Species Limits, and Evolutionary History of Glassfrogs

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Abstract

Recognizing the mechanisms of speciation and the limits of species is essential to understand the origin of biodiversity and how to conserve it. The general aims of my investigations during my doctoral studies were two-fold: to study evolutionary patterns and processes, and to provide specific and superspecific taxonomic classifications that try to reflect evolutionary history. I have focused my research on anurans in their biodiversity hotspot, the American Tropics.

I have used morphological, behavioral (mating calls), and genetic (DNA sequences) characters to study species boundaries between frogs of the genus Pristimantis and the family Centrolenidae (glassfrogs). The results show that the exclusive use of single lines of evidence or the application of arbitrary thresholds impair and bias our ability to recognize new species and limit the possibility to understand evolutionary processes. Only an integrative approach combining every source of evidence provides the necessary feedback to discover all species and test their identity by comparing independent sets of data. This approach further allows identifying those species that probably represent stable comparative units (well supported species hypotheses) and to flag taxa that require further assessment.

Phylogenetic reconstructions based on seven nuclear and mitochondrial genes for about 100 species of glassfrogs revealed that previous hypotheses of relationships were mislead by rampant convergent evolution at the phenotypic level. None of the previously suggested classifications fit with the reconstructed evolutionary history. Consequently, we proposed a new classification consistent with this phylogeny.

I also studied the tempo and mode of diversification among glassfrogs. Based on sequences from ten genes in 87 species, I estimated species divergence times, age-range correlation between sister species, and reconstructed ancestral areas and dispersal/vicariance events. The results revealed a complex model of diversification where geographical isolation seems to be the dominant scenario for speciation and only clades of altitudinal generalists have been able to spread across the Neotropical rainforests.

Keywords: Amphibia, Anura, Biogeography, Centrolenidae, Diversification, Frogs, Molecular Phylogenetics, Neotropics, Pristimantis, Speciation, Species, Systematics, Taxonomy

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Mathematical representation is inevitably simplistic, and occasionally one has to be brutal in forcing it to suit a reality that can only be very complex. And yet, there is a beauty about trees because of the simplicity with which they allow you to describe a series of events [...]. But one must ask whether one is justified simplifying reality to the extent necessary to represent it as a tree.

Cavalli-Sforza, Genes, People, and Languages (2001)

The universe is no narrow thing and the order within it is not constrained by any latitude in its conception to repeat what exists in one part in any other part. Even in this world more things exist without our knowledge than with it and the order in creation which you see is that which you have put there, like a string in a maze, so that you shall no lose your way. For existence has its own order and that no man’s mind can compass, that mind itself being but a fact among others.

The judge

McCarthy, Blood Meridian, or the Evening Redness in the West (1985)

These foul and loathsome animals [...] are abhorrent because of their cold body, pale color, cartilaginous skeleton, filthy skin, fierce aspect, calculating eye, offensive smell, harsh voice, squalid habitation, and terrible venom; and so their Creator has not exerted His powers to make many of them.

Carl von Linne opinion on amphibians

Linnaeus, Systema Naturae (1758)
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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In **Paper I,** SCF participated in collecting the data in the field, generating the molecular data in the lab, analyzing the molecular data, and in the writing and interpretation. In **Paper II,** SCF participated in collecting the data in the field, generated all the molecular, morphological, and bioacoustic data, led the analyses, writing, and interpretation. In **Paper III,** SCF collected in the field and generated in the lab half of the data, participated in the analyses, writing and interpretation. In **Paper IV,** SCF shared equally with JMG gathering the data, writing and interpretation. In **Paper V,** SCF generated and analyzed all the data, and did the majority of writing and interpretation. In **Paper VI,** SCF participated in collecting the data in the field, analyzing it, and led writing and interpretation. In **Paper VII,** SCF participated in collecting the data in the field, produced and analyzed all the molecular and bio-acoustic data, and half of the morphological in the lab, and shared writing and interpretation. In **Paper VIII,** SCF participated in collecting the data in the field and led data analyses, writing, and interpretation. In **Paper IX,** SCF shared collecting and analyzing the data, writing, and interpretation. In **Paper X,** SCF participated in collecting the data in the field and led data analyses, writing, and interpretation. In **Paper XI,** SCF participated in collecting the data in the field and led getting the data in the lab, data analyses, writing, and interpretation. In **Paper XII,** SCF participated in collecting the data in the field and led getting the data in the lab, data analyses, writing, and interpretation.
The following papers were writing during the course of my doctoral studies but are not part of the present dissertation:


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<tr>
<td>ARC</td>
<td>Age-range correlation</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<td>BM</td>
<td>Bayesian methods</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ESP</td>
<td>Evolutionary Species Concept</td>
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<td>ICNB</td>
<td>International Code of Nomenclature of Bacteria</td>
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<td>ICZN</td>
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<td>LS</td>
<td>Linnaean System</td>
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<td>Maximum Parsimony</td>
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<td>Maximum Likelihood</td>
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<td>My</td>
<td>Million years</td>
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<td>PC</td>
<td>Phylocode</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>TOL</td>
<td>Tree of Life</td>
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Introduction

The mere presence of life makes our planet, as far as we know, unique in the universe. The myriad of organisms that have populated the Earth, with their different forms, sizes, and lifestyles are hardly imaginable. This diversity has always puzzled humans and has given rise to all sorts of explanations for its origins from mythological to rational. I will argue here that understanding this diversity from a scientific perspective is a process with, at least, two steps, linked by the theory of evolution: (1) to know its breadth and (2) to appreciate the mechanisms and processes generating it and maintaining it.

The first of these activities involves the creation of a system of classification that allows both communication (e.g., through rules of nomenclature) and the recognition and assignation of elements into the classification (e.g., through shared characters). Classifying and organizing life probably represents one of the oldest human activities, because our dependence on other living beings. It is important to note that no system of classification needs to be based on scientific paradigms and many examples of these nonscientific classifications are used today (e.g., toads vs. frogs).

The great achievement of creating the skeleton upon which an international system of nomenclatural classification was built can undoubtedly be credited to the Swedish scientist Carl Linnaeus. The binomial Linnaean System (LS) of nomenclature (Linnaeus, 1758) has been the framework within which biologists and non-biologists have discussed biological diversity for 250 years. The three codes regulating nomenclature are the botanical (Greuter et al., 1998), zoological (International Code of Zoological Nomenclature ICZN, 1999), and bacteriological (International Code of Nomenclature of Bacteria ICNB, 1992). Although these codes include elements not present in Linnaeus’ work, they are Linnaean in fundamental concepts and principles (i.e. binomial species and hierarchical taxonomic ranks).

This hierarchical system implies a chain of relationships among different ranks, where for example species within one genus are more related to each other than to any other species of a different genus. Note that relationships here are not used in an evolutionary context of descend from a common ancestor. In fact, Linnaeus thought that this arrangement of similarity was the fruit of God’s divine plan. One important implication of this reflection is that current LS is “theory free”, this meaning that governs taxa names (nomenclature) but not how these taxa are constructed. Nevertheless, this hierarchical arrangement of life was, together with biogeography, paleontology, and
animal and plant breeding, one of the main lines of evidence for the postulation of the theory of Evolution by Charles Darwin and to a lesser extend Alfred Russel Wallace.

One hundred and fifty years after the publication of *On the Origin of Species* (Darwin, 1859), there is almost unanimous agreement among scientists that the theory of evolution represents the most powerful rational account to explain the origin of the diversity of life. Therefore, it is inevitably logical that classifications should reflect our current knowledge about evolutionary history. It is only when taxonomy is done under a scientific frame (like the theory of evolution) that can be considered a scientific activity.

Although there was a need for taxonomies to reflect evolutionary history, it was not until the second half of the XX century that the German entomologist Willi Hennig provided the necessary tools to reconstruct the evolutionary patterns (phylogenies) resulting from the evolutionary process of speciation. For that, he proposed the use of shared derived characters (synapomorphies) (see Hennig [1966] for a compendium of his major contributions during the 50s and 60s). Although it has not escaped criticisms (e.g., Mayr, 1974), phylogenetic systematics and its bifurcating diagrams representing the tree of life (independently of the method used to infer relationships) has overtaken the scientific community to the point that many people wrongly assume nowadays that all taxonomies reflect evolutionary patterns. Unfortunately, hundreds of years of inertia still persist among many taxonomists and a great fraction of classifications have not been phylogenetically evaluated since their proposal.

The second part of this two-step process requires understanding which mechanisms are involved in shaping, generating and maintaining the observed diversity. It was Darwin who proposed that the main mechanism behind evolution was natural selection acting upon phenotypic variability. However, it was not till the Evolutionary Synthesis (Huxley, 1942; Mayr and Provine, 1980) combined Darwin’s theory with Mendel’s genetic laws (Mendel, 1866) that genetic mutations were proposed as the units subject to selection. In addition to natural selection, genetic drift was identified as a new mechanism involved in creating the observed diversity. The scientific debate today is not whether natural selection exists or not, but rather what kinds of selection occur, their preponderance over drift, to which levels of organization they affect, how different geographical and biological settings influence divergence (e.g. allopatry versus divergence with gene flow), the genetic architecture of phenotypes, etc.

As indicated above, these two steps are clearly connected through the theory of evolution and are complementary. Many researchers worldwide are working to complete the Tree of Life (TOL). Although this is a mere descriptive tool (though very relevant), it is setting the basis for deeper understanding of the evolutionary process and the origin of biodiversity. At the same time, it is difficult to imagine how to study many of these processes
without a robust phylogenetic hypothesis and a clear and efficient system to name and communicate the different sections of the TOL.

It is in the context that I present my doctoral thesis. I have focused my research on anuran diversity in their biodiversity hotspot, the American Tropics. I have focused my work on glassfrogs (Centrolenidae) and, to a lesser extent, frogs of the genus *Pristimantis*. My study has been centered on their taxonomy, both at the specific and supraspecific level, phylogenetic relationships, and the geography and tempo of their diversification.
Study organisms

Caudata (salamanders and newts), Gymnophiona (caecilians), and Salienta (frogs and toads) are thought to form a clade called Lissamphibia that also includes some extinct amphibian lineages. They are a derived group of mainly terrestrial tetrapods that have been present for more than 200 My (Marjanovic and Laurin, 2007) and with representatives in all continents except Antarctica. The bulk of the more than 6000 described species of extant amphibians corresponds to anurans, the only clade within Salienta with surviving representatives (Duellman and Trueb, 1994). Within anurans, the vast majority of species correspond to the clade Neobatrachia (modern frogs), which is particularly diverse in tropical rainforests.

Amphibians are central for biologists studying vertebrate evolution and their diversity is thought to be largely underestimated (Köhler et al., 2005). They probably represent the group of tetrapods with more new species described every year but, unfortunately, also the one with more threatened species (> 30%) and has become a priority for conservation purposes (Stuart et al., 2004). Also their phylogenetic relationships have been poorly resolved probably because their derived and specialized bauplan left little phylogenetic signal at the morphological level to solve the complex relationships among the species (Hoegg et al., 2004).

Among vertebrates, amphibians constitute excellent subjects for the study of biogeography and diversification because of their limited dispersal capabilities and strong habitat dependence. Also, their species-rich clades are distributed through large areas, so by studying their evolutionary history we could gain insight into the history of their habitats and of the other inhabitants (Crawford et al., 2007; Crawford and Smith, 2005; van der Meijden et al., 2007; Wollenberg et al., 2008; Zeisset and Beebee, 2008).

During my doctoral studies I have investigated frogs of the family Centrolenidae and to a lesser extent of the genus *Pristimantis*. In the following paragraphs I will very briefly summarize some of their main biological attributes and our current knowledge on their diversity and relationships.

Glassfrogs

Glassfrogs (also known as centrolenids) represent a monophyletic group within Neobatrachia assigned to the family-group rank (family Centroleni-
dae or subfamily Centroleninae depending on the authors, see Frost, 2009). Although, they are nested within Noblebatrachia (Frost et al. 2006) and their sister taxon is *Allophryne ruthveni* their relationships with other members of Noblebatrachia are contentious. The current number of recognized species is 147 (Frost, 2009). Glassfrogs are exclusively Neotropical and their center of diversity is situated in the northern Andes of Colombia and Ecuador.

Centrolenids are restricted to humid forests of Central and South America. Although the majority of glassfrogs are montane species (500–2000 m) there are representatives from sea level to 3500 m. They are relatively small (snout to vent length = 18–80 mm) nocturnal frogs and most are arboreal and epiphyllous with a few saxicolous species (Cisneros-Heredia and McDiarmid, 2007). Glassfrogs owe their name to the fact that they have total or partial transparent ventral skin a unique character among tetrapods, only shared with some species of the frog genus *Boophis,* and that allows seeing the internal organs *in vivo.*

Glassfrogs reproduce outside the water and females deposit their eggs on leaves, branches, and mosses overhanging water or on stones next to streams and waterfalls. When the eggs hatch, tadpoles fall in the water where they live buried in leaf packs. Males have single vocal sacs and produce mating calls to attract females. Many species of glassfrogs show parental care where the male stays close by or over the egg clutch. Female parental care has only been observed in one species. Two different combat behaviors have been observed between males (although this has been described in < 10% of species). In some species, males fight dangling upside down while holding on to the vegetation with their hind legs, grasping one another venter-to-venter. This fighting behavior is considered to be derived given that it has been observed only within Centrolenidae. In contrast, in other species males have an amplexus-like fighting behavior, which is considered primitive.

Clear milestones in centrolenid systematics are the works of Ruiz-Carranza and Lynch (1991, 1995, 1998). They presented the first phylogenetic analysis, build the current supraspecific classification, and described ~22% of the known species of glassfrogs. Between 1991 and 1995, approximately 40% of the known species of glassfrogs were described (Fig. 1). This burst in species numbers is linked to the classification of Ruiz-Carranza and Lynch (1991), highlighting the deep impact of scientific classifications in the study of biodiversity.
Figure 1. Cumulative number of valid species of glassfrogs through time organized in periods of five years. Between 1991–1995 circa 40% of the species were described, this burst in species description followed the new classification of Ruiz-Carranza and Lynch (1991).

Pristimantis

The genus *Pristimantis* belongs to the family Strabomantidae, which clusters together with other Neotropical frogs with direct development forming the clade Terrarana (Hedges et al., 2008). Their phylogenetic position within Noblebatrachia is problematic and no strong hypothesis of relationships is available (Frost et al., 2006; Hedges et al., 2008; Heinicke et al., 2007; Roe-lants et al., 2007). *Pristimantis* frogs are distributed touth all mesic and humid forests of Central and South America from Mexico to Bolivia; however, they do not occur in the Brazilian Atlantic forest. Currently, there are 434 recognized species (Frost, 2009) and, similarly to glassfrogs, *Pristimantis* reach a peak in their diversity in the Andes of northern South America (Colombia, Ecuador, and Peru).

These frogs are relatively small (total length = 13–73 mm) but robust, mainly nocturnal and terrestrial (non-aquatic) with species adapted to all kinds of strata (from complete ground-dwelling to arboreal). *Pristimantis* frogs are a very important component of the vertebrate faunas in Neotropical forest and likely the most abundant terrestrial vertebrates of South America (Lynch and Duellman, 1997). In many species males exhibit a vocal sac and
attract females by emitting calls. Females lay eggs outside the water and, in the few species where they have been observed, embryos go through direct development (they skip the tadpole stage) and hatch into froglets.

Pristimantis originated between 17.30–34.82 Mya (Heinicke et al., 2007) and might represent the largest evolutionary radiation of tetrapods when compared to clades of similar age. Nonetheless their species diversity is not paralleled by a great number of morphologically informative characters and convergent evolution seems rampant (Hedges et al., 2008). Therefore, comparing different lines of evidence to test species in the genus Pristimantis is challenging. Pristimantis share the burdening characteristics of many taxonomically complex tropical groups: individuals are difficult to detect—resulting in few sampled individuals for species descriptions—, they possess subtle morphological differences, and commonly show high levels of intraspecific polymorphism. Thus, the study of their phylogeny and systematics can allow the development of approaches useful for the study of other complex groups.
Species boundaries

One of the oldest scientific enterprises is to estimate how many species inhabit this planet. In spite of the clear benefits and intellectual challenges of answering such a question, we are far from an answer and estimates differ in various orders of magnitude.

Background

Current estimates of species diversity among eukaryotes range from 3–100 million of which ~1.8–1.9 millions (most likely less than 1.6 if we removed synonyms) are known to science (May and Harvey, 2009). Among these “known” species < 1% have been addressed with more than a cursory anatomical description (Wilson, 2005). The implications of this go beyond those of a failed academic exercise. Without a clear idea of diversity of life, a complete research program in biology is hardly possible (Wilson, 2005), and practical economic aspects deeply affect our societies. The multiple services that ecosystems provide are vital for our survival, both as species and individuals, and many have been identified to be degrading (Mindell, 2009). Microorganisms and invertebrates of which we basically know nothing deliver a great deal of these services (Chivian and Bernstein, 2008). It would be impossible for us to mimic or substitute their activities in case of their extinction, a scenario sadly likely as explained below.

We are witnessing the sixth mass extinction (Pimm and Raven, 2000; Thomas et al., 2004). Our best-known organisms (vertebrates) currently show extinction rates 100–1000 times faster than those estimated from the fossil record and a 10-fold increase is foreseen during the next century (May and Harvey, 2009). Although such dramatic extinctions have happened before, two main aspects should be taken into account. This is the first one provoked by the activities of a single species (humans) rather than by extrinsic environmental changes. Through their evolutionary history, humans have no previous experience whatsoever in surviving the challenges associated with such mass extinction.

Amphibians, the group studied here, clearly exemplify this situation of combined extinction and lack of knowledge. More than 30% of the described species of amphibians are threatened with extinction, for another 30% their conservation status is unknown due to lack of data, and still a great propor-
tion of amphibian species are to be discovered, possibly a number even larger than the number of species currently known, 6474 (Köhler et al., 2005; Stuart et al., 2004).

The species problem(s)

Species are one of the most important units of comparison and organization in biology, comparable to lower levels like genes, cells, and organisms; but, as clearly explained by de Queiroz (2007), a fundamental temporal difference exits that makes their study and observation less straight forward.

[...] because species exist at higher levels of organization than the humans observing them, species also are generally much larger and longer lived than their human observers. Moreover, the connections among their parts (i.e., organisms) are ephemeral. This makes it more or less impossible for humans to perceive entire species simply by looking at them, as they do for cells and organisms, which is why biologists have symposia devoted to the topic of species delimitation.

In spite of the importance of species and their extended use among non-biologists, few topics in evolutionary biology have led to so many discussions with so little agreement than the nature of species, the so-called species problem [I would like to clarify that species do not have a problem but rather that humans have a problem knowing what species are]. Arguably, one of the main sources of confusion has been to consider distinct problems concerning species as a single one. de Queiroz (2005a) reviewed this issue and identified different but related problems that can be boiled down into three questions: What is a species?, how do we recognize species?, and which processes are responsible for the existence of species?

One main problem seems to be the confusion between the species concept and the criteria we use to differentiate them. According to Hey (2006) it was Mayr (1942) who elevated different approaches to species identification to the level of concept, thus equating the concept of species with operational definitions of species based on contingent criteria (biological properties) acquired through the process of divergence. This led to a plethora of different operational definitions, no fewer than 24, based on criteria that in many cases are partially or totally incompatible and therefore sparking conflict about the species concept (Mayden, 1997). It was not till the 90s that this problem was clearly identified and solutions inspired in the Evolutionary Species Concept (ESP; Simpson, 1951; 1961; Wiley, 1978) were proposed on the basis of shared aspects among all species concepts and definitions recognized by biologists (de Queiroz, 1998, 1999; Frost and Kluge, 1994; Mayden, 1997).
de Queiroz (1998) reviewed this species problem and presented evidences that all species concepts and definitions represent variants of a single general concept of species just differing in the criteria described to identify or delimit species taxa. This general species concept states that a species is a temporal segment of a populational or metapopulational lineage independently evolving from other lineages, and that no trait alone (e.g. morphological or ecological differentiation, reproductive incompatibility) can be considered as a biological property that a species must show to be recognized as such. The logic behind is that these different criteria correspond to different events that occur during lineage separation and divergence (Fig. 2). Furthermore, given the complexity and uniqueness of each speciation process (affected by mutation, natural selection, gene flow, and genetic drift), the characters affected are very diverse and involve many different aspects of the organismal biology so that we can not expect these characters neither to occur at the same time, or to happen in a certain order and rate, or even to ever happen if the lineage goes extinct before lineage divergence is complete for a certain trait. The implications of this concept to different branches of evolutionary biology have been further discussed during the last decade (de Queiroz 2005a, b; 2007; Sites and Marshall 2003, 2004; Wiens, 2004, 2007) and Dayrat (2005) labeled the taxonomic research that explicitly accepts the implications of the ESC as “Integrative Taxonomy”.

The third problem involves the identification of the process(es) responsible for the origin of species. Traditionally, emphasis has been placed on the role of sexual reproduction (gene flow) on maintaining the cohesion of species (Dobzhanski, 1937; Mayr, 1942; Wright, 1940). This would imply that asexual organisms are not organized as species. However, there are strong evidences supporting the maintenance of metapopulation lineages that exchange a great deal of genes freely (Fitzpatrick et al., 2008a) in asexual organisms and that they are organized according to the expectations derived from fragments of independently evolving lineages (Fontaneto et al., 2007). Thus, mechanisms like ecological mediated natural selection and developmental constraints inherited by common ancestry are likely involved in the cohesion of species (de Queiroz, 2005a).

Species delimitation

That a unified and general species concept has been proposed does not mean that all problems are solved. Now the problem has shift to how to recognize different independently evolving segments of metapopulation lineages (Wiens, 2007). The disagreement between other species concepts and definitions was related to the number and diversity of the species recognized. Since we keep using the same criteria we will expect in principle to have the same disagreements. Nonetheless, a major theoretical reinterpretation of the
meaning and origin of the different biological traits allows the existence of discordance between criteria. Basically, under the general lineage species concept the presence of any of these different characteristics is an evidence for the divergence of lineages but the absence of any of them is not evidence for the absence of two different species (Fig. 2).

Figure 2. Schematic diagram of lineage split and divergence. Lineage split (= speciation) is indicated by the dashed rectangle. The gradation in shades of grey represent the daughter lineages diverging through time. Criterion 1–4 refers to different biological properties acquired by the lineages at different times and used as criteria in different species definitions (e.g., reciprocally monophyletic, phenotypically distinguishable, reproductively incompatible, ecologically different, etc.). The fixation of none of these characteristics needs to be necessarily coupled with the speciation event. Modified from de Queiroz (2007).

Species are real entities of the natural world and not mere constructions of the human mind (Coyne and Orr, 2004). We conceptualize these real “species” through our species concepts and propose hypothesis of species taxa (a group of organisms that we believe constitute an independently evolving segment of a metapopulational lineage) and assign them to the species category (the Linnaean rank). The evidences used for these hypotheses are biological properties derived from the process of lineage divergence that, for example, include reproductive isolation, monophyly, phenotypic differentiation, ecological distinctiveness, etc. As explained above, divergence between lineages does not require by any means any particular order or rate of character fixation; thus, all properties are equally valid to evaluate lineage divergence and species hypothesis (de Queiroz, 2007). This does not imply that these evidences are infallible as they depend on the quality of the data, the strength of the test used, and the interpretation of the results. For example, most species hypotheses have been built implicitly or explicitly on the presence of distinct morphological characters. However, for many organisms like tropical amphibians, the validity of these characters has just been examined in very few specimens or the results could reflect sampling biases (e.g., sam-
pling just part of a continuous phenotypic diversity, different ontogenic stages) rather than real differences between lineages. On the other hand, the mere absence of one or more of these properties cannot be used to reject the species hypothesis and should be interpreted either as sample bias or the decoupling of the evolution of different character during lineage divergence. Only the absence of all properties can be invoked to reject two independently evolving metapopulation lineages as separate species (de Queiroz, 2007). Of course, the more lines of evidence support a species the more stable we can expect that hypothesis to be in the face of new data. The theoretical advances presented above, both in concepts and species delimitations, do not imply that all problems are solved. Several conflicting points can still be identified:

1. Only lineages that have diverged enough can be identified effectively. During the period of time comprised from the speciation event to the appearance of the first fixed and traceable divergent trait, different species will be treated as one.
2. It allows the identification of anagenetic species (transformation through a lineage without splitting), a very conflictive model of speciation.
3. The nature of populations creates misleading situations. Populations can be isolated for relatively short periods of time and, depending on the conditions of isolation, can show some degree of divergence during that time that could be interpreted as evidence for speciation.

Characters used for species delimitation

A renaissance in the interest of delimiting species limits has occurred during the last years analytical methods have been developed to infer species boundaries (reviewed in Marshal and Sites 2003; 2004) and new tools have been added to the palette of evolutionary biologists trying to identify species (Cardoso et al., 2009; Wiens 2007). In this section I will briefly explain the main sets of characters used in my research and how they are relevant to identify species.

Phenotypic characters

The phenotype is the product of the interaction between the genetic composition and the environment. Thus, the study of the phenotypes to separate species has represented, for a very long time, a way to study their genetic differentiation at functional traits. Fixed divergences in phenotypes represent separate evolutionary lineages. Studies of phenotypes are usually centered on morphological or behavioral aspects.
Morphology

Morphological characters are the oldest and most used evidence for species descriptions and, still today, morphology plays a central role in taxonomy (Schlick-Steiner et al., 2007). Both continuous and discrete characters are useful to detect lineage divergence. In amphibian taxonomy there is probably a bias to use external characters based on soft tissues (e.g., the integument), although numerous examples of internal characters exist through the literature (for example, skeletal differences).

As summarized by Vences and Wake (2007): “When two distinct morphs, identifiable by at least two unrelated characters, occur in a single population with age and sex taken into account, they are considered species”. This reflects an ideal situation because in many cases few specimens are available for comparison. Further, individuals available for study may be of a single sex and/or age class, and only one character is used to indicate divergence (which, according to the general lineage species concept, should be enough). The underlying assumption is that fixed differences in morphology might be strong evidence of reduced or absent gene flow (Wiens and Servedio, 2000), and those differences usually coincide with separate units defined by reproductive gaps (Rieseberg et al., 2006) and molecular divergences (Avise and Walker, 1999), constituting thus evidence of lineage divergence. Following Wiens and Servedio (2000), if a character is fixed if a sample of at least ten specimens, there is 95% probability or higher that the trait is fixed for the species.

Behavior

Behavioral traits have been less exploited as source of evidence for determining lineage divergence, probably because opportunities for direct observation are usually rare. In some of the papers here presented, I have used some behavioral characters like position of calling males and antagonistic interactions between males as characters for species descriptions. Nonetheless, the most commonly used behavioral evidence to delimit species boundaries among anurans is their advertisement calls (Schneider and Sinsch, 2007).

Frogs emit a variety of sounds but those involved in reproduction are more relevant for species identification. The most general situation is that males produce a mating call that is recognized by conspecific females. Therefore, the underlying assumption is that fixed differences in advertisement calls evidence the likely existence of prezygotic reproductive barriers (Gerhardt 1994), because the neurological structure controlling the female auditory system is adapted to detect and select calls of conspecific males (Ryan 1988). The suggestion of prezygotic reproductive barrier should not be confounded with evidence of reproductive incompatibility, because spe-
cies showing differences in advertisement call can and do hybridize (Pfenning, 2007).

Genetic characters

There are several other facts, also shared with nDNA, including incomplete lineage sorting, introgression, gene duplications, nuclear insertions, vastly different mutation and/or substitution rates among lineages that can mislead the interpretation of results.

Two main sets of approaches use genetic information for species delimitation, tree based and non-tree based methods (Sites and Marshall, 2003). In their simplest form, tree based methods reconstruct a tree of haplotypes using one or several algorithms. The expectation is that different species should be reciprocally monophyletic. This is based on the assumption that coalescent patterns in gene genealogies are related to the historical processes that originate separate lineages (e.g., Avise, 2000; Knowles and Carstens, 2007). Non-tree based methods are mainly performed through the estimation of genetic distances and assume that genetic differentiation between populations within a species tends to be relatively small because of gene flow, whereas divergence between species increases with time and reduced gene flow. This led to the idea that universal thresholds of genetic distances could be used to identify species throughout the tree of life (e.g., DNA barcoding). Although the simplicity of this idea is undoubtedly attractive and it has potential for preliminary surveys of large datasets, the assumptions are so strong and so likely to be violated that it can not be used as a reliable unique tool. Its usefulness is analogous to the strict molecular clock, it might be correctly used under some cases and is straightforward to apply but should be used and interpreted with extreme caution.
Phylogenetics

We study the relationships of both extinct and current species using heritable characters, a branch of biology called phylogenetics. Phylogenetic studies (independently of the method used) infer clades, monophyletic groups of species that include their most recent common ancestor and its descendants. Many clades are either nested or mutually exclusive but phenomena like hybridization, species fusion, and symbiogenesis can result in overlapping clades. Clades, as species, are biological entities and both are products of the evolution that have an existence regardless of whether we study and name them.

Phylogenetic classifications

As explained above, the LS of nomenclature has been the principal tool to communicate biological diversity during the last 250 years and there is consensus among biologists that taxonomic classifications should reflect evolutionary history and relationships between organisms. However, it is difficult to imagine a classification system that could capture all the complexity of the processes involved in the evolution of life. Here I direct my attention to LS and the PC (Cantino and de Queiroz, 2007), an alternate nomenclatural convention designed for the specific purpose of naming supraspecific clades, and briefly compare them.

In LS, each species bears a combination of two names—the generic name followed by the specific epithet (e.g., Rana temporaria); thus, in traditional nomenclature, the genus is a mandatory rank. Species are organized hierarchically into successive suprageneric ranks (family, order, class, phylum, kingdom), and the names of these ranks are tied to designated type genera or type species. Several of these ranks have specific suffix so a hierarchical relationship can be transmitted without the need of a diagram (e.g., a tree). For example, the name “Ranidae” corresponds to the zoological taxonomic rank of Family, containing the type genus Rana; similarly, the name “Rana” is the name of the genus containing the type species Rana temporaria. In the ICZN, this principle of typification does not extend above the level of family-group rank (e.g., superfamily, family, subfamily).

de Queiroz and Cantino (2001) argued that the fundamental difference between PC and the LS is the way in which names are associated with clades.
Phylocode ties names directly to clades, whereas the LS links names indirectly to taxa by means of the ranks to which the taxa are assigned. Thus, the same clade can have different names because of shifts in ranks (e.g., Centrolenidae sensu Taylor, 1951, and Centroleninae sensu Frost et al., 2006). Phylocode incorporates phylogenetic definitions to link a taxon name explicitly to a particular clade. In the PC, a clade is defined by two or more reference points (i.e., specifiers or anchors; e.g., species, apomorphies; a clade can be defined as the most recent common ancestor of species A and B and all its descendants or by all species that have a certain apomorphy) that “point” to a clade in a particular phylogenetic hypothesis. Also, by directly associating clades with names, the PC provides a more direct link between nomenclature and taxonomy than LS.

PC is an unranked system; thus avoids changes in clade names due solely to shifts in rank. In a group in which the standard ranks are in use, naming a newly discovered clade requires either the use of an unconventional rank or the shifting of ranks causing a cascade of name changes. Also, names in the LS can only be applied to mutually exclusive clades, while in PC other evolutionary processes than speciation by lineage splitting (e.g., hybridization, fusion) can be incorporated.

Both systems have advantages and some are complementary, thus it might be useful to combine the beneficial aspects of each system. In my thesis, I have followed the ICZN guidelines for the LS, and included phylogenetic definitions based on the PC when appropriate.

Phylogenetic methods and characters

Dozens of methods for phylogenetic inference have been developed and they can be classified in two categories. In distance-based methods, distances are calculated from pairwise comparison (e.g., comparison of sequences), building a distance matrix which is used to construct a tree attending to pairwise similarities. Most commonly, a clustering algorithm is used to transform the matrix into a phylogenetic tree like in neighbor joining. Character-based methods attempt to fit all the character changes in all species to a tree. Maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods (BM) are the most used character-based methods. See Felsenstein (2004) for a review of methods.

Characters used in phylogenetic studies have to be heritable and variable but their nature can vary. Traditionally, most phylogenetic works have inferred trees on the basis on morphological characters but behavioral and molecular data, especially the later, have gained popularity. In fact, the use of molecular data has supposed a revolution in phylogenetic inference. The most frequently used molecular characters in phylogenetics are the bases of DNA sequences. However, the number of possible characters conveyed in
any given genome is so large and the technological advances are allowing recovering them at such rate, that the main concerns in phylogenetic inference are computational (Avise, 2008).

Throughout my doctoral studies I have used DNA sequences from mitochondrial and nuclear genes. Data have been analyzed using a variety of distance- and character-based methods. I have not used phenotypic data because previous studies have already explore their relevance in the studied organisms, the number of available characters is too few to solve relationships among our datasets, and many of the proposed characters are likely to be adaptive providing more opportunities for convergent evolution (Guayasamin et al., 2006; Ruiz-Carranza and Lynch, 1991).
Diversification

Biodiversity is not evenly distributed throughout our planet and understanding the causes of diversity patterns (rather than just their correlations) requires and evolutionary (historical) perspective. The ultimate processes changing the species diversity within an area are speciation, extinction and dispersal (Wiens and Donoghue, 2004). Insight into these processes is thus better gained by considering phylogenetic relationships among species to infer biogeographic and speciation patterns.

The New World tropics are one of the most species rich areas in the planet. However, it has been scarcely studied in the recent literature how this diversity has originated. Furthermore, as biodiversity is globally threatened and we enter a unique human-induced era of extinctions, the need of grasping how this diversity arises and is maintained becomes a priority for sensible and successful conservation strategies. In the following sections I will (very) briefly introduce the most popular diversification scenarios, that try to explain species diversity in the Neotropics, focusing on how to study them using phylogenies.

Geographic isolation

Many researchers have shown that, in most of the speciation events studied, the lineages involved have gone through periods of complete geographic isolation. This has led some authors (Coyne and Orr, 2004) to propose allopatric speciation as the null hypothesis when evaluating the geographic setting of the speciation process. Nonetheless, demonstrating complete allopatry is, in many cases, as difficult as proving speciation in total sympatry.

The geography of the speciation process is better studied as a dynamic continuum where complete allopatry and sympatry are the extremes (Butlin et al., 2008; Fitzpatrick et al., 2008b). Nevertheless, it is worth studying these extremes and to try to determine their relative frequency. The idea that the biogeographic component of the speciation process can be explored by combining phylogenetic relationships and current distribution ranges of extant species has been present for long among biologists (Barraclough and Vogler, 2000; Fitzpatrick and Turelli, 2006; Lynch, 1989; Phillimore et al., 2008 and references therein). However, it is expected that changes in the distribution of species after speciation events will blur the relationship be-
tween the geography of speciation and contemporary distribution ranges (Chesser and Zink, 1994; Losos and Glor, 2003). The so-called age-range correlation (ARC) methods try to overcome problems derived from shifts in species distributions by taking into account the age of the split of lineages (reviewed in Fitzpatrick and Turelli, 2006). This is made by plotting estimations of both the degree of overlap between the ranges of two taxa against their divergence time. Therefore, if allopatry has been the predominant geographic scenario for speciation we expect a positive relationship between percentage of range overlap and age of the split, with an intercept approximately at cero (for species pairs that appeared in complete allopatry, some range overlap is more likely as the time since divergence increases). On the other hand, if sympatry has been the most common geographic setting for lineage divergence a negative relationship is expected with an interception close to one.

Tropical areas have been traditionally thought to be stable when compared to temperate ecosystems. It was not until the second half of the last century that tropical rainforests were seen as more dynamic historic landscapes that have changed their extension and connectivity under the effects of environmental changes. Haffer (1969) proposed an attractive and sound model to explain species diversification in the Neotropics in which glaciations during the Quaternary induced periods of drier and colder weather that restricted rainforests to certain humid pockets, refugia, where fauna contracted and diverged in isolation. Interglacial periods of warmer and more humid weather produced range expansions and connections of faunas and floras.

This model, although elegant, simple and very attractive, has received little support from empirical data and has generated much criticism (Bush, 1994; Colinhaux and De Oliveira, 2000; Colinhaux et al., 2001). As alternative, geological events from the Tertiary like the uplift of the Andes, marine incursions, and the closure of the Panama Isthmus have been proposed as main forces leading to isolation and speciation (Albert et al., 2006; Elmer et al., 2007; Noonan and Wray, 2006; Nores, 1999, 2004).

Ecological gradients
Alternatively to geographic isolation, it has been suggested that ecological gradients have shaped the current diversity in tropical areas. This model predicts divergence selection across strong environmental gradients and does not require suppression of gene flow. The expected outcome is sister species adapted to different but adjacent habitats like rainforests and savannas or lowlands and highlands (Moritz et al., 2000).

There is strong evidence that ecological divergence plays a central role in speciation (Funk et al., 2006). However, the relevance of ecological gradi-
ents in speciation is less clear. It is obvious and there are many examples of species distributed through ecological gradients showing local adaptations and some degree of population differentiation (Schneider et al., 1999; Smith et al., 2005; Smith et al., 1997) but this is not necessarily speciation. Few studies based on phylogenetic relationships and species distributions and ecology have favored speciation through gradients (Graham et al., 2004).

The study of centrolenid frogs, which inhabit a wide range of altitudes and habitats, offer a unique opportunity to investigate if ecological gradients may have played an important role in speciation along ecological gradients.
Research goals

The general aims of the investigations during my doctoral studies are two-fold:

- To study patterns and processes involved in the origin of biodiversity of Neotropical anurans.
- To provide taxonomic classifications at both levels that try to reflect the gained knowledge on the evolutionary history.

Specific aims

More specifically I have:

- Evaluate different criteria to delimit species boundaries under the framework of the general lineage species concept in a group of frogs from the genus *Pristimantis* and several species of Centrolenidae.
- Contribute to the revision of the species diversity of Centrolenidae and their taxonomy.
- Reconstruct the phylogeny of the family Centrolenidae using diverse molecular markers.
- Propose taxonomic classifications at supraspecific levels that convey as much as possible information on the evolutionary history of Centrolenidae.
- Investigate the tempo and mode of diversification in Centrolenidae.
Results

In the following paragraphs I summarize the main results of the twelve papers included in my thesis. For the sake of brevity and clarity I will group them by general topics.

Papers I and II

Recent conceptual advances in the problem of species concepts and delimitation have provided a specific theoretical framework in which to analyze and interpret species boundaries as inferred from different lines of evidence, the so-called “Integrative Taxonomy”. In Papers I and II we have investigated species limits in two groups of Neotropical anurans with a complex taxonomic history.

Paper I focuses on 26 species of frogs of the genus Pristimantis. This species-rich genus is challenging because individuals are difficult to detect— resulting in few sampled individuals for species descriptions —, they possess subtle morphological differences, and commonly show high levels of intra-specific polymorphism. We started by comparing the external morphology of 1538 specimens belonging to the 26 species (including type specimens). In this way we assessed both, the accurateness of the original description and the validity of each species based on morphological characters. In other words, we evaluated that each species has diverged in morphology from all other species occurring in the area. We also compared the advertisement calls of 14 of these 26 species to determine whether prezygotic reproductive barriers were likely to exist. Finally, we performed phylogenetic analyses of a fragment of the 16S mtDNA gene to assess their monophyly and divergence of each species in this gene genealogy. Our data set allows us to test the status of 15 species of Pristimantis for which at least two lines of evidence were available.

Of the 26 nominal species analyzed for morphological divergences, 13 show overlap in character states. Only nine of the 15 species selected for the comparison of the different lines of evidence have diverged in morphological characters, although 11 of them were originally described on the basis of morphology only. Moreover, our analysis of morphological divergence seems to be the most restrictive approach because it does not allow the discovery of some sibling monophyletic species well distinguished by their
advertisement calls. Full congruence between lines of evidence is restricted to only four out of the 15 species. Five species show congruence of two lines of evidence, whereas the remaining six are supported by only one. The separate analysis of differences in advertisement calls (evidence of reproductive isolation) or of phylogenetic data alone also shows limitations, because they do not support some morphological species.

These results exemplify several important implications of using an integrative approach to delimit species boundaries: (i) discordance between lines of evidence are expected under the general lineage concept of species, closely related species are not expected to show similar levels of divergence for all characters; (ii) the use of universal thresholds to delimit species (e.g., DNA barcoding) is theoretically and practically flawed because the degree of divergence at some character is dependent on the specific conditions of the speciation process; (iii) using different and independent lines of evidence allows taxonomists to communicate the degree of stability that users of species names can expect.

Paper II focuses on *Hyalinobatrachium* glassfrogs from the Guiana Shield. Delimiting species limits within frogs of this genus is particularly difficult because of their small size, arboreal habits, inaccessibility of localities, restricted distribution, and low density of most of the species, what result in very limited sample sizes. Also, high levels of phenotypic homoplasy, and subtle morphological differentiation between species complicate their identification. This situation is clearly exemplified in the Guiana Shield where at least five of the nine species recognized for the area have been identified through the literature to possess unresolved taxonomic problems. We compared species limits using morphological characters, morphometry, advertisement calls, and monophyly of mtDNA markers. Our results allowed us to discover two new species of which we described one, a resurrected species, four junior synonyms, several misidentifications including type material of some species, and new country records.

All six recognized species in this work show clear signs for divergence at several of our criteria. By far, the use of body proportions is the most limited method of the ones here explored here. Two possible evolutionary scenarios could account, at least partially, for this repeated lack of divergence at body proportions across *Hyalinobatrachium* species. We hypothesized that the fact that all members of *Hyalinobatrachium* lay their eggs on the underside of leaves could play an important role in their concerted phenotypic evolution because this reproductive strategy, almost restricted to them among centrolenids, could in theory impose morphological restrictions. Alternatively, the general *bauplan* of *Hyalinobatrachium* could be extremely conserved across the clade because of limitations on developmental patterns or reduced genetic variability underlying the studied characters.
In spite of our limited sample size, we suggest that there seems to be indications of a north to south genetic differentiation within species that is coincident with an altitudinal gradient.

Paper III

Glassfrogs represent an exceptionally diverse group among Neotropical anurans, but their evolutionary relationships have never been assessed from a molecular perspective. Mitochondrial and nuclear markers were used to develop a novel hypothesis of centrolenid phylogeny. Ingroup sampling included 100 terminals, with 78 (53%) of the named species in the family, representing most of the phenotypic diversity described for the group. Thirty-five species, representing taxa traditionally associated with glassfrogs, were used as outgroups. Gene sampling consisted of complete or partial sequences of three mitochondrial (12S, 16S, ND1) and three nuclear markers (c-myc exon 2, RAG1, POMC) for a total of 4362 bp. Phylogenies were estimated using MP, ML, and BM for individual genes and combined datasets.

The separate analysis of mitochondrial and nuclear datasets allowed us to clarify the relationships within glassfrogs. Also, we corroborated the sister-group relationship between *Allophryne ruthveni* and glassfrogs. The new phylogeny differed significantly from all previous morphology-based hypotheses of relationships, and showed that hypotheses based on few traits are likely to misrepresent evolutionary history. Traits previously hypothesized as unambiguous synapomorphies are shown to be homoplastic, and all genera in the current taxonomy (*Centrolene, Cochranella, Hyalinobatrachium, Nymphargus*) were found to be poly- or paraphyletic. The new topology implies a South American origin of glassfrogs and suggests allopatric speciation as the most important speciation mechanism. The phylogeny profoundly affects the traditional interpretations of glassfrog taxonomy, character evolution, and biogeography—topics that now require more extensive evaluation in future studies.

Paper IV

Based on the molecular phylogeny presented in paper III, a new phylogenetic taxonomy that is compatible with both the International Code of Zoological Nomenclature (ICZN) and the PhyloCode was proposed for Glassfrogs and their sister taxon, *Allophryne ruthveni*. The arrangement emphasizes the recognition of clades having (i) significant statistical support and congruence among phylogenetic estimation methods (i.e., parsimony, maximum likelihood, and Bayesian inference criteria), (ii) congruence among
genetic markers, (iii) morphological and/or behavioral distinctiveness, and (iv) preserving, when possible, the names and contents of the most generally accepted previous classifications.

The evolutionary proximity of Centrolenidae and Allophrynidae was recognized by combining these families into an unranked taxon, a proposal that maintains the traditional names and species contents of Centrolenidae and Allophrynidae. We arranged centrolenid diversity in two subfamilies and 11 genera of which seven are new. In one subfamily, the diagnosis and species content of the genera *Centrolene*, *Cochranella*, and *Nymphargus* was modified; another was resurrected and modified, and five other clades were proposed as new genera. The other subfamily, contained a new genus and a modified *Hyalinobatrachium* that fully corresponded to the former *fleischmanni* Group. Additionally, another genus was described and could not be assigned with confidence to either subfamily and it was placed as *incertae sedis* in Centrolenidae. The data at hand suggested that the later genus is a lineage as old as the two subfamilies.

The revised taxonomy differed markedly from previous arrangements, which were based on phenetics and few morphological characters. Most of the genera defined herein were confined to distinct biogeographic regions, highlighting the importance of geography in the speciation of Glassfrogs. The principal limitation of this proposal is that it was based on an incomplete sampling of taxa (54% of the recognized glassfrogs). Although diagnoses were based on phenotypic traits, there were several cases (16% of all species) in which the allocation of species was ambiguous because of morphological homoplasy and the lack of molecular data.

**Paper V**

Tropical areas harbor the most biodiverse biomes in the world. However, molecular studies assessing the patterns and processes of diversification within the tropics are still relatively scarce. Here we study the tempo and mode of diversification among glassfrogs, a clade of ~150 arboreal species distributed throughout all major highland and lowland rainforests of the Neotropics with a peak in their species diversity in the northern Andes of Colombia and Ecuador. Based on seven nuclear and three mitochondrial gene sequences (> 6400 bp) from 87 species, we reconstructed phylogenetic relationships, estimated divergence times, age-range correlation (ARC) of sister species, and reconstructed ancestral areas and dispersal/vicariance events. Our main results showed that: (i) the Guiana Shield was a core area in the origin of glassfrogs and the Amazon lowlands can be ruled out as a center of origin for Andean clades; (ii) the family appeared during the Oligocene and most genera already appeared during the early-middle Miocene (23.7–11.2 Mya); (iii) there is a progressive specialization of taxa with a
broad altitudinal distribution into specialist of either lowlands or highlands and once a taxon becomes a specialist does not normally reverse into an altitudinal generalist; (iv) dynamics of altitudinal distributions have played an important role in the current patterns of diversity and distributions among glassfrogs and mainly clades including altitudinal generalist have been able to spread across Neotropical rainforests; (v) diversification among glassfrogs predominantly occurred through pulses within single biogeographic areas; (vi) both complete isolation and ecological gradients seem to have been involved in the speciation of glassfrogs. Our work constitutes and important example illustrating that at different evolutionary times and in different biogeographical areas, alternative competing models of diversification of Neotropical faunas can occur. This highlights the importance of studying large evolutionary radiations at a continental scale.

Papers VI–XII

Throughout these papers I addressed specific taxonomic problems at the species level within Centrolenidae. In Papers VI and XII we described two new species of Hyalinobatrachium. In particular, Paper XII evaluated the diversity of Hyalinobatrachium in the eastern versant of the Andes and based on different data (morphology, mating calls, and genetics) we also proposed a previously recognized species as a junior synonym of Hyalinobatrachium pellucidum and extend its distribution to the eastern versant of the Andes in central Peru. In Papers VII–IX we evaluated the validity of several nominal species based on different criteria and revisited their distribution. We resurrected one species (Hyalinobatrachium orocostale), detected a putative new species from the Caribbean montane forests of Venezuela, identified four junior synonyms, corrected previous erroneous identifications, and provided new country records for Hyalinobatrachium nouraguense and Hyalinobatrachium ignioculus.
Conclusions and prospects

This dissertation has provided the following novel data and insights on questions related with taxonomy, phylogenetics, classification, and diversification in the Neotropics with special relevance for amphibians:

• The exclusive use of single lines of evidence or the application of arbitrary thresholds to identify new species does not only impair and bias our potential for discoveries, but also limits the possibility to understand evolutionary processes. Only an integrative approach combining every source of evidence provides the necessary feedback to test and discover all species. This approach further allows identifying those species that are probably representing stable comparative units and to flag species pending reassessment.

• Integrative taxonomists should provide users of species names with information about the degree of stability that they can expect from nominal species. This stability can be important, for example, for the design of management and conservation programs that depend on an accurate knowledge of the diversity in a region.

• The alpha-diversity of both Centrolenidae and Pristimantis is far from being stable and we can expect many more new species to be described and several new synonyms to be recognized.

• Previous hypothesis of relationships among centrolenid and hence their classifications frogs were mislead by rampant convergent evolution at the phenotypic level. This illustrates the relevance in phylogenetic studies of analyzing different datasets independently and under different tree building methods.

• A new suprageneric classification based on phylogenetic hypotheses of glassfrogs and their sister taxon is proposed based on their evolutionary history. The new classification follows specific criteria to assign clades to ranks and incorporates phylogenetic definitions.

• Our results support a complex model of diversification which started in the early Neogene and that probably has been influenced by major tectonic, orogenic, and climatic changes during the last 30 My. Glassfrogs expanded early to all major rainforests of South America, likely originating from an ancestor from the Guiana Shield. As glassfrogs diversified, many became specialists of highlands or lowlands and colonized different biogeography regions where they further diversified, perhaps includ-
ing some instances of parapatric speciation events along ecological gradients.

The results presented above are neither conclusive nor complete and more research is needed to confirm their validity. The origin of the diversity in the Neotropics is an important and complex question that I expect will drive the attention of many researchers in the future. The results of this thesis allow me to identify a number of relevant questions that should be addressed in future studies:

- There have been a number of the new tools recently developed to identify species limits and that incorporate recent theoretical and technological advances. An integrative taxonomic approach will definitively benefit from exploring such lines of evidence. For example the coalescence theory could help to detect incomplete lineage sorting and niche modeling to statistically assess ecological divergence.
- A more complete taxon sampling within Centrolenidae is needed to place all the species in the molecular phylogeny since phenotypic traits alone are not enough to prove relationship between species and affiliation to the new genera described.
- The phylogenetic framework created by our studies provides an ideal arena to study different questions regarding the evolution of complex phenotypes using Centrolenidae as model organisms. Comparative analyses correcting for phylogenetic relationships seem to be the right approach to assess such questions. Specific relevant issues would include elucidating the causes for the concerted evolution within *Hyalinobatrachium*, studying if characters potentially under strong sexual selective forces (i.e., mating calls) evolve at different rates than other characters, investigate the evolution of transparency and its correlation with other traits, and study possible relationships between environmental conditions and phenotypic traits and diversification rates.
- To better understand the diversification of amphibians in their biodiversity hotspots, integrative and comparative studies of their temporal, geographical, and biological origins should be performed. There are several large phylogenies available (e.g., families Bufonidae, Centrolenidae, Hylidae, Dendrobatidae, Strabomantidae) but no study has tried to identify generalities in their evolutionary history. Specifically, it seems necessary to investigate if there is a relationship between diversification rates within different and highly diverse anuran groups, and in different ecological areas, time periods and biological properties (namely, different modes of reproduction).
Svensk sammanfattning

Själva förekomsten av liv gör vår planet unik i universum, åtminstone så vitt vi vet i dagsläget. Den mångfald av organismer i olika storlekar, former och livsstil som finns och har funnits på jorden är nästan ofattbar. Mångfalden har alltid fascinerat och engagerat människor och förklaringarna till mångfaldens upphov har varit allt från rent mytologiska till mer rationella. Ur ett vetenskapligt perspektiv kan biologisk mångfald studeras genom deskriptiva och kvantitativa metoder för att kartlägga organismers och arters förekomst och antal, och studier som syftar till att förstå de mekanismer och processer som genererar och bibehåller mångfalden. I det sammanhanget presenterar jag min avhandling. I avhandlingen har jag fokuserat på groddjurens diversitet i en hotspot för mångfald, tropiska Amerika. Jag har framförallt studerat glasgrodor (Centrolenidae) och i mindre utsträckning grodor av släktet *Pristimantis*. Under min forskning har jag samarbetat med flera högskolor och studerat dessa grodors taxonomi på flera taxonomiska nivåer, deras fyllogenetiska relationer och geografiska utbredning samt hastigheten med vilken diversifiering har skett.

Groddjur är betydelsefulla och utmanande organismer för de biologer som studerar vertebrat-evolution och mångfald. Antalet beskrivna arter anses vara en kraftig underskattning av antalet förekommande arter (för närvarande är >6000 arter beskrivna). De utgör förmodligen den grupp av tetrapoder där flest nya arter beskrivs varje år. Tyvärr är många av dessa (>30%) hotade arter och flera har varit av högsta prioriteten för bevarandebiologiska insatser (Stuart et al., 2004). Även deras fyllogenetiska relationer har varit dåligt upplösta. Amfibier är en utmärkt grupp för att studera biogeografi och diversifiering eftersom det i allmänhet är arter som uppvisar begränsad spridningsförmåga och starka habitatberoenden. Artrika amfibiegrupper har utbredningsområden som inkluderar vida geografiska områden och genom att studera deras evolutionära historia kan vi få inblick i historien om anpassningar till den naturliga livsmiljön.

Trots den stora betydelsen av begreppet ”art” och begreppets utökade användning bland icke-biologer, har få ämnen i evolutionsbiologi lett till så omfattande diskussioner och skriftliga argumentationer med så lite konsensus. Definitionen av begreppet art är alltså ett vida diskuterat tema. Kärnfrågor i sammanhanget inkluderar; vad är en art, hur kan vi känna igen en art, och vilka är de evolutionära processer som bidrar till artbildning? Det största problemet tycks vara en sammanblandning av art som begrepp och de krite-
rierver vi använder för att skilja arter åt. För att lösa detta har ett allmänt art-koncept föreslagits; en art är ett temporärt urval av individer som för tillfället representerar en populations eller metapopulations utvecklingslinje vilken utvecklas oberoende av andra utvecklingslinjer och ingen enskild egenskap (t.ex. morfologisk eller ekologisk) skall betraktas som en artspecifik karaktär som en art måste uppovisa att erkännas som sådan. Logiken bakom detta resonemang är att dessa olika kriterier motsvarar olika händelser som inträffar under utvecklingslinjernas evolution (Fig. 2). Olika karaktärer kan alltså användas för att upptäcka skillnader mellan utvecklingslinjer. I min avhandling har jag använt en kombination av fenotypiska (morfologi och beteenden) och genetiska (DNA-sekvenser) karaktärer. En annan viktig aspekt av studier av den biologiska mångfalden är förhållandet mellan arter. Genom att bygga studierna på principen om att senare utvecklingslinjer har gemensamma anor kan vi studera inbördes släktskapsrelationer mellan såväl utdöda som existerande arter genom att titta på ärtliga karaktärer, denna gren av biologin brukar kallas fylogeni. Fylogenetiska studier (oberoende av vilken metod eller vilka karaktärer som används) resulterar i att vi kan dra slutsatser om utvecklingslinjers gemensamma ursprung. Under mina studier har jag använt DNA-sekvenser från mitokondriella och nukleära gener. Data har analyserats med hjälp av olika distans- och parsimonimetoder och stöd för beräknade topologier har uppskattats. Jag har inte använt fenotypiska data eftersom tidigare studier redan har undersökt deras betydelse i de studerade organismerna; antalet tillgängliga fenotypiska karaktärer är generellt för få för att få tillräcklig upplösning och flera fenotypiska karaktärer kan vara resultat av konvergerade evolution.

Den biologiska mångfalden är inte jämnt fördelad över hela vår planet och för att förstå orsakerna till olika mönster kräves ett evolutionärt perspektiv. Slutmålet är att få förståelse för de processer som förändrar artrikedom inom ett område, artbildning, utdöende och spridning. Inblick i dessa processer ges genom fylogenetiska studier där förhållandet mellan arter och biogeografiska och artbildningsprocesser studeras (utdöende har också studerats i detta sammanhang, men dess inflytande på biologisk mångfald har inte undersömts i mina doktorandstudier). Tropikerna i nya världen är planetens mest artrika område men hur denna mångfald har uppkommit har nästan inte alls beskrivits i den senaste litteraturen och allmänna mönster för diversifiering inom nya världens tropiska regioner är fortfarande dåligt utforskade. Eftersom den biologiska mångfalden är globalt hotad och vi går in i en unik era av utdöenden orsakad av mänsklig verksamhet är behovet av att förstå hur denna mångfald uppstår och upprätthålls viktig för utformandet av vettiga och framgångsrika bevarandestrategier. I följande avsnitt kommer jag att (mycket) kort presentera de mest populära diversifieringsscenarierna i sin enklaste form och att försöka förklara artmångfalden i nya världens tropiska regioner. Jag fokuserar på hur man kan studera detta med fylogenetiska-istället för populationsgenetiska metoder.
Artbildning genom geografisk isolering kan inkludera fysiska hinder för att isolera populationer, stoppa genflödet och därmed möjliggöra en uppdellungen av utvecklingslinjerna. Fysiska hinder kan orsaka barriärer mellan i övrigt kontinuerliga populationer och om den fysiska barriären endast tillåter spridning av en eller ett fåtal individer kan det leda till grundandet av en ny isolerad population. För att studera barriärer och spridningshändelser använder vi fylogenie och prover från lämpliga geografiska områden (t.ex. områden där endemiska arter förekommer) för att uppskatta ursprungliga utbredningsområden. I bästa fall bör dessa rekonstruktioner inkludera fylogeniernas grenlängder för att ge bättre tolkningar av divergenttider och mer realistiska paleogeografiska rekonstruktioner.


De övergripande målen för mina undersökningar under mina doktorandstudier är av två huvudtyper: att studera mönster och processer på artnivå och på högre taxonomiska nivåer och att bidra till taxonomiska indelningar som återspeglar den evolutionära historia av organismerna som studeras. Jag har fokuserat mina ansträngningar på groddjur i den neotropiska regionen. Först har jag utvärderat olika kriterier för att avgöra arter inom ramen för det allmänna artbegrepp som inkluderar en fristående utvecklingslinje. Det har jag studerat i en grupp av grodor från släktet Pristimantis och flera arter av Centrolenidae. Jag har också jämfört fylogenetiska hypoteser som grun-

Denna avhandling har bidragit med följande nya uppgifter och insikter om frågor i samband med taxonomi, fylogeni, klassificering och diversifiering i den Neotropiska regionen med särskilt fokus på groddjur, mer specifikt:

den nuvarande mångfalden av glasgrodor (Paper V). Dynamik i altitudinella utbredningar har spelat en viktig roll för den nuvarande mångfalden och utbredningen av glasgrodor och i princip har endast grupper som inkluderat altitudinella generalister kunnat spridas över de neotropiska regnskogarna (Paper V). Det har varit en successiv specialisering i artsammansättningen med altitudinella anpassningar till specialister i antingen lågland eller högland (Paper V). Diversifieringen bland glasgrodor har främst skett genom diversifiering inom enskilda biogeografiska områden (Paper V).

Resultaten som presenteras ovan är varken slutgiltiga eller heltäckande och fler undersökningar kommer att behövas för att bekräfta deras giltighet. Resultaten har dock tillåtit mig att identifiera ett antal relevanta frågor som bör behandlas i kommande studier:

Nyligen har ett antal verktyg utvecklats för identifiering av arter och deras avgränsningar och dessa införlivar de senaste teoretiska och tekniska metoderna. En integrativ taxonomisk strategi kommer definitivt att ha nytta av ny tekniker som coalescens-teori och nischmodellering. En mer fullständig provtagning inom Centrolenidae kommer att bli nödvändigt att få större tillförlitlighet i deras taxonomiska relationer. Det fylogenetiska ramverk som skapats av våra studier är en idealisk arena för att studera olika frågor kring evolutionsen av complexa fenotyper inom Centrolenidae. Jämförande analyser korrigerade för fylogenetiska relationer verkar vara rätt metod för att besvara sådana frågor. Särskilt viktiga frågor att klargöra är bland annat orsakerna till den konvergerande evolutionen inom Hyalinobatrachium, att undersöka om karaktärer potentiellt under stark sexuell selektion (till exempel parningslåten) utvecklas annorlunda än andra karaktärer, att undersöka evolutionen av genomskinlighet och dess korrelation med andra egenskaper och att undersöka eventuella samband mellan miljöfaktorer och fenotypiska egenskaper och diversifieringshastigheterna. För att bättre förstå diversifiering av groddjur i deras biologiska diversitetshotspots behövs integrativa och jämförande studier av arternas tidsmässiga, geografiska och biologiska ur sprung. Det finns flera stora utarbetade fylogener tillgängliga (t.ex. familjerna Bufonidae, Centrolenidae, Hylidae, Dendrobatidae, och Strabomantidae) men ingen studie har försökt identifiera generella mönster i deras evoluti onära historia. Specifikt kommer jag att undersöka om det finns ett samband mellan diversifieringshastighet inom och mellan olika artrika groddjursgrupper grupper, mellan olika ekologiska områden, över evolutionära tidsskalor och mellan biologiska egenskaper.
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I am afraid I have probably forgotten someone so please write your name here _______________________. Thank you!

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