with the microsatellite data, mtDNA provides no statistical support for genetic differentiation on the basis of trophic morphology in any study lake (table 2).

Further investigation of within- and between-lake variation of color morphs was conducted via a hierarchical AMOVA (table 3). As suggested by the haplotype network, a large proportion of the genetic diversity (18.4%) is found between lakes, while within-lake variation of color morphs is responsible for only 2.4% of total genetic variation (table 3). These results illustrate that the diversification of color morphs is relatively recent in comparison to lake colonization, implying independent and repeated radiations of color morphs in each of the lakes.

Overall, our results demonstrate strong population subdivision between the lakes due to geographic isolation. In addition to among-lake variation, differentiation between color morphs in Lakes Apoyo and Nicaragua suggests that assortative mating of cichlids within lakes may also be driving diversification and possibly speciation. The significant positive F_{IS} values found for each subpopulation (figure 3) suggest further population subdivision, possibly due to non-random mating in natural populations more strongly based on color differences than on pharyngeal jaw differences.

3.5 DISCUSSION

Our microsatellite markers and mtDNA sequence data reveal consistently high genetic differentiation between *A. citrinellum* populations from different lakes. This finding is consistent with topographic data indicating that the three crater lakes sampled are highly isolated and are not connected by any current river systems (Barlow 1976). This genetic differentiation also reflects the high degree of morphological variation between *A. citrinellum* populations from different lakes, where phenotypic differences have been documented (table 1; Barlow 1976; Meyer 1990b). There is a much higher variation in coloration of gold morphs of Midas cichlids (ranging from white to orange) from more turbid lakes (e.g. Lake Nicaragua) than that found in lakes with clear water (such as from Lake Apoyo) (Barlow 1976), possibly indicative of an influence of turbidity on the evolution of coloration and sexual selection, as has been suggested for Lake Victoria cichlids from Africa (Seehausen *et al.*, 1997). While variation in gold morphs is much larger in Lake Nicaragua than in Lake Apoyo, the absolute proportion of gold morphs in all four study lakes is remarkably similar (table 1), suggesting that frequency-dependent

selection may be contributing to color metamorphosis. The fundamental differences in water transparency, surface area and depth profiles (table 1) in these lakes offer further opportunities to test the impact of visibility on sexual selection in cichlid species.

All four *A. citrinellum* populations were found to deviate significantly from Hardy Weinberg expectations, always indicating substantial heterozygote deficits. These results suggest that some form of within-lake reproductive isolation may be playing a role in mating patterns of Midas cichlids in Nicaraguan lakes. While our data suggest that assortative mating on the basis of color may be playing a role in divergence of populations within Lakes Nicaragua and Apoyo, (Schliewen *et al.*, 1994) found a different pattern in a species flock of cichlids in Cameroon, where trophic specialization has apparently played the key role in diversification in sympatry. This contrast in patterns of diversification in Old and New World crater lake cichlids highlights the complexity of factors promoting sympatric speciation in nature.

The two color morphs of this species show a strong tendency to mate assortatively with respect to color (Barlow & Munsey 1976; Barlow *et al*. 1977; McKaye 1980), and within Lake Jiloa, have also been observed to breed at different depths (McKaye 1980). Our molecular data support these ecological findings, demonstrating significant population structuring of Midas cichlid populations on the basis of coloration (table 2) in both Lake Nicaragua and Lake Apoyo. While Lake Nicaragua has a greater diversity of color morphs of *A. citrinellum* (see above), it appears to hold similar levels of genetic diversity of Midas cichlids as those found in Lake Apoyo (table 2), suggesting that although intrinsic factors may be responsible for the proportion of fish that undergo metamorphosis (see above) environmental

factors (possibly variation in carotenoid content of diet (Witte *et al.*, 1997)) may contribute to subtle variation of gold morphs. In contrast to Lakes Nicaragua and Apoyo, color morphs were not strongly differentiated in the smaller Lake Masaya, perhaps suggestive of a role of lake size (see table 1) on assortative mating in *A. citrinellum*. As the presence of genetic structuring in color morphs in Lakes Nicaragua and Apoyo may be significant, further study of fish collected in 1999 from a diverse size range of habitats may help to reveal the significance of micro-topographic factors on mating patterns of Midas cichlids (Hrbek, Wilson & Meyer, unpubl. data).

The Midas cichlid also exhibits a trophic polymorphism of its pharyngeal jaws and the two trophic morphs have been shown to be ecologically separated (Meyer 1989; 1990b; Meyer 1990a). Meyer (1990b) argued that if mate choice and pair formation took place in each trophic morphs' respective habitat one might expect to find genetic differentiation between the two trophic morphs. However, our data reveal no significant genetic variation between trophic morphs within a sub-population (table 2). Furthermore, while a previous study of color and pharyngeal jaw morphs (Meyer 1990b) suggested that the color morph and pharyngeal jaw morphology covary, the present study provides no genetic evidence to support this hypothesis. While our data fail to support the hypotheses of reproductive isolation on the basis of trophic polymorphisms and its ecological consequences, the significant heterozygote deficiencies in all four lakes suggest that non-random mating may be having a significant impact on the population genetic structure of *Amphilophus citrinellum* in Nicaraguan lakes, potentially promoting species-level divergence through sympatric speciation where assortative mating based on color differences (see above) might lead to a reinforcement of ecological variation.

Barlow (1998) presents an argument to explain why the Midas cichlid has failed to speciate, even in the presence of presumably powerful reproductive isolating mechanisms such as strong assortative mating. Detailed laboratory study of the timing of melanophore loss and consequent color metamorphosis has revealed that while the majority of juveniles change color prior to reproductive age (18 months), some adults may not metamorphose until they are two or more years old. Therefore, more slowly metamorphosing gold individuals could possibly mate with genetically normal individuals for at least one to two reproductive seasons before they breed “true” to their color. Even if these aberrant individuals are rare, Barlow (1998) argues that the potential impact of individuals mating first with normal morphs and then later in their life-span with golds could be enough to break down genetic structuring based on coloration, even in the presence of generally strong assortative mating in the field where more than 90% of all pairs were of the same coloration (McKaye, 1986).

The high degree of genetic differentiation (0.25-0.60% mtDNA control region sequence divergence within populations) in populations of *Amphilophus citrinellum* suggests that, in addition to observed morphological variation within each lake, extensive genetic variation has been achieved within each of the four lacustrine populations since their colonization and has apparently not resulted in speciation. The genetic diversity of *A. citrinellum* is remarkably similar to that found in the cichlids of the Cameroon crater lakes (Schliewen *et al.*, 1994) (Schliewen *et al.* 1994), where comparable levels of sequence divergence in the mtDNA control region (Lake Bermin: 0.59%) and population structuring (as quantified by F_{ST}) delineate what are considered to be morphologically well-separated cichlid species flocks (Schliewen *et al.*, 1994).

There are several alternative explanations for these contrasting patterns of speciation, one being differences in rates of molecular evolution between New World and Old World cichlids (Farias *et al.*, 1999). (Farias *et al.*, 1999) documented higher rates of molecular evolution in Geophagine cichlids from South America than those found in lineages of African cichlids. They proposed that radically different climatic histories, in combination with biotic factors, might explain this rate acceleration. If *A. citrinellum* has experienced a similar acceleration in its molecular evolution, the ages of the Nicaraguan crater lake cichlid populations might actually be younger than those of the Cameroon crater lakes, even though their levels of mtDNA sequence divergence are quite comparable.

In addition to biological interpretations of this difference between Neotropical and African patterns of cichlid evolution, there remains a fundamental difference between the largely phylogenetically-based studies of African cichlids and the more ecological approach that has characterized the study of Neotropical cichlids such as *A. citrinellum*. With the exception of long term ecological study of Lake Victoria cichlids by Witte and coworkers (ex. (Witte *et al.*, 1997)), many molecular studies of African cichlids (including Schliewen *et al.* 1994) have been conducted on groups for which little ecological data are available. Furthermore, the sample sizes in many of these molecular studies have generally been limited to only a few representatives of each putative species (e.g. (Meyer *et al.*, 1990; Schliewen *et al.*, 1994); but see (Van Oppen *et al.*, 1998)), possibly obscuring genetic and ecological variation bridging assumed species boundaries. As highlighted above, levels of sequence divergence and F_{ST} estimates for *A. citrinellum* are comparable, and in some cases exceed, that observed in cichlid species from the Cameroon crater lakes, where (Schliewen *et al.*, 1994) genetically characterized a monophyletic lineage of six

endemic species of Tilapia in Lake Bermin on the basis of a single representative of each putative species. This significant discordance in experimental approach may be partially responsible for apparent differences in diversification in Old and New World cichlids.

While high levels of phenotypic plasticity and low rates of speciation appear to be common in Neotropical cichlids (Kornfield *et al.*, 1982; Meyer, 1987; Meyer, 1990a), the reverse is generally assumed for African cichlids (Fryer & Iles, 1969; Meyer, 1987; Witte, 1984); but see (Hoogerhoud, 1986; Witte *et al.*, 1997). While this may also reflect differences in experimental emphasis in the study of African and Neotropical cichlids, the striking contrast in rates of molecular evolution (Farias *et al.*, 1999) and apparent mechanisms of speciation may be partly explained by intrinsic differences that have accrued since the divergence of the two groups, or alternatively, reflect dissimilar environments encountered by Old and New World cichlids. Characterization of genetic factors (i.e. speciation genes: (Coyne, 1992; Ting *et al.*, 2000)) possibly underlying observed phenotypic variation in cichlids in their Old and New World habitats will be an important next step in research efforts to bridge the gap between genotype and phenotype and clarify the relative significance of intrinsic and extrinsic factors on speciation in aquatic environments.

Table 1. Physical characteristics of the four study lakes and distribution of previously reported color morphs (Barlow 1976) and pharyngeal jaw morphs (Meyer 1990b) of *A. citrinellum*. Snail abundance (Meyer 1990b) has also been included for comparison with distribution of pharyngeal jaw morphs.

	Nicaragua		*Apoyo*	*Masaya*	*Jiloa*
Physical characteristics (Barlow 1976)					
Surface area (km^2)	7740		21.2	8.4	3.8
Maximum depth (m)	50		>92	82	92
Water transparency (Secchi disc, m)	0.25-0.35		3.5-9.5	3-5	0.23-1.0
Color morphology (Barlow 1976)					
% Normal	90.7%		92.5%	91.9%	≅ 90%
% Gold	9.3%		7.6%	8.1%	≅ 10%
N	**1565**		**79**	**99**	**N/A**
Jaw morphology (Meyer 1990b)	shore	islands			
% Molariform	67%	7%	17%	0%	50%
% Papilliform	29%	93%	70%	100%	49%
% Intermediate	4%	0%	13%	0%	1%
N	**155**	**40**	**53**	**68**	**136**
Snail Abundance (Meyer 1990b) (#/15cm X 15cm quadrat)	N/A	0	75.5	0	111.5

Table 2. F_{st} and R_{ST} estimates over all loci and significance values for a) all pair-wise combinations of lake populations; b) comparison of color morphs within lakes and; c) comparison of trophic morphs within lakes. Microsatellite sample sizes are given in square brackets. Probability values: *P<0.05; **P<0.001.

(F_{ST} estimates calculated according to Weir & Cockerham (1984) and R_{ST} calculated following Slatkin (1995) as implemented by Arlequin V2.0 (Schneider et al. 1997). Significance estimates based on 10,000 permutations of the data set following sequential Bonferroni correction. P-value of the test is the proportion of permutations leading to an estimate equal or larger to that observed (Schneider et al. 1997). Kimura 2 parameter distance estimates used to calculate mtDNA F_{ST} values. For mtDNA population sizes, see figure 4.)

			mtDNA
	F_{ST}	**R_{ST}**	**F_{ST}**
a) Between lakes			
Nicaragua [51] - Jiloa [51]	0.065**	0.051*	0.141**
Nicaragua [51]- Masaya [15]	0.048**	0.001	0.129*
Nicaragua [51] - Apoyo [24]	0.183**	0.203**	0.223**
Jiloa [51] – Masaya [15]	0.092**	0.139*	0.015
Jiloa [51] – Apoyo [24]	0.306**	0.413**	0.320**
Masaya [15] – Apoyo [24]	0.181**	0.184**	0.344**
b) Within lakes separated by color			
Nicaragua golds [25] - Nicaragua normals [16]	0.033*	0.065*	0.315**
Apoyo golds [4] - Apoyo normals [20]	0.003	0.003	0.199*
Masaya golds [5] – Masaya normals [10]	-0.026	-0.058	0.000
c) Within lakes separated by jaw morphology			
Nicaragua papilliform [19] – Nicaragua molariform [31]	-0.002	0.032	-0.086
Jiloa papilliform [33] – Jiloa molariform [5]	0.015	-0.030	0.067

Table 3. Analysis of Molecular Variance (AMOVA) hierarchical genetic analysis on populations of color morphs based on mtDNA control region sequence data.

(Kimura 2-parameter distance estimates calculated from sequence data as implemented by Arlequin V2.0 (Schneider et al. 2000).

Source of variation	df	variance	% total
Between color morphs	1	0.0247	2.42
Among populations / Similar color morphs	4	0.1877	18.39
Within populations	36	0.8081	79.18

Group 1 (Normal): Apoyo, Nicaragua, Masaya
Group 2 (Gold): Apoyo, Nicaragua, Masaya

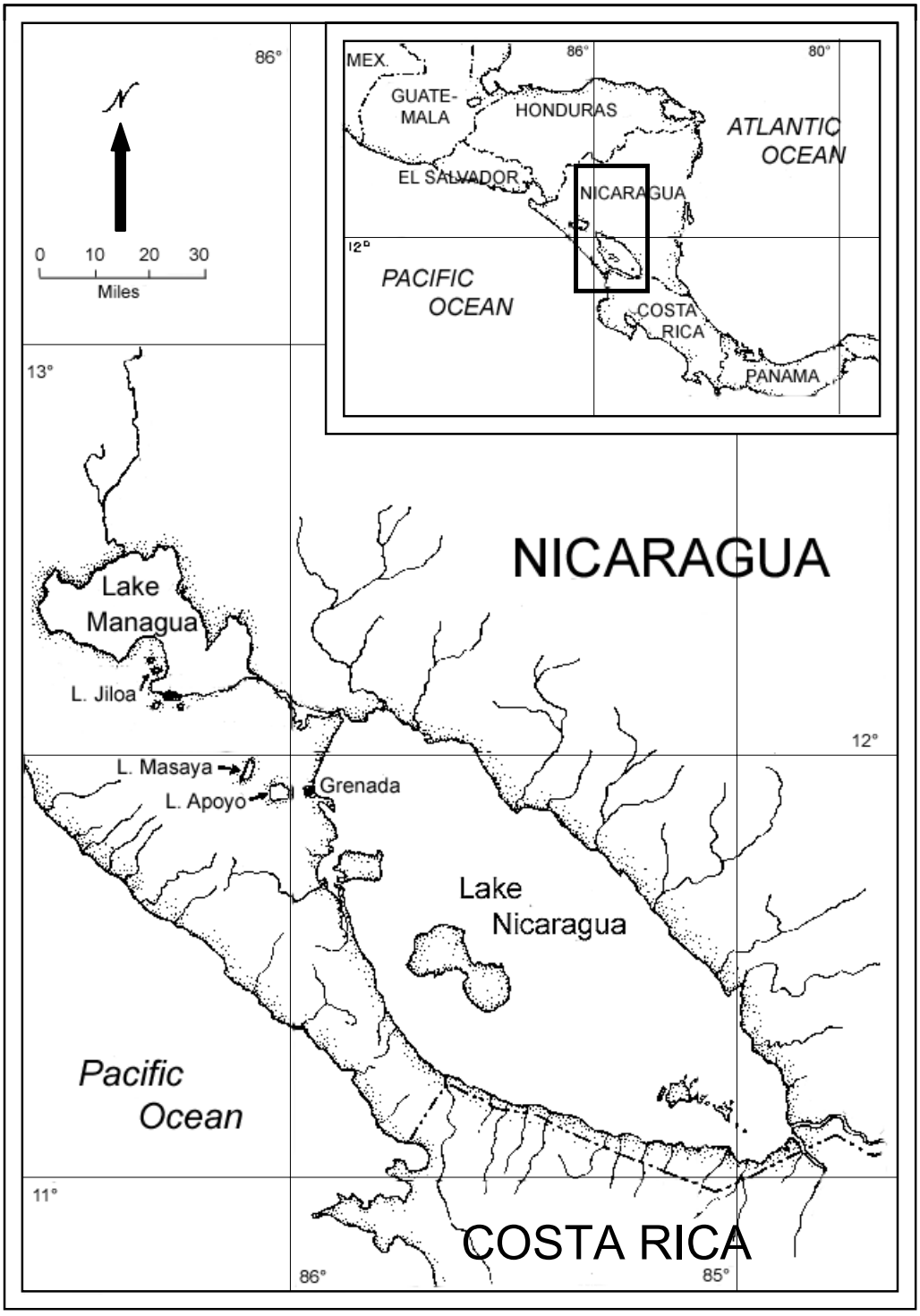
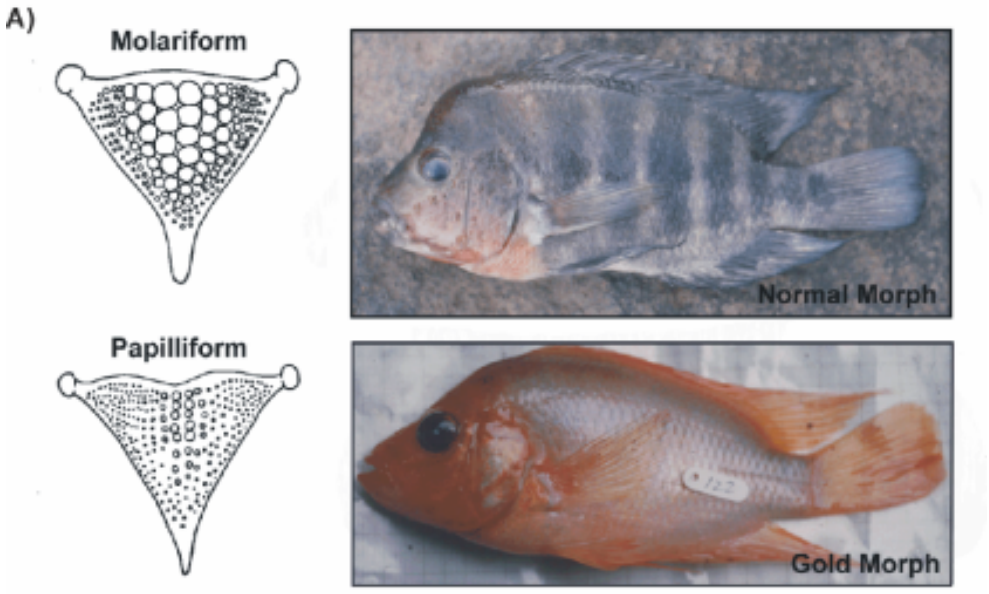


Figure 1. Map of Nicaragua showing the location of the lakes sampled (from Barlow, 1976; Figure 1, reproduced with permission).



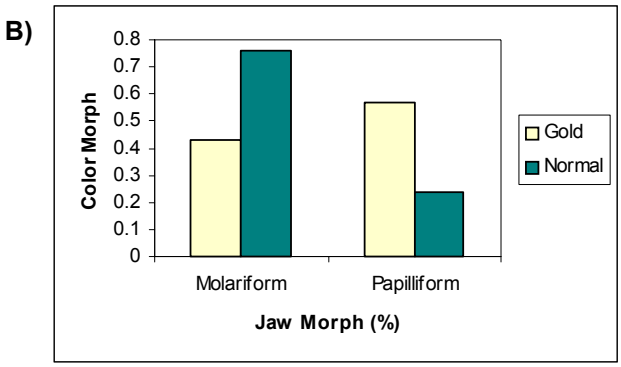


Figure 2. Comparative morphology of *Amphilophus citrinellum* (Adapted from Meyer 1990b). A) Left: Variation in pharyngeal jaw morphology; Right: Color morphs. B) Association of jaw morphology with color in *A. citrinellum* from Lake Nicaragua (prepared from table 2, Meyer 1990b).

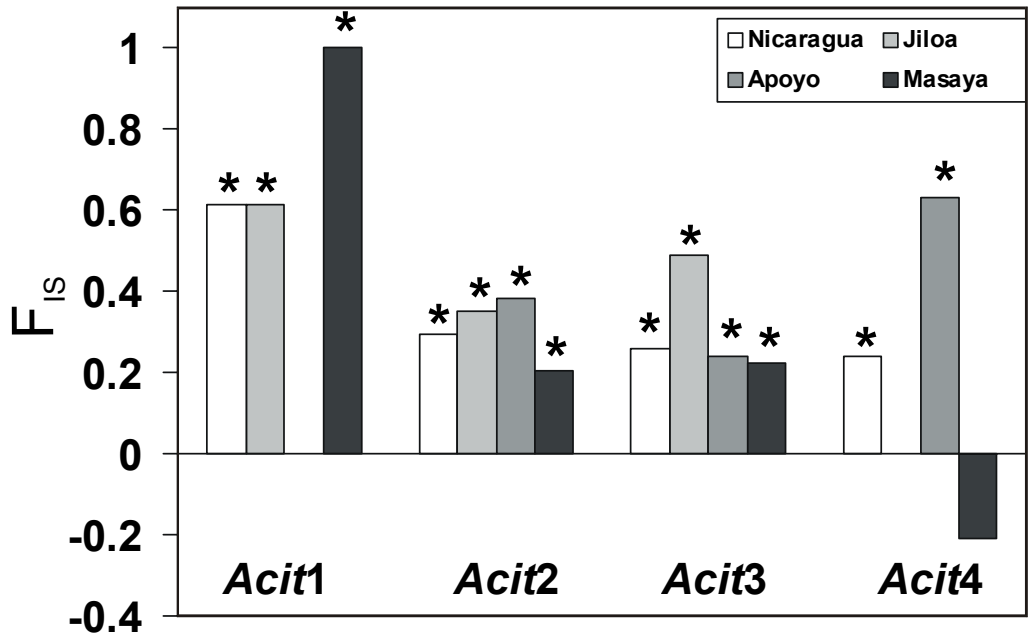


Figure 3. Hardy-Weinberg exact test for each locus in each population calculated by GENEPOP V3.1d (Raymond and Rousset 1995). * indicates a significant heterozygote deficiency (P<0.05). All four populations indicated significant deviations from H-W equilibrium (P<0.001). Note: Lake Apoyo is fixed for a single allele at *Acit1*.

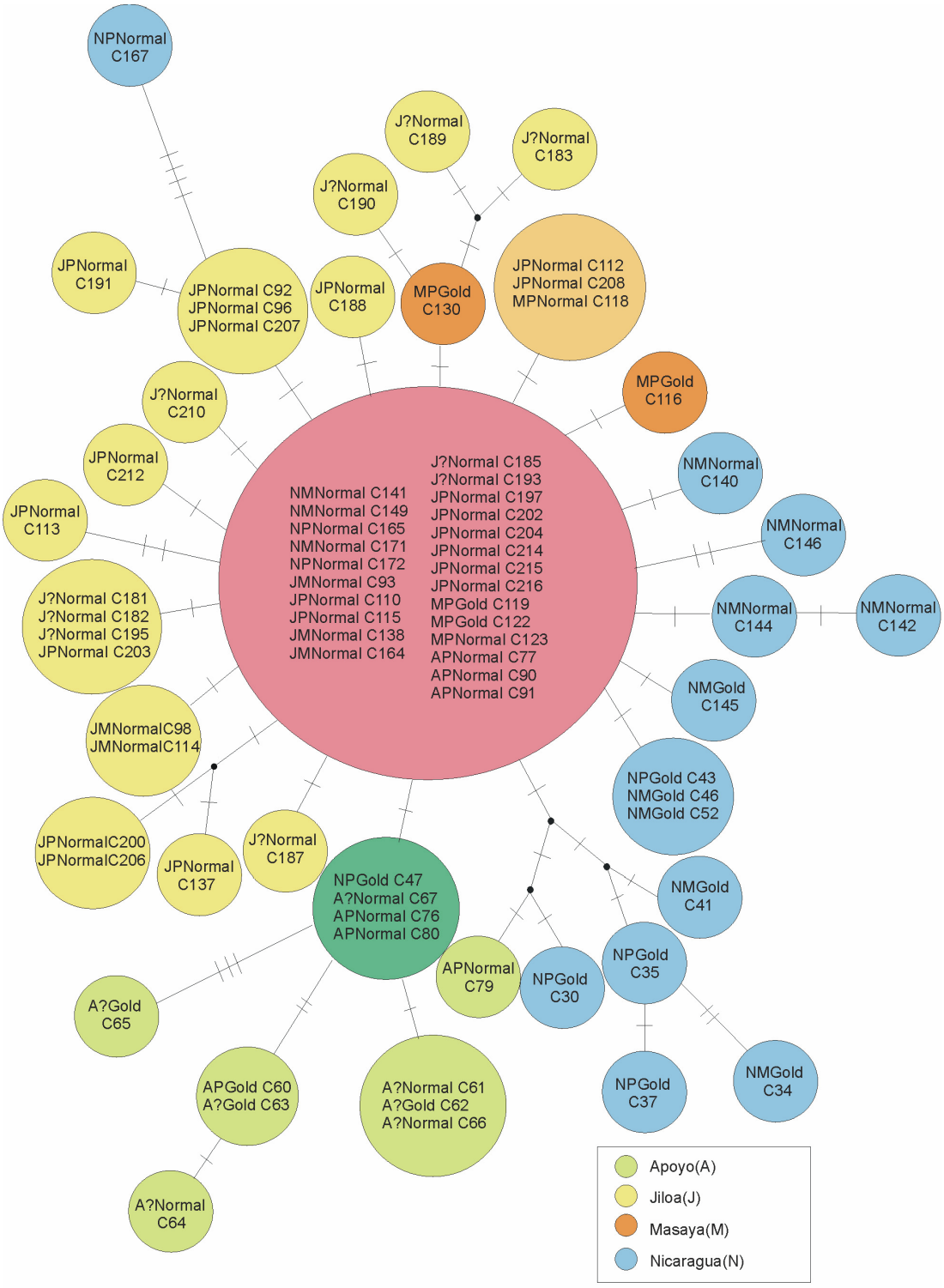


Figure 4. Haplotype network constructed from mtDNA control region haplotype data. Colours of haplotype groupings reflect geographic affinities. Jaw Morphology: P=Papilliform jaw morphology; M=Molariform jaw morphology; ?=Jaw Morphology unknown. Color morphology: Gold=Gold morph; Normal=Normal morph. Haplotype network was generated following a star decomposition search based on the parsimony algorithm as implemented by PAUP*V4.0b4a (Swofford 1998).

CHAPTER 4

Male pregnancy in seahorses and pipefishes (Family Syngnathidae): Rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny

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Male pregnancy in seahorses and pipefishes (Family Syngnathidae): Rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny

4.1 ABSTRACT

In contrast to the majority of vertebrate species, primary male parental care is common in fishes and encompasses a remarkable diversity of adaptations. Seahorses and pipefishes (Family Syngnathidae) exhibit some of the most specialized forms of paternal care amongst animals and so are ideally suited to the study of the evolution of male parental care. During mating, female syngnathids transfer eggs to specialized morphological structures that are located on either the abdomen or tail of the male. The male provides all post-fertilization parental care and has morphological and physiological adaptations to osmoregulate, aerate and even nourish the developing embryos. While all syngnathid species are adapted for paternal care, the brooding structure with which this is accomplished varies between species, from simple ventral gluing areas to much more complex structures such as the completely enclosed pouches of the seahorses. Our combined cytochrome *b*-, 12S rDNA- and 16S rDNA-based molecular phylogeny of syngnathid fishes demonstrates that rapid diversification of male brooding structures has been associated with the major evolutionary radiation of the group, suggesting that development and diversification of structures involved in paternal care may have been key evolutionary innovations of the Syngnathidae. Molecular analyses also highlight geographical centers

of biodiversity and suggest inter-oceanic migration of *Syngnathus* pipefishes from their center of origin in the Pacific.

4.2 INTRODUCTION

Evolutionary theory predicts that organisms should attempt to maximize reproductive success by monopolizing resources and mates and optimizing costs and benefits of parental care (Darwin, 1871; Emlen & Oring, 1977; Clutton-Brock, 1991; Andersson, 1994). Female parental care far exceeds that of males in many vertebrates, but this pattern is reversed in fishes where, in addition to gametic investment, males often provide the majority of parental care (Blumer, 1982). Fish are exceptional in their wide variety of parental care behaviours (Baylis, 1981), and have been instrumental in increasing our understanding of the evolutionary origins of parental care (Baylis, 1981; Gross & Sargent, 1985).

The Order Gasterosteiformes includes fishes with a remarkable diversity of reproductive behaviours (Breder & Rosen, 1966; Clutton-Brock & Vincent, 1991). The Family Syngnathidae (pipefishes and seahorses) are characterized by especially pronounced adaptations for male parental care, with the female depositing eggs directly to a specialized incubatory area or brood pouch on either the tail (Type A: Subfamily Urophori) or the trunk (Type B: Subfamily Gastrophori) of the male (Herald, 1959). This key morphological innovation ensures a male complete confidence in the paternity of its offspring (Jones & Avise, 1997; Jones *et al.*, 1999), but at a cost of paternal care that exceeds that of most other vertebrates (Clutton-Brock & Vincent, 1991).

Primary taxonomic groupings within the Family Syngnathidae reflect the location and development of the male brood pouch (Duncker, 1915; Herald, 1959)

(Figure 1): Type B1: Eggs are loosely attached to the ventral side of the male and are completely unprotected by a brood pouch (*Entelurus*, *Nerophis*); Types A2, B2: Eggs are placed into individual membranous egg-compartments (*Solegnathus*, *Doryrhamphus*); Type B3: Eggs are incubated in a well-defined pouch and protected by pouch plates (ventral extensions of the lateral plates of the trunk or tail rings) (*Oostethus*); Type A4: Eggs are placed into a well-defined pouch, with fleshy bilateral pouch folds that meet on the ventral midline of the pouch, and partially or fully enclose the eggs (*Syngnathus*); and Type A5: Eggs are incubated in a completely enclosed sac-like fleshy pouch, which opens through an anteromesial slit or pore (*Hippocampus*). Although neither A1 or A3 pouch types are known from the fossil record, the subfamilies of both the Gastrophori and Urophori are hypothesized to have evolved through successive development of the brood pouch (Herald, 1959); Figure 1).

The extreme degree of specialization for paternal care in the Syngnathidae is accompanied by a notable increase in species-level diversity over that of closely related groups. The Syngnathidae are by far the most diverse Family in the Order Gasterosteiformes, with approximately 230 described species (Dawson, 1985), while their close relatives, the stickleback Family, is comprised of only seven species (Wootton, 1984). However, whereas most species of sticklebacks have a circumpolar distribution concentrated in the northern hemisphere (Wootton, 1984), the highest diversity of the syngnathids is concentrated in a relatively small region of the southwest Pacific (Dawson, 1985). As the Gasterosteidae are believed to be closely related to the Syngnathidae (Bowne, 1984), this striking difference in both species-level diversity and geographic distribution is particularly

notable. Evidently, lineage-specific factors have been responsible for the clear differences in patterns of speciation observed between these two groups.

Molecular methods have proven useful in delineating the relative significance of intrinsic and extrinsic isolating factors in the speciation process (Lynch, 1989; Barraclough *et al.*, 1998; Howard & Berlocher, 1998). Molecular markers, and mitochondrial DNA in particular, have also yielded profound insights into the distribution and evolution of a broad array of animal taxa (Avise *et al.*, 1987; Avise, 2000) and have given us a better understanding of both the approximate timing and relative rates of diversification in many fish species (Bermingham *et al.*, 1997; Bernatchez & Wilson, 1998; Meyer *et al.*, 1990; Meyer, 1993a). In the present study, we use fragments of mitochondrial 12S rDNA and 16S rDNA genes and the complete cytochrome *b* mitochondrial gene to clarify syngnathid phylogeography and investigate the evolution of morphological specializations for paternal care in the Syngnathidae.

Previous morphology-based taxonomic revisions of the Family have stressed the importance of the male brood pouch in the syngnathid radiation and have made male reproductive biology a key taxonomic character in defining the group (Duncker, 1915; Herald, 1959; Dawson, 1985). Our molecular investigation investigates this assumption with a suite of three mitochondrial gene fragments. Strong congruency between Herald's proposed model of paternal care evolution (Herald, 1959) and the present molecular phylogenetic study would provide support for the significance of brood pouch diversification on the evolution of these fishes. Alternatively, conflicts between Herald's morphology-based model and our molecular-based phylogeny might indicate that alternative factors have been responsible for the radiation of the Syngnathidae.

4.3 MATERIALS & METHODS

Samples

Forty-four samples, including representatives of 34 species, were collected at sites across the entire geographic range of the Syngnathidae (Table 1; Figure 2). Archived syngnathid samples are housed at the Evolutionary Biology Center (Uppsala). Specimens used as outgroups (collection locality) were three members of the stickleback Family Gasterosteidae (threespine stickleback, *Gasterosteus aculeatus*, New York, USA; blackspotted stickleback, *Gasterosteus wheatlandii*, Rhode Island, USA; and ninespine stickleback, *Pungitius pungitius*, Scotland, UK); and the Japanese tubenose (*Aulichthys japonicus*, Kanagawa, Japan), a member of the Family Aulorhynchidae.

DNA Extraction / MtDNA sequencing

Specimens were preserved in 70% ethanol and total genomic DNA was extracted from white muscle or liver tissue by proteinase K/SDS digestion and purified by phenol-chloroform extraction and ethanol precipitation (Kocher *et al.*, 1989). Fragments of 12S rDNA and 16S rDNA genes and the complete cytochrome *b* gene were PCR-amplified with primers under previously published reaction conditions (Table 2). Approximately 0.2 μg of QIAquick (Qiagen) PCR Purification Kit-purified product from this PCR reaction was cycle-sequenced in both forward and reverse directions with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (V1.0) in 10 μL volumes following manufacturer's instructions (Applied

Biosystems), with 5 pmol primer and 2 μL Terminator Ready Reaction Mix. The cycling profile for the sequencing reaction consisted of 25 cycles of 96°C for 10 sec, 45°C for 5 sec, and 60°C for 4 min. Ethanol/sodium acetate purified cycle sequencing products were analyzed on an ABI 377 Automated Sequencer (Applied Biosystems). Sequences have been deposited in GenBank (see Table 1 for accession numbers).

Phylogenetic analysis of sequence data

The orthologous DNA sequences obtained were aligned, using default settings, by CLUSTALW (Thompson *et al.*, 1994) and optimized by eye. Preliminary sequence analysis by DAMBEV4.0.17 (Xia, 2000) was used to investigate the ratios of transitions to transversions as a measure of sequence saturation. PAUPV4b3b (Swofford, 2000) and MODELTEST V3.0 (Posada & Crandall, 1998) were used to estimate rate heterogeneity (as estimated by the gamma parameter Γ) and to determine phylogenetic models appropriate for both single gene and combined analyses.

Preliminary sequence analyses indicated that all three gene fragments best fit a HKY model of substitution (Hasegawa *et al.*, 1985) (Figure 3). Neighbour-joining distance (100 bootstrap replicates) and maximum parsimony analyses were performed with PAUPV4b3b (Swofford, 2000), with indels coded as missing data. Parsimony minimal trees were determined with full heuristic searches (100 bootstrap replicates) using random addition (10 replicates), the TBR branch swapping algorithm and the MULPARS option. Quartet puzzling maximum likelihood estimates (10000 puzzling steps) were calculated by TREE-PUZZLE V4.02 (Strimmer & von Haesler, 1999) with

transition/transversion ratio and nucleotide frequencies estimated from the dataset.

4.4 RESULTS

Preliminary sequence analyses were conducted on aligned 12S rDNA (339 bp) and 16S rDNA (497 bp) gene fragments and the complete cytochrome *b* gene (1149 bp) to investigate base composition bias, distribution of sequence variation, and possible saturation of transitions and transversions. While frequencies of bases were approximately equal for 12S rDNA and 16S rDNA gene fragments, cytochrome *b* exhibited significantly lower frequencies of glycine ($P<0.0001$), a widespread pattern amongst vertebrate taxa (Johns & Avise, 1998), and fishes in particular (Meyer, 1993a). While significant rate heterogeneity was detected for all three genes (Table 3), no taxon-specific base compositional biases were detected for any of the three gene fragments (chi-square test: $P>0.1$ for all genes). Although 12S and 16S did not exhibit saturation of transitions, cytochrome *b* gene transitions at third codon positions were saturated (Figure 3). Third codon positions of cytochrome *b* were removed from subsequent phylogenetic analyses.

Single Gene Analyses

While sequence data for all three genes were collected for the majority of specimens, a subset of species failed to amplify at one or more gene fragments due to the poor quality of the collected material. In an effort to include as broad an array of taxa as possible and to identify possible

differences in rates of molecular evolution across different genes, we conducted separate neighbour-joining, maximum parsimony and maximum likelihood analyses on each of the three mitochondrial gene fragments.

All three gene fragments provide good within-pouch type resolution, and indicate that the initial origin of pouch types occurred extremely rapidly, as evidenced by the low level of resolution of basal relationships between pouch types (Figure 4). High levels of resolution both above and below this point on the phylogeny (Figure 4) strongly suggest that the polytomy inferred at all three gene fragments reflects an increased rate of diversification during this time period. Judging from brooding structures of extant species, morphological diversification appears to have evolved early in the evolution of each major lineage and been strongly conserved over subsequent within-lineage evolution (but see A4e/s pouch type transitions discussed below; Figure 4). In addition to this extremely rapid diversification of pouch varieties, all single gene analyses indicate a clear split between the Urophori (pouch under tail) and Gastrophori (pouch under abdomen) (Figure 4), consistent with predictions from Herald's postulated evolution of syngnathid brooding structures (Herald, 1959) and supporting the taxonomic division of the Syngnathidae into 2 separate subfamilies (Figure 1) .

Maximum-likelihood phylogenetic analyses were conducted both with the assumption of constant rates of molecular evolution (i.e., molecular clock) and with rates of molecular evolution free to vary across lineages. In all three cases, the log-likelihood of the trees estimated without a molecular clock constraint was significantly greater than those assuming clocklike behaviour (likelihood ratio test: $P<0.001$), indicating that all three gene fragments exhibit

significant rate heterogeneity in these fishes. However, the addition or removal of additional taxa did not affect the basic tree topology, which was consistent across all three gene fragments (Figure 4), demonstrating congruency of molecular data. The three gene fragments were therefore combined for further analyses.

Total Molecular Evidence

Neighbour-joining distance, maximum parsimony and quartet puzzling maximum likelihood phylogenetic analyses conducted on the combined dataset of 1602 bp supported the single gene analyses (Figure 5). As expected (de Queiroz *et al.*, 1995), while the tree topology of the combined analysis remained essentially identical to the analysis of individual gene fragments, the larger number of characters present in the combined dataset provided greater confidence of inference, as measured by bootstrap support for critical branches (Figure 5). Once again, the rapid diversification of the Syngnathidae is clear from the combined analysis, and this analysis clarifies the basal branching of the abdominal-brooding pipefishes (Type B: Gastrophori) from tail-brooding lineages (Type A: Urophori) (Figure 5). All three methods of phylogenetic analysis indicate that the division of the ancestral pipefish into abdominal- and tail-brooding forms preceded a rapid diversification of pouch types, and that an increase in pouch complexity occurred independently in the Urophori and Gastrophori (Figure 5).

The total evidence molecular analysis also provides insight into the evolution of *Hippocampus* seahorses. In contrast to Herald's model of pouch evolution, which suggested a transition from the everted abdominal pouch

(type A4e) to the completely enclosed pouch of the seahorses (type A5) ((Herald, 1959); Figure 1), the total evidence phylogeny instead strongly supports a sister-group relationship between the seahorses and *Syngnathus* pipefishes (inverted pouch type A4i) (Figure 4b).

Phylogeography of Syngnathidae

We included biogeographical data on our consensus phylogenetic tree in an effort to infer historical biogeographic partitioning which might be partially responsible for the diversification of these fishes. Several interesting patterns are observed in the within-lineage diversification of the Subfamily Urophori. There is a clear split of *Syngnathus* pipefishes into North American and European lineages (Figure 5), and while significant species-level diversification has occurred, there has been no corresponding diversification of pouch types within the genus.

Although male pouch types appear to have been established during the formation of most major syngnathid lineages, there is a significant amount of variability in pouch structure in a small group of pipefishes endemic to south-eastern Australia. While *Solegnathus hardwickii* (type A2) is widespread in south-east Asia, *Urocampus carinirostris* (type A4s) and the other species on this branch of the tree are all Australian endemics that have apparently evolved both the semi (A4s) and everted (A4e) pouch types multiple times (Figure 5). A Kishino-Hasegawa test rejected the null hypothesis of pouch monophyly (t-test: $P<0.001$), supporting the replicated evolution of A4s and A4e pouch types in this lineage.

4.5 DISCUSSION

Rapid Diversification and Independent Evolution of Syngnathid Brooding Structures

Both single gene and total molecular evidence analyses indicate a rapid radiation of major lineages and, concomitantly, also of pouch types in the Syngnathidae. Tail- and abdominal-brooding lineages evolved independently, with remarkable increases in pouch morphological complexity in both subfamilies, an evolutionary series that reached its greatest development in the completely enclosed pouches of the seahorses (Subfamily Urophori). While our study provides some evidence for repeated evolution of a number of pouch types (pouch types A4e and A4s: Figure 5), the morphological variation between these pouch types represents a relatively minor proportion of the total brood pouch diversity found in these fishes (Figure 1). Our molecular data confirm much of Herald's morphological scenario of brood pouch evolution (Herald, 1959), suggesting that the evolution of the paternal brood pouch has been closely associated with the rapid diversification in both subfamilies of the Family Syngnathidae.

Given the nest building, maintenance, and considerable territorial guarding behaviour of the Gasterosteidae (Breder & Rosen, 1966), the exceptional development of the syngnathid brood pouch may not be entirely unexpected (Baylis, 1981). As gasterosteoid nests face predation and attacks by neighbours, and protected eggs may be fertilized by sneaky males (Wootton, 1984), the evolution of male brooding in the Syngnathidae presumably resulted in a net increase in reproductive success, as measured by the proportion of fertilized offspring that survive to reproduce. One may speculate that the benefits of carrying embryos away from predators and

sneakers may have led to the simple attachment of embryos to the ventral surface in a pre-pipefish ancestor (Baylis, 1981). Subsequent within-lineage diversification and closure of the brooding structure could help to explain the diversity of pouch types observed today.

Evolutionary Origin of *Hippocampus*: The "Birth" of Seahorses

Total molecular analyses provide strong evidence for a sister-group relationship between *Syngnathus* and *Hippocampus*, suggesting that the A4i (inverted pouch type) pipefishes and A5 (sealed pouch) seahorses have a common ancestor. While Herald believed that the A4e (everted pouch) pipefishes gave rise to *Hippocampus* seahorses (Herald, 1959), our molecular analyses unequivocally group *Syngnathus* and *Hippocampus* (Figure 5).

In his study on the evolution of syngnathid brooding structures, Herald paid particular attention to the evolution of the seahorse brood pouch, and based on a suite of shared characters, proposed a hypothetical evolutionary sequence from an everted pouch ancestor to the completely sealed pouch of the seahorse (Herald, 1959). Unfortunately, many of the characters used in his analyses (raised dorsal fin base, absence of caudal fin, prehensile tail), have multiple independent origins in the Syngnathidae (Duncker, 1915; Dawson, 1985), possibly confounding his attempts to identify the ancestors of the sealed pouch seahorses. Herald (1959) believed that an Atlantic subspecies of *Acentronura* was the closest extant relative of *Hippocampus*, yet acknowledged several important differences between the genus *Acentronura* and *Hippocampus*, including absence of lateral tail ridges in *Acentronura* and a complete lack of protecting pouch plates in *Hippocampus*.

Based on these differences, Herald (1959) concluded that although these two genera resemble each other superficially, they did not evolve in the same manner. Further morphological comparisons of reproductive characters within this Family are clearly necessary in light of our molecular phylogenetic analyses.

The genera *Hippocampus* and *Syngnathus*, with some of the most developed male brooding structures in the Syngnathidae, are by far the most species-rich genera in the Family (Dawson, 1985). The close phylogenetic relationship between *Hippocampus* and *Syngnathus* as inferred from our molecular phylogeny suggests that shared characteristics, possibly in addition to complex paternal care structures, have played an important role in the diversification of these two groups. *Syngnathus* and *Hippocampus* have also been especially successful at achieving wide geographic distributions (Dawson, 1985; Lourie *et al.*, 1999) indicating that dispersal capability in these two genera may be higher than that of other members of the Syngnathidae (see *Syngnathus* discussion below).

Syngnathus biogeography: A Pacific origin?

Due to the broad geographic distribution of many of the genera analyzed in the present study, it remains difficult to elucidate centers of origin of the species-rich clades, including *Hippocampus* and *Syngnathus*. Although *Syngnathus* has a cosmopolitan distribution and species are found not only in marine, but also in freshwater and estuarine environments, Atlantic Ocean lineages of *Syngnathus* are clearly disjunct from the less speciose Indo-Pacific lineage (Dawson, 1985). Moreover, two distinct configurations of body ridges distinguish the European species from those found in the western

Atlantic and eastern Pacific (Dawson, 1985). Our total evidence molecular results clearly separate European and western Atlantic species and demonstrate a close relationship between *Syngnathus leptorhynchus*, an eastern Pacific pipefish, and both the Atlantic Ocean *Syngnathus* pipefishes and *Syngnathus schlegeli*, a representative of the disjunct Indo-Pacific lineage. Inter-oceanic dispersal of a syngnathid ancestor may be responsible for seeding the Atlantic radiation of *Syngnathus*, a pattern that has also been detected in *Gasterosteus aculeatus* (Orti *et al.*, 1994), a species whose center of molecular diversity is found in the Pacific Ocean. Whereas body ridge configuration has varied over the history of the genus *Syngnathus* (Dawson, 1985), brood pouch structure has been remarkably conserved.

Parental Investment and Sexual Selection: Insights from the Syngnathidae

It is now commonly believed that relative parental investment of sexes in their young is a key factor responsible for sexual selection (Clutton-Brock & Parker, 1992; Trivers, 1972). Given the complete, but still variable, paternal care of the Syngnathidae, pipefishes and seahorses offer an exceptionally well-suited system to investigate sexual selection in relation to parental investment. In contrast to predictions from parental investment theory (Trivers, 1972), the variation in sexual dimorphism among syngnathids (Vincent, 1994; Berglund *et al.*, 1986b) does not appear to be associated with the degree of pouch development (Berglund *et al.*, 1986b; Vincent *et al.*, 1992). Instead, accumulating evidence indicates that environmental variables as well as anatomical and physiological constraints may strongly influence differences in potential reproductive rates between sexes (Vincent *et al.*,

1992; Ahnesjö, 1995; Kvarnemo & Ahnesjö, 1996), thereby influencing mate competition and ultimately sexual selection. Furthermore, ongoing molecular studies suggest that sex role reversal has had multiple independent origins uncorrelated with brood pouch development (Wilson, Vincent, Ahnesjö, and Meyer, submitted), indicating that environmental factors have played a significant role in influencing sex-role reversal and sexual selection in syngnathid fishes.

4.6 CONCLUSIONS

The rapid diversification of male pregnancy in the Syngnathidae and increasing complexity of pouch structure in both major lineages of the Family indicate that highly developed male parental care has been closely associated with the syngnathid radiation. Although much of previous morphological analyses are supported by our molecular data, significant discrepancies between molecular and morphological work suggest that further examination of pouch development and/or taxonomic revision of the group may be necessary. Our molecular results shed new light on the phylogeography of the Family, suggesting a Pacific origin for *Syngnathus* pipefishes and indicating regional concentrations of genetic biodiversity in the Western Indo-Pacific.

With the present molecular phylogenetic framework in place, future studies of syngnathid species should aim to further characterize behavioural and morphological variation within the Family and to clarify this variation in relation to established phylogenetic relationships. At the same time, more detailed species-level phylogenetic studies will help to increase our understanding of the influence of mating

systems on evolution of these fascinating creatures. The marriage of population genetic (Jones & Avise, 2001) and phylogenetic data will continue to broaden our perspective on the relationship between the evolution of mating and parental care systems and the diversification of syngnathiform fishes.

Table 1. Syngnathid specimens included in this study. See Figure 2 for geographic distribution of the Family and the origin of individual samples (Sample ID (S#) after species name).

Species (ID#)	Pouch	Collection Locality	Collector
Urophori (type A: tail pouch)			
Solegnathus hardwickii (S52)	A2	Australia	C. Linaker
Syngnathus abaster (S23)	A4i	West Sicily, Italy	P. Franzoi
S. acus (S2)	A4i	Sweden	I. Ahnesjö
S. acus (S46)	A4i	North Wales, Britain	C. Linaker
S. floridae (S21)	A4i	Virginia, USA	R.L. Teixeira
S. floridae (S41)	A4i	Florida, USA	R. Ruiz-Carus
S. fuscus (S19)	A4i	Virginia, USA	R.L. Teixeira
S. leptorhynchus (S33)	A4i	Humboldt, USA	R. Fritzsche
S. louisianae (S42)	A4i	Florida, USA	R. Ruiz-Carus
S. rostellatus (S3)	A4i	Sweden	I. Ahnesjö
S. schlegeli (S14)	A4i	Pearl River Estuary, China	F. Leung
S. scovelli (S40)	A4i	Florida, USA	R. Ruiz-Carus
S. typhle (S4)	A4i	Sweden	I. Ahnesjö
S. typhle (S22)	A4i	West Sicily, Italy	P. Franzoi
S. taenionotus (S24)	A4i	Po Delta, Italy	P. Franzoi
Corythoichthys intestinalis (S15)	A4s	Ambon, Indonesia	A. Vincent
C.intestinalis (S18)	A4s	Tumon Bay,Guam	C. Dayton
Stigmatopora argus (S8)	A4s	Dunalley Bay, Tasmania	A. Jordan
S. argus (S37)	A4s	Botany Bay, Australia	C. King
S. argus (S50)	A4s	Australia	C. Linaker
S. nigra (S38)	A4s	Botany Bay, Australia	C. King
S. nigra (S51)	A4s	Australia	C. Linaker
Urocampus carinirostris (S39)	A4s	Botany Bay, Australia	C. King
Vanacampus phillipi (S48)	A4s	Australia	C. Linaker
V. poecilolaemus (S45)	A4s	Australia	C. Linaker
Halicampus grayi (S29)	A4e	Vietnam	I. Ahnesjö
H. grayi (S30)	A4e	Vietnam	I. Ahnesjö
Hippichthys penicillus (S16)	A4e	Kuwait Bay, Kuwait	A. Vincent
Hypselognathus rostratus (S44)	A4e	Australia	C. Linaker
Kaupus costatus (S49)	A4e	Australia	C. Linaker
Trachyrhampus serratus (S54)	A4e	Australia	C. Linaker
Hippocampus abdominalis (S35)	A5	Hobart, Tasmania	A. Vincent
H. barbouri (S11)	A5	Philippines	A. Vincent
H. comes (S12)	A5	Philippines	A. Vincent
H. erectus (S20)	A5	Virginia, USA	R.L. Teixeira
H. kuda (S31)	A5	Taiwan	Fang
H. kuda (S32)	A5	Taiwan	Fang
H. sp. (S17)	A5	Kuwait Bay, Kuwait	A. Vincent
H. zosterae (S36)	A5	USA	H. Masonjones
H. zosterae (S43)	A5	Florida, USA	R. Ruiz-Carus
Gastrophori (type B: abdominal pouch)			
Entelurus aequareus (S6)	B1	Sweden	I. Ahnesjö
Nerophis ophidion (S5)	B1	Sweden	I. Ahnesjö
Doryrhamphus dactyliophorus (S10)	B2	Philippines	A. Vincent
Oostethus brachyrus (S9)	B3	Puerto Barrios, Guatemala	D. Reznick

Table 2. Polymerase Chain Reaction (PCR) primers for seahorse 12S rDNA, 16S rDNA and cytochrome *b* mitochondrial genome fragments. Primer names follow the convention of naming the primer by the most 3' position of the primer in the human mtDNA sequence (Kocher *et al.*, 1989).

Primer	Sequence	Reference
12S		
L1091	5'-AAACTGGGATTAGATACCCCACTA-3'	(Kocher *et al.*, 1989)
H1478	5'-GAGGGTGACGGGCGGTGTGT-3'	(Kocher *et al.*, 1989)
H2001	5'-AACCAGCTATCACCAGGCTCG-3'	
16S		
L2510	5'-CGCCTGTTTATCAAAAACAT-3'	(Palumbi *et al.*, 1991)
H3058	5'-CCGGTCTGAACTCAGATCACGT-3'	(Palumbi *et al.*, 1991)
Cytochrome *b*		
L14725	5'-CGAAGCTTGATATGAAAAACCATCGTTG-3'	(Pääbo *et al.*, 1991)
L15162	5'-GCAAGCTTCTACCATGAGGACAAATATC-3'	(Taberlet *et al.*, 1992)
H15240	5'-TTRTCTACNGARAANCCNCCTCA-3'	
H15915	5'-TCATCTCCGGTTTACAAGAC-3'	(Irwin *et al.*, 1991)
H15926	5'-AAGGGKGGATTTTAACCTCCG-3'	(This study)

Table 3. Hierarchical likelihood ratio test of phylogenetic model as implemented by MODELTEST V3.0 (Posada & Crandall, 1998). * = selected model of evolution (P<0.01).

Model	Parameters	-Ln (Likelihood)	Gamma (Γ)	Proportion of Invariable Sites (I)
12S				
JC	1	4832.9736		
F81	4	4806.0513		
HKY	5	4692.2627		
HKY + Γ	6	4285.8623*	0.4329	0
HKY + Γ + I	7	4285.7329		
16S				
JC	1	5792.5630		
F81	4	5781.4595		
HKY	5	5670.0010		
HKY + Γ	6	5021.7520		
HKY + Γ + I	7	5015.7070*	0.6219	0.3880
Cytochrome *b*				
JC	1	5992.3228		
F81	4	5941.6152		
HKY	5	5747.3623		
HKY + Γ	6	5197.8252		
HKY + Γ + I	7	5185.4873*	0.4590	0.3946
Combined analysis				
JC	1	17239.4961		
F81	4	17176.9199		
HKY	5	16765.9062		
HKY + Γ	6	14996.7080		
HKY + Γ + I	7	14981.1475*	0.4333	0.2755

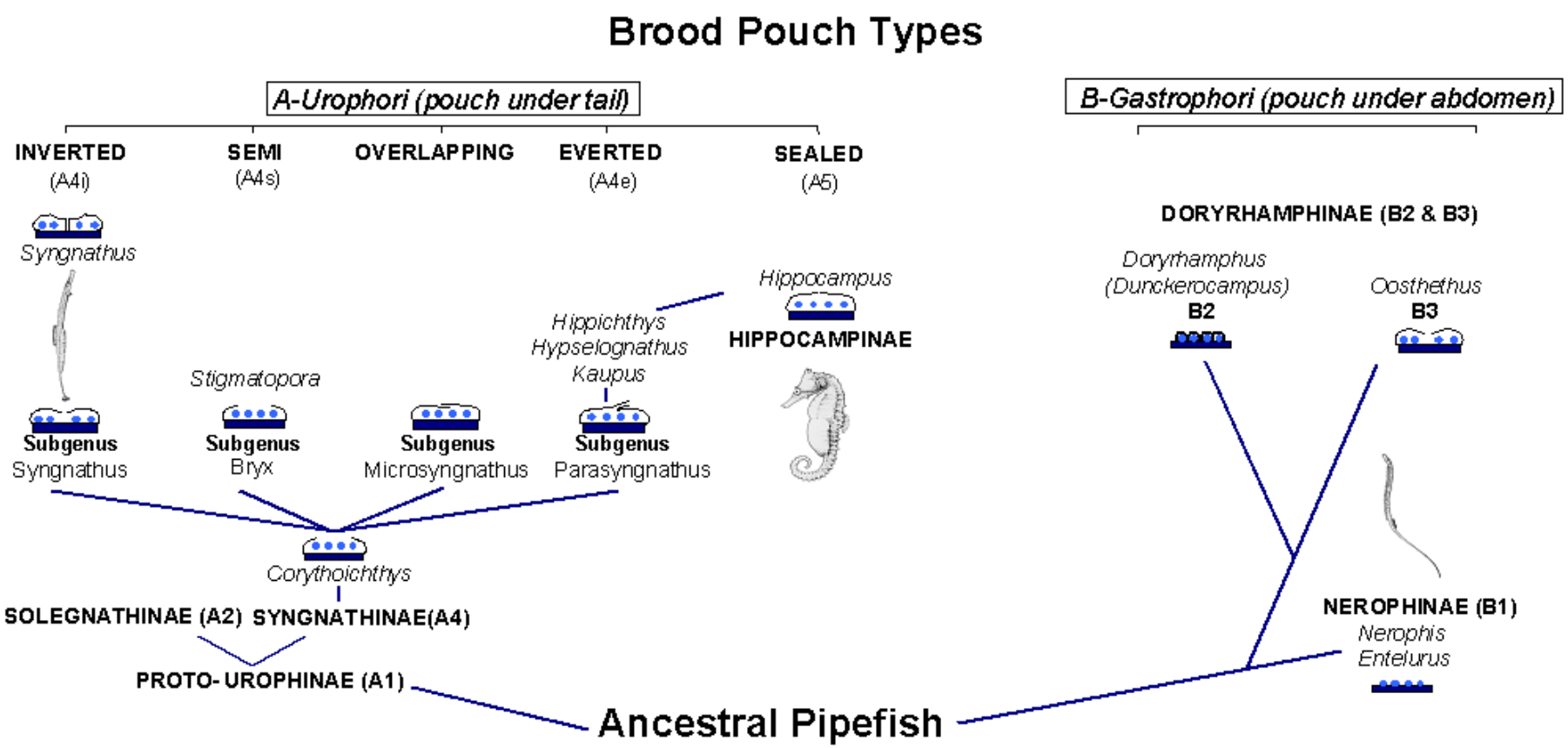

Figure 1. Hypothetical evolutionary development of the syngnathid brood pouch (after Herald, 1959), detailing independent radiations of Urophori and Gastrophori and the diversification of pouch types. For descriptions of pouch type variation, see text. Generic names (in italics) represent taxonomic sampling of the present study. Shown are schematic cross-sections through the male brood pouch (Herald, 1959), showing general pouch design and the extent of pouch enclosure.

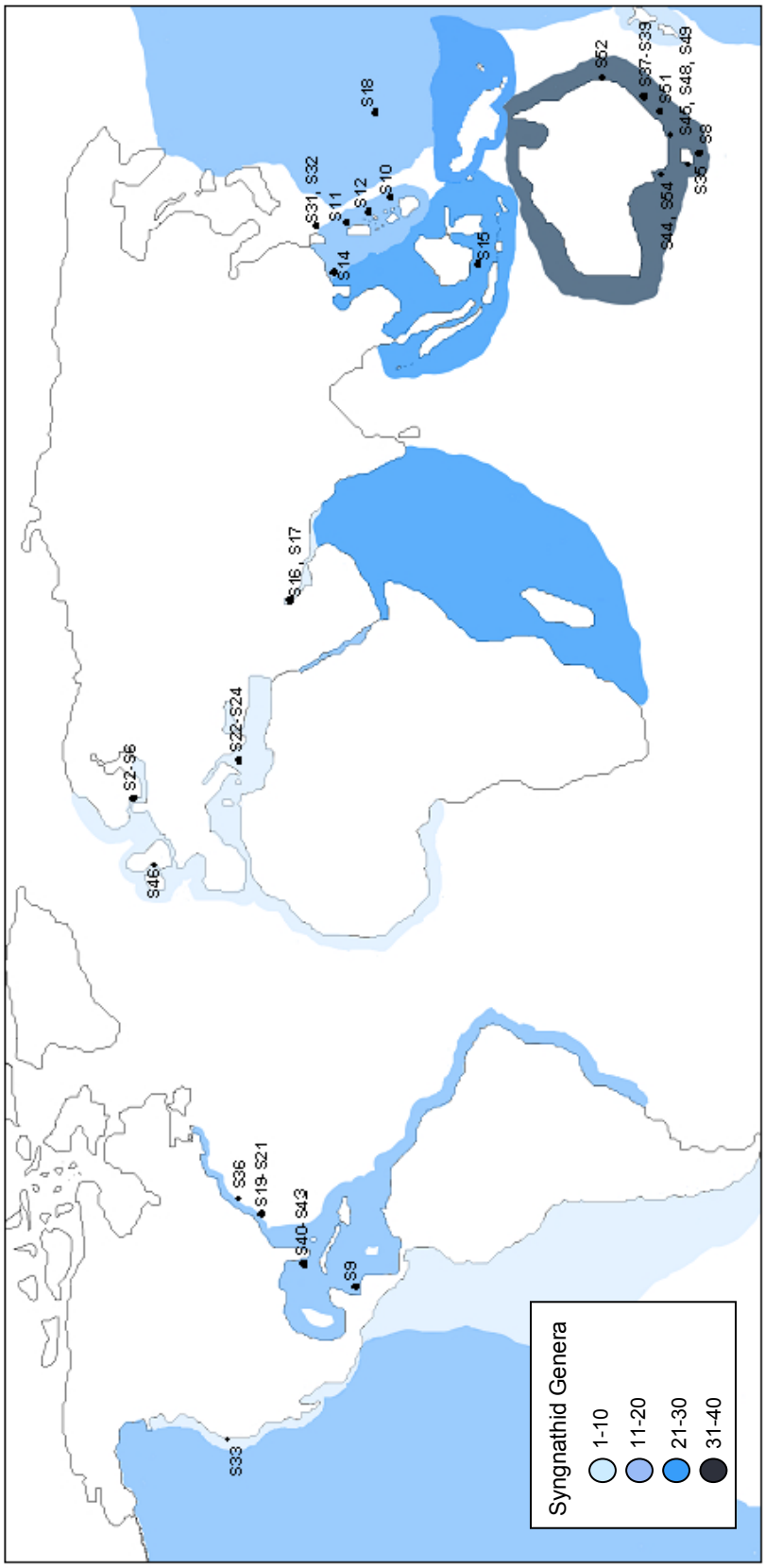

Figure 2. World map detailing seahorse and pipefish sample collection localities and the global distribution of syngnathid genera (Dawson, 1985). See Table 2 for species identification and collection information.

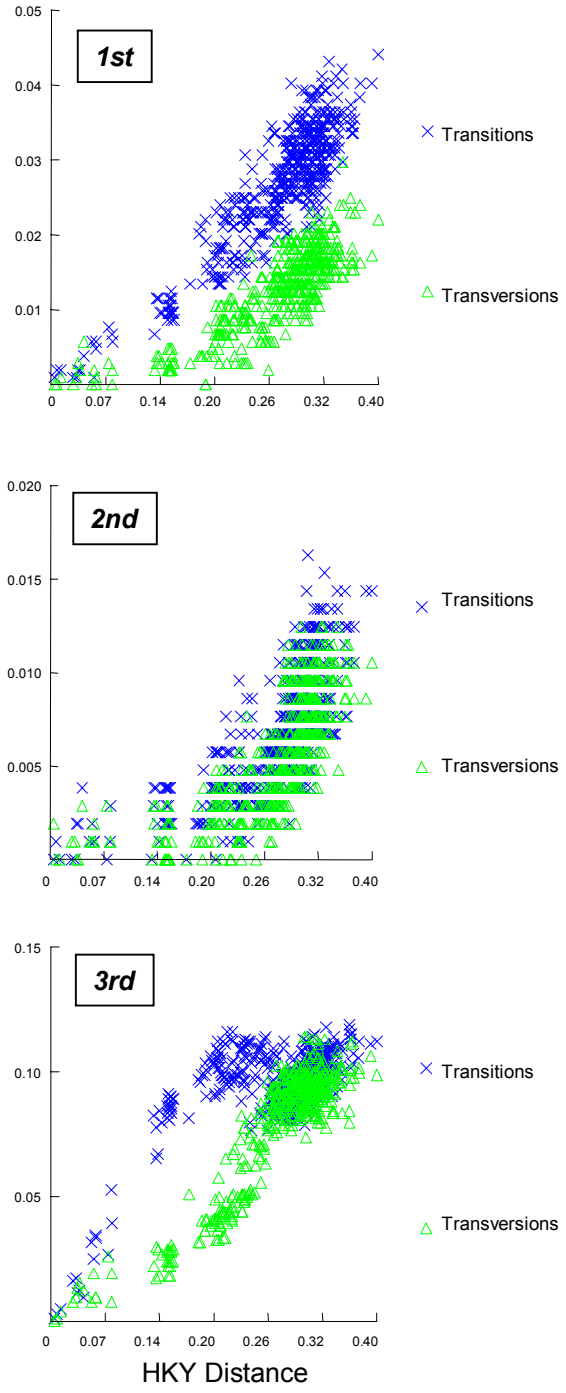

Figure 3. Transitions / transversions plotted against HKY distance for the complete cytochrome *b* gene (1149 bp). Plots were generated by DAMBE V4.0.17 (Xia, 2000). Transitions at third codon positions of cytochrome *b* are saturated.

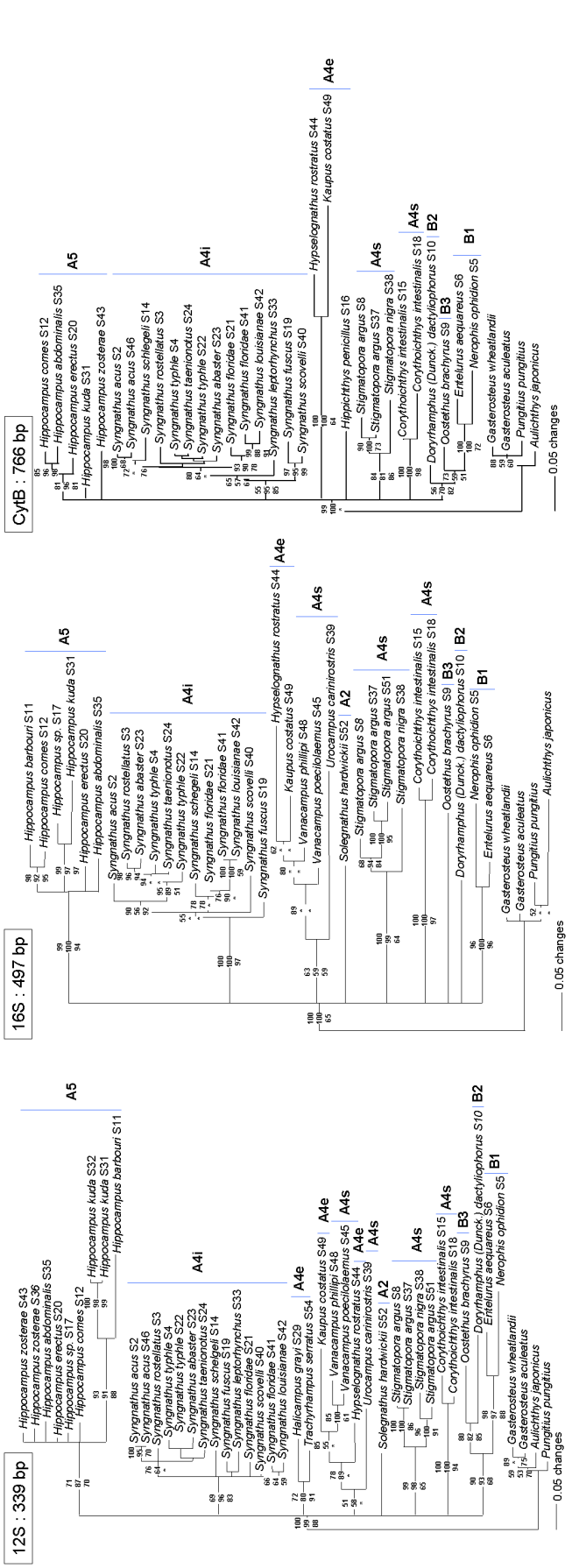

Figure 4. Consensus phylogenetic tree for (a) 12S rDNA; (b)16S rDNA; (c) cytochrome *b* sequence data with branch lengths as estimated from neighbour-joining HKY distances. Numbers on branches represent bootstrap values/puzzling support from distance/parsimony/likelihood analysis (asterixes indicate collapsed branches).

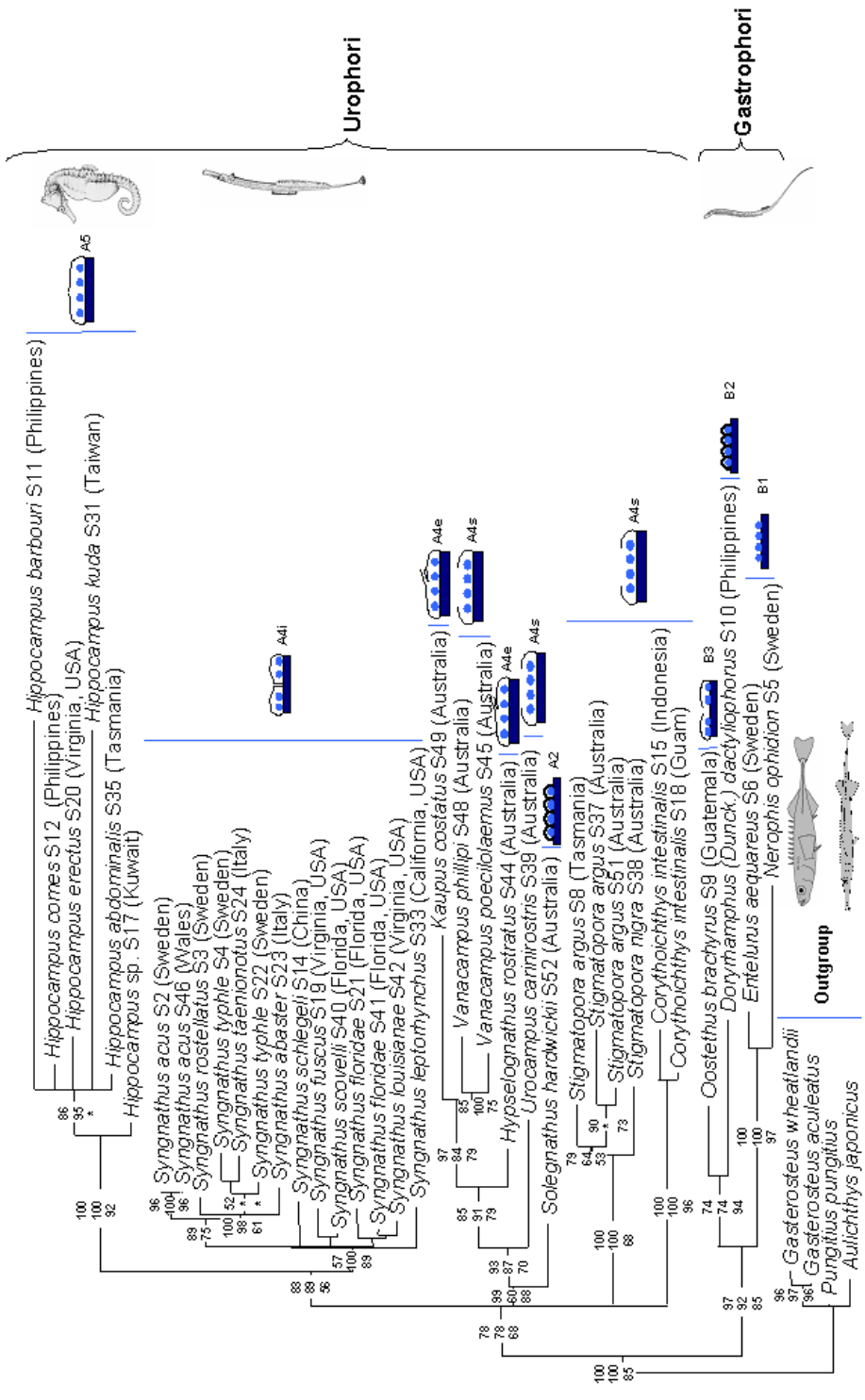

Figure 5. Consensus phylogenetic tree constructed from neighbor-joining distance, maximum parsimony, and quartet puzzling maximum-likelihood analyses based on the combined dataset of 1602 bp with branch lengths as estimated from neighbor-joining distances. Numbers on branches represent bootstrap values/puzzling support from distance/parsimony/likelihood analysis (asterisks indicate collapsed branches). Syngnathid diagrams adapted from Froese and Pauly (2000), Nelson (1994), and Vincent *et al.* (1992)

CHAPTER 5

Correlated evolution of sex-roles and mating systems in male brooding seahorses and pipefishes

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Correlated evolution of sex-roles and mating systems in male brooding seahorses and pipefishes

5.1 ABSTRACT

Modern theory views relative parental investment of the sexes in their young as a key factor responsible for sexual selection. Seahorses and pipefishes (family Syngnathidae) are exceptional amongst fishes in their remarkable adaptations for paternal care and frequent occurrences of sex-role reversals, characterized by female-female competition for access to mates. During mating, the female transfers eggs into specialized egg-brooding structures that are located on either the male's abdomen or its tail, where they are osmoregulated, aerated and nourished by specially adapted structures. All syngnathid males exhibit this form of parental care and the complexity of the brooding structure generally reflects the degree of paternal investment, ranging from the simple ventral gluing areas of some pipefish to the completely enclosed pouches found in seahorses. We present a molecular phylogeny that indicates that the diversification of pouch types is correlated with the major evolutionary radiation of the group, suggesting that this extreme development and diversification of paternal care may have been a key evolutionary innovation of the Syngnathidae. In contrast to the prediction that sex-role reversals should increase with increasing pouch complexity, a parsimony-based reconstruction of the evolution of sex-role reversal in pipefishes and seahorses suggests multiple shifts in sex-roles in the group, independent from the degree of brood pouch development. Further analysis of these data demonstrates that sex-

role reversal in syngnathid fishes is tightly correlated with polygamous systems of mating, supporting the hypothesis that potential reproductive rates may play an important role in defining sex-roles in natural populations.

5.2 INTRODUCTION

In the vast majority of animals, the male's sole contribution to the survival of his offspring are his sperm (Trivers, 1972). As a result, the operational sex ratio is often biased toward males and males almost universally compete more strongly for mates (Darwin, 1871; Emlen & Oring, 1977), while females typically exert greater mate choice. Although most fishes leave their eggs unprotected (Clutton-Brock, 1991), sole male care is the predominant pattern in those species that care for their young (Blumer, 1982). Paternal care is likely to increase offspring fitness, but at the cost of the father's ability to invest in other offspring (i.e. a parental investment *sensu* Trivers, 1972). If male parental investment reduces their potential reproductive rate below that of females, the operational sex ratio (Emlen & Oring, 1977) may become female-biased, resulting in a reversal of traditional sex-roles with females competing more strongly for mates (Clutton-Brock & Parker, 1992; Parker & Simmons, 1996; Kvarnemo & Ahnesjö, 2002). Variation among species in paternal care and variation in the occurrence of sex-role reversals offer unique opportunities to explore hypotheses concerning the relationship between parental investment, sex-roles and sexual selection (Trivers, 1972; Parker & Simmons, 1996).

The family Syngnathidae (pipefishes and seahorses) is characterized by remarkable adaptations for paternal care. The female deposits eggs directly onto a specialized brooding area or into a pouch located under the abdomen or the tail of the male (Breder & Rosen, 1966). This evolutionary innovation ensures males

complete confidence of paternity, but at a level of paternal investment that exceeds that of most other vertebrates (Breder & Rosen, 1966). Thereafter the embryos are nourished, osmoregulated and protected during a lengthy period of male pregnancy (Vincent *et al.*, 1992; Berglund *et al.*, 1986a; Berglund *et al.*, 1989). The complexity of brooding structures ranges, in five steps, from: (1) a simple unprotected ventral area for gluing, (2) individual membranous egg-compartments, (3) protection of eggs in a pouch with pouch plates, (4) bilateral pouch folds that grow together into a closed pouch, to (5) the most complex and completely enclosed brooding pouch of seahorses (Dawson, 1985). There is a further difference among species in that brooding may occur on the tail (Urophori: A-type) or on the abdomen (Gastrophori: B-type) (Herald, 1959).

Parental investment in the true sense of Trivers (1972) is extremely complex to assess, involving the assessment of ultimate fitness costs from expenditures in different currencies (time, energy, predation risks). Although studies on parental investment in syngnathid fishes have been limited, males of species with less complex brooding structures (e.g. *Nerophis ophidion*) spend less energy on their young than do those brooding embryos in enclosed pouches with placenta-like structures (e.g. *Syngnathus typhle*) (Berglund *et al.*, 1986b). Assuming that increasing pouch complexity results in a general increase in male parental investment relative to females, we would expect to see more frequent sex-role reversals in species with more complex brood pouches. Intense mating competition between females should also favour the evolution of sexual dimorphism, where females are larger and more colourful than males. True to predictions, although some species of seahorses and pipefishes retain traditional sex-roles (e.g. most *Hippocampus* spp., *Hippichthys penicillus*), females of

several sex-role reversed species (e.g. *Nerophis ophidion, Stigmatopora nigra, Syngnathus typhle*) are more vividly coloured and striped than males (Table 1) (Berglund *et al.*, 1997; Kvarnemo & Ahnesjö, 1996).

In order to reconstruct the evolution of male brooding structures and to study evolutionary patterns of sex-role reversal in the Syngnathidae, we sequenced three mitochondrial genes from a global sampling of syngnathid species, representing all major male pouch types. If pouch complexity accurately reflects paternal investment, sexual selection theory (Trivers, 1972) predicts that sex-role reversal should be most prevalent in syngnathids with an increased complexity of pouch development. At the same time, we investigate the hypothesis that an increase in potential reproductive rate realized through increased access to mates may also strongly influence sex-roles (Clutton-Brock & Vincent, 1991; Clutton-Brock & Parker, 1992). If mating patterns influence mating competition and impact on sexual selection, we predict a correlation between polygamous mating and sex-role reversals among syngnathid fishes (Vincent *et al.*, 1992).

5.3 MATERIALS & METHODS

Sample Collection, PCR Amplification and DNA Sequencing

We sampled 43 specimens from across the entire geographic range of the family representing all major pouch types (Table 1; for localities, see Fig. 2). In addition, we included three outgroup species from the sticklebacks (family Gasterosteidae): the threespine stickleback (*Gasterosteus aculeatus*: New York, USA), the blackspotted stickleback (*Gasterosteus wheatlandii*: Rhode Island, USA), and the ninespine stickleback (*Pungitius pungitius*: Scotland, UK); and the

Japanese tubesnout (*Aulichthys japonicus*: Kanagawa, Japan) (family Aulorhynchidae). Specimens were preserved in 70% ethanol and total genomic DNA was extracted by proteinase K/SDS digestion and purified by phenol-chloroform extraction and ethanol precipitation (Kocher *et al.*, 1989).

The polymerase chain reaction (PCR) was used to amplify a 484 bp segment of the large subunit (16S) mitochondrial ribosomal gene, a 352 bp segment of the small subunit (12S) mitochondrial ribosomal gene and the complete (1149 bp) mitochondrial gene coding for cytochrome *b*: details of the protocol and the primer sequences are published (Kocher *et al.*, 1989; Palumbi *et al.*, 1991; Taberlet *et al.*, 1992; Pääbo *et al.*, 1991; Wilson *et al.*, 2001). DNA Sequences have been submitted to GenBank (Accession numbers: AF354940-AF355033, AF356040-AF356081, AF356539).

Sequence Alignment and Phylogenetic Reconstruction

The orthologous DNA sequences obtained were aligned, using default settings, by CLUSTALW (Thompson *et al.*, 1994) and optimized by eye. Preliminary sequence analysis revealed saturation of transitions at third codon positions of cytochrome *b*. Subsequent analysis was based on up to 1,602 bp of sequence data for each individual. Neighbour-joining distance and maximum parsimony analyses were performed with PAUPV4b3b (Swofford, 2000), with indels coded as missing data. Quartet puzzling maximum likelihood estimates (25000 puzzling steps) were calculated by TREE-PUZZLE V5.0 (Strimmer & von Haesler, 1996). Parsimony minimal trees were performed with full heuristic searches (500 bootstrap replicates) using random addition (10 replicates), the TBR branch swapping algorithm and the MULPARS option. For parsimony

analyses, a transversion/ transition weighting of two was used. Both neighbour-joining analyses (500 bootstrap replicates) and quartet puzzling maximum likelihood applied a HKY model of substitution (Hasegawa *et al.*, 1985), with transition/transversion ratio (1.88), gamma shape parameter (0.49), proportion of invariable sites (0.30) and nucleotide frequencies (A: 0.3029; C: 0.2360; G: 0.1581; T: 0.3031) estimated from the dataset using Modeltest V3.06 (Posada & Crandall, 1998).

To investigate the evolution of sex-role reversal in the Syngnathidae, distance and parsimony analyses were repeated constraining sex-role reversed, non-role reversed, and outgroup species (Table 1) to be monophyletic groups (backbone constraint under PAUP V4b3b (Swofford, 2000), with all other parameters as outlined above. The branching order of these constrained trees was compared with that of the unconstrained distance and parsimony trees using a likelihood-based Kishino-Hasegawa (KH) test (Kishino & Hasegawa, 1989) with likelihood parameters specified as estimated by Modeltest V3.06 (Posada & Crandall, 1998).

A two-tailed Spearman rank correlation test was used to test for a correlation between brood pouch development (with rankings corresponding to the degree of pouch development (see above)) and sex-role reversal in the family Syngnathidae for those species for which these data were available. The statistical significance of this correlation was tested by comparison with a t-distribution (df=34).

Available empirical data on both sex-roles and mating systems for each species of syngnathid included in our phylogenetic analyses (Table 1) were mapped onto our molecular phylogeny in order to reconstruct the most

parsimonious evolution of each of these characters in the family, using MacClade V3.08a (Maddison & Maddison, 1992) and including all equally parsimonious reconstructions of character evolution. A two-tailed Pearson's correlation coefficient test quantified the correlation between sex-role reversal and mating systems within the Syngnathidae.

5.4 RESULTS

Molecular Phylogeny of Syngnathid Fishes

Mitochondrial sequences of 12S rDNA, 16S rDNA and cytochrome *b* were collected, collated and aligned for 48, 45 and 40 specimens, respectively, resulting in a total sequence length of up to 1,985 bp per specimen. Analyses of cytochrome *b* sequence data revealed third codon saturation of transitions for Kimura-2-parameter distances greater than 0.20. Subsequent analysis was based on up to 1,602 bp of sequence data for each individual.

Concatenated DNA sequences analyzed with neighbor-joining distance, maximum parsimony and quartet-puzzling maximum likelihood analyses resulted in identical topologies for major groups of syngnathid fishes (Fig. 1). All three analyses support monophyly of the Urophori (tail brooding) and Gastrophori (abdominal brooding) lineages of syngnathid fishes. While variable phylogenetic resolution was possible within major lineages, monophyly of *Solegnathus* and *Phyllopteryx* (single egg membrane compartments type A2), *Stigmatopora* and *Corythoichthys* (semi-inverted pouch type A4s), *Syngnathus* (inverted pouch type A4i), and *Hippocampus* (completely enclosed pouch type A5) were all supported by our multi-gene analyses (Fig. 1). Although pouch type variation is evident in a

group of syngnathid pipefishes endemic to Australia (Fig. 1), specific brooding structures are generally restricted to the monophyletic groups outlined above.

Phylogenetic Reconstruction of Sex-Role and Mating System Evolution

Mapping sex-roles on the consensus tree constructed from genetic data suggests between 4-7 changes in sex-roles within the Syngnathidae (Fig. 2a). The log-likelihood estimates of both distance and parsimony trees estimated without the constraint of sex-role monophyly are significantly better than those estimated with sex-role reversed and non-role reversed species constrained as monophyletic lineages (One-tailed KH-test: ln-likelihood for distance trees: -16285.35 vs. –16307.61; MP trees: -16197.35 vs. –16255.88; both significant at $p<0.05$). A two-tailed Spearman rank correlation test also failed to support a relationship between sex-roles and male pouch development (rho=0.288; t-test distribution$_{(df=25)}$, p=0.145). Although our phylogeny indicates that male brooding structures have been remarkably conserved over the evolutionary history of the group (Fig. 1), reversed sex-roles appear to have evolved multiple times, independent of the degree of pouch complexity within the family (Fig. 2a).

We tested further for a correlation between sex-role reversal and mating patterns (Fig. 2b) in syngnathid fishes. We performed a two-tailed Pearson's correlation coefficient test on ecological data on sex-roles and mating patterns collected from the literature (Table 1), Results were highly significant (Pearson $X^2_{(1, n=27)}=20.35$, $p<0.001$, $\Phi=0.66$), indicating a strong correlation between sex-roles and mating patterns in the Syngnathidae, where sex-role reversed species are generally polygamous and species with non-reversed patterns of mating competition are monogamous.

5.5 DISCUSSION

Mitochondrial Phylogeny Supports Parallel Evolution of Major Pouch Lineages

In accordance with the assumed evolutionary significance of male brooding structures, phylogenetic analyses indicate that distinct pouch morphologies each represent monophyletic lineages of species. Our total molecular evidence dataset indicates that each major pouch type arose once in a relatively short burst of morphological change and speciation early in the evolution of the seahorses and their relatives (Fig. 1). Our molecular data also support Herald's (1959) classification of these fishes into tail- and abdominal brooders, demonstrating the independent radiations of morphological structures in the Urophori (A-type: tail pouch) and Gastrophori (B-type: abdominal pouch). Our phylogeny suggests that the primary split between these two lineages occurred at the same time or shortly before the major morphological radiation of male brooding structures and the associated radiation of species. The early diversification of the ancestral syngnathid into tail and abdominal brooders is consistent with results from a karyotypic study, which also highlights a possible total-genome duplication in the abdominal-brooding lineage (Vitturi *et al.*, 1998). Brooding structures within these two lineages independently increased in complexity, culminating in the completely enclosed brood pouches located on the tail of seahorses (Herald, 1959) (type A5) and the well-defined abdominal pouch of *Oostethus brachyrus* (type B3), the most highly developed abdominal pouch type.

Repeated Shifts in Sex-roles

Detailed behavioral and ecological studies of a subset of syngnathid species has revealed substantial variation in patterns of sex-roles (estimated as the predominant competitor for access to mates, where traditional refers to

predominantly male-male competition and sex-role reversal refers to female-female competition) in the family (Vincent *et al.*, 1992). The independence of sex-role reversal and degree of pouch complexity demonstrated in our study suggests that the relationship between parental care, sex-roles and sexual selection in these fishes may not be as straight-forward as predicted. Our conclusions are based on the assumption that pouch complexity accurately reflects relative parental investment. While it is likely that energy expenditures are correlated with increasing pouch complexity (Berglund *et al.*, 1986b), the assessment of true parental investment (*sensu*-Trivers (1972)) is more complex, involving the ultimate fitness costs of both energy- and time-expenditures in both sexes. Independent of this assumption, however, it is clear that it is not possible to map sex-roles on the consensus phylogeny of syngnathids without allowing multiple appearances of role reversal (Fig. 2a; see results of KH test above).

Our results are, at the same time, consistent with recent studies which demonstrate that sex-roles may be flexible and influenced by environmental factors (Kvarnemo & Ahnesjö, 1996; Ahnesjö *et al.*, 2001; Kvarnemo & Ahnesjö, 2002). Studies on the sex-role reversed pipefish *Syngnathus typhle* illustrate how an environmental factor may influence the intensity of mating competition. During a warm breeding season, males have shorter pregnancies and are available more frequently for mating than under colder ambient water temperatures (Vincent *et al.*, 1994). Consequently, even though females always compete for access to mates, they will compete even more intensely when temperatures are reduced, due to a more female-biased operational sex ratio caused by the extended period of male pregnancy (Vincent *et al.*, 1994; Ahnesjö, 1995). Similar environmental influences on sex-roles have been demonstrated in other ectothermic animals

(Kvarnemo & Ahnesjö, 1996; Kvarnemo & Ahnesjö, 2002). While our results are consistent with the view that sex-roles are flexible and influenced by environmental factors at the species and even the population level (Kvarnemo & Ahnesjö, 1996; Ahnesjö *et al.*, 2001; Kvarnemo & Ahnesjö, 2002), there is a intriguing consistency in the similarity of sex-roles and pouch type at higher taxonomic levels (e.g. *Syngnathus* and *Hippocampus*) (Table 1; Figs. 1, 2a).

Correlated evolution of sex-roles and mating patterns

While male pouch development does not predict the presence of sex-role reversal in the Syngnathidae, analysis of our data highlights a strong correlation between sex-role reversal and mating patterns in syngnathid species. Syngnathids exhibiting sex-role reversal are generally polygamous, whereas those species with non-reversed patterns of mating competition are monogamous. Vincent et al. (1992) have suggested that, as a polygamous mating system may increase the reproductive rate of males and skew the operational sex ratio, a shift from a monogamous to a polygamous system of mating may influence the intensity of sexual selection and help to shape sex-roles in the wild. Although a skewed operational sex ratio or differences in quality of potential mates may create competition for mates in monogamous species, considerable mating competition in polygamous animals generally results in more extensive sexual dimorphisms as compared to monogamous species (Andersson, 1994). This close correlation between sex-role reversal and multiple mating has also been identified in several species of the avian orders Gruiformes and Charadriiformes (Emlen & Oring, 1977), highlighting the potential significance of polygamous mating patterns on sex-roles in natural populations.

5.6 CONCLUSIONS

Our molecular data demonstrate a rapid radiation of seahorses and pipefishes, leading to extensive morphological divergence in lineages with diverse and highly specialized adaptations for paternal care. In syngnathids, the evolution of increased pouch development occurred in parallel in both abdominal and tail-brooding lineages, leading to the remarkable diversity of male brooding structures observed today. At the same time, multiple evolution of sex-role reversal within the family Syngnathidae suggests that brood pouch complexity may not directly reflect relative parental investment, or that the relationship between pouch complexity and the sexual difference in potential reproductive rates (and thereby the operational sex ratio) is more dynamic rather than being a direct consequence of their brooding structure. In contrast, the strong association between sex-roles and mating patterns in syngnathid species highlights the relationship between polygamous mating and sex-role reversal (Vincent & Sadler, 1995). Clearly there are evolutionary benefits of the specialized adaptations for paternal brood care in the Syngnathidae family, which may, by the influence on the sexual difference in potential reproductive rates, result in a reversal of sex-roles (Berglund *et al.*, 1989). At the same time, there are also important environmental influences on potential reproductive rates, operational sex ratios and mating patterns which may have dramatic influences on sexual selection in the wild (Kvarnemo & Ahnesjö, 1996; Kvarnemo & Ahnesjö, 2002).

Table 1. Syngnathid species and pouch type[1], with sex-role (traditional: male-male competition for mates; reversed: female-female competition for mates) and mating pattern from literature.

Species	Pouch type	Sex-role	Mating pattern
Hippocampus abdominalis	A5	Traditional (Vincent *et al.*, 1992)	Monogamous (Vincent *et al.*, 1992)
Hippocampus barbouri	A5	Unknown	Unknown
Hippocampus comes	A5	Traditional (Lourie *et al.*, 1999)	Monogamous (Lourie *et al.*, 1999)
Hippocampus erectus	A5	Unknown	Unknown
Hippocampus fuscus	A5	Traditional (Vincent, 1994)	Monogamous (Vincent, 1994)
Hippocampus kuda	A5	Unknown	Unknown
Hippocampus reidi	A5	Traditional (Vincent *et al.*, 1992)	Monogamous (Lourie *et al.*, 1999)
Hippocampus subelongatus	A5	Traditional (Kvarnemo *et al.*, 2000)	Monogamous (Jones *et al.*, 1998)
Hippocampus whitei	A5	Traditional (Vincent & Sadler, 1995)	Monogamous (Vincent & Sadler, 1995)
Hippocampus zosterae	A5	Traditional (Masonjones & Lewis, 2000)	Monogamous (Masonjones & Lewis, 2000)
Syngnathus abaster	A4i	Reversed (Fiedler, 1955)	Polygamous (Fiedler, 1955)
Syngnathus acus	A4i	Reversed (Vincent *et al.*, 1995)	Polygamous (Vincent *et al.*, 1995)
Syngnathus floridae	A4i	Reversed (Jones & Avise, 2001)	Polygamous (Jones & Avise, 2001)
Syngnathus fuscus	A4i	Reversed (Roelke & Sogard, 1993)	Unknown
Syngnathus leptorhynchus	A4i	Reversed (Bayer, 1980)	Polygamous (Bayer, 1980)
Syngnathus louisianae	A4i	Unknown	Unknown
Syngnathus rostellatus	A4i	Reversed (Berglund *et al.*, 1988)	Polygamous (Vincent *et al.*, 1995)
Syngnathus schlegeli	A4i	Reversed (Watanabe *et al.*, 2000)	Polygamous (Watanabe & Watanabe, 2001)
Syngnathus scovelli	A4i	Reversed (Jones & Avise, 1997)	Polygamous (Jones & Avise, 1997)
Syngnathus taenionotus	A4i	Unknown	Unknown
Syngnathus typhle	A4i	Reversed (Berglund *et al.*, 1986b)	Polygamous (Vincent *et al.*, 1992)
Hippichthys penicillus	A4e	Traditional (Watanabe *et al.*, 1997)	Monogamous (Watanabe *et al.*, 1997)
Hypselognathus rostratus	A4e	Unknown	Unknown
Kaupus costatus	A4e	Unknown	Unknown
Urocampus carinirostris	A4e	Unknown	Unknown
Pugnaso curtirostris	A4s	Unknown	Unknown
Vanacampus phillipi	A4s	Unknown	Unknown
Vanacampus poecilolaemus	A4s	Unknown	Unknown
Stigmatopora argus	A4s	Reversed (Kendrick, pers. comm.)	Polygamous (Vincent *et al.*, 1992)
Stigmatopora nigra	A4s	Reversed (Kendrick, pers. comm.)	Polygamous (Vincent *et al.*, 1992)
Corythoichthys intestinalis	A4s	Traditional (Gronell, 1984)	Monogamous (Gronell, 1984)
Corythoichthys haematopterus	A4s	Reversed (Matsumoto & Yanagisawa, 2001)	Monogamous (Matsumoto & Yanagisawa, 2001)
Phyllopteryx taeniolatus	A2	Unknown	Unknown
Solegnathus hardwickii	A2	Unknown	Unknown
Entelurus aequareus	B1	Reversed (Vincent *et al.*, 1995)	Polygamous (Vincent *et al.*, 1995)
Nerophis ophidion	B1	Reversed (Berglund *et al.*, 1986b)	Polygamous (Berglund *et al.*, 1986b)
Doryrhamphus (Dunckerocampus) chapmani	B2	Traditional (Vincent *et al.*, 1992)	Monogamous (Vincent *et al.*, 1992)
Doryrhamphus (Dunckerocampus) dactyliophorus	B2	Unknown	Unknown
Doryrhamphus (Doryrhamphus) excisus	B3	Unknown	Unknown
Doryrhamphus (Doryrhamphus) japonicus	B3	Traditional (Vincent *et al.*, 1992)	Monogamous (Vincent *et al.*, 1992)
Oostethus brachyrus	B3	Unknown	Unknown

1. Pouch type legend – A=tail-brooder; B=abdominal-brooder; 1-5=increasing pouch complexity (see text); I=inverted pouch folds, e=everted pouch folds, s=semi-inverted pouch folds (only applicable to the A4 pouch type).

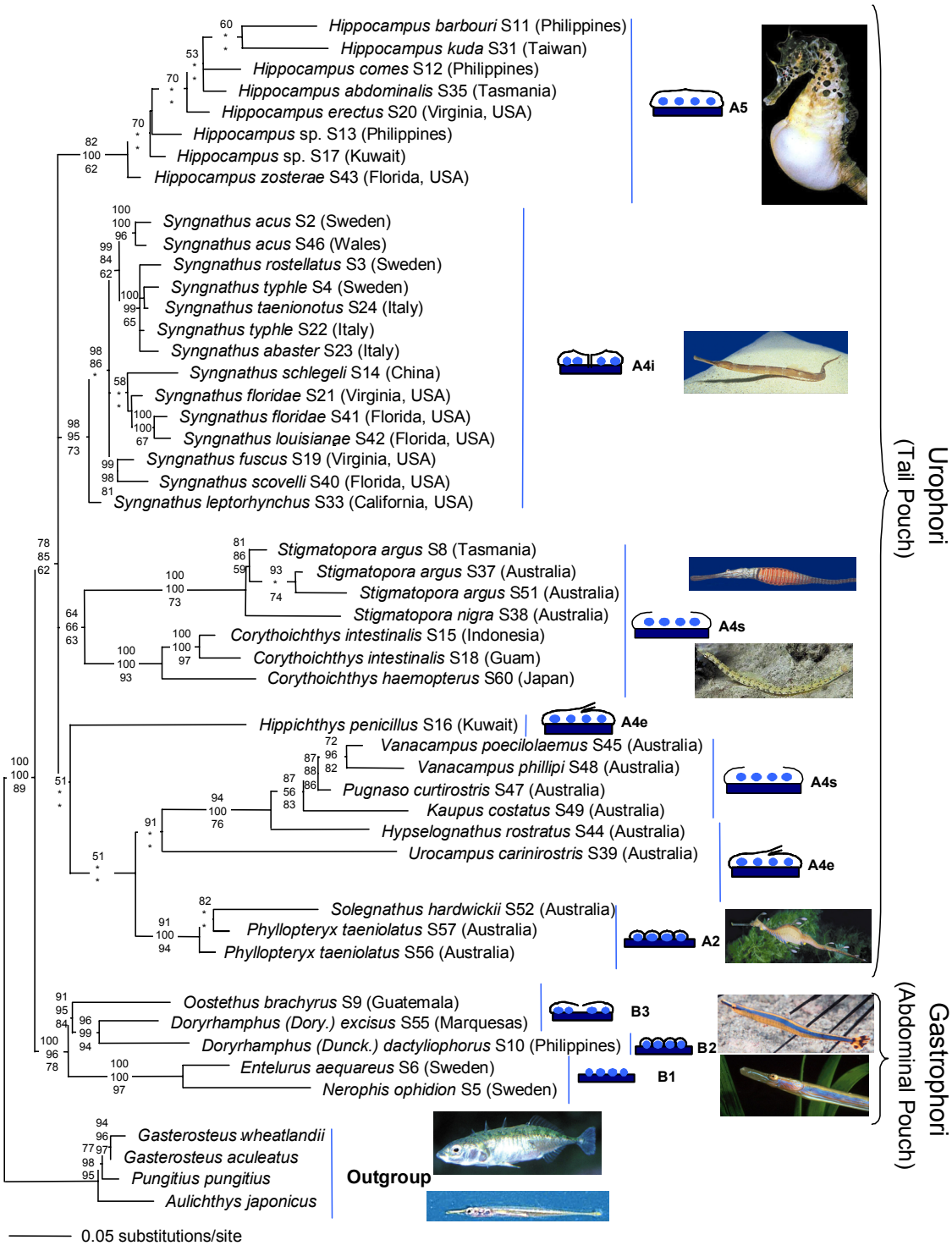

Figure1. Consensus phylogenetic tree constructed from neighbour-joining distance (Saitou & Nei, 1987), maximum parsimony (Swofford & Berlocher, 1987) and quartet puzzling maximum likelihood (Strimmer & von Haesler, 1996) analyses based on the combined dataset of 1,602bp with branch lengths as estimated from neighbour-joining HKY + I + Γ distances. Numbers on branches represent bootstrap values from distance/parsimony/likelihood analysis (asterixes indicate collapsed branches). Photographs reprinted with permission from Kuiter (2001).

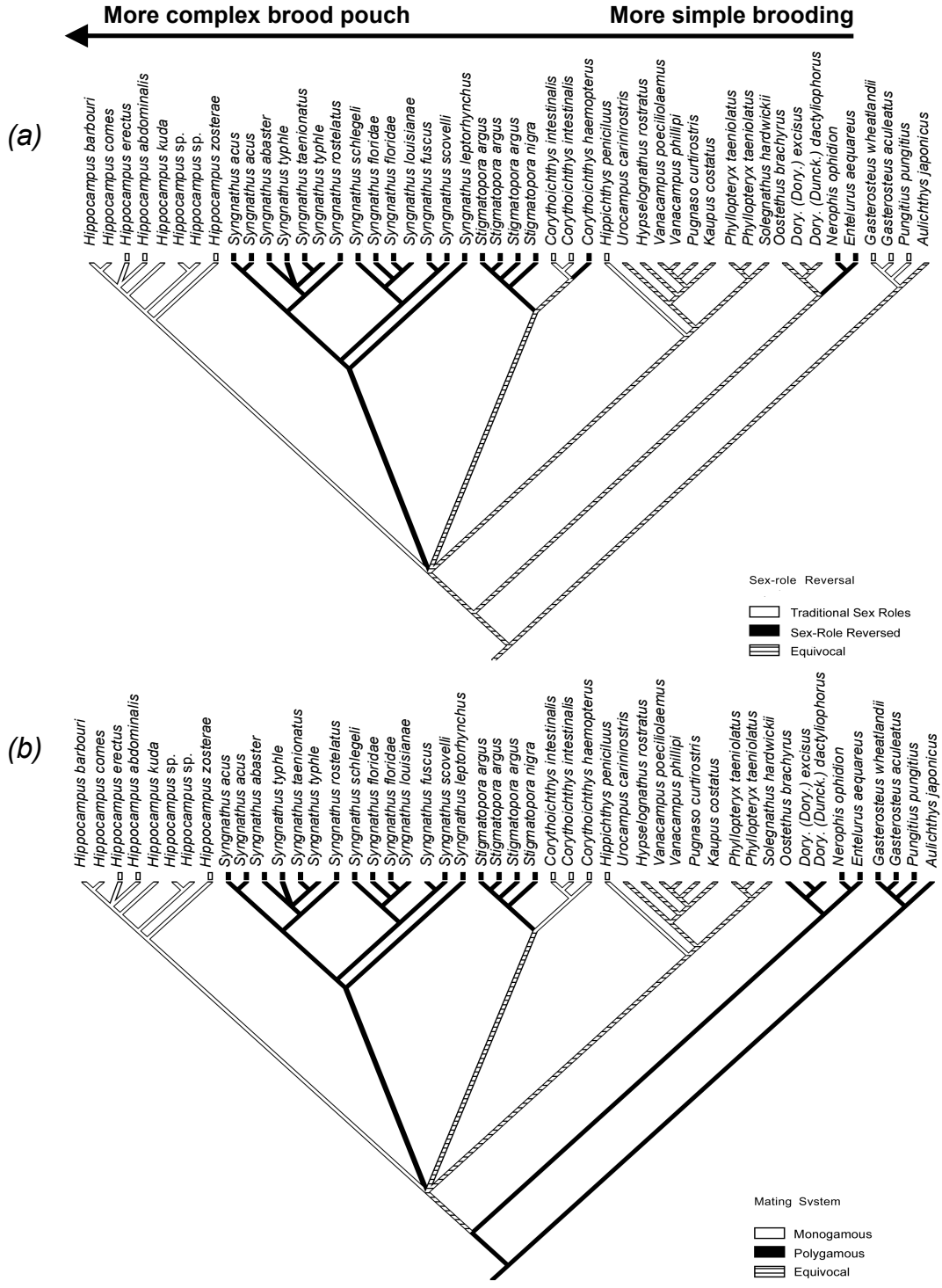

Figure 2. Most parsimonious reconstruction of (a) sex-role reversal and (b) mating system mapped on the consensus phylogenetic tree (Fig. 1) using MacClade V3.08a (Maddison & Maddison, 1992) with empirical data coded where available. Character states for outgroups from references in McLennan *et al*. (1988) The most parsimonious reconstruction of these two characters involves multiple independent origins of sex-roles (4 steps) and mating systems (3 steps) within the Syngnathidae. Sex-role reversal is uncorrelated with degree of male brood pouch development (Two-tailed spearman rank correlation test (p=0.102).

CHAPTER 6

Ancient lakes as evolutionary reservoirs: Evidence from the thalassoid gastropods of Lake Tanganyika

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Ancient lakes as evolutionary reservoirs: Evidence from the thalassoid gastropods of Lake Tanganyika

6.1 ABSTRACT

Ancient lakes are often collectively viewed as evolutionary hotspots of diversification. East Africa's Lake Tanganyika has long been the subject of scientific interest due to dramatic levels of endemism in species as diverse as cichlid fishes, paludomid gastropods, decapod and ostracod crustaceans and poriferan sponges (Coulter, 1991). As the largest and deepest of the African rift lakes, its endemic fauna has been presented with a stable inland environment for over 10 MY (Cohen *et al.*, 1993), offering unique opportunities for within-lake diversification. Although astonishing diversification has been documented in the endemic cichlid fauna of the lake (Nakai *et al.*, 1994; Rossiter, 1995; Verheyen *et al.*, 1996; Sturmbauer *et al.*, 1994), similar patterns of rapid diversification have long been assumed for other groups. In contrast to this hypothesis of rapid speciation, there is no evidence for an accelerated rate of diversification in the endemic thalassoid gastropods of the lake. While within-lake speciation has occurred, the dramatic diversity of gastropods presently found within the lake has evolved from at least five major lineages that pre-date its formation. At the same time, a widespread group of African gastropods appears to have evolved from taxa presently found in the lake. While Lake Tanganyika has been a cradle of speciation for cichlid fishes, the lake has also been an

important evolutionary reservoir of gastropod lineages that have been extirpated outside the basin.

6.2 INTRODUCTION

The fossil gastropods of East Africa have served as some of the strongest empirical evidence supporting the theory of punctuated equilibria, the idea that evolution occurs in rapid bursts of change that are often followed by long periods of evolutionary stasis (Eldredge & Gould, 1972). Williamson's classic study on the molluscs of the Turkana Basin (Williamson, 1981) (Fig. 1) attracted critical interest due to his contention that the system provided strong evidence of punctuated equilibria operating across multiple lineages. While this work provoked considerable debate (Fryer *et al.*, 1983; Cohen & Schwartz, 1983; Kat & Davis, 1983), recent studies on the Lusso Beds of Zaire (Morris, 1996) and the Lake Albert Basin (Van Damme *et al.*, 2001) (Fig. 1) support the idea that rates and patterns of change in African gastropods have deviated significantly from a model of phyletic gradualism. Although these studies are provocative, detailed investigations of extant lineages of African gastropods are clearly necessary for a more general understanding of patterns and processes driving evolution in aquatic ecosystems (Benton & Pearson, 2001; Glaubrecht, 1999).

Speke and Burton's legendary expeditions in search of the source of the Nile resulted in the discovery of the East African Lake Tanganyika in 1858 (Burton, 1860). Even before the first published accounts of the Lake's discovery appeared, Speke's collections of gastropods from Tanganyika attracted considerable scientific interest (Woodward, 1859), and further collections highlighted the particularly spectacular diversity (25-70 species

(West & Michel, 2000; Brown, 1994); 62% endemism) and thalassoid (marine-like) form of the gastropods of the Lake (Fig. 2) (Moore, 1897; Smith, 1906). The identification of marine-like gastropods, with spines, strong shells and morphologies uncommon among freshwater snails, in Lake Tanganyika led to the suggestion that the lake was once directly connected to the ocean (Moore, 1898; Moore, 1903; Nicolas, 1898). However, more recent geological data indicate that the lake was formed by rifting in the African sub-continent and could not have had oceanic ancestry (Tiercelin & Mondeguer, 1991; Cohen *et al.*, 1993; Cohen *et al.*, 1997). While the cichlid fishes of the East African Great Lakes have long been recognized as key model organisms in the study of rapid evolution in freshwater, virtually nothing is known about the evolution of the other members of the rich aquatic fauna, and, as a result, these lakes have often been viewed collectively as “evolutionary hotspots” (Coulter, 1991; Rossiter, 1995). Although it is clear that Lake Tanganyika is home to a disproportionately large number of freshwater endemics (Coulter, 1991), understanding the evolutionary history of these animals is critical to clarify the relative importance that the lacustrine environment and intrinsic properties of the animals themselves have played in their evolution.

6.3 MATERIALS & METHODS

Sample Preparation and DNA Amplification

Specimens were collected by hand using SCUBA, sorted and cracked and preserved in 70% EtOH. DNA was extracted from ethanol-preserved foot tissue using a CTAB protocol (Winnepenninckx *et al.*, 1993). The polymerase chain reaction (PCR) was used to amplify a 944 bp segment of the large

subunit (16S) mitochondrial ribosomal gene and a 658 bp fragment of the mtDNA gene coding for cytochrome oxidase subunit I (COI): details of the protocol and the primer sequences for amplifying COI have been published (Folmer *et al.*, 1994). Long range PCR (using the Expand Long Template PCR System (Boehringer Mannheim)) was used with previously published primers 12SA (Kocher *et al.*, 1989) and 16SBr (Palumbi *et al.*, 1991) to amplify a 1967 bp fragment from a subset of gastropod individuals. These sequences were used to design an internal primer pair with widespead applicability among Tanganyikan gastropods (16SF: 5'-CCGCACTAGTGATAGCTAGTTTC-3', H3059: 5'-CCGGTYTGAACTCAGATCATGT-3'). Sequences have been deposited in GENBANK under accession numbers:

Phylogenetic Analyses

The orthologous DNA sequences obtained were aligned, using default settings, by CLUSTALW (Thompson *et al.*, 1994) and optimized by eye. Third-codon positions of cytochrome oxidase were saturated at distance values greater than 0.15 and were eliminated from further analyses. Neighbor-joining distance, quartet-puzzling maximum likelihood (Strimmer & von Haesler, 1996) and maximum parsimony analyses were performed on the combined dataset of 1021 bp using PAUPV4b3b (Swofford, 2000), with indels coded as missing data. Bootstrapped neighbor-joining (500 replicates) and quartet-puzzling likelihood analyses (10000 puzzling steps) were performed under a GTR + Γ + I model of substitution with parameters estimated by ModeltestV5.0 (Posada & Crandall, 1998). Parsimony minimal trees were performed with full heuristic searches (500 bootstrap replicates) using random

addition (10 replicates), the TBR branch swapping algorithm and the MULPARS option.

Phylogenetic relationships were also approximated following a Bayesian method of phylogenetic inference as implemented by MrBayesV2.01 (Huelsenbeck & Ronquist, 2001). Posterior probabilities of phylogenetic trees were estimated by a 100000 generation Metropolis-coupled Markov chain Monte Carlo (4 chains, chain temperature=0.2) under the GTR + Γ + I model of substitution, with parameters estimated from the dataset. A 50% majority-rule consensus tree was constructed following a 25000 generation burn-in to allow likelihood values to reach stationarity.

Molecular Clock

While significant rate heterogeneity was observed for the COI gene fragment across multiple lineages (branch length X^2 test (Takezaki *et al.*, 1995)), a test of branch length as implemented by LINTREE (Takezaki *et al.*, 1995) highlighted only two taxa that deviated from a molecular clock based on the 16S dataset. Following the elimination of these taxa, genetic distances were recalculated for the 16S dataset with a molecular clock constraint (Takezaki *et al.*, 1995) and a molecular calibration for transitions and transversions of 16S rDNA of caenogastropods (Reid *et al.*, 1996) was used to estimate a molecular clock for Tanganyikan snails.

Modeling lineage diversification

A lineage-through-time plot was constructed from the linearized 16S phylogenetic tree. While *Lavigeria limnaea* exhibited a reduced rate of cladogenesis based on the relative cladogenesis statistic (Nee *et al.*, 1994b), the remainder of the phylogeny did not exhibit significant variation in

speciation and extinction rates among lineages. A Monte Carlo constant-rates test (Pybus & Harvey, 2000) failed to reject the null hypothesis of constant speciation and extinction rates through time for the phylogeny (critical value of γ (at 5% value) for 23 species randomly sampled from a phylogeny containing 100 species (25000 replicates) = -3.182860 (Pybus, 2000); γ-statistic calculated for phylogeny shown in Figure 3: -0.181; P>0.95).

6.4 RESULTS & DISCUSSION

Molecular phylogenetic analyses were undertaken on samples of gastropods collected from sites located in the southern basin of Lake Tanganyika and neighboring Lake Mweru in 1999 and 2000. Individual analyses of mitochondrial cytochrome oxidase I (COI) and large subunit (16S) rDNA gene fragments show essentially the same phylogenetic pattern and reflect the large distances (average HKY distances: COI=0.196; 16S=0.112) separating Tanganyikan gastropods (data not shown). Better resolution of basal relationships among gastropods is provided by 16S rDNA, suggesting that, as previously discussed (West & Michel, 2000), COI may not provide adequate resolution of relationships among Tanganyikan gastropods. While an explosive within-lake radiation of Tanganyikan gastropods has been proposed based on COI data (West & Michel, 2000), multi-gene analyses indicate that these conclusions may have been biased by their choice of molecular marker.

Analysis of the combined 16S and COI dataset is consistent with individual gene analyses and supports five major clades of Tanganyikan gastropods (Fig. 2). These major lineages as delineated by molecular data

correspond closely to conchological (shell morphology)-based taxonomic groupings of Tanganyika taxa (Strong & Glaubrecht, unpub. data) and include: (1) the minute Syrnolopsidae with *Syrnolopsis, Anceya,* and *Martelia;* (2) profundal taxa *Limnotrochus, Tiphobia* and *Paramelania*; (3) a nearshore rock-dwelling group including *Spekia*, *Bridouxia*, *Stormsia*, *Cleopatra* and *Reymondia*; (4) the *Lavigeria* species flock (Michel, 2000); and (5) *Lavigeria limnaea*, a species found in rock and cobble that is highly genetically divergent from other members of the genus (Fig. 2).

Representatives of both the Thiaridae *sensu stricto* (*Melanoides* spp.) and Paludomidae were included in the analysis as outgroup species. Interestingly, although *Paludomus siamensis* (Thailand, Asia) clusters at the base of the Tanganyikan species, *Cleopatra johnsoni*, a paludomid found in nearby Lake Mweru, clusters within the Tanganyika group (Fig. 2). *Cleopatra ferrugina*, a cosmopolitan African species, also clusters within the Tanganyikan taxa (West & Michel, 2000). Taken together, these data suggest that *Cleopatra*, a genus which is now widely distributed in Africa, may have originated in Lake Tanganyika and have reached its current range of distribution in as little as 8 million years (see molecular clock analyses below). The placement of *Cleopatra* within the Tanganyikan endemics supports recent work that suggests that the Tanganyikan gastropods are not Thiaridae *sensu stricto* as often assumed in the literature (West & Michel, 2000; Brown, 1994), but should instead be recognized as members of the Paludomidae, a distinct gastropod family with an oriental distribution and exceptionally wide range of shell variation (Glaubrecht, 1999; Strong & Glaubrecht, 2002).

Reflection seismic-radiocarbon dating work indicates that the Tanganyika basin began to form as a series of swamupy protolakes between 9 and 12 MYA and developed into a large lacustrine basin between 5 and 6 MYA (Cohen *et al.*, 1993; Cohen *et al.*, 1997). In an effort to clarify the importance of within-lake diversification, we conservatively assume that snails present in these protolakes 12 MYA gave rise to the present-day diversity of Tanganyika gastropods. After calculating standard errors of molecular evolution for transitions and transversions of the 16S rDNA gene, up to five major lineages still clearly predate the formation of Lake Tanganyika by as much as 10 million years (Fig. 3).

Although phylogenetic trees reflect relationships among extant taxa, lineage-through-time plots offer a means to detect differential proliferation of lineages through evolutionary time (Goldstein & Harvey, 1999). A lineages-through-time plot of diversification in endemic Tanganyikan gastropods does not deviate significantly from a constant rates process with no extinction (Goldstein & Harvey, 1999), indicating that the unique environment of Lake Tanganyika has not led to a significant acceleration in lineage splitting and speciation in its endemic gastropod fauna. While additional taxa remain to be described from the Tanganyika basin (Michel, 1994), theoretical models demonstrate that a lineages-through-time plot including as few as 10% of extant taxa may be adequate for the estimation of speciation and extinction coefficients from phylogenetic data (Nee *et al.*, 1994a).

While a poor fossil record has made it difficult to identify the origins of the Tanganyikan cichlid radiation, our evidence indicates that a large proportion of the molecular diversity of the endemic gastropods presently

found in Lake Tanganyika predates the formation of the lake basin and ancient diversification events correlate with the majority of the ecological diversity presently found in the Lake. While it is clear that diversification has taken place since the formation of Lake Tanganyika, the conservative morphological evolution of the *Lavigeria* gastropods (Michel, 2000), coupled with the lack of evidence of conchological intermediates, strongly suggests that morphological differentiation has not been a major engine of speciation in Tanganyikan gastropods. As Lake Tanganyika is believed to have been divided into at least three major basins during its history and has experienced repeated episodes of desiccation (Tiercelin & Mondeguer, 1991; Cohen *et al.*, 1997), allopatric speciation may at least partially explain the within-lake diversification of the endemic gastropods, as has been demonstrated for cichlid fishes (Verheyen *et al.*, 1996). The identification of gastropod fossils from two now-endemic Tanganyikan genera (*Lavigeria* and *Neothauma*) outside the lake basin (Fuchs, 1936; Cox, 1939), coupled with the identification of new species affinities with *Cleopatra* (see above) indicates that species that are now restricted to the lake once had distributions extending beyond the Tanganyika basin and suggests that Lake Tanganyika has played an important role as an evolutionary reservoir of ancient lineages that have since spread outside the lake to colonize surrounding waterways.

The long-term persistence of the Tanganyikan gastropod fauna may be due to the extreme depth and stability of this unique aquatic environment**.** If this is the case, then within-lake predator-prey coevolution (West & Cohen, 1994) is unlikely to explain the present-day species-level diversity of the Tanganyikan gastropods. Alternatively, as major changes in gastropod

morphology need not necessarily reflect speciation (Palmer, 1985), the unique lacustrine environment may have contributed to the evolution of unique morphologies in the absence of an increase in speciation rates. Our data suggest that the unique lacustrine environment has not fueled a major adaptive radiation (Schluter, 2000) of gastropod species. While the Great Lakes of East Africa have served as evolutionary hotspots for the diverse flocks of cichlid fishes, Lake Tanganyika has also been an evolutionary reservoir of an ancient group of gastropods that have since been extirpated outside the lake. The extreme depth and stability of this inland sea have played an important role in the persistence of these living fossils.

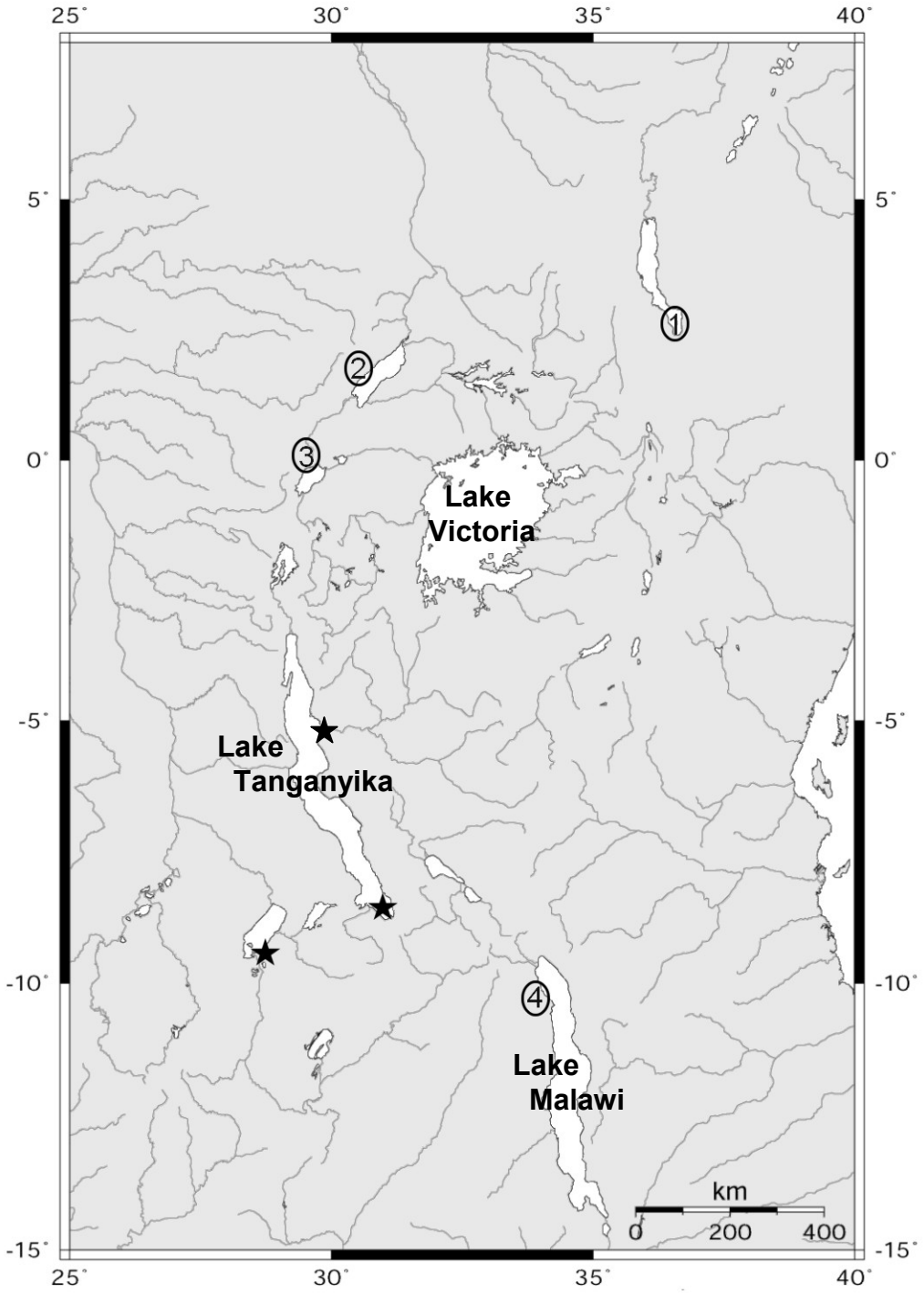

Figure1. Map of Lake Tanganyika and major river systems. 34 samples, representing 19 species, were collected from sites near the southern end of Lake Tanganyika, from the Malagarasi Delta and from neighboring Lake Mweru (indicated with stars). Archived samples and data on specimen collections are housed at the Museum für Naturkunde (Berlin). Sites of recent fossil gastropod investigations: 1) Lake Turkana (Williamson, 1981); 2 & 3) Albertine Basin (Morris, 1996; Van Damme & Pickford, 1999; Van Damme *et al.*, 2001) and 4) Lake Malawi (Van Damme *et al.*, 2001).

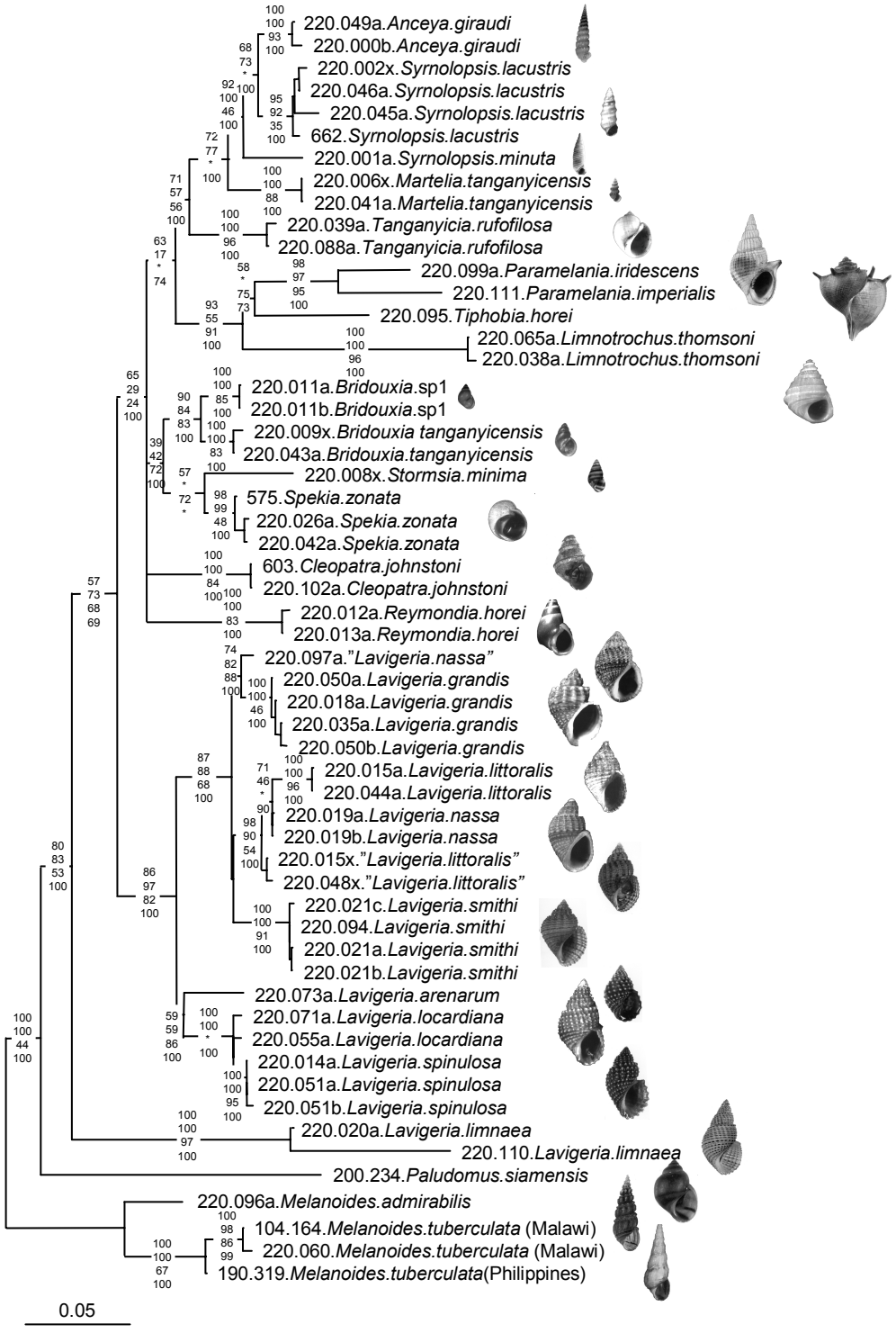

Figure 2. Combined 16S and COI mitochondrial gene analysis constructed from neighbor-joining distance (Saitou & Nei, 1987), maximum parsimony (Swofford & Berlocher, 1987) , quartet puzzling maximum likelihood (Strimmer & von Haesler, 1996), and Bayesian inference of phylogeny (Huelsenbeck & Ronquist, 2001) with branch lengths as estimated from neighbor-joining distances. Numbers on branches represent bootstrap values/puzzling support (likelihood)/posterior probabilities (Bayesian analysis) from distance/parsimony/likelihood/ Bayesian reconstructions of phylogeny (asterixes indicate collapsed branches).

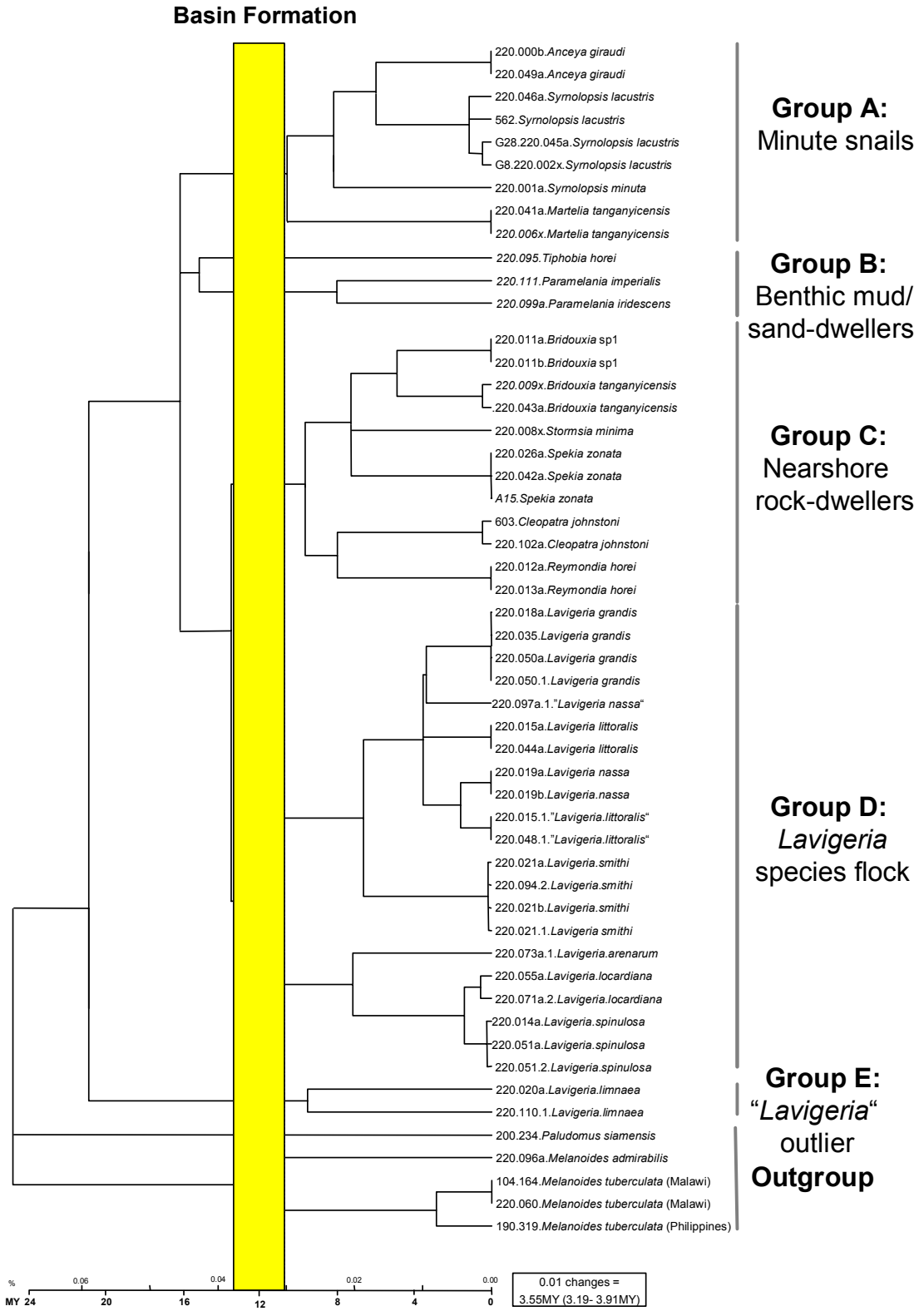

Figure 3. Linearized tree for 423bp of 16S alignment based on Kimura-2-Parameter distances constructed using MegaV2.1 (Kumar *et al.*, 2001), using molecular clock calibration for caenogastropods (Reid *et al.*, 1996).

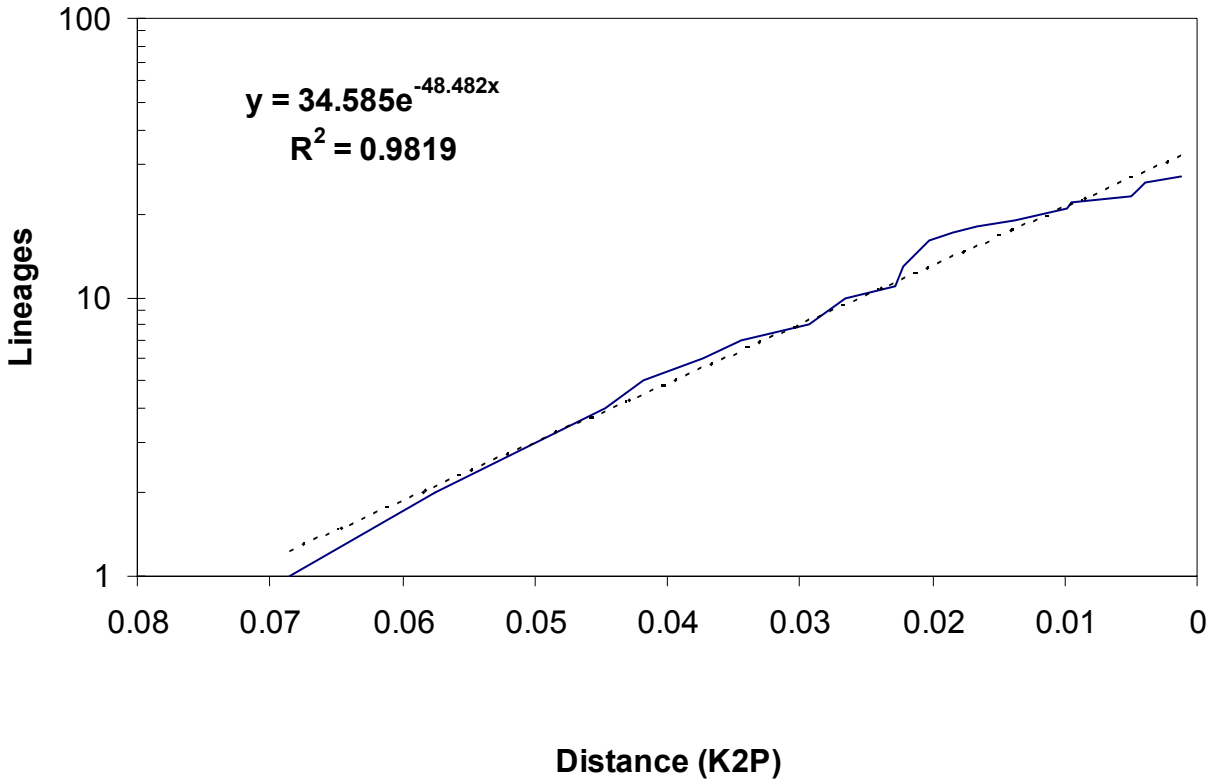

Figure 4. Lineages-through-time plot (Nee *et al.*, 1992) for species of endemic Tanganyikan gastropods constructed from linearized phylogenetic tree (Fig. 3). Dashed line represents best-fit curve (R2=0.9819; P<0.001).

CHAPTER 7

Discussion – The Way Forward

DISCUSSION

Modern molecular methods have revolutionized the study of evolution. Never before have we been able to gain such intimate insights into the patterns and processes underlying evolutionary biology. However, with new technologies come new challenges - long-held paradigms have and continue to change. The present thesis illustrates several applications of molecular methods to questions that are currently in debate amongst evolutionary biologists, namely, i) What is the role of sympatric speciation in evolution?; ii) How important are mechanisms of sexual selection in the speciation process? And iii) Are micro-evolutionary patterns adequate for the explanation of macro-evolutionary trends?

Sexual Selection and Sympatric Speciation in the Midas Cichlid –

Although both sexual selection and ecological partitioning have been implicated in the speciation process, this study on sympatric speciation in the Midas cichlid, *Amphilophus citrinellus*, found significant population-level differentiation only in coloration, suggesting that sexual selection may be playing a more important role during the early stages of sympatric speciation in this species (Chapter 3). These data are consistent with research on the haplochromine cichlid species flock of Lake Victoria, Africa, which suggests that changes in male nuptial coloration influenced by sexual selection has played an important role in the rapid radiation of these species (Seehausen *et al.*, 1999; Seehausen & van Alphen, 1999). Sexual selection has also been shown to be important in reproductive isolation of cichlids endemic to Lake Malawi (Turner, 1994; Knight *et al.*, 1998).

In contrast, work on the crater lakes of Cameroon indicates that ecological diversification may have played a more important role in the initial stages of speciation in the endemic cichlid species flocks of Lakes Bermin and Barombi Mbo (Schliewen *et al.*, 1994) and Lake Ejagham (Schliewen *et al.*, 2001). Molecular study of the Eretmodine cichlids of Lake Tanganyika also suggests that trophic specialization has been critical to the adaptive radiation of these species (Ruber *et al.*, 1999). A recent review by Danley and Kocher (2001) highlights that trophic partitioning and sexual selection have both independently contributed to at least three separate radiations of cichlids in Lake Malawi. A divergence with gene flow model of speciation appears well suited to explain the diversification of Malawian cichlids (Danley & Kocher, 2001).

These results highlight how sexual selection and resource specialization can both contribute to the sympatric diversification of aquatic species, even within a single family of fishes. Theoretical studies illustrate that both assortative mating linked to disruptive selection on ecological variation (Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999) and sexual selection alone (Higashi *et al.*, 1999) are capable of generating divergent species in sympatry. Empirical research using molecular markers, in combination with the development of robust theoretical models, have provided strong evidence to indicate that sympatric speciation has often played an important role in rapid adaptive radiations. While sympatric speciation has clearly been important in several model systems, the general importance of sympatric diversification relative to allopatric speciation in nature awaits the careful accumulation of further molecular studies.

Sex-role Reversal and Paternal Care in Syngnathid Fishes –

My work on syngnathid fishes has further investigated the role of sexual selection as a catalyst in the speciation process. I have used molecular phylogenetic techniques to illuminate historical patterns and rates of diversification based on extant taxa of syngnathid fishes. My molecular results have demonstrated that a diversification of male brooding structures occurred within a short burst of morphological innovation early in the evolution of the group and that pouch types independently increased in complexity in abdominal- and tail-brooding lineages of seahorses and pipefishes (Chapter 4). These results are largely in support of Herald's (1959) morphological work on the group and strongly suggest that development and diversification of paternal brooding structures in syngnathid fishes has played an important role in speciation in this family.

Building on these molecular phylogenetic results, I have used the comparative method to demonstrate that sex-role reversal has had multiple independent origins in this family, independent from the degree of brood pouch development (Chapter 5). These results are contrary to the expectations of theory that predicts that parental investment should be tightly associated with mating competition and sexual selection (Trivers, 1972). As outlined above (Chapter 5), Trivers' (1972) predicted relationship between parental investment and sexual selection depends on the relative investment of parents in their young, an extremely difficult to quantify variable that integrates expenditures of both time and energy. While brood pouch development correlates with paternal energy investment in all syngnathids

studied to date (Berglund *et al.*, 1986b), ongoing experimental study of these fishes aims to further quantify relative energetic investments and to estimate potential reproductive rate in terms of "reproductive time-outs" (Masonjones & Lewis, 2000)

Although sex-role reversal appears to be uncorrelated with paternal investment as measured by the level of brood pouch development, there is a strong correlation between sex-roles and mating systems (defined as the general behavioral strategy employed in obtaining mates (Emlen & Oring, 1977)) in the group, suggesting that mating systems may strongly impact on the intensity of sexual selection in the wild. A shift from a monogamous to a polygamous system of mating may skew the operational sex ratio and, consequently, moderate or intensify mating competition (Vincent *et al.*, 1992). This correlation between mating system and sex-role reversal has also been observed in several groups of birds (Emlen & Oring, 1977), indicating that reproductive behavior is often intimately associated with competition for mates in wild populations. Clearly, empirical investigations into the development and diversification of mating systems and their impact on mating competition offer some of the most exciting areas for future research (ie. DeWoody & Avise, 2001).

Lake Tanganyika as an Evolutionary Reservoir for Freshwater Gastropods –

While experimental work has suggested that the high diversity of both gastropods and their predators in Lake Tanganyika has resulted from within-basin predator-prey coevolution, my molecular reconstruction of evolution in

this group of snails indicates that up to five of the major lineages of gastropods found in Lake Tanganyika are older than the Lake basin itself. Clearly, although substantial morphological divergence in these animals may have occurred following colonization of the Tanganyika basin, this does not appear to have led to corresponding increases in the rate of speciation. At least one major group of gastropods appears to have originated in Lake Tanganyika and rapidly expanded its range throughout most of Africa. Tanganyika has thus served as an important evolutionary reservoir of gastropod lineages that have been extirpated outside the Lake (Chapter 6).

The high level of morphological variation in the gastropods of Lake Tanganyika does not appear to reflect increased rates of speciation following the development of the lake basin. These results support research which indicate that major changes in gastropod morphology may occur in the absence of speciation (Palmer, 1985). While the fossil gastropods of East Africa have served as some of the most significant evidence in support of the theory of punctuated equilibria, my research provides additional evidence that indicates that the interpretation of speciation from series of fossil gastropods may be complicated by ecophenotypic variation unrelated to speciation.

Molecular methods have helped clarify patterns of diversification and speciation of the gastropod snails of Lake Tanganyika and suggest that the pattern of diversification in the endemic gastropods of Lake Tanganyika differs significantly from the rapid speciation of the cichlid species flocks of Lakes Victoria (Meyer *et al.*, 1990) and Malawi (Albertson *et al.*, 1999). While the cichlids of Lake Tanganyika have also diversified within the lake (Salzburger *et al.*, 2002), there is increasing evidence that the present cichlid fauna of

Lake Tanganyika represents multiple colonization events (Salzburger *et al.*, 2002; Salzburger, pers. comm.) and the lake has also been an important evolutionary reservoir for cichlid lineages that have spread to seed the radiations in Lakes Victoria and Malawi (Meyer *et al.*, 1990; Nishida, 1991; Meyer *et al.*, 1994a). Work on Lake Baikal supports a similar pattern: while there has been considerable within-lake diversification, several of the most species-rich lineages appear to have been seeded by multiple colonization events (Sherbakov, 1999). While ancient lakes will continue to be important natural laboratories for the study of evolution (Rossiter & Kawanabe, 2000), recent molecular work indicates that rapid, within-lake adaptive radiations must be tested in a phylogenetic context. Comparative molecular phylogenetics will help to identify the true role that these long-lived lakes have played in the diversification of their endemic faunas.

Conclusions – The Way Forward

In this thesis, I have presented three model systems well suited for detailed investigations of organismic evolution. Molecular population genetic and phylogenetic methods have helped to illuminate processes underlying diversification in each of these groups. While the relative significance of natural and sexual selection in these three examples is variable, each of these systems consistently demonstrates that micro-evolutionary change is capable of explaining broad-scale patterns in evolution.

Molecular techniques developed over the past twenty years will continue to offer intimate insights into the interactions between organisms and their environments. At the same time, researchers have continued to make

significant advances in molecular biology over the past decade and these developments have quickly been integrated into evolutionary biology. New insights gained from developmental biology and functional genomics have done much to illuminate processes underlying the relationship between genotype and phenotype, but at the same time, they have highlighted just how complicated the genetic landscape is, with duplicated genes, differential transcription and regulation pathways and variation in cell competence all contributing to the complex relationship between genome and environment (Scott, 2000). The combination of data at the genomic level, in association with physiological and developmental work characterizing gene function, provide great opportunities for researchers working in all fields of evolutionary biology to tackle ambitious research projects which, until recently, were impossible.

The availability of large volumes of genetic data in online databases has spawned a “mini-revolution” itself, sparking the field of bioinformatics and encouraging researchers to use genetic studies on model organisms to investigate gene and genome evolution in a wider context. Today, although neutral molecular variation remains an important resource for researchers in evolutionary biology, scientists are becoming increasingly interested in the role of the genes themselves and how minor genetic changes directly influence phenotypic change. This revitalization of classical genetics will continue to dominate evolutionary biology over the decades to come.

Notwithstanding terrific advances in molecular biology and genetics, a balance between genome-oriented evolutionary biology and traditional morphology, paleontology and ecology is critical for further development of

the field. Molecular phylogenetic work has proven so powerful precisely because of the multitude of well-studied systems that predated the development of these techniques. Molecular genetic study has complemented and, in many cases, fundamentally changed our interpretation of these classical studies, but non-molecular studies will continue to be critical to the development of evolutionary research.

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WEB ADDRESSES FOR PHYLOGENETIC SOFTWARE

Package	Author	Web Address
Arlequin (V2.001)	Stefan Schneider David Roessli Laurent Excoffier	http://lgb.unige.ch/arlequin/
Bioedit (V5.09)	Tom Hall	http://www.mbio.ncsu.edu/BioEdit/changes.html
ClustalW (V1.7)	Toby Gibson Des Higgins Julie Thompson	http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/
DAMBE (V4.0.17)	Xuhua Xia	http://web.hku.hk/~xxia/software/software.htm
Genepop	Michel Raymond Francois Rousset	http://wbiomed.curtin.edu.au/genepop/
GENIE (V3.0)	Oliver Pybus Andrew Rambaut	http://evolve.zoo.ox.ac.uk/software/Genie/main.html
LINTREE	Naoko Takezaki	http://shanghai.bio.psu.edu/lintree.html
MacClade (V3.08a)	David Maddison Wayne Maddison	http://macclade.org/macclade.html
MCCRTest	Oliver Pybus	http://evolve.zoo.ox.ac.uk/software/MCCRTest/main.html
Mega (V2.1)	Sudhir Kumar Koichiro Tamura Ingrid Jakobsen Masatoshi Nei	http://www.megasoftware.net/
Modeltest (V5.0)	David Posada	http://zoology.byu.edu/crandall_lab/modeltest.htm
MrBayes (V2.01)	John Huelsenbeck Fredrik Ronquist	http://morphbank.ebc.uu.se/mrbayes/
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- **Vienna, Austria 2001** - Ancient lakes as evolutionary reservoirs: Evidence from the gastropods of Lake Tanganyika (World Congress of Malacology 2001)
- **Vienna, Austria 2001** - Reconstructing an invasion: Colonization and spread of invasive zebra and quagga mussels in the Laurentian Great Lakes as revealed by microsatellite markers (World Congress of Malacology 2001) (**Student Award**)
- **Edinburgh, Scotland 2000** - Ancient lakes as evolutionary reservoirs: Evidence from the thalassoid gastropods of Lake Tanganyika. (BBSRC - Workshop on Molecular Evolution and Diversity)
- **Barcelona, Spain 1999** - Detailed mtDNA phylogeny of seahorses and their relatives: Inferences on the evolution of male breeding structures (VII European Society for Evolutionary Biology Congress)
- **Roscoff, France 1999** - Polymorphic microsatellites highlight multiple dispersal strategies in the invasive quagga mussel (Dreissena bugensis). (CNRS/NSF - The Evolution of Dispersal)
- **Hamilton, Canada 1998** - Population structure of invasive zebra and quagga mussels in Lake Ontario as revealed by polymorphic microsatellite markers. (International Association for Great Lakes Research)
- **Fredericton, Canada 1996** - Genetic investigations of Corophium volutator in the North Atlantic. (Atlantic Undergraduate Biology Conference)