Diversity, Phylogeography and Conservation of two groups of anurans of the family Hylidae in Mesoamerica.

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Thesis Abstract

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Diversity, Phylogeography and Conservation of two groups of anurans of the family Hylidae in Mesoamerica.

The amphibian fauna of Mesoamerica is one the most diverse, yet the most endangered in the area, perfectly exemplifying why the region is one of the world biodiversity hotspots. I carried out analyses from three interrelated perspectives: Conservation Genetics, species delimitation and Comparative Phylogeography of two genera of the family Hylidae in most of Mesoamerica: *Agalychnis* and *Dendropsophus*. First I performed a species delimitation analysis for the genus *Agalychnis*, with the specific aim of testing for cryptic diversity within the species *A. callidryas*, which exhibits wide morphological variation and the uncertainty of whether or not it represents more than one species has not been solved. Secondly, I did a conservation analysis of the genus *Agalychnis* adopting a comparative approach between endangered and non-endangered species and integrating ecological modelling and genetic information, with the aim of gathering key information for a better management and conservation planning at a regional scale of this genus in particular and Hylid frogs in general. This analysis resulted in the suggestion of short-term alternatives for conservation of critically endangered species and the management of non-endangered species at regional scale. Finally, I applied a comparative phylogeographic analysis between the two genera *Agalychnis* and *Dendropsophus* with the general objective of identifying factors and processes underlying species origin and dispersal within Mesoamerica. I proposed hypotheses for the colonization and dispersal of species of these two genera within Mesoamerica, on the basis of genetic data, geographical information, and ecological niche modelling. As part of data gathering for the latter analysis a new country record leading to the considerable extension of the geographical distribution of *D. phlebodes* is reported.
Declaration
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It is hard to mention in a few lines all the people involved directly and indirectly in this project and with the whole process of my PhD to who I would like to thank for contributing to my personal and professional progress and improvement. But briefly I want to thank every person that offered me help during my time in Manchester, those that supported me from Colombia in the distance and those who gave me a hand in the different countries in Central America.

The opportunity of being in Manchester and studying for this degree may have never been possible without the acceptation of my Supervisor Cathy Walton, who allowed me to be part of her team and develop this project in her lab. Thanks to her for the trust and support during the course of my PhD. I acknowledge the support and trust of my advisor Chris Klingenberg, who never stopped believing in me and the possibility for this project to be successful, thank you for the advice and for being always very welcoming with me. I thank the company and friendship of members of Walton’s lab and other graduate students and staff at the University of Manchester who I could account with through the different stages of my time at the University. Very critical, particularly during the early periods of my studies in Manchester, was the help of the family Hushion, in whose house I lodged for most of my time in the UK, especial thanks for welcoming me in their property and guide me through the different aspects of establishing myself in the country.

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Thank you to my family and close friends that were supporting me always from Colombia in the distance, for believing in me and for their trust, patience and understanding.
Remarks on authorship

This thesis has been submitted in an alternative format in order to facilitate rapid dissemination of the data in the wider academic and scientific community. To this end, all chapters of this thesis were prepared for submission to peer reviewed journals. All studies were designed and executed in co-operation with my Supervisor Catherine Walton. She is co-author on all the manuscripts presented here, which have been reviewed by her and she has presented her contributions to the writing. Other listed authors have contributed with their herpetological knowledge and experience for the preparation and execution of fieldwork and have contributed with samples collected by them through the years. All of the analyses, figures, tables, text and data have been generated by the Author following registration at the University of Manchester.

The thesis is aimed at improve our understanding of the diversity and phylogeographic patterns of amphibians in general and the genera Agalychnis and Dendropsophus in particular, in Mesoamerica. It aims at having the final impact of contributing to the conservation of critically endangered and management of non-endangered Hylid frogs in the region, particularly of the genus Agalychnis.

Chapter 2 is a paper prepared for Conservation Biology. It presents a comparative analysis of the genetic and ecological characteristics of three species of Agalychnis, two critically endangered species, A. lemur and A. moreletii, and one non-endangered species, A. callidryas. The final aim of this paper is to identify key factors for the effective conservation of these species at a regional scale in Mesoamerica.

Chapter 3 is a paper prepared for PLos Biology. It is a comparative phylogeographic analysis of the two genera Agalychnis and Dendropsophus in Mesoamerica. It aims at assessing the effects of historical climate change and geographical barriers on the generation of biodiversity and on shaping its patterns in the region.

Chapter 4 is a small note prepared for Check List. It aims at reporting a new country record and the consequent extension of the geographical distribution of D. phlebodes by 400Km north the currently recognized northern limit.
Chapter 1.

General Introduction
Mesoamerica is a biodiversity hotspot (Myers et al. 2000). Among all taxa the amphibian fauna is a perfect example of why the region is a hotspot, since this is one of the most diverse groups of vertebrates in the region, yet the most threatened (Wilson et al. 2010). Since it has been recognized that the aim of conservation should finally be the maintenance of the processes that have generated the diversity in the wild (Beebee 2005; Beebee & Griffiths 2005), understanding the processes that have generated such high amphibian diversity and identifying the factors influencing those processes is an important step towards the better design of effective conservation plans.

The following sections aim at giving a context to the research project presented here, which is concerned to the amphibian fauna of Mesoamerica, with particular emphasis on two genera of the family Hylidae. A broad view of the tropical biodiversity is given, as well as the particularities of Mesoamerican diversity. Moreover, because of the importance of the geological history of the region in shaping the patterns of Biodiversity, a brief description of the formation of Mesoamerica is presented and the hypotheses, derived from such geomorphological evolution, that have been proposed to explain the origin of Mesoamerican diversity. I further present an overview of the characteristics and conservation status of Mesoamerican amphibians in general and the genera under study in particular. Finally, a brief description of approaches and data useful in the study of diversity are presented, with a context of the applicability of such approaches, to then describe the goal of my research project.

1. Biodiversity.

Biodiversity as the contracted form of biological diversity became frequently used around the 80’s (Ghilarov 1996); however, about 85 concepts exist to define it (DeLong Jr 1996), and a high volume of research has been carried out for decades from different perspectives and disciplines to better understand the biodiversity, its origins and patterns around the globe (Pianka 1966; Ghilarov 1996). Given that my research project is about Biodiversity, I consider useful to start this section by suggesting the concept that better describe the scope of my thesis: ‘Evolution is genetic alteration through time, Biodiversity is genetic diversity’ (Avise 2008 p.11564). Therefore, the analyses I will present are based on genetic data as the main source of information and intend to interpret evolutionary processes originating biodiversity in Middle America and shaping its distributional patterns, with focal attention to amphibians. In the following
subsections I will give a background on the aspects of Biodiversity that motivated this project, especially in the study area.

1-1. Global Biodiversity Patterns.

The distribution of biodiversity exhibits patterns at large and small scale on earth. The first well recognized pattern at a global scale is the latitudinal gradient, which consists in higher species richness at lower latitudes like the tropical area, compared to temperate regions (Gaston 2000; Willig et al. 2003; Gaston 2007). In amphibians this gradient is clearly observed: The highest density of species is present in the forest of South America (Figure 1), where amphibians represent more than 36% of the diversity (Young et al. 2004). Such a distributional pattern has also been observed in birds (Cook 1969) and mammals (Simpson 1964).

It is difficult to formulate a general explanation for the latitudinal gradient, but several models have been proposed to explain it, and were recently categorized from three perspectives (Mittelbach et al. 2007): ecological (Figure 2a), evolutionary (Figure 2b), and historical (Figure 2c). Earlier ideas were based mainly on ecological principles and the interpretation of the geological history (Fischer 1960; Pianka 1966). In this context, it was suggested that high tropical species richness is due to a bigger offer of niches (Dobzhansky 1950; Boulière 1957; Simpson 1964) and the existence of tropical and
temperate-like niches within the tropics as a result of the complex relief (Simpson 1964; Cook 1969). Moreover, Dobzhansky, T. 1950 proposed that when a high number of habitats are available for species in absence of competition versatility in adaptation is possible; but when there is competition between species, the number of habitats available becomes limited and species go into specialization. Therefore, evolution of tropical organisms is determined by the conflict that generates a high number of habitats and inhabitants (Dobzhansky 1950). Similar ideas indicated that environmental stability in the tropics, especially of factors like temperature, influences speciation by promoting physiological and structural variation (Fischer 1960), the development of finer adaptations and specializations (Dobzhansky 1950; Pianka 1966; Stehli et al. 1969). Furthermore, it was suggested that long-term stability made tropical biotas ‘mature’; while temperate regions have a sequence of ‘mature’ biotas, extinction, and new ‘immature’ biotas; However, biotas of tropical mountains are not as mature as the rest in the tropics, because those places are relatively younger in geological time (Fischer 1960 p.79).

In the ecological context, recent models have focused on the role of ecological gradients on the evolution of species; an example is the hypothesis of Ecotones, which states that selection is the key process in generating biodiversity and it is highly promoted by the wide range of niches in the tropics (Moritz et al. 2000). This idea is compatible with the concept of niche conservatism, which claims that the relationship between environmental factors, like temperature, and the species richness is due to ancestral niches’ maintenance when adaptive shifts occurred in the evolutionary history, rather than the result of higher energy available in the tropics (Diniz-Filho et al. 2007). Evidences for this model have been found in several taxa, including bats (Stevens

Figure 2. Mechanisms supporting hypotheses to explain latitudinal gradients of diversity. (a) Ecological model: Diversification rate is similar along the gradient, so the difference in richness is due to ecological factors. (b) Diversification rate in the tropics is higher, and so is the richness. (c) The tropics have had more time for diversification than temperate areas (Figure taken from Mittelbach et al. 2007 p. 316).
Evolutionary mechanisms, on the other hand, have focused on differences between speciation and extinction rates in temperate and tropical regions (Gaston 2000; Gaston 2007), and have been categorized into three models (Arita & Vázquez-Domínguez 2008): ‘The tropics as a cradle’, assumes that the tropics have higher origination rates and constant extinction rates; ‘the tropics as a museum’, assumes constant origination rates but lower extinction rates; and ‘the out of the tropics’ model hypothesizes higher origination rates, lower extinction rates, and migration of species from tropical to ‘extra-tropical’ areas. In tropical *Eleutherochactylus* frogs the tropics seem to have functioned as ‘museums’ more than ‘cradles’ (Crawford 2003), while the model of ‘out of the tropics’ has been supported by the 11 million years history of marine bivalves (Jablonski et al. 2006).

It has also been proposed, that the evolutionary activity decreased in temperate zones because processes of selection under severe climatic conditions, like glaciations, reduced the number of species living and evolving in the area (Brieger 1969). However, it is difficult to think that glaciations did not affect the tropics, especially if we consider that the most famous hypothesis so far proposed to explain the high Neotropical biodiversity, known as the Refugia Hypothesis, is about the effects of glaciations in the Amazon during the Pleistocene (Haffer 1969). This theory is a very old idea, which seems to predate Darwin’s work (Haffer & Prance 2001) (Mayr & O’Hara 1986), but just after it was published by Haffer (1969) it became more popular, and lately polemic.

The modern version of the refugia hypothesis states that during the Pleistocene and post-Pleistocene, dry climatic conditions in the Amazon lead to the formation of arid non-forested areas and the confinement of the forest in small patches, which served as refuges for animal populations that were isolated and deviated from one another during the isolation period. The forest was again continuous all over the Amazon during humid climates, and animal populations extended their ranges (Haffer 1969 p.131). The idea has received support by studies attempting to explain the origin of some taxa in Africa (Mayr & O’Hara 1986; Anthony et al. 2007), Asia (FaGen & NianHe 2005; Zhang et al. 2006), birds (Smith et al. 1997; Hawkins et al. 2006; Ricklefs 2006; Sánchez-González & Navarro-Sigüenza 2009) and frogs (Schneider et al. 1999; Navas 2002; Wiens et al. 2006; Guarnizo et al. 2009). In the latter, climate seems to be important in delimiting niches, promoting isolation and speciation (Kozak & Wiens 2007).
2008; Zuccon et al. 2008), Madagascar (Richard & Dewar 1991), South America (Hubert et al. 2007), and also out of the tropics, in Australia (Shapcott 2000; Koetz et al. 2007), east Greenland (70°-74°N) (Funder 1979), Europe (Boller et al. 1976; Thorpe 1982; Thorpe 1984; Ribera & Vogler 2004; DeChaine & Martin 2006), New Zealand (Neiman & Lively 2004), and North America (McCoy & Connor 1980; Schmidtling et al. 1999). Also, evidence from specific taxa includes: tropical butterflies (Brown et al. 1974; Turner 1976; Turner 1981; Hammond 1990), birds (Feduccia 1977) and beetles (Noonan 1985; Ratcliffe & Jameson 1989; Jameson 1990).

Recently, however, the original idea of refuges has been debated (Colinvaux 1998; Colinvaux et al. 2000; Colinvaux et al. 2001) due to the availability of data on Paleoclimate (Colinvaux & Liu 1987), Paleoecology (Bush & Colinvaux 1988), and Palaeontology (Rancy 2000), which allows questioning the basis of the theory and its applicability, especially in the Amazonia. The proposal of aridity and the fragmentation of the forest during the Pleistocene glaciations (Haffer 1969) has been contrasted by the idea that persistent forest during glaciations included montane-like species, which later during the Holocene were displaced out of the lowlands for not tolerating the heat (Colinvaux et al. 2000). Evidence against the hypothesis has also been found on specific taxa, including South American frogs, whose phylogenetics is incompatible with the refugia (Heyer & Maxson 1982; Lynch 1982; Hedges 1989). The origin of Amazonian mammals during the Pleistocene in areas previously suggested as patches of savannah among forested areas (Haffer 1969), also disagreed with the refugia, and instead it indicated that Pleistocene forest relicts more likely existed in edges of rivers (Rancy 2000; Webb 2000). The latter supported an alternative idea, although not mutually exclusive with the refugia, known as the river-created habitats (Remsen & Parker III 1983).

Modifications of the original refugia theory (Rull 2005; Solomon et al. 2008) and its combination with historical phenomena, like historical sea levels fluctuations (Gascon et al. 1998; Cabanne et al. 2007), as well as completely novel ideas (Bush 1994; Colinvaux et al. 2000; Allen et al. 2006; Mazzoleni et al. 2007) have given new perspectives to the study of the origin and evolution of tropical biodiversity. The Disturbance-Vicariance hypothesis (DV), is a modern idea based on changes in temperature and CO2, and proposes that speciation occurs by diversity-instability and is maintained by intermediate disturbance and habitat heterogeneity (Bush 1994;
Colinvaux et al. 2000). So, rather than aridity, high endemism and stability during glacial periods (Haffer 1969), places with high species movement most likely showed isolation and speciation (Bush 1994). The effects of Miocene marine incursions and tropical rivers as barriers to gene flow for terrestrial taxa have also been studied (Haffer 2008). In the family of frogs Ranidae (Evans et al. 2003) and subfamily Eleutherodactylinae (Heinicke et al. 2007) evidence has been presented that show the influences of ancient fluctuations of sea level on their dispersal patterns and so their current distribution (Figure 3).

Overall, the mechanisms postulated to explain the high species diversity in the tropics from an evolutionary point of view range from dispersal, vicariance, their combination and variations, but, the interpretation of the tempo and mode of speciation of Neotropical biodiversity based on those mechanisms remains unclear (Chown & Gaston 2000), and a large debate on this topic has been active for decades, which is evident in the numerous discussions published over the years (Pielou 1981; Abele 1982; Nimis

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**Figure 3. Oceanic dispersal of Eleutherodactylinae frogs.**

a. Dispersal over water from South America (SA) to Middle America (MA) 42-31 Mya originate the Middle American Clade (MAC), and to Proto-Antilles (PA) 47-29 Mya originate the Caribbean Clade (CC). b. Higher sea level and land subsidence (~30 Mya) isolated two clades from the CC: Western Caribbean Clade (WCC) on Cuba (Cu) and Eastern Caribbean Clade (ECC) on Hispaniola and Puerto Rico (H/PR). c. (~20 Mya) Radiation of Syrrhopus from Cu to Southern North America (NA) and Northern MA; colonization of Lesser Antilles (LA) by individuals of ECC and South American Clade (SAC). d. Interchange of species all along the mainland (from NA to SA) (Figure taken from Heinicke et al. 2007 p. 10095).
I think that the present trend is to integrate principles of different areas including geology, ecology, geography and evolution that complement each other, rather than being polarized and follow principles of only one of these disciplines. Adapting this kind of integrative approaches would take us closer to understand the processes underlying the origin and shaping of biodiversity patterns, even so it is not a magic solution that could give us a general answer, it would take into account as much data as possible produced by the different disciplines that would otherwise remain ignored when trying to answer general questions using only one type of data (i.e. ecological, genetic, morphological).

1-2. Processes that generate Tropical Biodiversity.

Complex geomorphological structure and fluctuating climate change are among the most commonly pointed factors to explain the generation of biodiversity in both the old and Neotropics (Mittelbach et al. 2007). Transforming geology and topographic structure are thought to be the main causes of allopatric isolation (Halffter 1987) and differentiation and vicariance that lead to speciation in a wide range of taxa (Noonan 1985; Colinvaux 1998; Watson & Peterson 1999; Cabanne et al. 2007; Guarnizo et al. 2009; Daza et al. 2010). Moreover these characteristics in places like the Neotropics have allowed the identification of effective barriers to gene flow that may have influenced the diversification of taxa, for example mountain ranges, types of forests, river formations, etc. One of such clear barriers are for example the Andes, which is thought to have driven allopatric diversification of many different taxa, including amphibians (Guarnizo et al. 2009), birds (R. Terry 2000). Another example of effective barrier is the replacement of different types of forest, like occurs in the Pacific versant of lower Central America. In this region, a mix of dry and wet forest is present and drives the isolation of taxa adapted to one or the other type of forest due the discontinuity of their habitat. Although the effect of this barrier have been observed mainly by direct analysis of taxa distribution (Watson & Peterson 1999), the explanation to its effects on the origin and dispersal patterns of diversity have rarely been deeply analyzed (Crawford et al. 2007). Moreover, the presence of extensive lowlands, like the case of Nicaraguan depression, the Motagua valley (in the collision
point between the Maya and the chortis block) is thought to be a major barrier to gene flow and to drive vicariance in some taxa (Castoe et al. 2009; Daza et al. 2010).

The formation of rivers and also the changes of river drainages have also played an important role in driving speciation in the Neotropics. Such dynamic formation of river systems is also closely related to the complex and constantly evolving geomorphology of the region. The effect of rivers as effective barriers to gene flow in some cases and as providers of microclimates that favour speciation has been suggested across the Neotropics from the limit between extra tropical and tropical areas in Mexico (Bryson et al. 2011) to the very tropical Amazon River (Gascon et al. 1998; Hayes & Sewlal 2004).

The effects of climate change have also been widely studied and tested as a main influencing factor in driving speciation in the tropics (Colinvaux & Liu 1987; Pennington et al. 2004; DeChaine & Martin 2006; Hawkins et al. 2006; Mittelbach et al. 2007; Daza et al. 2010; Bryson et al. 2011). In the American continent, overall, the effects have been clearly established, particularly by the identification of refugia, where many taxa have confined during glaciations and diverged into allopatric species. This appears to be the case in wide range of taxa in South America, particularly the Amazonia (Haffer 1969; Mayr & O'Hara 1986; Haffer & Prance 2001; Haffer 2008). Also, another effect of climate change has been in the mediation of dispersion across geographic areas after speciation has occurred. For example climate change has been proved to influence the formation of new taxa in Andean hills that during favourable climate have come down and colonized the Amazonia, feeding in this way the highly diverse biota with taxa originated in extra Amazonian regions, and leading to further speciation processes in situ (Santos et al. 2009).

**1-3. Middle America: Biodiversity and Biogeography.**

The Neotropics is one of the richest and most diverse regions on the planet (Muñoz & Mondini 2008). Its range of habitats is so wide, containing a large percentage of the global rainforest, some of the driest deserts in the world (Burnham & Graham 1999; Muñoz & Mondini 2008), the greatest wetland, largest mountain chain and richest ocean current (Stotz 1996). Altitudes vary from sea level to ~6,000 meters above and temperatures range from above 30°C to below 10°C (Burnham & Graham 1999). This variety of habitats and environmental conditions explains the complex arrangement of
Neotropical biotic communities. My research project is about biodiversity in the Neotropical region, specifically in Middle America (as defined by Winker 2011) where I gave emphasis to Continental Central America.

Within the Neotropics Middle America is an outstanding region due to the rich diversity and degree of endemism it harbours (Campbell 1999; Myers et al. 2000; WWF 2009a), which is perfectly exemplified by its herpetofauna (Campbell 1999; Wilson et al. 2010) and it is a remarkable fact especially given its small geographical area. Based on the definition of Eco-region as “a large unit of land or water containing a geographically distinct assemblage of species, natural communities, and environmental conditions” World Wildlife Fund for Nature (WWF) identified 867 Eco-regions on earth (WWF 2009a). Furthermore, in a program called Global 200 (G200) WWF grouped several of the originally recognized Eco-regions to create the list of G200 Eco-regions, considered the most outstanding places on earth and first priorities for conservation.

The G200 program assumes that by concentrating the conservation strategies on unique areas, it is possible to preserve the global biodiversity (WWF 2009b). Sixteen G200 Eco-regions are located in Middle America (WWF 2009b), and all of them extend into at least one of the seven Central American countries and Mexico. Nine are categorized as critically endangered, three vulnerable and only the remaining three are considered stable or “intact” (WWF 2009b). A similar idea for conservation has lead to the identification of hotspots, defined as places where an exceptional number of species occur, but also a high and fast habitat loss threatens their existence (Myers et al. 2000). 34 Biodiversity hotspots have been identified around the world and Middle America (referred in this program as Mesoamerica) almost in its total extent is one of them (Conservation-International 2007).

Middle America is one of the world’s Biodiversity hotspots, which is clearly supported by the levels of endemism found in such a small geographical region. Middle America harbours more than 17,000 species of plants, of which 2,491 are endemic to the region; some 1,113 species of birds, 440 species of mammals (Conservation-International 2007), 1,148 species of reptiles and 731 species of amphibians (Wilson et al. 2010). Unfortunately, a high percentage of species occur in areas under high pressure and habitat disturbance as result of human colonization, rapid expansion and growth in Middle America (Conservation-International 2007). This is similar across biological
groups. Threatened species include 36 endemic birds and 36 endemic mammals endemic to the region (Conservation-International 2007), and the alarming figure of 291 species of amphibians (Wilson et al. 2010), 232 of which are endemic to the region (Conservation-International 2007; Wilson et al. 2010). With this in mind, research that conducts to a better understanding of the processes and factors that have originated and would maintain Biodiversity in Middle America are crucial in designing effective conservation plans.

Overall the distribution of biodiversity in Middle America follows a latitudinal gradient evidenced by a high number of species in southern countries like Costa Rica and Panama (nearer the Equator). However, when looking at a smaller scale or at specific biotic groups within the region other patterns can be identified (Campbell 1999). In amphibians, there is a high diversity in the southern area; but factors different from latitude need to be taken into account to explain, among others, the comparable richness of amphibian species found in the south (Panama and Costa Rica) and the north-west (Mexico and Guatemala) of Middle America (Campbell 1999; Wilson et al. 2010), or the drastic drop in species richness found in Nicaragua (Wilson et al. 2010). Consequently, a complex group of factors including latitude, altitude, vegetation formations, the geological and climatic history of the region, as well as biological characteristics of the biotic groups need to be taken into account when trying to explain their distributional patterns in this region.

Relatively few studies have exclusively attempted at understanding the biogeographic processes within Middle America (Poelchau & Hamrick 2011), but the majority have mainly focused on the migration of stocks from North or South America into Middle America, and those trying to understand diversification processes within Middle America, have been conducted either in the extreme north limit, like northern Guatemala and Mexico (Bryson et al. 2011; Sullivan et al. 2000), or in the south, including Panama and the transition Colombia-Panama (Bermingham & Martin 1998; Crawford 2003; Crawford et al. 2007), and to a lesser extent in Costa Rica (Crawford 2003). Such studies have led to conclusions on the clear mixing of taxa from both surrounding big landmasses but little information on the origin of Middle America native groups, once the stocks have colonized, the processes underlying this origin, and the effect of different factors involved, i.e. climatic change, geomorphological characteristics, historical effects. It is therefore clear that the knowledge about the
evolutionary history of taxa in Middle America is very limited compared to that about the South American biodiversity (particularly the Amazonia and the Andes) (Haffer 1969; Remsen & Parker III 1983; Bush 1994; Colinvaux 1998; Gascon et al. 1998; Lougheed et al. 1999; Terry 2000; Haffer & Prance 2001; Navas 2002; Hayes & Sewlal 2004; Haffer 2008; Solomon et al. 2008; Guarnizo et al. 2009; Santos et al. 2009).

Studies concerning phylogeography and trying to explain how species of particular biotic groups have shaped their current distributional patterns in Mesoamerica have included small rodents (Sullivan et al. 2000), trees (Poelchau & Hamrick 2011) and to a lesser extent amphibians (Crawford 2003). It has been acknowledged the effects that past historical events may have had on the phylogeography of species (Poelchau & Hamrick 201; Sullivan et al. 2000), but also the variable responses that different biotic groups and even independent species have had to those historical events and changes (Sullivan et al. 2000), with which generalized phylogeographic patterns have not been suggested in either of the studied groups. Even so it has been accepted that co-distributed taxa would share more commonalities on their history (Crawford 2003; Poelchau & Hamrick 2011; Sullivan et al. 2000) the hypothesis of complete congruence in the phylogeographic patterns of different species has not been considered the most likely scenario in Mesoamerica (Sullivan et al. 2000).

Climate change has been one of the important historical events identified as influential on shaping the distributional patterns today observed in Mesoamerican species, particularly the vegetation (Poelchau & Hamrick 2011), followed by geomorphological events, especially important to explain the phylogeography of highland species, like rodents that are today confined to certain high mountains in Mexico, and most likely corresponded to species once continuously distributed across a geographical range, which then was interrupted by the appearance of mountain ranges, and led to speciation (Sullivan et al. 2000). Even so such newly originated species are closely related and allopatric, their subsequent response to a particular historical event, like climate change, may not be the same (Sullivan et al. 2000). Also, in Mesoamerica, the climate pattern like the strong and frequent occurrence of hurricanes has been pointed as one factor that powerfully influences the phylogeography of species as may have been the intense and rapid changes in the landscape by human activity, especially trees, (Lemes et al. 2010). Moreover, it is not likely that just one historical or recent event is enough to explain the
phylogeography of any species or group of species (Poelchau & Hamrick 2011; Sullivan et. al. 2000).

Finally, in general in temperate and tropical regions it has been identified the existence of refugial areas where forests confined several times along the history (Hewitt 2004). In Mesoamerica it has also been suggested the existence of refugia during the Pleistocene and also islands during the Pliocene, which are similar areas to those identified as refugia in the Amazonia (Haffer 1969). Such areas may have offered stable habitats for species to move to during extreme climates, like glaciations. Phylogeographic studies on tree species of Central America have identified such Pleistocene refugia particularly along the Pacific versant and in the current San Juan River area shared between Costa Rica and Nicaragua, and Pliocene islands have also been suggested along the Pacific from El Salvador to Panama (Poelchau & Hamrick 2011).


The study of the biodiversity and its distributional patterns in a region requires some knowledge of the geological history of the place. In Middle America, several big main areas can be identified, the south eastern of Mexico, Nuclear Central America (NCA), Lower Central America (LCA), the latter two connected by the big land mass of Nicaragua (Winker 2011). The formation of each one of these areas involved complex geological processes.

Five main tectonic plates were involved in the formation of the Central American isthmus as we know it today, the Maya, Chortis, Chorotega, and Chocó blocks and to a lesser extent the Caribbean Plate (Savage 2002). The first two blocks correspond to the northernmost area of Middle America, and it is estimated that they collided approximately 38 Mya leading to the formation of the current NCA and most of Nicaragua, the latter being the last portion of the Chortis block. Such collision it is thought to have had high effects on the region’s topology, by leading to volcanic processes through most of the latest Miocene and the Pliocene, especially on the Pacific side of countries like, Guatemala, El Salvador, Honduras and Nicaragua, probably allowing the uplifting of mountain chains (Savage 2002).
Continuing southwards through the region, the middle area covering the last southern portion of the Chortis block, which correspond to the current south areas of Nicaragua, it is thought to have collided with the northern limit of the Chorotega block around 5.3 to 1.8 Mya, the resulting area of the collision is currently known as the Nicaraguan depression and is the geological limit between NCA and LCA (Bolaños et al. 2008). Parallel to these collision processes there has been a subduction of the Cocos Plate under the Caribbean Plate trough most of the Quaternary, which is thought to be still undergoing, particularly at the area known as the Middle American trench. Such subduction is believed that originated the Pacific volcanic chain as we see it today and that in relatively short time (less than 1 Mya) has allowed the formation of large number of big and small volcanoes whose distribution extends from Guatemala through El Salvador to Nicaragua and to a less extent to northern Costa Rica (Savage 2002; Carr et al. 2007). A good number of such young volcanoes are currently active.

Within LCA, the last part of the bridge that connects the whole of Central America with South America involved long processes of shortening, uplifting and migration of the Central American Volcanic arc (Coates & Obando 1996). The initial collision of Southeast Panama with the Northwest of Colombia started ca. 12.8 to 9.5 Mya (Coates et al. 2004). According to geological and palaeoecological evidence, the process of collision seemed to finish around 8.6 Mya, and it was followed by the uplifting, which would last at least until ca 4.8 Mya (Coates et al. 2004); but it seems that only 3.1-2.8 Mya the isthmus became a complete land bridge for terrestrial species (Webb 1991) and a barrier to marine species (Woodring 1966; Coates & Obando 1996). Such bridge apparently has only been interrupted by risings of the sea levels that have formed temporary marine corridors through time (Coates & Obando 1996).

In aquatic ecosystems the presence of the new landmass stopped the interaction between two big systems, the Pacific and the Atlantic, and changed the oceanic circulation, freshwater balance, geochemical cycles, and ocean-atmospheric feedback, modifying the climatic regime of the area (Haug et al. 2001). For terrestrial species, the bridge mainly influenced the dispersal by allowing the Great American Interchange (GAI) (Marshall et al. 1982; Webb 1991), whose effects varied among biotic groups (Vanzolini & Heyer 1985; Vermeij 1991). In mammals, it allowed to increase the number of genera in North and South America (Marshall et al. 1982), but also promoted extinction (MacFadden 2006). North American species spread and diversified at high
rates once they reached South America, even replacing native species (Marshall et al. 1982), and that is why many South American species have North American origin (Lessa et al. 1997). In North America a few groups settled down, but the extinction rate of non-natives was higher and speciation hardly happened (Marshall 1988).

For groups with high ability to cross barriers the occurrence of migrations strictly after the emergence of the isthmus was initially doubted (Vuilleumier 1984), but it was recently recognized that the GAI influenced the evolution of Neotropical avian-fauna (Dacosta & Klicka 2008), especially of large birds (Barker 2007). However, some warnings have been stated about these conclusions because research on the topic is still basic (Barker 2007; Ricklefs 2002). Similarly, it is still controversial the role of the isthmus in the dispersal patterns of herpetofauna (Vanzolini & Heyer 1985).

Early studies recognized that very few herpetological species took part on the Interchange when the bridge just formed (Vanzolini & Heyer 1985), and recent phylogenetic studies on snakes suggested that there was colonization of South America by northern central American immigrants, but it was a slow process that was not accelerated by the presence of the land bridge (Wuster et al. 2005). In Hylidae frogs, it has been suggested that originated in South America and a subsequent dispersal to Central America was facilitated by the formation of the bridge (Duellman 2001); whereas in Craugastoridae frogs very old lineages have been identified in Central America, which predate the GIA (Crawford 2003). In Eleutherodactylinae frogs an ancient oceanic dispersal, mediated by water rather than the land-bridge, has been suggested to explain the migration of South American ancestors to the Caribbean Islands and Central America (Heinicke et al. 2007). All these contradicting evidences suggest that much more research is needed to clarify the role of the Panama isthmus on the Neotropical amphibians, and also that is expected a different scenario among groups, and even species.

2. Middle American Amphibians: The Study System.

Middle America harbours around 731 species of amphibians of which around two-thirds are endemic to the country of occurrence (Wilson et al. 2010 p.43). The diversity of amphibians is currently classified into 21 families and 89 genera mainly present in (1) tropical rainforest and (2) highlands (Wilson et al. 2010). Such distribution of species
Richness may explain the high number of species found in the forests of Costa Rica and Panama, and the highlands of Guatemala, and Honduras, in contrast to the low diversity reported in Nicaragua and El Salvador, countries with marked dry regimes and mostly dominated by lowlands (Sunyer Mac Lennan 2009; Wilson et al. 2010). However, it is important to note that the uneven distribution of habitats and therefore species along Middle America also corresponds to an uneven herpetological work carried out in the region, with some countries accounting with considerably better and historically more intense surveys of their species and ecosystems than others (Sunyer Mac Lennan 2009; Wilson et al. 2010).

2-1. Genus *Agalychnis* (Cope, 1864).

The genus *Agalychnis* was first described from a type species corresponding to the currently recognized *A. moreletii*, although frequently in the literature the generotype has been attributed to *A. callidryas* (Duellman 2001). It currently contains fourteen species (Faivovich et al. 2010). However, within this big group the previously recognized *Agalychnis* species are those now included in the *A. callidryas* group, characterized by moderate to extensive webbing in hands and feet, absence of bicoloured iris, egg-laying in open surfaces, particularly leaves overhanging ponds or other sources of water (Duellman 2001; Jungfer et al. 2004; Pounds et al. 2004; Santos-Barrera et al. 2004; Solis et al. 2004a; Faivovich et al. 2010).

The genus is currently represented in Middle America by five species of the former *A. callidryas* group: *A. annae*, *A. callidryas*, *A. moreletii*, *A. saltator*, *A. spurrelli* and the unassigned to any group: *A. dacnicolor* and *A. lemur* (Faivovich et al. 2010). This project is mainly concern to the *A. callidryas* group and the species *A. lemur* (six species). These species are distributed across the whole of Middle America, with some of them having very restricted distributions, i.e. *A. lemur*, *A. moreletii* and *A. annae*, and another one, *A. callidryas*, being widespread through the region. Among these frogs there are pond breeders, like most of the *A. callidryas* group and a mainly stream breeder, *A. lemur*. In terms of altitude *A. callidryas* and *A. saltator* occur at a relatively wide range of altitudes from sea level to almost 1,300m (Jungfer et al. 2004; Solis et al. 2004a); *A. annae*, *A. lemur*, and *A. moreletii* are highland species with lowest altitudes being at about 500m and tolerating the highest altitudes among the species of this genus (Pounds et al. 2004; Santos-Barrera et al. 2004; Solis et al. 2004b); and, *A. spurrelli* is
mainly a lowland species, with its highest altitudinal limit being around 700m (Jungfer et al. 2004).

Wide morphological variation has been observed mainly in *A. callidryas*, which has motivated controversial discussion through the years (Duellman 2001). However, equal evidence has been presented to support the recognition of more than one species within this taxon (Funkhouser 1957) than to accept the current taxonomic status of just one widespread species (Savage & Heyer 1967). Still, the question is opened and no deep analyses have been carried out that allow accepting either taxonomic status, by combining evidences from different sources of data. Therefore, analyses intended to test for the presence of cryptic diversity within this taxon are much needed. Moreover, if these uncertainties exist within one of the species, it is possible that similar cases occur in the other taxa, particularly those of which little is known compared to *A. callidryas* (i.e. *A. saltator* whose better known populations are in Costa Rica, but some records exist from Nicaragua and Honduras, with very little information about such populations) (Jungfer et al. 2004).

Following the IUCN categories of threat (IUCN 2010) two out of the six species referred to in this thesis are critically endangered (CR), *A. lemur* and *A. moraletii* (Santos-Barrera et al. 2004; Solis et al. 2004b), one is endangered (EN), *A. annae* (Pounds et al. 2004) and the remaining three are of least concern for conservation (LC), *A. callidryas, A. saltator* and *A. spurrelli,* although for the latter three species populations are also considered to be decreasing in the wild (Jungfer et al. 2004; Solis et al. 2004a). Better surveys of the different populations within all these species, would help in clarifying their conservation status and defining their real occurrence ranges, because except for *A. callidryas* the species have not been exhaustively surveyed across their currently accepted geographic distributional ranges. The latter represents a difficulty for policy changing and decision making, particularly for conservation and management.

2-2. Genus *Dendropsophus.*

Species of the currently named genus *Dendropsophus* were initially part of the genus *Hyla*, until they were recently recognized as another genus within the family Hylidae containing the species of *Hyla* known to have 30 chromosomes (Faivovich et al. 2005).
Species of this genus are distributed from Southern Mexico through Central America to Northern Argentina and Uruguay (Frost 2009). The genus contains ninety-two species assigned to nine species groups based on some morphological similarities (Faivovich et al. 2005). Although the phylogenetic relationships within the genus are better known than for other genera of frogs uncertainties remain on this and other aspects and further research is necessary to increase our knowledge of the species, species groups and to clarify the phylogenetics of the genus (Faivovich et al. 2005).

*Dendropsophus* is overall a genus of least concern for conservation, with one species Critically Endangered, two endangered and another one vulnerable (IUCN 2010). Populations are generally stable, however a considerable number of species experience some kind of threat, mainly by agriculture, aquaculture and the use of biological resources which could change notoriously the status of conservation of several species in the short term (IUCN 2010). A warning also exists on species classified as Data Deficient, because their situation in the wild is very uncertain (IUCN 2010) and by the time information is gathered they could be in the highest levels of threat.

2-2.1. *Dendropsophus ebraccatus* (Cope, 1874)

The Hourglass tree frog, *Dendropsophus ebraccatus* (Frost 2009), belongs to the *D. leucophyllatus* species group (Faivovich et al. 2005). This species occurs in tropical forest and forest edges, but also in open, no-forested or disturbed areas (Jungfer et al. 2010). It is distributed from Southern Mexico in Middle America, where it has a patchy occurrence, to Colombia and some areas of Northern Ecuador in South America, where it seems to have a more continuous range (Jungfer et al. 2010). Its populations tend to be stable along its distributional range, so it is considered a common or abundant species, of least concern for conservation, and it is highly favoured by occurring in several protected areas (Jungfer et al. 2010). Potential threats include agricultural development, and particularly the pollution resulting from spraying illegal crop plantations (Jungfer et al. 2010).

*Dendropsophus ebraccatus*, is a very interesting species for ecological and evolutionary studies (Touchon & Warkentin 2008b; Robertson et al. 2009), because it has terrestrial and aquatic oviposition, which seems to be induced by environmental conditions and can vary at the individual level (Touchon & Warkentin 2008b). Furthermore, it has been
suggested that this is a suitable species to study the ecological factors influencing the evolution of terrestrial and aquatic reproduction (Touchon & Warkentin 2008b), which is an important systematic character in amphibians (Faivovich et al. 2005). This species seems to be an intermediate stage in the evolution of terrestrial oviposition within the *D. leucophyllatus* group, by presenting what is considered the ancestral aquatic oviposition and also what could be a derived terrestrial oviposition (Touchon & Warkentin 2008b). Tadpoles of *D. ebraccatus* also show particular responses to predators (i.e. fishes and dragonflies) and develop different phenotypes specially related with the tail’s colour and size, being the phenotypes also specific for the predator. So, it has been suggested that balancing selection in the presence of different predators in a particular breeding site would lead to genetic variation for developmental plasticity on this species (Touchon & Warkentin 2008a).

2-2.2. *Dendropsophus microcephalus* species group

*Dendropsophus microcephalus* (Cope, 1886) is the species after which its group is named. Within Middle America this group is represented by four species including the *D. microcephalus* itself, *D. phlebodes, D. sartori* and *D. robertmertensi*, and in general the group contains 33 species (Faivovich et al. 2005). *Dendropsophus microcephalus* is a lowland species that prefers savannah and forests edges, but is also found in marshy areas, clear areas in the middle of the forest and in highly disturbed zones (Bolaños et al. 2004). It has a wide geographical distribution, overall continuous from Mexico to Brazil (Bolaños et al. 2004). Populations of this species are stable and big and, like in not many other amphibians, are predicted to be increasing (IUCN 2010). Recently, a new record was added that not only increases the list of localities for the species but also the range of distribution towards the south Pacific lowlands of Colombia (Bolivar-G. et al. 2009). Moreover, in South America *D. microcephalus* is found breeding year round (Armesto et al. 2009).

As expected, this species is in the category of Least Concern (LC) for conservation, mainly due to its wide distribution range and big population sizes, which are not expected to decrease fast enough (IUCN 2010). Moreover, the species occurs in many protected areas (IUCN 2010) and seems to be very adaptable to disturbed areas where deforestation has taken place (Bolaños et al. 2004); In fact, studies on the effects of deforestation over the composition and structure of amphibian communities in the
Neotropics have predicted that in places of Central America, where the forest is facing rapid and devastating changes due to human activities, this species would become one of the dominant taxa of new or surviving communities (Furlani et al. 2009).

Apart from *D. microcephalus*, I will make emphasis on two additional species of this species group that occur in Middle America, *D. phlebodes* and *D. robertmertensi*. These are thought to have originated from a common ancestor with *D. microcephalus* (Duellman 2001). *Dendropsophus phlebodes* is distributed along the Caribbean coast of Costa Rica and Nicaragua (Solís et al. 2010) and *D. robertmertensi* is only present in the Pacific coast of northern El Salvador, Guatemala and parts of Mexico (Santos-Barrera et al. 2010). Morphologically both species are not very distinct from *D. microcephalus*, with some dorsal markings being the key to differentiate among them. Both species are pond breeders that form aggregations during rainy seasons, and prefer open grasslands with shallow ponds that can be temporal or permanent and, similar to *D. microcephalus*, they both tolerate disturbed landscapes (Santos-Barrera et al. 2010; Solís et al. 2010). Both, *D. phlebodes* and *D. robertmertensi* are of least concern for conservation (Santos-Barrera et al. 2010; Solís et al. 2010); however, these are understudied taxa, therefore there are reasons to keep the question opened regarding their conservation status, especially given their relatively restricted geographic ranges, compared to their closely related species *D. microcephalus*.


Amphibians in the biodiversity hotspot of Middle America are under very high levels of threat. A total of 731 species of amphibians occur in this area (Wilson et al. 2010) of which 291 species are under threat (Wilson et al. 2010) and it is likely that many species disappear before we discover them and describe them. Moreover, the number of described species has increased in around 133 species since 1999 (Campbell 1999), and is expected to keep increasing as better surveys are carried out, which is a real need in this region where characterization of the biodiversity is one of the crucial steps towards its conservation (Wilson et al. 2010).

Main threats to amphibians in Middle America are caused by human activities, particularly due to the increase in human population and the need for space for urbanization and agriculture. High levels of deforestation, which increase at fast rates
across the region, are one of main causes of habitat loss (Wilson et al. 2010). This summed to the emergence of lethal diseases, like Chytridiomycosis, have extirpated almost whole assemblages of amphibians in short time across the region, and this is known for the most famously studied areas like Panama, Costa Rica (Lips et al. 2003; Crawford et al. 2010) and Mexico (Lips et al. 2003; Lips et al. 2004), therefore scenarios in other areas are uncertain and there is no reason to think they could be any better but by contrast they could be worse, especially given the known high rate of habitat destruction in countries like the under-surveyed Nicaragua (Sunyer Mac Lennan 2009) or the highly deforested Honduras (Wilson et al. 2010).

3. Integrative approaches for the study of Biodiversity.

Research on biodiversity, either to answer questions of main biological importance or to gather necessary data for the effective management and conservation of species, i.e. for policy changing or decision making, requires basic knowledge to be acquired about the taxa under study as part of any research, for example clarifying the taxonomic status of the species. For such investigations to get closer to answer the questions, while acquiring all the necessary data and to avoid extrapolations or mere speculations, it is very useful to adopt integrative approaches that allow different types of data to be analyzed simultaneously and to answer different types of questions as part of the same research. The outcome is then more likely to be applicable to real solutions for conservation and of course would offer a much richer answer for biological questions.

The following sections describe briefly some types of data and related approaches that are useful for the study of biodiversity, particularly when not only the science but the conservation of the species is contemplated.

3-1. Methodologies and types of data.

This section describes different sources and types of data that result useful when studying biodiversity and the approaches or fields that use such data.
3-1.1. Morphology.

The use of morphological data is among the oldest approaches in science, being the main source of information for taxonomic studies since the beginning of times. Although modern times have brought a variety of methodologies aiming at accelerating the process of gathering data about species richness, it is not possible to think that traditional taxonomy can be completely replaced or displaced by, for example, modern molecular taxonomy like DNA barcoding (Ebach & Holdrege 2005). Even when the need for cataloguing biodiversity is high, particularly in fields different from the purely taxonomic, i.e. conservation and ecology (de Carvalho et al. 2007). However, this view may be different when studying microscopic organisms (Kaya et al. 2009), in which traditional morphological analysis is tedious, probably subjective and may lead to underestimation of the diversity (Kaya et al. 2009).

Other than for traditional taxonomic questions, especially in the search for those key morphological characters that allow the differentiation of one species from another, the morphology can be used in conjunction with other data and within multidisciplinary approaches as in ecological, evolutionary and genetic studies. Examples are studies aiming at explaining evolutionary and ecological patterns (Miles & Ricklefs 1984; Wainwright 1988; Wainwright & Richard 1995; Ohmer et al. 2009), studying sexual selection (Butler & Losos 2002), and habitat use (Wainwright & Richard 1995), etc.

3-1.2. Genetics: Phylogenetics and Phylogeography.

Among the most widely used types of data in biodiversity studies currently is the genetic data. For example in the study of the processes that generate biodiversity, like the vast Neotropical amphibian diversity, three disciplines based on the usage of genetic data have proved to be efficient and facilitating tools that could be used separately or together as a complex approach. Phylogenetics allows to infer the evolutionary history of groups of organisms (Moret & Warnow 2005; Ye 2007), at macro- and micro-evolutionary level (Avise 1987). Phylogenies are visually represented on trees (Hall 2004) that we estimate based on assumptions (Models) of the processes that originated the data under analysis (i.e. nucleotide/amino acid sequences) (Kelchner & Thomas 2007).
Population genetics, concerned with heredity and microevolution (Avise 1987), is a mix between making predictions (hypotheses) about populations based on Mendelian inheritance, and finding out how our predictions meet the genotype and allele frequencies in the populations that inspired the predictions in the first place (Hamilton 2009). Moreover, the development of the coalescent theory gave population genetics a much needed mathematical boost making it statistically flexible and adaptable (Emerson et al. 2001). But it was the introduction of Phylogeography what brought these two independent fields together for the study of Evolution (Avise 1987). So, Phylogeography is concerned with historical processes and principles underlying the geographic distribution of gene lineages (Avise 2000). Thus, it is one of the most integrative disciplines in Biology using concepts of population genetics, phylogenetics, and ecology (Avise 1998) to understand the influences of climate, geography and ecology on communities’ composition and evolution (Hickerson et al. 2010). Therefore, the application of the three disciplines, are the powerful tool in building the puzzle of Neotropical Amphibians’ origin and evolution (Avise 1998; Emerson et al. 2001; Hickerson et al. 2010).

In the context of systematics and taxonomy the usage of genetic data, have motivated the development of statistical tools for species delimitation based on genetic-only information, like the General Mixed Yule Coalescent algorithm for species delimitation (GMYC) (Pons et al. 2006; Monaghan et al. 2009). This algorithm, in particular, is aimed at detecting the transition point from speciation processes to coalescent processes in a phylogeny (Pons et al. 2006), and has proved to be very efficient in the identification of cryptic diversity across many taxa (Stuart et al. 2006; Bickford et al. 2007; Monaghan et al. 2009).

### 3-1.3. Geography and Geology: Species Distribution models.

Geographic data related to the taxa (i.e. e. georeferenced occurrence data) and geological information on study areas allow in principle the establishment of distributional ranges of species, particularly when combined through the usage of especial software and methodologies like the package ArcGIS and the maximum entropy algorithm, Maxent (Phillips et al. 2006), respectively. Moreover, when analyzed in conjunction with ecological and climatic data, the geographical data becomes very powerful information that allows modelling of species distributions under different
combinations of environmental conditions, widely used in ecological studies (Pearson et al. 2007; Elith et al. 2011). Furthermore, when placed into context of evolutionary biology, species distribution models (SDMs) can be projected into past climatic conditions, to predict paleodistribution of species (Chan et al. 2011). This way it opens the door to the proposal of geographically explicit hypotheses on the origin and evolution of taxa in a given region that otherwise would be very difficult to propose and evaluate (Thomé et al. 2010; Chan et al. 2011). This is a very important approach in evolutionary biology.

Species distribution models (SDMs) can also be projected into future environmental conditions, becoming an important tool to predict the effects of some factors, particularly those that mainly affect the survival of species in years to come (Beaumont et al. 2008). This is a useful approach in conservation, even though the approaches for its application still need revision and improvement (Beaumont et al. 2008; White et al. 2010) and the data available, particularly for climate conditions, varies widely among proposed models (Gordon & O'Farrell 1997; Collins et al. 2004; Hasumi & Emori 2004; Beaumont et al. 2008).


This section aims at presenting in context some of the previously described types of data and approaches, to highlight their applicability and usefulness in studies of biodiversity from the evolutionary point of view and for the conservation of species, which are topics of main interest in my study. Furthermore, when possible, emphasis will be done on Neotropical amphibians.

3-2.1. Species delimitations.

The delimitation of species is one of the most important, yet complicated processes in biology, especially when there is not clear consensus on the concept of species (De Queiroz 2007). However, millions of species have been described, mostly by the application of traditional taxonomic methodologies and in all cases without paying much attention to the processes behind the species formation. Most recently the
integration of genetic data, although not to replace traditional taxonomy (Ebach & Holdrege 2005), have facilitated the identification of independently evolving entities that may represent cryptic species within traditionally recognized species (that exhibit conservative morphological characteristics) (Pons et al. 2006; Monaghan et al. 2009; Crawford et al. 2010; Francis et al. 2010). Especially useful has been the application of an approach known as the DNA barcoding (Hebert et al. 2003).

DNA barcoding works under the concept that the variation of a selected short sequence of DNA between species (specifically, the mitochondrial Cytochrome Oxidase subunit 1 (COI) in animals), can be used as their barcode (Hebert et al. 2003). DNA barcoding has proved useful mainly because it can speed up the process of species identification compared to only traditional methods, which would take long time (DeSalle & Amato 2004; Waugh 2007), also because it facilitates the discovery of cryptic, synonym or extinct species (Crawford et al. 2010), the match of adults with immature specimens, and the monitoring of species with or without scientific aims (Savolainen et al. 2005; Waugh 2007). However, DNA barcodes should not be treated as absolute evidence, but only as a complement to the information obtained from nuclear DNA, morphology and ecology (DeSalle & Amato 2004; Rubinoff et al. 2006).

In amphibians, COI has been found to work well for the fundamental aims of DNA barcoding (Smith et al. 2008), even though some studies have identified some downfalls for COI as the amphibians barcode, including high variability of priming sites, and overlapping of inter- and intra-specific divergence values (Vences et al. 2005a). An alternative to minimize risks of inaccurate results is to combine the analysis of COI with other mitochondrial and nuclear genes (Vences et al. 2005a). The mitochondrial 16S rRNA gene has been suggested as a very good marker, because it is a gene that allows the usage of truly universal primers, and separate species unambiguously, proving to be far more efficient than COI or the other commonly used marker Cytb (Vences et al. 2005b). However, the inclusion of amphibians on the database of the DNA barcoding initiative imply using the standardized sequence (COI), and it has been recommended to do so, because this marker provides the needed information for the aims of the DNA barcoding project (Smith et al. 2008). On Neotropical Hylids the COI barcode has allowed to clarify the taxonomic identity of populations within a particular species (Hawkins et al. 2007), and 16S has been used to discover cryptic species in amphibian communities on specific areas (Fouquet et al. 2007). The overall result of these studies
confirmed the underestimation of the amphibian diversity in the Neotropics and the applicability of the DNA barcoding approach to carry out those kinds of assessments.

Overall, in Neotropical amphibians even though the existence of widespread species has been confirmed (Fouquet et al. 2007), studies on historical gene-flow have evidenced the underestimation of frogs diversity in the Neotropics, when finding long-term complete reproductive isolation between populations considered of the same species due to their morphological conservatism (Elmer et al. 2007b). These studies have been done in just a few species and not in their entire geographic distribution (Elmer et al. 2007b), which means there is a lot of work ahead of us before we really know how big is the already considered —Vast Neotropical amphibian diversity.

3.2.2. Reconstruction of the Evolutionary history.

Studies of amphibian evolution relied for long time on morphological and ecological data only (Duellman 1967; Duellman 1973), except for pioneer works that used albumin analysis to infer phylogenies on frogs (Heyer & Maxson 1982), or allozymes analysis to study population genetics (Gascon et al. 1998). As in those early studies, similar parameters of genetic diversity are analysed today, but now we have a better idea of which parameters provide the most useful information about populations (Beebee & Griffiths 2005), and have the benefit of a greater range of molecular markers and improved statistical analyses (Storfer et al. 2009), which has allowed to do better Phylogenetic reconstructions (Wiens et al. 2005; Noonan & Wray 2006), systematic reviews (Faivovich et al. 2005; Faivovich et al. 2010), and studies of the biogeographic patterns of amphibians (Noonan & Wray 2006; Wiens et al. 2006; Smith et al. 2007).

Genetic exchange is vital for long-term survival of populations (Blaustein et al. 1994). In Neotropical amphibians, fine-scale studies at intra-specific (Gascon et al. 1998) (Nielson et al. 2006), subpopulation (Robertson et al. 2008) and inter-individual scales (Elmer et al. 2007a), have found that gene-flow in amphibians tends to be affected by geographic distance (Elmer et al. 2007a), but it really depends on the dispersal ability of the species, and how that ability is affected by environmental conditions more than by the presence of assumed geographical barriers (Nielson et al. 2006; Noonan & Wray 2006; Elmer et al. 2007b; Robertson et al. 2008).
Phylogenetic analyses have also been used to complement identification of cryptic amphibian species, since it allows the recognition of paraphyletic and polyphyletic gene lineages among species (Fouquet et al. 2007). However, the most important contribution of Phylogenetics favoured by the integration of molecular markers, especially Mitochondrial DNA (mtDNA) (Darst & Cannatella 2004; Beebee & Griffiths 2005; Hickerson et al. 2010), has been the improvement of Systematics and Phylogenetic reconstructions (Hoegg et al. 2004). The family Hylidae has received special attention, and these kind of analyses have been done for the whole family (Darst & Cannatella 2004; Faivovich et al. 2005), in particular geographical areas (Salducci et al. 2005), at subfamily (Faivovich et al. 2010) and genus level (Chek et al. 2001). For instance, a recent phylogeny of the subfamily Phyllomedusinae used up to 12 genes and intervening rRNA’s, morphological and ecological evidence to present what is the best-supported hypothesis of the Phylogenetic relationships within the subfamily so far, as well as insights on the evolution of several important characters related to the reproduction and physiology of these frogs (Faivovich et al. 2010), therefore it is a good base for further studies on aspects of the evolutionary history of the group.

Identifying the processes and factors underlying Neotropical amphibians’ evolution has been facilitated by the use of phylogeographic approaches (Avise 1998; Hickerson et al. 2010), and has been mainly based on two concepts: vicariance and dispersal (Vences & Köhler 2008). Several hypotheses have been tested to explain phylogeographic patterns of Andean, Amazonian and other lowland species, and the results seem to vary among areas depending on their history, but also between species, from different areas and within the same area. For the family Hylidae in particular the processes identified to underline the evolutionary history are: Genetic drift and local selection, which seems to explain large diversification at small geographic scale in the lowlands (Ohmer et al. 2009) and Vicariance (Disturbance-Vicariance) in the highlands, as a result of the Andes and minor mountain ranges’ uplift and historical climate change that lead to adaptations to ecotones and altitudinal movements, respectively (Rull 2005; Guarnizo et al. 2009; Koscinski et al. 2009).
3.2.3. Conservation of Biodiversity.

Amphibians are considered very good models for genetic studies because of their low mobility that makes them easy to sample, promotes population structure and because controlled crosses are possible due to their external fertilization (Beebee 2005). But, it is mainly the conservation concern, especially in the tropical areas (Stuart et al. 2004; Beebee 2005), what is focusing research towards this group, especially to try and design effective strategies for their conservation. The latter requires great amount of information on the species, which explains a lot of research is needed to prioritize what we need to conserve, where, and more importantly how, particularly in a group that is incredibly diverse, in the region where the most of its species occur (the Neotropics harbour 59% of the world total) and are already declining at a rather accelerated rate (39% of species are threatened of extinction) (Young et al. 2004).

Despite the strong ecological direction of the conservation Biology, the importance of genetics in the process of species conservation has been highlighted for long time (Varvio et al. 1986; Moritz 1994; Frankham 1995), especially because it has been recognized that many genetic issues in populations are causes of species extinction (Frankham 1995; DeSalle & Amato 2004). Better knowledge of the impact that genetic issues have on the endangerment of wildlife in nature and captivity, bigger range of molecular approaches and markers, and the improvement of statistical analysis tools (Hedrick & Miller 1992; Frankham 1995; Avise 1996; Hedrick 2001; Caballero & Toro 2002; Neigel 2002; DeSalle & Amato 2004), have brought up the importance of using genetics, and approaches that integrate genetics with other disciplines for conservation decisions (DeSalle & Amato 2004). Furthermore, alternative (no-invasive) methods have been developed to obtain big sample sizes for genetic analysis without causing a big impact in the populations and still conserving the quality of the DNA (Poschadel & Möller 2004; Broquet et al. 2007), which is especially useful when working on highly endangered species.

Overall, the application of genetics for the conservation of amphibians has especially benefited from population genetics, with approaches that allow determining effective population sizes (recent and historical), population structure and hybridization; Phylogeography, which have allowed to identify important events on the evolutionary
history of species and track amphibians' routes of dispersal; and quantitative genetics, which has been used to study adaptive variations, plasticity, and fitness traits (Beebee 2005; Smith et al. 2007). More recently, landscape genetics (Manel & Segelbacher 2009), has been used to determine the role of habitat distribution in shaping patterns of genetic diversity in single amphibian species (Koscinski et al. 2009).

4. PhD Goals.

I carried out analyses from three perspectives: Conservation Genetics, species delimitation and Comparative Phylogeography. My study systems were two groups of anurans of the family Hylidae in most of Mesoamerica: the genera *Agalychnis* and *Dendropsophus*. The specific aims of my study were to:

1. Test for the existence of cryptic diversity within the genus *Agalychnis*, especially in *A. callidryas*, which exhibits wide morphological variation but the uncertainty of whether or not it represents more than one species has not been solved (Duellman 2001). To do so I applied a DNA barcoding-like approach using mtDNA.
2. Identify the relationship between intra-specific genetic diversity and degree of endangerment in *Agalychnis* species, adopting a comparative approach between endangered and non-endangered species.
3. Estimate the levels of connectivity between wild populations of *Agalychnis* species. To do so, I applied geographic information systems (GIS) tools to integrate ecological modelling and genetic information.
4. Assess the effects of climate change on the dispersal patterns of the genera *Agalychnis* and *Dendropsophus* throughout Mesoamerica.

I hope the results from this study can contribute key information for a better management and conservation planning at a regional scale of *Agalychnis* in particular and Hylid frogs in general in Mesoamerica.


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Chapter 2

Comparative genetics and ecology for the regional scale conservation of *Agalychnis* (Hylidae: Phyllomedusinae) in Mesoamerica

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Comparative genetics and ecology for the regional scale conservation of

*Agalychnis* (Hylidae: Phyllomedusinae) in Mesoamerica

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Abstract

Conservation of highly threatened species is challenging in places where not only the biodiversity but also their habitat are being lost at a fast rate. Comparative studies involving endangered and non-endangered closely related species are a strategy that allows gathering necessary information in short time, to help planning effective conservation. We integrated genetic techniques and GIS tools to combine mitochondrial DNA (mtDNA) data with ecological, climatic and geographical information to study three species of the genus *Agalychnis* in Mesoamerica: one non-endangered, *A. callidryas* and two critically endangered, *A. lemur* and *A. moreletii*. We assessed intra-specific genetic diversity, tested for cryptic species presence, modeled their current and future distribution under different scenarios of climate change, and estimated current and historical connectivity between populations of endangered species to identify conservation corridors. We found that *A. callidryas* and *A. lemur* are complexes of five and two independent entities, respectively, and suggested them to be treated as independent units for conservation purposes. Further research is also suggested to clarify the taxonomic status of such entities, and if needed to elevate their taxonomic status. On the basis of the distribution models for the three species for current conditions and onto the future, a decrease and patchiness of the habitat suitability was predicted for the following decades. To avoid the extinction in the wild of the remaining populations of *A. lemur* and *A. moreletii* and to improve the management of *A. callidryas* at a regional scale in Mesoamerica we proposed the establishment of conservation corridors. Immediate conservation action should start by ecological restoration projects and further monitoring inside the conservation corridors and preferably bi-national strategies should be implemented.

Keywords:
Biodiversity; cryptic diversity; connectivity; climate change; conservation corridors.
Introduction

Effective conservation plans require comprehensive knowledge of the taxa, including clear taxonomic status, distributional ranges and habitat requirements and the extent of intraspecific variation (both genetic and ecological). Integrating data from different sources, geographical, genetic and ecological, as well as predictive modeling is therefore key to the development of better management and conservation plans. An important component of this integrative approach is the use of molecular data, for example, to the discovery of cryptic diversity (Bickford et al. 2007; Stuart et al. 2006), the estimation of effective population sizes and the effects of habitat alteration on species fitness (Beebee & Griffiths 2005; Kraaijeveld-Smit et al. 2006).

Despite its importance, the process of gathering data is often not straightforward, due to difficulty in accessing and studying rare taxa, particularly in biodiversity hotspots, where most of forest and biodiversity occurs and undergoes the highest levels of endangerment (Myers et al. 2000). Nowhere is this more true than for the Mesoamerican amphibian fauna, currently composed of 731 described species, two thirds of which are endemic to the region (Wilson et al. 2010), which is remarkable given its small land surface. However, amphibian populations are declining so fast that almost whole assemblages of species have disappeared in a short time across the region (Crawford et al. 2010; Lips et al. 2004; Lips et al. 2003) and 291 species are threatened, mainly due to human impacts (particularly leading to habitat loss) and diseases (IUCN 2010; Wilson et al. 2010). Furthermore, chances of minimizing the effects of all threats in the short term are very low, especially because the dynamics of amphibians’ populations and the way threats operate are poorly understood (Beebee & Griffiths 2005), ultimately becoming a real challenge for in situ and ex situ conservation (i.e. captive breeding programs) (Griffiths & Pavajeau 2008; Kraaijeveld-Smit et al. 2006).

In planning the conservation of communities containing species under different levels of threat, a useful strategy is to compare endangered species alongside closely related non-endangered species (Burne et al. 2003; Ge et al. 1999). This enable us to gain a good understanding of the general factors impacting all taxa and specific factors determining the conservation status of each species, particularly those endangered.
This is the approach we applied here to study a group of frogs of the family Hylidae, the most diverse yet highly endangered, in Mesoamerica (Duellman 2001): 166 species occur in the region and 104 are under some category of threat (IUCN 2010). Agalychnis (Cope, 1864) (Phyllomedusinae) is one of Hylidae genera currently represented by seven species in Mesoamerica (Faivovich et al. 2010), three of which are endangered: A. annae, A. moreletii, and A. lemur, and one, A. callidryas, that is non-endangered and is widespread through almost the entire region. Even this taxon, however, is of potential conservation concern since it exhibits wide morphological variation that the possibility of it representing more than one species or subspecies has been extensively discussed (Funkhouser 1957; Savage & Heyer 1967), but it is a question that remains unanswered (Duellman 2001).

We analyzed and compared the genetic diversity and ecological characteristics of three species of Agalychnis, A. callidryas, A. lemur, and A. moreletii, in Mesoamerica to improve our understanding of the interaction between these factors at the species level and provide key information for better conservation practices. Our goals were to: (1) Test for the existence of cryptic diversity and identify genetically unique units within our putative species through the application of a DNA barcoding-like approach (Francis et al. 2010; Pons et al. 2006) and (2) assess intra-specific genetic diversity, using mitochondrial DNA (mtDNA). (3) To obtain species distributions models (SDM’s) on the basis of current and future climatic data and geographic information using GIS tools and the maximum entropy algorithm; and finally (4) to estimate levels of connectivity between populations given their genetic relatedness and ecological characteristics to help guide the management and conservation of the genus at regional scale.

Methods

Sample Collection

We collected genetic samples from three focal species of frogs of the genus Agalychnis, A. callidryas, A. lemur, and A. moreletii, through most of Mesoamerica (Figure 1 & supporting information) using a mouth swabbing technique (Poschadel & Möller 2004). Agalychnis callidryas is a widespread non endangered species, which
occurs from Mexico through Central America to the Pacific of Colombia (Solís et al. 2004a), and was sampled from 34 localities in six countries at elevations that varied from sea level to 1,300m. For the other two focal species, both critically endangered, we visited sites where they were known to occur historically and searched for their remaining wild populations. *Agalychnis lemur* is currently patchily distributed in Costa Rica and Panama (Solís et al. 2004b). We sampled the species from one locality in Costa Rica and obtained tissue samples donated by El Valle Amphibian Conservation Center (EVACC) from two localities in Panama; elevations varied from 540m to 950m. *Agalychnis moreletti* is patchily distributed in Mexico, El Salvador, Belize, Guatemala and Honduras (Santos-Barrera et al. 2004) and was sampled in the latter three countries from seven localities at elevations between 600m and 1,500m. These populations were represented by a minimum of one and a maximum of 83 individuals (Supporting information). Additionally we sampled a fourth related species, *A. spurrelli*, in one locality in Costa Rica (Supporting information).

**DNA extraction, Amplification and Sequencing**

We used the ISOLATE Genomic DNA Mini Kit (Bioline, London, United Kingdom) to extract DNA from swabs and liver tissue samples. A 753bp fragment of the mitochondrial gene Cythocrome b (Cytb) was amplified and sequenced using either published (Moritz et al. 1992) or redesigned primers (Supporting information) that typically generated higher yields of PCR products. All sequences were manually checked in the program Geneious (Drummond et al. 2011) and aligned in Clustal X2 (Larkin et al. 2007) followed by minor checking and trimming in MacClade (Maddison & Maddison 2000).

**Species Delimitation Analysis**

To evaluate the existence of cryptic species within the putative taxa of the genus *Agalychnis* in Mesoamerica we first built median joining haplotype networks in Network v 4.6.1.0 (Fluxus Technology Ltd) using default settings, to initially identify any distinct genetic cluster within species. Secondly, we employed the Pons et al (2006) ‘general mixed Yule coalescent’ (GMYC) algorithm for species delimitation as implemented in the package ‘splits’ (SPecies LImits by Threshold Statistics
The GMYC tests for species boundaries by identifying the threshold value at the transition point between the processes of speciation and coalescence in a phylogeny and requires a fully resolved topology (with no polytomies) of the putative species with branch lengths as input (Pons et al. 2006). We determine that the evolutionary model that best fit our data using the AIC criterion in JModeltest (Posada 2008) was the GTR+G model, so it was subsequently used in tree estimation.

To obtain a fully dichotomous tree we removed all identical haplotypes in DNASP (Librado & Rozas 2009). Given our interest in inferring the phylogeny using the full length amplified for the Cytb gene (753bp with no gaps), it was not possible to root the tree using a suitable outgroup from published sequences; therefore we used the midpoint rooting for the phylogenies as implemented in FigTree v1.3.1. We used the Bayesian Markov Chain Monte Carlo (MCMC) (Drummond et al. 2002) method as implemented in BEAST v1.6.2 (Drummond & Rambaut 2007) to infer a tree topology and estimate posterior probabilities. In BEAST is necessary to assign the specimens to taxa as a prior and we did so following the clusters defined by the haplotype networks. We used two evolutionary rates known for Cytb in amphibians: the lowest, 0.8%/Mya and the highest 2.5%/Mya (as used by Lougheed et al. 1999), and additionally an intermediate rate of 1.5%/Mya.

BEAST analysis included 4 independent runs per rate: 2 assuming a strict molecular clock (strict clock) and 2 using a relaxed uncorrelated lognormal clock (relaxed clock). Every run consisted of 10,000,000 generations with the first 1,000,000 discarded as burnin and parameter values sampled every 1,000 generations. We used LogCombiner v1.6.2 (Drummond & Rambaut 2007) to combine the two independent runs and resulting trees for every rate and clock. Convergence in combined results was checked in Tracer v1.5 (Rambaut & Drummond 2007 -a) and TreeAnnotator v1.6.2 (Rambaut & Drummond 2007 -b). We used the function ‘compute.brln’ as implemented in the package ‘APE v. 2.8’ (Paradis et al. 2004) run in R (http://www.R-project.org/) to make the final trees ultrametric and use them as input for the species delimitation analysis using the GMYC algorithm. We applied both the single and multiple threshold methods to the GMYC algorithm (Monaghan et al. 2009; Pons et al. 2006).
Figure 1. Sample sites, measured from the known geographical distribution of the three focal species (IUCN 2010), the sampling sites per species for general and specific localities (colored circles). A. lemur and A. moreletii were not found at the moment of sampling (colored crosses).
Species Distribution Models: Current and Future

Distribution modeling was performed on the basis of the currently recognized taxonomic status of the putative species to estimate their current geographical distribution given the climatic conditions. We gathered species occurrence data from different Museums and collections (Accessed through the Global Biodiversity Information Facility, GBIF Data Portal, data.gbif.org, 2011-11-22) and from the literature (Robertson et al. 2009) to complement our presence data and generate species distribution models (SDM’s) using the maximum entropy algorithm implemented in Maxent v 3.3.3k. (Phillips et al. 2006). The total number of unique presence records compiled was 218 for *A. callidryas*, 98 for *A. moreletii* and 32 for *A. lemur*. To model the current distribution of the three species we used three climatic variables: precipitation, minimum temperature and maximum temperature available from http://www.worldclim.org. Such variables are in close relationship with amphibian biology (Carey & Alexander 2003). We partitioned the presence data into two sets: one training set consisting of 75% of occurrence data used for prediction of the model, and the remaining 25% was the test data used to validate the models and calculate statistics.

We used default settings in Maxent except for restricting model building to ‘hinge features’, which allow a smoother model (Elith et al. 2011); ‘calculating response curves’ to assess the probability of the species occurring at a specific geographical point given different values of the predictors (environmental variables) (Phillips et al. 2006); ‘performing jackknife to measure variable importance’, and choosing the function of ‘don’t extrapolate’ to avoid bogus projections (Thomé et al. 2010). We used the ‘minimum training presence’ criterion to define the threshold for habitat suitability (Thomé et al. 2010; Webber et al. 2011). The resulting model from Maxent attempts at ranking locations within a given geographical area according to their suitability for the occurrence of the focal species (Phillips et al. 2006). We based the evaluation of the obtained distribution models on the calculation of the area under the curve (AUC) of the Receiver Operating Characteristics Curve (ROC), a threshold independent index commonly used to compare model performance (Phillips et al. 2006; Thuiller et al. 2004). Perfect discrimination of classifiers reflects on AUC values of 1; however, when using presence-only data the maximum value of AUC is less than 1 (Wiley et al. 2003), which applies to our models.
To estimate the potential future distribution of the species we projected the obtained model for the current climatic conditions into one of the internationally recognized general climate models (GCM’s) for future climate change, the CSIRO model (Commonwealth Scientific and Industrial research Organization) (Gordon & O'Farrell 1997) which in combination with the A2a emission scenario was recently found to be a realistic scenario that considers the world to change at regional scale rather than being globally homogeneous (White et al. 2010). For comparison we also projected SDM’s into a second more environmentally friendly scenario of CO$_2$ emission, the B2a. Projections were modeled for three time scales: 2020, 2050 and 2080, using the same settings in Maxent as for the current conditions.

Population connectivity

To visualize the connectivity between populations we used the SDM’s modeled for the current conditions and the haplotype networks obtained per species. We inverted the SDM’s in ArcGIS to create “dispersal cost layers” (Chan et al. 2011) i.e. regions with high probability of occurrence would now correspond to low dispersal cost regions and regions where species do not occur or where the probability of occurrence is low would represent high cost to dispersal through that region. We calculated least-cost paths (LCP) between populations (here, sampling geographic localities) in ArcGIS on the basis of their haplotypic connection using shared and sister haplotypes, and using the dispersal cost as friction layer. Given the influence that habitat heterogeneity has on the capacity for dispersal LCPs between populations were further classified into three categories (lowest 1%, lowest 2% and lowest 5%) (Chan et al. 2011). Finally a population connectivity map per species was obtained by summing all the LCP generated.

Additionally, we calculated LCPs between our sampled localities and georeferenced localities gathered from databases (GBIF 2010) and published literature (Robertson et al. 2009) of the two critically endangered species, *A. lemur* and *A. moreletii*. A total of 10 historical presence localities of *A. lemur* and 23 of *A. moreletii* were assessed for connectivity with our sampled localities on the basis of ecological-only connectivity. Such LCPs between genetically known populations and surrounding historically occupied areas would help in targeting paths that offer easier dispersal, where ecological restoration could
mediate progressive natural recovery of wild populations and periodical monitoring should be established. We will refer to these paths as ‘Conservation corridors’.

Genetic Diversity analysis

We used DNASP v.5.10.01 (Librado & Rozas 2009) to calculate descriptive statistics. Intra-specific subdivisions were based on haplotype clusters, the species delimitation analysis and the level of connectivity between them. This is especially useful given that some populations, especially of the endangered species *A. lemur* and *A. moreletii*, are represented by just one individual. We estimated the number of haplotypes (h), segregating sites (S), haplotype diversity (Hd) and nucleotide diversity (π) (Nei 1987). Pairwise genetic distances between groups were assessed by estimating $F_{ST}$ values.

Results

Species delimitation

The haplotype networks for all three described species revealed highly divergent clusters within species that were generally grouped geographically (Figure 2-4). Within *A. callidryas* 70 haplotypes (Table 2) were distributed across seven clusters with a minimum distance between clusters of 26 mutational steps (AcNor to AcPan) (Figure 2). Within clusters haplotypes were separated by only few mutational steps, except for two rare haplotypes from the AcCR group that were 18 mutational steps or more away from the rest of the cluster (AcVie in Figure 2d). Furthermore, populations from Pacific Costa Rica (AcOsa and AcQue) did not share any haplotypes between them or with any other region and were separated from each other by 45 steps, similar to the distance between the northernmost (AcNor) and southernmost (AcPan) clusters.

Among the endangered species the lowest number of haplotypes was found in *A. lemur* (Table 2 and Figure 3).
Figure 2. Species habitat suitability, haplotype relationship and population connectivity of Agalychnis callidryas. a) Current habitat suitability as modeled in Maxent. b) Map of population connectivity as suggested by estimated least-cost paths (LCP). Population groups are indicated within colored squares. c) Phylogenetic tree showing on branches the bootstrap values from Maximum likelihood analysis. d) Haplotype networks with georeferenced color coded localities depicting the distribution of haplotypes.
Distant clusters were evident with Costa Rica (AcCR) and Panama (AcPan) separated by more than 41 mutational steps (Figure 3). Fifteen haplotypes were found in *A. moreletii* the majority in Belize (Figure 4 and Table 2). Haplotypes were distributed into three clusters: AmGuá separated by 16 steps from the other two clusters, AmBel and AmHon. Within clusters the distance between haplotypes was never more than two steps.
In the GMYC analysis applied to the four putative species under the two available methods, the single threshold, which reflects one single transition in time from speciation to population split processes across the whole phylogeny, and multiple thresholds, which allows for different thresholds to be applied at different parts of the phylogeny independently, overall, the single threshold method applied better to our data as indicated by the chi square ($X^2$) tests; however, in all cases this method failed to identify *A. spurrelli* as a GMYC species (Figure 5a). Moreover, applying different clock rates and models...
(0.8%/Mya, 1.5%/Mya, 2.5%/Mya and strict or relaxed uncorrelated lognormal) the existence of 7 (with confidence intervals ranging from 5-10) independent entities (GMYC species) in our dataset (Table 1) was consistent under the different clocks and models. Threshold time under the single threshold method was consistent at 0.440 on the different molecular clocks and rates, with two exceptions: 2.5% strict and 0.8% relaxed lognormal molecular clocks, and under these two models the estimation of GMYC species was also higher, 8 (confidence interval 7-11) and 11 (confidence interval 7-14), respectively, compare to the rest of applied models.

The multiple threshold method overestimated the number of entities, particularly adding new thresholds towards the tips and suggesting about seven times more entities than the single threshold in the majority of cases. The only exception was the relaxed lognormal with 1.5% rate clock where the single threshold method was not preferred over the multiple, this is the model we present here. The deepest transition from speciation to coalescence in this model was consistent in both the single and multiple threshold methods. In the latter method two additional (more recent) transition points were identified (Figure 5b). Within species clustering was similar in GMYC analysis and the haplotype network, except for some over-splitting of A. moreletii in the GMYC analysis under the multiple threshold method (Figure 5b). With respect to haplotype networks, within A. callidryas five and six GMYC species were identified under single and multiple threshold methods, respectively, with most of splits happening before the deepest threshold point (Figure 5).

The GMYC species corresponded to observed morphological variation, especially in colour (Figure 5b); some groups have geographically restricted distributions, such as

Table 1. Summary statistics of the GMYC algorithm for species delimitation.

<table>
<thead>
<tr>
<th>Clock model</th>
<th>Rate</th>
<th>Method single</th>
<th>Method multiple</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N_{GMYC}^a</td>
<td>(CI)^b</td>
<td>T^c</td>
<td>N_{GMYC}^d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strict</td>
<td>0.8%</td>
<td>7</td>
<td>(6 - 8)</td>
<td>-0.440</td>
</tr>
<tr>
<td></td>
<td>1.5%</td>
<td>7</td>
<td>(5 - 10)</td>
<td>-0.440</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>8</td>
<td>(7 - 11)</td>
<td>-0.226</td>
</tr>
<tr>
<td>Relaxed</td>
<td>0.8%</td>
<td>11</td>
<td>(7 - 14)</td>
<td>-0.172</td>
</tr>
<tr>
<td>Lognormal</td>
<td>1.5%</td>
<td>7</td>
<td>(5 - 9)</td>
<td>-0.440</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>7</td>
<td>(6 - 10)</td>
<td>-0.440</td>
</tr>
</tbody>
</table>

^a Number of GMYC entities identified.
^b Confidence interval.
^c Threshold value where transition from speciation to coalescent processes occurred.
^d Single threshold was not preferred over multiple threshold method.
AcQue and AcOsa, whereas other groups are widespread, such as the group containing of AcNic, AcCen, AcCR, whose populations are distributed continuously in three countries (Figure 2).

Figure 5. Phylogenetic trees showing the GMYC species for a) single and b) multiple thresholds as estimated by the relaxed lognormal 1.5%/Mya rate clock. Lineage through time plots are shown below the trees.
Finally, in *A. lemur* the two GMYC species identified by the multiple threshold method corresponded to the haplotype clusters (Figure 3). The split between these GMYC species happened before the most recent threshold point (Figure 5b).

**Genetic diversity and population connectivity**

We found 94 unique haplotypes in total. Seventy in the non-endangered species *A. callidryas*, two in *A. spurrelli* and the remaining 22 distributed between *A. lemur* (7) and *A. moreletii* (15) (Table 2 and Figure 2-4). Thirteen of the total 94 haplotypes were shared among individuals from different localities. To estimate genetic indices populations within species were grouped on the basis of the results of haplotype networks, species delimitation analysis and levels of connectivity found in our previous analysis (Figure 2-4). Consequently, *A. lemur* was represented by 2 groups (Figure 3b): Costa Rica (AlCR) and Panama (AlPan); *A. moreletii* was represented by three groups (Figure 4b): Hondurans (AmHon), Pacific Guatemala (AmGua) and Belize-Caribbean Guatemala (AmBel); *A. callidryas* was represented by the following groups of populations (Figure 2b): AcNor (contains Belize, Guatemala and western Honduras); AcCen (grouping eastern Honduras, Caribbean Nicaragua and some individuals from Northern Costa Rica); AcCR (Caribbean Costa Rica); AcNic (Pacific Nicaragua); the two populations from Pacific Costa Rica remained independent: AcQue and AcOsa; finally, Panamanian populations were grouped together: AcPan.

Within all species, both endangered and non-endangered, there was a considerable range of genetic variation among the populations. In *A. callidryas* the highest and lowest values were found in AcCR and AcCen, respectively, both groups are from the Caribbean versant, but the latter being much more widespread from Costa Rica to the north of Honduras (Figure 2). Comparing the two endangered species, haplotype and nucleotide diversities at the species and population levels were higher in *A. lemur* than *A. moreletii* (Table 2). Furthermore, the Costa Rican population of *A. lemur* was more diverse than the cluster of Panamanian populations (AlPan). Apart from the Belizean population, *A. moreletii* had very low haplotype and nucleotide diversities, much lower than *A. lemur* or any of the groups of *A. callidryas*.
the two populations from the Pacific of Costa Rica. Found between distant populations from the North (AcNor) and South (AcPan) as very high between Pacific and Caribbean populations, than between the two Caribbean species. F values were close to 1 in the majority of population pairs (Table 3). Within A. callidryas the lowest genetic differentiation was found within the complex formed by: AcCen, AcCR and AcNic, but very high between the newly identified GMYC species. Similarly high F values were found between distant populations from the North (AcNor) and South (AcPan) as between the two populations from the Pacific of Costa Rica. Within A. moreletti differentiation was higher between Pacific and Caribbean populations, than between the two Caribbean populations. In A. lemur Costa Rica and Panama were highly differentiated from each other.

Table 2. Intra-specific genetic diversity.

<table>
<thead>
<tr>
<th>Group</th>
<th>n²</th>
<th>h⁰</th>
<th>S²</th>
<th>Hd²</th>
<th>π²(± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. callidryas</td>
<td>266</td>
<td>70</td>
<td>151</td>
<td>0.950</td>
<td>0.02490 (± 0.000013)</td>
</tr>
<tr>
<td>AcPan</td>
<td>13</td>
<td>8</td>
<td>16</td>
<td>0.910</td>
<td>0.06633 (± 0.00076)</td>
</tr>
<tr>
<td>AcCosa</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>0.800</td>
<td>0.00212 (± 0.00063)</td>
</tr>
<tr>
<td>AcCque</td>
<td>26</td>
<td>5</td>
<td>5</td>
<td>0.810</td>
<td>0.00204 (± 0.00026)</td>
</tr>
<tr>
<td>AcCen</td>
<td>56</td>
<td>14</td>
<td>19</td>
<td>0.560</td>
<td>0.00121 (± 0.00029)</td>
</tr>
<tr>
<td>AcCR</td>
<td>57</td>
<td>24</td>
<td>56</td>
<td>0.919</td>
<td>0.00503 (± 0.00133)</td>
</tr>
<tr>
<td>AcNic</td>
<td>46</td>
<td>6</td>
<td>7</td>
<td>0.584</td>
<td>0.00156 (± 0.00021)</td>
</tr>
<tr>
<td>AcNor</td>
<td>62</td>
<td>9</td>
<td>9</td>
<td>0.753</td>
<td>0.00174 (± 0.00110)</td>
</tr>
<tr>
<td>A. lemur</td>
<td>12</td>
<td>7</td>
<td>69</td>
<td>0.909</td>
<td>0.04248 (± 0.00526)</td>
</tr>
<tr>
<td>AlPan</td>
<td>5</td>
<td>3</td>
<td>20</td>
<td>0.700</td>
<td>0.01062 (± 0.00594)</td>
</tr>
<tr>
<td>AlCR</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>0.857</td>
<td>0.00759 (± 0.00127)</td>
</tr>
<tr>
<td>A. moreletti</td>
<td>116</td>
<td>15</td>
<td>31</td>
<td>0.83688</td>
<td>0.000863 (± 0.00098)</td>
</tr>
<tr>
<td>AmBel</td>
<td>84</td>
<td>10</td>
<td>11</td>
<td>0.74154</td>
<td>0.00204 (± 0.00019)</td>
</tr>
<tr>
<td>AmGua</td>
<td>19</td>
<td>3</td>
<td>3</td>
<td>0.29240</td>
<td>0.00067 (± 0.00031)</td>
</tr>
<tr>
<td>AmHon</td>
<td>13</td>
<td>2</td>
<td>1</td>
<td>0.15385</td>
<td>0.00020 (± 0.00017)</td>
</tr>
</tbody>
</table>

²Number of individuals in the analysis; ³Number of haplotypes; ⁴Segregating sites

Overall genetic differentiation was high within putative species. F values were close to 1 in the majority of population pairs (Table 3). Within A. callidryas the lowest genetic differentiation was found within the complex formed by: AcCen, AcCR and AcNic, but very high between the newly identified GMYC species. Similarly high F values were found between distant populations from the North (AcNor) and South (AcPan) as between the two populations from the Pacific of Costa Rica. Within A. moreletti differentiation was higher between Pacific and Caribbean populations, than between the two Caribbean populations. In A. lemur Costa Rica and Panama were highly differentiated from each other.

Table 3. Intra-specific genetic differentiation as estimated by the pairwise F values.

Intra-specific genetic differentiation

<table>
<thead>
<tr>
<th>Agalychnis callidryas</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcNor</td>
</tr>
<tr>
<td>AcNor</td>
</tr>
<tr>
<td>AcQue</td>
</tr>
<tr>
<td>AcOsa</td>
</tr>
<tr>
<td>AcNic</td>
</tr>
<tr>
<td>AcCen</td>
</tr>
<tr>
<td>AcCR</td>
</tr>
<tr>
<td>AcPan</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agalychnis lemur</th>
<th>Agalychnis moreletti</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlPan</td>
<td>AmHon</td>
</tr>
<tr>
<td>AICR 0.874</td>
<td>AmHon</td>
</tr>
<tr>
<td></td>
<td>AmBel</td>
</tr>
</tbody>
</table>
Population connectivity reflected the effect of habitat suitability and heterogeneity on the capacity for dispersal, especially for the endangered species. Also, connectivity was higher between populations within haplotype clusters, which in most cases coincided with their close geographical location. Among all species *A. callidryas* showed the highest levels of connectivity even across large distances, except between the two populations from the Pacific of Costa Rica (AcOsa and AcQue), despite there being suitable habitat connecting them (Figure 2a).

![Figure 6. Conservation corridors. Corridors of connectivity as estimated by the sum of least cost paths (LCP) between sampled sites (stars) and surrounding historical occurrence localities (circles) of a) *A. lemur* and b) *A. moreletii*. Green arrows point to suggested conservation corridors.](image)
In *A. moreletii* higher connectivity was observed between populations from the Pacific, where high habitat suitability was found (Figure 4b). Similarly populations in the Caribbean region also appeared to be connected with the locality of El Estor being a key point for connection between Belizean and Honduran frogs (i.e. no other route was considered likely between them when calculating the LCPs). But, there was a very low probability of this species moving in either direction between Pacific and Caribbean regions under current climatic conditions. Finally, no connectivity was found between Costa Rican and Panamanian populations of *A. lemur*, but some connectivity was observed within the Panamanian cluster (Figure 3b).

Corridors calculated between historical locations of occurrence and our sampled sites for *A. lemur* (Figure 6a) revealed one main area in the Caribbean versant of Costa Rica that would represent least cost for the species to disperse through. In Panama, even though the analysis indicated certain level of connectivity between localities; the distances are large so their likelihood of being effective corridors for recolonization needs to be treated cautiously. For *A. moreletii* we identified four regions of high ecological connectivity between historical populations (Figure 6b): Mexico, Guatemala - El Salvador, Belize–Mexico, and to a lesser extent Honduras.

**Species distribution Models: current and future**

Current distribution models obtained for the three focal species always performed better than random models. AUC values indicated high levels of discrimination: *A. callidryas* AUC 0.848 (SD 0.023), *A. lemur* AUC 0.986 (SD 0.005) and *A. moreletii* AUC 0.878 (SD 0.042). SDMs were generated after minimum training presence thresholds of 0.037 (*A. callidryas*), 0.040 (*A. lemur*) and 0.077 (*A. moreletii*). A jackknife test of variable importance indicated that variables with the highest explanatory power for the current distribution were precipitation for *A. callidryas* and *A. lemur* and minimum temperature for *A. moreletii*. The most suitable areas predicted by the SDMs were compatible with the known distribution of the species (Figs. 7, 8, 9). The only overprediction was found in middle and southern area of Mesoamerica for *A. moreletii* as this species currently only occurs in the northern region (Figure 9).
The projection of SDMs onto future scenarios of climate change predicted a progressive decrease of suitable habitat for all the three species (Figs. 7, 8, 9). Results for the A2a and B2a scenarios were similar; although the latter under a more environmentally friendly
scenario, seemed more optimistic. Suitable areas for *A. callidryas* will decrease soon, and are expected to force a north-south division, because currently suitable areas in south and Caribbean Nicaragua and northern Costa Rica were predicted to substantially decrease (Figure 7). Moreover, easternmost Honduras, northern Guatemala, middle Panama and most of Belize were predicted to become unsuitable by 2080. Also, a progressive displacement of suitability towards the Pacific was observed.

![Current distribution model of *Agalychnis lemur* as modeled in Maxent and future projection for three time periods (2020, 2050, 2080) under the CSIRO model of climatic change and two emission scenarios (A2a and B2a).](image_url)
For *A. lemur* future models predicted that suitable areas in the northern and southern limits of its current distribution will become fragmented. A progressive movement of suitable habitat towards higher elevations was also observed with most of the suitable remaining areas being in Costa Rica while in Panama a substantial decrease over time was predicted (Figure 8).

![Current distribution model of Agalychnis moreletii as modeled in maxent and future projection for three time periods (2020, 2050, 2080) under the CSIRO model of climatic change and two emission scenarios (A2a and B2a).](image)

Figure 9. Current distribution model of Agalychnis moreletii as modeled in maxent and future projection for three time periods (2020, 2050, 2080) under the CSIRO model of climatic change and two emission scenarios (A2a and B2a).
*Agalychnis moreletii* showed more obvious changes and declines in habitat suitability, particularly Belize. Pacific Guatemala and El Salvador were predicted to become progressively less suitable and remaining suitable areas appeared as discontinuous patches, whereas in Mexico a slightly bigger and continuous area was predicted to remain suitable (Figure 9).

**Discussion**

Conservation action plans should conclude with effective strategies that guide natural populations’ recovery and maintenance in, and along with, their natural environment. This is an ambitious and difficult task given the real situation of habitat and biodiversity loss, perfectly exemplified by Mesoamerican amphibians: one of the most diverse and endangered faunas in the world, inhabiting a very small land surface with one of the highest rates of habitat loss (Wilson et al. 2010). A big issue for the conservation of this fauna is the lack of knowledge about the different taxa particularly those that are more endangered, which are also the biggest priority for conservation. One strategy to help solve this problem is to study endangered species along with close non-endangered relatives (Burne et al. 2003; Ge et al. 1999). We studied three species of *Agalychnis*, including endangered and non-endangered species, to identify genetic and ecological factors that could contribute to their management and conservation. To do so we (1) quantified the genetic variation and identified cryptic diversity within species; (2) modeled current and future distribution and identified areas that allow connectivity between populations to (3) help guide the effective conservation and monitoring plans that would facilitate the natural recovery of endangered species and management of non-endangered species of frogs in Mesoamerica.

**Cryptic diversity**

Based on haplotype networks and GMYC analysis we found more than one entity within our putative taxa, particularly *A. callidryas* (Figure 5). Wide morphological variation has been observed in this species for a long time, and there has been considerable interest to know if it represents more than one species: equal evidence has been presented to both support (Funkhouser 1957) as well as refute (Savage & Heyer 1967) the recognition of more than one species or subspecies. Although this remains an open question, currently
only a single species is considered valid (Duellman 2001). The GMYC species identified here corresponded to observed morphological “types”, particularly in color, in the field. Moreover, the assessment of connectivity on the basis of genetic and ecological relatedness between populations resulted in high connectivity within GMYC species but low to zero connectivity between clusters that correspond to independent GMYC species (Figure 2), supporting the idea of several isolated entities.

Based on our results we recognize the existence of five different entities within A. callidryas: (1) “Northern” (AcNor) that extends from western Honduras, through Guatemala and Belize (and probably Mexico), similar to the subspecies recognized by Funkhouser (1957) as A. callidryas taylori; (2) “Middle” (AcCen, AcCR, AcNic) is the most widespread, present from northeast Honduras, through Nicaragua to the Caribbean of Costa Rica, and it has color patterns, particularly flanks, similar to those described for the once called A. helena (Duellman 2001); (3) “Pacific” (AcQue) present in the Pacific region of Costa Rica, presumably all along that coast, except in the osa peninsula, where (4) “Osa” (AcOsa) occurs; (5) finally, the “Panamanian” (AcPan) present in middle Panama, probably near to the border with Costa Rica, along the Pacific versant judging by the closer relationship with the populations from Pacific Costa Rica compared to the Caribbean ones. Although, we do not elevate these entities to a different taxonomic status here, we do recognize their potential to be different species or subspecies and therefore suggest further investigating this uncertainty by adding molecular evidence, like nuclear markers, analyzing morphological data in depth, and if possible better describe the ecological characteristics of the GMYC species.

In A. lemur, one of the critically endangered species, we did not observe marked morphological differentiation concordant with the different GMYC species suggested by the analyses, but we think this needs further investigation. For example in the case of A. lemur, we only saw wild individuals from Costa Rica. Therefore, we think it is necessary to gather morphological data from different localities, which would probably require the review of museum material given the current rarity of the species in the wild. Furthermore, in A. lemur there was no historical connectivity between Costa Rican and Panamanian populations suggesting long term isolation, which explains the 41 steps between them in the haplotype networks and the two species resulting in the GMYC analysis (Figure 5b). However, the split between GMYC species in both A. lemur and A. moreletii were found in
both cases to be much more recent than those within *A. callidryas*. So given their status of being critically endangered and the fact that their populations have been almost extirpated from many places through their geographical ranges (Santos-Barrera et al. 2004; Solis et al. 2004b), the results of the analyses indicating genetic differentiation to the point of speciation in such short time could be the consequence of diversity loss and prevalence of rare haplotypes in different populations. Clarify the taxonomic status of taxa is of major importance in conservation so we suggest to confirm these uncertainties and to establish the geographical ranges of the final valid species. The latter would radically change the conservation status of the “new species”.

**Conservation Corridors**

For the critically endangered species we identified paths with the highest potential for natural population recolonization on the basis of the connectivity between sampled populations and historical occurrence points. We called those paths conservation corridors (Figure 6. green arrows). These corridors are predicted to offer the most suitable and least costly paths for natural dispersal of individuals, given good habitat conditions. Moreover, climate change is one of the challenging factors for adaptation in amphibians (Carey & Alexander 2003); based on our results of niche modeling in the following decades the suitable habitat for the focal species will tend to decrease and move geographically; conservation corridors give an opportunity for frogs to progressively move and colonize more favorable areas. Therefore we propose conservation corridors to be given priority in habitat protection policies, at least at country levels. Furthermore, ecological restoration projects should be promoted within the corridors to help in the recuperation of the habitat that species will need to recolonize. Conservation corridors could also be good areas for monitoring amphibian populations, and for the experimental re-introduction of captive bred animals of these species, provided that correct control measures are taken. Finally, areas within and near the conservation corridors would be key and strategic for environmental education and for the identification and inclusion of stakeholders directly related to the conservation of amphibians in general and these frogs in particular.
Implications for Conservation

Our results can improve the conservation and management of frogs of the genus *Agalychnis* in Mesoamerica because they (1) help to prioritize populations and geographic areas for *in situ* conservation plans; (2) highlight factors that will challenge the species survival and (3) provide solutions to minimize the impact of such factors on these species in the near future; (4) help to target stakeholders whose action would directly benefit the frogs and their ecosystems and are therefore key to effective conservation plans. Although we do not yet present formal taxonomic changes, the possibility for such modifications and further re-assessment of the conservation status of species is strongly recommended. For immediate conservation action, all the “potential species” we identified should be treated separately and conservation actions within the conservation corridors should be prioritized to benefit all three species; establishment of these corridors will require local and national government support and a long term strategy of expansion and periodic monitoring. Given that decision making is generally done by each country separately, the findings of this study will be made available to the relevant authorities of the six countries under study highlighting factors of particular interest to each one of them; however, we think that bi-national strategies would be more effective, especially given the limited resources. Finally, we recognize the high cost of genetic studies but also its evident benefit for conservation. We therefore suggest that as new populations are encountered that they should be assessed genetically and, using the analytical tools used here, placed within clusters or species to progressively establish the geographic limits of each management unit and species.

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We did sampling expeditions of 2 to 5 months through Mesoamerica during the rainy seasons of 2009, 2010 and 2011, when frogs are expected to form aggregations around their reproductive sites, particularly the pond breeders like *A. calliclava* and *A. morleyi*.

Appendix S1, Fieldwork Information.

<table>
<thead>
<tr>
<th>Localities</th>
<th>Human activity</th>
<th>Code</th>
<th>Species</th>
<th>Latitude Longitude</th>
<th>Habitat</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belize: Cockscomb Basin Wildlife Sanctuary, &quot;Cockscomb Basin Wildlife Sanctuary&quot;</td>
<td>Tourism observation</td>
<td>B: Cockscomb Basin Wildlife Sanctuary</td>
<td>22.13230 -88.75375</td>
<td>Forest pond</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belize: Caal Caves Research Station</td>
<td>Research observation</td>
<td>B: Caal Caves Research Station</td>
<td>18.72238 -88.95310</td>
<td>Forest pond</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B: Calle &quot;Casa El Arenal&quot;</td>
<td>Tourism observation</td>
<td>B: Casa El Arenal</td>
<td>10.37678 -84.37561</td>
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<td>B: La Marina Reserve Centre, &quot;La Marina Reserve Centre&quot;</td>
<td>Tourism observation</td>
<td>B: La Marina Reserve Centre</td>
<td>16.21350 -88.75481</td>
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<td>Research - Tourism observation</td>
<td>CR: CRARC</td>
<td>10.05411 -83.55181</td>
<td>Forest pond</td>
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<td>Research - Tourism observation</td>
<td>CR: CRARC</td>
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<td>Forest pond</td>
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</table>

Table S1. Geographic location data and habitat information.
Puntarenas: Península de Osa, “Corcovado Natural Park - Control Point El Tigre”.

Cosa: 6.8.3906 - 83.9503

Canelo and Nautical Pond

Tourism, Agriculture

Tourism, Agriculture

Tourism Observation

Tourism Observation

Tourism

Rosario pond

Natural pond

Hotel Espadilla.

Quepos-Manuel Antonio, “Hotel Espadilla”.

CR: Natural Pond- Control Point El Tigre

Nivel: 11.8.3314 - 83.98022

Natural Wetland: Hotel Rana Roja.

Cortesía: “Hotel Rana Roja”.

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<tr>
<td>Matagalpa</td>
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<td>Primane</td>
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<td>Primary Premontane wet forest, tourism</td>
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<td>Natural wetland</td>
<td>Stream-side</td>
<td>10.93101, -83.72604</td>
<td>Natural wetland, urbanism, tourism</td>
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<tr>
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<td>Natural wetland</td>
<td>Primane</td>
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<td>Matagalpa</td>
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Agalychnis spurrelli

**B: Belize**

<table>
<thead>
<tr>
<th>Location</th>
<th>Activity/Species</th>
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<th>Number of Genetic Samples</th>
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<tbody>
<tr>
<td>Las Cuevas</td>
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<tr>
<td>Club El Río</td>
<td>Forest pond, research/observation</td>
<td>14.43806, -90.46586</td>
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<tr>
<td>University of El Valle</td>
<td>Artificial pond, urbanism and research</td>
<td>14.60550, -90.49375</td>
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<tr>
<td>Zone 2</td>
<td>Natural wetland, urbanism and transportation</td>
<td>14.66269, -90.51643</td>
<td>14</td>
</tr>
<tr>
<td>El Estor</td>
<td>Roadside pond, mining excavation</td>
<td>15.54667, -89.29033</td>
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</tr>
<tr>
<td>Private Natural Reserve Los Andes</td>
<td>Artificial pond, tourism and coffee/tea farming</td>
<td>14.52806, -91.19100</td>
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<td>La Roda</td>
<td>Coffee plantation pond, cattle and cattle ranching</td>
<td>14.51744, -88.11086</td>
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Agalychnis spurrelli

**CR: Costa Rica**

<table>
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<tbody>
<tr>
<td>Costa Rica Amphibian Research Center</td>
<td>Artificial pond, research-tourism</td>
<td>8.83511, -83.55118</td>
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<td>CRARC</td>
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**G: Guatemala**

<table>
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<td>13.33, -88.1086</td>
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<td>14.5244, -88.9967</td>
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<tr>
<td>El Estor</td>
<td>Roadside pond, mining excavation</td>
<td>15.54667, -89.29033</td>
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<tr>
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<tr>
<td>La Roda</td>
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**H: Honduras**

<table>
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<tr>
<td>Intibucá Jesús de Otoro, San Jerónimo</td>
<td>Coffee plantation pond, tourism and research</td>
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**N: Nicaragua**

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**P: Panama**

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</table>

**Countries are represented by one capital letter: B: Belize; CR: Costa Rica; G: Guatemala; H: Honduras; N: Nicaragua; P: Panama. Underlined are the district/provinces within each country. Each sampling point is written within quotes.**

*Human activities we observed happening directly in or nearby the sampling pond/stream.*
Polymerase chain reactions used the primers in Table 1. Every reaction was performed to a final volume of 50µl containing: 1x Buffer NH4 (Bioline), 2.5mM MgCl2 (Bioline), 0.25mM dNTP mix (Bioline), 0.1µM of each primer, and 2.5 units of Taq polymerase (Biotaq from Bioline) and 1-10ng of DNA.

### Table 1

<table>
<thead>
<tr>
<th>Step</th>
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<th>Primer Sequence</th>
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<tr>
<td>1st cycle</td>
<td>94°C 2min</td>
<td>MVZ15</td>
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<td></td>
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<td>MVZ3</td>
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<td></td>
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<td></td>
<td></td>
<td>35 cycles</td>
<td>ƒ&amp;'</td>
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<th>Primer Sequence</th>
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### Footnotes
- a (Moritz et al. 1992).
- b This study.
- c Acalychnis calidris (Belize, Honduras, Guatemala).
- d Acalychnis calidris (Nicaragua, Costa Rica).
- e Acalychnis calidris (Panama);
- f Acalychnis calidris (Belize, Honduras).
- g Acalychnis calidris (Guatemala).
- h Acalychnis calidris (Costa Rica).
References


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Chapter 3

Comparative Phylogeography and environmental modeling of two genera of Hylid frogs in Mesoamerica

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Comparative Phylogeography and environmental modeling of two genera of Hylid frogs in Mesoamerica

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Abstract

Mesoamerica despite its size is one of the world’s biodiversity hotspots and contains a large percentage of the Neotropical amphibian diversity. However, the processes involved in the generation of the high Mesoamerican biological diversity in general and of amphibians in particular are poorly understood, partly because they have been overlooked, and main research on this topic have concentrated in the southern or northern limit of the region (i.e., near the extra-tropical northern area in Mexico, or closer to South America in Panama and to a lesser extent Costa Rica). Using an integrative approach that combines the use of genetic data with geospatially explicit information and climatic data, we attempt at proposing hypotheses to explain the processes of colonization and dispersal of two genera of frogs, *Agalychnis* and *Dendropsophus* in particular and in a general context of the Hylid frogs throughout most of Mesoamerica. A first observation that worth attention was the intra-specific variation found in several of the species under study, which suggested further investigation is needed to evaluate the validity of more than one taxonomic entity within such species. This has important implications for conservation and management of the species at the regional scale. Moreover, coupling a detailed species delimitation analysis with the phylogeographic analysis presented would allow the practice of conservation under the ideal strategy that aims at maintaining the processes that have generated the diversity. On the basis of genetic diversity, differentiation and relatedness at the intra- and inter-generic levels, dissimilar hypotheses were proposed, although highlighting common barriers to dispersal across the region. Furthermore, it is hypothesized that different responses to general historical events will be exhibited by the two genera when testing the applicability of the proposed models of colonization and dispersal. Overall, the influences of climatic fluctuations and the complex process of the relatively young Mesoamerica on genetic diversity were anticipated. Finally, the space and ways of integrating further analyses to test the proposed hypothesis and find the most likely model of origin and dispersal of Mesoamerican frogs are given.
Introduction

Effects of climate change on genetic diversity both during Pliocene and, particularly, during the Pleistocene have been extremely well studied in extra-tropical regions like North America and Europe (Funder 1979; Hewitt 2004; Kappelle et al. 1999; Taberlet & Cheddadi 2002; Martínez-Meyer et al. 2004; Ribera & Vogler 2004). However, where the most of the world’s diversity occurs, the tropical area, effects of climatic fluctuations on genetic diversity are still poorly understood, and the few existing studies have mainly focused on South America (Brown et al. 1974; Lynch 1982; Solomon et al. 2008; Santos et al. 2009; Thomé et al. 2010) Africa (Peter B 2004; Anthony et al. 2007) and Southeast Asia (Gathorne-Hardy et al. 2002; Evans et al. 2003; O’Loughlin et al. 2008). Similar studies are lacking for Mesoamerica and those attempting at explaining the origin and evolution of taxa in the region have mostly focused to either Mexico (Halffter 1987; Castoe et al. 2009; Daza et al. 2010; Bryson et al. 2011) or lower Central America, in Panama and to a lesser extent Costa Rica (Crawford 2003; Crawford et al. 2007). Applying a comparative phylogeographic approach of the genera *Agalychnis* and *Dendropsophus* we aimed at identifying the effects of climate change on genetic diversity during the Pleistocene and hypothesized dispersal routes of these Hylid frogs throughout Mesoamerica.

Different geomorphological events have shape the formation of Mesoamerica as we know it today (Coates & Obando 1996; Coates et al. 2004), and several events have been predicted to have occurred throughout its history, like marine incursions and the formation of mountainous ranges, which is expected to have influenced the patterns of biodiversity in the region (Coates & Obando 1996; Bermingham & Martin 1998; Coates et al. 2004; Crawford et al. 2007). Specific barriers to gene flow in Mesoamerica have been suggested and in some taxa, particularly trees and some snakes groups their effectiveness have been tested (Castoe et al. 2009; Robertson et al. 2009; Daza et al. 2010; Bryson et al. 2011; Poelchau & Hamrick 2011); however, overall patterns of the diversity in the region are still understudied.

Among applied approaches the comparative phylogeographic studies have proved to be powerful strategies to understand the effects of different factors including climate change on related and non-related taxa, this way allowing the elucidation of general patterns of diversification and dispersal across regions (Castoe et al. 2009; Poelchau &
Hamrick 2011). Such studies allow testing the hypothesis that similar factor or barriers to gene flow have been equally effective across taxa. In applying this kind of approach the use of integrative analyses that involve data gathered from different sources (i.e. ecology, geography, geology, genetics), and allow the proposal of explicit hypotheses that better reflect the history of the area and of the organisms (Chan et al. 2011).

In this study we used seven species that have varying ecological characteristics and go from widespread (A. callidryas and D. microcephalus) to very geographically restricted (A. lemur, D. robertmertensi); have different levels of forest dependency that varies from completely forest dependant in some species of Agalychnis (Santos-Barrera et al. 2004; Solis et al. 2004a; Solis et al. 2004b) to very tolerant to highly disturbed areas in some Dendropsophus (Bolaños et al. 2004b; Jungfer et al. 2010; Santos-Barrera et al. 2010; Solis et al. 2010). Their levels of endangerment also vary, from mainly endangered in Agalychnis to completely least concern for conservation in Dendropsophus. Given that Agalychnis frogs are, between the two genera, the most forest dependent species, it is expected that climatic fluctuations had greater effects on them and particularly on those highly endangered.

Our goals were to assess the effects of climate change on the origin and dispersal patterns of the two mentioned genera, to establish whether the different taxa had similar responses to the different historical effects. We aimed at proposing hypotheses that would ultimately lead to the understanding of the evolutionary pattern of these genera in particular and of Hylid frogs in general in Mesoamerica. In doing so, we adopted an integrative approach that involved the use of current and historical climatic data, genetic information and their combination through ecological niche modeling, and comparative phylogenetic and phylogeographic analyses. Such kind of approaches have proved to be very powerful tools in evolutionary studies (Thomé et al. 2010; Chan et al. 2011; Elith et al. 2011; Webber et al. 2011) and that is the reason for us to apply it in this case.

Results

We focused the analyses on seven species from two genera belonging to different subfamilies within the family Hylidae: the genus Agalychnis (Phyllomedusinae), currently represented by six species in Mesoamerica, of which we included three focal
species here, *A. callidryas*, *A. lemur*, *A. moreletii*; and the genus *Dendropsophus* (Hyliinae) with five species in Mesoamerica, of which we focused on two species *D. ebraccatus* and *D. microcephalus*, and additionally gathered data for two closely related species, *D. phlebodes* and *D. robertmertensi*. For comparison purposes we also performed some of the analyses, particularly environmental modeling on additional species from the subfamily Phyllomedusinae that occur in Mesoamerica, *A. annae*, *A. saltator* *A. spurrelli* and *Cruziohyla calcarifer*. Results of these analyses are mainly presented in Supporting Information. No genetic data were gathered in this study for these species, except from one locality in Costa Rica for *A. spurrelli*.

**Species distribution models**

We obtained current distribution models for all seven species under analysis (Figure 1 – 7). To test the model performance we used the calculation of the area under the curve (AUC) of the Receiver Operating Characteristics Curve (ROC). Perfect discrimination of classifiers reflects on AUC values of 1; however, when using presence-only data the maximum value of AUC is less than 1 (Wiley et al. 2003). We used presence-only data, and therefore expected the AUC values to be less than 1 even for perfect discrimination. In all species, the obtained models always performed better than random models. Statistics and minimum presence threshold after which predictive maps were generated are shown in Table 1.

The modeled distributions for the current climatic conditions were compatible with the known distribution of the species (Figure 1 – 7). Despite the no extrapolation option chosen for model estimation in Maxent, some over prediction was observed in southern Mesoamerica (Costa Rica) for *A. moreletii*, which is currently only known to occur from Honduras west and northwards to Mexico (Figure 3). By contrast, some under prediction was observed in the distribution obtained for *A. spurrelli* under the current climatic conditions compared to the known distribution (Figure S4).
<table>
<thead>
<tr>
<th>Species</th>
<th>SDM</th>
<th>Threshold</th>
<th>AUC</th>
<th>SD</th>
<th>Best Environmental predictor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. callidryas</em></td>
<td>Current</td>
<td>0.046</td>
<td>0.842</td>
<td>0.021</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.041</td>
<td>0.845</td>
<td>0.022</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
<td>0.043</td>
<td>0.845</td>
<td>0.021</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.04</td>
<td>0.850</td>
<td>0.021</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>A. lemur</em></td>
<td>Current</td>
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<td>0.987</td>
<td>0.004</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.039</td>
<td>0.987</td>
<td>0.004</td>
<td>Precipitation of the coldest quarter</td>
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<td></td>
<td>LGM-MIROC</td>
<td>0.039</td>
<td>0.987</td>
<td>0.004</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.039</td>
<td>0.987</td>
<td>0.004</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>A. moreletii</em></td>
<td>Current</td>
<td>0.017</td>
<td>0.848</td>
<td>0.060</td>
<td>Min Temperature of the coldest month</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.017</td>
<td>0.848</td>
<td>0.060</td>
<td>Min Temperature of the coldest month</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
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<td>0.848</td>
<td>0.060</td>
<td>Min Temperature of the coldest month</td>
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<td></td>
<td>LGM-CCSM</td>
<td>0.017</td>
<td>0.848</td>
<td>0.060</td>
<td>Min Temperature of the coldest month</td>
</tr>
<tr>
<td><em>D. ebraccatus</em></td>
<td>Current</td>
<td>0.008</td>
<td>0.915</td>
<td>0.019</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.008</td>
<td>0.915</td>
<td>0.019</td>
<td>Precipitation of the coldest quarter</td>
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<td>0.019</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>D. microcephalus</em></td>
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<td>0.032</td>
<td>0.823</td>
<td>0.013</td>
<td>Precipitation of the coldest quarter</td>
</tr>
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<td></td>
<td>LIG</td>
<td>0.032</td>
<td>0.823</td>
<td>0.013</td>
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<td>LGM-MIROC</td>
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<td>0.823</td>
<td>0.013</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.032</td>
<td>0.823</td>
<td>0.013</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>D. phlebodes</em></td>
<td>Current</td>
<td>0.189</td>
<td>0.990</td>
<td>0.003</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.189</td>
<td>0.990</td>
<td>0.003</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
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<td>LGM-MIROC</td>
<td>0.189</td>
<td>0.990</td>
<td>0.003</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.189</td>
<td>0.990</td>
<td>0.003</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>D. robertmertensi</em></td>
<td>Current</td>
<td>0.039</td>
<td>0.967</td>
<td>0.010</td>
<td>Precipitation of the driest month</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.039</td>
<td>0.967</td>
<td>0.010</td>
<td>Precipitation of the driest month</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
<td>0.039</td>
<td>0.967</td>
<td>0.010</td>
<td>Precipitation of the driest month</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.039</td>
<td>0.967</td>
<td>0.010</td>
<td>Precipitation of the driest month</td>
</tr>
</tbody>
</table>
Figure 1. Modelled distribution of Agalychnis callidryas for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Solís et al. 2004a).

In Dendropsophus phlebodes (Figure 6), Agalychnis saltator (Figure S3) and Cruziohyla calcarifer (Figure S5) the predicted distribution for the current time indicated a high probability of occurrence of such species out of their currently known distributions. Points outside their geographical ranges were suggested particularly in Nicaragua, which is currently appearing as a gap in their geographical distribution (IUCN 2010) (black continuous lines in Figure 6, S3 and S5). We found historical records that backed up the results of the models (Table S5). Particularly in this study was confirmed the presence of *D. phlebodes* in a locality significantly far north from the currently known northern limit of the species distribution (black continuous line in Figure 6). Such locality was Wisconsin in Nicaragua (Nwis) (Table S5 and Figure 6), near the border
with Honduras but currently the limit of distribution is recognized to be in the south of Nicaragua (Solís et al. 2010).

![Map of Agalychnis lemur distribution](image)

**Figure 2.** Modelled distribution of Agalychnis lemur for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Solís et al. 2004b).

In terms of geographical extent at the regional level the projected models onto past climatic conditions for the LIG and the LGM predicted overall similar distributions to those obtained for the current climatic conditions. Even though to a lesser extent, over predictions observed in the current models (as in *A. moreletii*) (Figure 3) and under predictions (as in *A. spurrelli*) (Figure S4) were again visible in the past. In *Agalychnis*, particularly among the LGM models, the MIROC model predicted more fragmentation and isolation of highly suitable areas for the focal species (Figure 1 – 3). Moreover,
LGM models for the four additional species, *Agalychnis annae*, *A. saltator*, *A. spurrelli* and *Cruziohyla calcarifer* resulted in similar distributions, being almost identical in the case of *A. spurrelli* (Figure S4). In the case of *A. annae* the LGM-CCSM predicted wider distributions with expansion towards the north, and similarly under the LGM-MIROC the distribution was wider compared to the current distribution but still no extrapolation out of the southern region was observed (Figure S2). For *A. saltator*, both models of the LGM resulted in very similar distributions, although slightly wider in Honduras under the LGM-CCSM (Figure S3). Similarly, for *C. calcarifer*, predictions under both models coincided almost entirely with some expansion in Honduras under LGM-CCSM (Figure S5).

![Figure 3. Modelled distribution of Agalychnis moreletii for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Santos-Barrera et al. 2004).](image-url)
During the LIG, distributions of *Agalychnis* species were overall more confined to the southern region. The Caribbean versant of Costa Rica, particularly, remained highly suitable for the species currently present in the southern region, i.e. *A. annae*, *A. lemur* *A. saltator*, *A. spurrelli*, *Cruziohyla calcarifer* and the widespread species *A. callidryas*. For *A. moreletii*, however, such movement was not as noticeable as for the rest of species and the distribution remained in nuclear Central America instead, but exhibited a clear movement towards the Pacific versant (Figure 3), with the region from Guatemala to Mexico remaining highly suitable for this species through the whole period of time modeled here (Figure 3). While the distribution of *A. lemur* during the LIG appeared within similar geographical limits to the current time distribution, *A. callidryas* did not go north from Nicaragua, and had low probability of occurring in southeastern Honduras (Figure 1); instead, the probability of occurrence increased throughout the entire of Panama.

In the *Dendropsophus* genus there was not a general pattern among the two models for the LGM in all the species. In *D. ebraccatus* and *D. robertmertensi* wider ranges were predicted under the CCSM model for the LGM, furthermore for *D. robertmertensi* this model over predicted some areas in the south Pacific (where the species currently does not occur) (Figure 7). Under the LGM-MIROC model the predicted distribution had less suitable areas for both species although the distribution was not as fragmented (Figure 4 – 7) compared to the *Agalychnis* species (Figure 1 – 3). For *D. microcephalus* (Figure 5) the LGM-CCSM predicted an overall continuous distribution compared to the LGM-MIROC, which predicted more fragmentation, even higher than that observed for *D. ebraccatus* (Figure 4). The distribution of *D. phlebodes* during the LGM under the LGM-CCSM was much narrower than under the LGM-MIROC model. The latter showed an expanded distribution much more southwards and with some over prediction along the Pacific, where the species currently is not known to occur (Figure 6).
Figure 4. Modelled distribution of Dendropsophus ebraccatus for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Jungfer et al. 2010).

In the models obtained for the distributions of Dendropsophus species during the LIG, there was not a general pattern for all species, and different from the genus Agalychnis an overall movement of distributions towards the southern region was not observed. For D. ebraccatus, there was a decrease of the distribution in the Pacific versant throughout the region. By contrast along the Caribbean versant the distribution expanded significantly. Moreover, the modeled suitable areas formed an almost continuous corridor from Panama northwards to Honduras along the Caribbean versant (Figure 4).
For *D. microcephalus* (Figure 5) we observed somehow the opposite pattern to that predicted for *D. ebraccatus* during the LIG. The highly suitable areas for *D. microcephalus* were mainly located along the Pacific versant, even though some suitable areas were found in Caribbean Costa Rica. Also, not presence was predicted for Belize and very little probability of occurrence was predicted for western Honduras, Guatemala and Mexico, where the species currently occurs. The latter pattern was somehow similar to that found in the *Agalychnis* species, showing the southwards movement of the distributional range during the LIG.
Figure 6. Modelled distribution of Dendropsophus phlebodes for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Solís et al. 2010). The white star in the current distribution model indicates the new locality where the species was recorded in this study.

Last Interglacial distribution predicted for *D. phlebodes* was very narrow but still conserving its Caribbean presence we see today. There was also zero probability of this species being in the north as from south Nicaragua, similarly to *A. annae* (Figure S2) and *A. spurrelli* (Figure S4). Finally, for *D. robertmertensi*, the distribution during the LIG was almost identical to that predicted for the current time, except for some over prediction towards the south throughout the Pacific to Costa Rica and Panama but with a gap in Nicaragua. However, compared to the other species of the same genus and to the *Agalychnis*, the distribution of *D. robertmertensi* throughout the last 120 Ky did not show extreme changes (Figure 7).
Figure 7. Modelled distribution of Dendropsophus robertmertensi for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Santos-Barrera et al. 2010).

Long term high stability areas: Likely Refugia

On the basis of the obtained species distribution models for the three periods, current, LGM (two models) and LIG, we identified long term stability areas that may have served as refugia for the species through the Pleistocene (particularly the last 120 Ky). This was done by intersecting the layers for the different periods and identifying areas that remained suitable in all the periods (see Materials and methods for details). Likely refugia were identified per species (Figure 8 – 9) and their locations were compared...
among species, this way we could identify areas that may have played important roles as refugia at the regional scale and not only for our species.

For *A. callidryas* the main refuge was located in the northern Caribbean of Costa Rica (Figure 8), which covered a big region on the eastern slope of the Cordillera de Talamanca, covering parts of the currently named Province of Limon, northwards to near the San Juan River (border between Nicaragua and Costa Rica). Additionally, two small refugia were identified in the north Pacific and northern Caribbean of Panama (near the border with Costa Rica) for this species. Based on the models obtained for *A. lemur*, a refuge for the species was overlapped with the main refuge of *A. callidryas*, but with a smaller extension, and with high altitudes not reaching the sea level (Figure 8). Moreover, two very small refugia for *A. lemur* were revealed in the northern end of the Cordillera de Talamanca and on a small isolated hill on the southeast of Costa Rica, both on the Caribbean slopes of the mountain range.

For *A. moreletii*, a considerably larger number of refugia were inferred (Figure 8), all of them on the Pacific versant, except for one tiny refuge located near the lake Izabal in Guatemala (which is near one of our sampled localities, Gesto, for details see Table S1). Refugia inferred for *A. moreletii*, were variable in size, with some isolated very small refugia distributed through the Pacific coast of Guatemala and the border between El Salvador and Honduras, and other rather larger refugia in the southwest pacific coast of Mexico and near the border of El Salvador with Guatemala.

Refugia identified for the additional species (Figure S6) were in very similar locations to those identified for the focal species. The Caribbean versant of Costa Rica, particularly towards the north of the country in the current province of Limon along the eastern slopes of the Cordillera de Talamanca was indicated as a refuge for all the species that currently occur in the southern region of Mesoamerica, with the exception of *A. spurrelli*, for which the Osa Peninsula was the only recovered refuge (Figure S6). For the highland species *A. annae*, other than the big refuge on the Caribbean versant of northern Costa Rica, small refugia were also inferred in the northernmost isolated hills of the mountain range of the Cordillera de Talamanca, in the area surrounding the lake El Arenal, which is also near several volcanoes, i.e. Arenal, Poas, Tenorio.
For *A. saltator*, the only refuge identified was large and continuous along the Caribbean region of Costa Rica starting from near the border with Nicaragua, excluding the current area of the Tortuguero, and southwards to the west of Panama (Figure S8). Similarly, the only refuge identified for *Cruziohyla calcarifer*, was continuous and extended through most of the Caribbean slope of the Cordillera de Talamanca, only in Costa Rica, and closer to the mountains more than the coastline.
In the genus *Dendropsophus* refugia were also identified mainly in Costa Rica, similarly to *Agalychnis*, except for the northern species *D. robertmertensi*, whose refugia were located in the Pacific coast of mainly Mexico and a couple in Guatemala (Figure 9). Moreover, the main refuge of this species formed overall a continuous area in Mexico with a very small extension into western Guatemala. Additionally, three isolated refugia were located, two in Mexico and one in Guatemala, the latter being the largest (Figure 9). Similar Pacific refugia were identified for *D. microcephalus*, although much larger than those for *D. robertmertensi*. Moreover, refugia for *D. microcephalus* were located in most of Costa Rica and to a lesser extent in Panama (Figure 9).

![Figure 9](image-url)"Identified likely Pleistocene Refugia (red areas) for the focal species of the genus *Dendropsophus* in Mesoamerica.

In the two remaining species, *Dendropsophus ebraccatus* and *D. phlebodes*, the refugia were located in the Caribbean versant and overlapped with those located for the most of *Agalychnis* species. In *D. ebraccatus* there was additionally very small patchy areas within the Osa Peninsula and in the very final north end of the Cordillera de Talamanca, but the largest refugia was more contracted than that identified for *D. phlebodes*, forming a large area that extended through most of the current Limon province of Costa Rica, except in a good part of the Tortuguero region. For *D. phlebodes* the largest area of refugia was distributed in similar regions of Costa Rica to that identified of *D.*
*ebraccatus*, but it was a group of refugia rather and a large continuous area. It extended from Costa Rica into Nicaragua to the region of the upper river San Juan. Within Costa Rica it extended to the Pacific in the Guanacaste region, and apparently not going to high elevations but rounding the hills. On the Caribbean Costa Rica, it seemed to form two corridors, one through the very coastal area (as it is currently) and the other one through the hills on the eastern slope of the Cordillera de Talamanca, going somehow out and higher than its currently known distribution.

**Haplotype Networks**

To visualize the haplotypic relationships between populations of our focal species, we first built median joining haplotype networks. These networks allowed us to identify groups (of populations) according to their genetic relatedness within species, and also to further establish any concordant geographic clustering of such groups of populations.

The *Agalychnis* populations were clearly clustered in all the focal species, which also corresponded to a geographical clustering (Figure 10). For *A. lemur* there was a clear division between populations from Costa Rica and Panama, separated by more than 41 mutational steps. In *A. moreletii*, the division was between Pacific and Atlantic populations, with Belize, Honduras, and the population from El Estor (Gesto) clustered together and separated from the Pacific cluster by sixteen mutational steps.

Within *A. callidryas* seven clusters were identified (Figure 10), which were consistent with the geographical distribution of the populations contained in each cluster: (1) a northern cluster (AcNor) that grouped the populations from Belize, Guatemala and western Honduras; (2) a cluster in the center of the geographical range (AcCen) covering populations from eastern Honduras, Caribbean and center Nicaragua and northern Caribbean Costa Rica; (3) a cluster (AcCR) dominated by populations from the Caribbean of Costa Rica but also including some individuals from the Southeast Nicaragua; (4) a Pacific Nicaraguan cluster (AcNic), grouping all populations from the Pacific versant of Nicaragua; two independent Pacific clusters in Costa Rica, which were well separated from each other with more than 40 mutational steps between them, (5) AcQue, containing the population from the Pacific Coast of Costa Rica, and (6) AcOsa containing the only population from the Osa Peninsula; (7) the panamanian cluster (AcPan) that grouped all the populations sampled in Panama.
Figure 10. Haplotype networks of the genus *Agalychnis* colours relate haplotypes. Pictures are not proportional to real size of the frogs. Diameter of the circles is proportional to the frequency of the haplotypes. Geographical distribution of the locations is shown in the map of Mesoamerica. Geographical location of the individuals corresponds to the geographical location of the map of Mesoamerica.
In the genus *Dendropsophus*, which similarly to *Agalychnis* is here represented by one species confined to the north (*D. robertmertensi*), one to the southern regions (*D. phlebodes*), and two widespread through the most of Measoamerica (*D. ebraccatus* and *D. microcephalus*), haplotype networks allowed to identify some groups of genetically more related populations, also clustered geographically (Figure 11).

The low number of distinct sequences did not allow the generation of a haplotype network for *D. robertmertensi*. In the haplotype network built for *D. ebraccatus*, there were clearly distinct haplotype clusters. Similarly to *A. callidryas*, the population of *D. ebraccatus* from the Osa Peninsula was distinct from any other surrounding area, even from the Pacific coast of Costa Rica (i.e. Cque) with over 30 mutational steps separating it from the closest cluster (Cvie). It is also clear a north–south division, with a separated cluster grouping the northern populations (Belize) and a very distant cluster containing the Panamanian populations. In the middle region (Nicaragua and Costa Rica) there was a distant Caribbean Costa Rican cluster (from the southeast of the country Cvie), which was clearly separated from the other Caribbean cluster that covers populations from north-eastern Costa Rica (Ccrarc/Ccumb) to the northeast of Nicaragua (Nwis). Particularly the separation of the Panamanian cluster with a minimum of 44 mutational steps away from the rest of haplotypes in the network is a very similar pattern to that previously observed in *A. callidryas* and *A. lemur*. Also, the separation of the northern cluster from the other groups follows a similar pattern to that found in *Agalychnis*.

Morphologically *D. ebraccatus* exhibits variation on the dorsal patterns across the whole region. However, there is not concordance between the observed colour patterns and the haplotype clusters, as all the different patterns seemed to be present in almost every population; Although in Nicaragua there is a clear dominance of the traditional hourglass pattern. Figure 11 shows the different dorsal colour patterns observed in this species, but they are shown for information only and their position in the figure does not exclusively relate to the nearest cluster. For all the other species the position of the pictures relate to the clusters.

*Dendropsophus phlebodes* showed connections between the populations of Costa Rica and Nicaragua, and had only one rare haplotype from the Caribbean of Costa Rica (Ccumb) that was separated from the rest of the cluster by 24 mutational steps.
*Dendropsophus microcephalus* also showed a north-south division of the populations in the haplotype networks (Figure 11). The populations of Panama (southern cluster, Pcanal, Pgamb, Pvant) are separated by more than 66 mutational steps from the northern cluster (connecting with the most northern populations in Belize). Moreover, within the northern cluster which starts from Costa Rica northwards, the Pacific of Costa Rica is separated from the rest of the northern cluster. The latter grouped populations from Nicaragua, Honduras, Belize and Guatemala, that appeared well mixed with each other. Populations from Belize, northern Guatemala (Gchin, Gesto, Gpete) and some individuals from Hmang (a population located in western Honduras) were somehow grouped closer with respect to the rest of the cluster. The rest of populations from Honduras were mostly close to those from Nicaragua and formed a cluster that covered a large geographic area (map in Figure 11). Moreover, the north south division coincide with a morphological differentiation of the frogs (see pictures for *D. microcephalus* in Figure 11). The northern form has a reticulated pattern in the dorsum that in the southern form is replaced by two dark lines.

Overall, the haplotype networks revealed greater signals of population expansion in the northern areas, in both the *Agalychnis* and the *Dendropsophus* genera, compared to the southern region, particularly Panama. In the middle area within Costa Rica, populations from the Pacific and the Caribbean did not share haplotypes and in most cases were separated by big numbers of mutational steps (Figures 10 – 11). Moreover, this middle region (Costa Rica) and the area where Honduras, Guatemala and Belize converge geographically (in the northern area), were the places where most of the diversity was found, particularly in the Caribbean versant. Whereas diversity in the Pacific region in both groups in general and in the species that occur in that area, in particular, was much lower.
Figure 11. Haplotype networks of the genus Dendropsophus. Colours relate to the geographical location of the individuals. Geographical distribution of the locations is shown in the map of Mesoamerica. Diameter of the circles is proportional to the frequency of the haplotypes. Pictures are not proportional to real size of the frogs.
Hypothesized colonization and dispersal processes of *Agalychnis* and *Dendropsophus* frogs through Mesoamerica

The identification of likely refugia, that is the areas that may have remained suitable for each species through most of the Pleistocene when environmental fluctuations associated with glaciations took place, could guide the proposal of geographically explicit hypothesis aiming at explaining how the populations within species have diverged and colonize the different areas of Mesoamerica where they are currently present. These hypotheses represent merely an interpretation of the geographical and climatic scenario in which the frogs under study have survived (Figure 8 – 9), according to our modeling, and of the mitochondrial genetic data analyzed through haplotype networks (Figure 10 – 11). We present here a general description of the observed refugia and their relationship with the geographic distributions and genetic relatedness per species, and propose one idea to explain how the processes of colonization and dispersal through Mesoamerica took place, but several further alternative hypotheses could derive from our insights.

The genus *Agalychnis* exhibited similar locations of the refugia in the south of Mesoamerica for *A. callidryas* and *A. lemur*, the northern Caribbean of Costa Rica seemed to be the area that remained suitable for both species (although at different altitudes) during the last 120Ky. For *A. callidryas*, based on the location of this big refuge, and the distribution of the suitable areas through Mesoamerica at different periods of time (during glacial and interglacial) we could propose the hypothesis that an early ancestor migrate from South America (much earlier than the LIG), probably when climatic conditions were similar to the interglacial period (Figure 12). The ancestor may have entered Panama and occupied both Caribbean and Pacific versants and reaching north to Costa Rica on the Caribbean side. Colonization of the Pacific versant of Costa Rica from Panama may have happened during glacial periods. Further spread through Nicaragua and nuclear Central America may have also been facilitated by glacial conditions, mainly through the Caribbean and from Nicaragua northwards, probably through the hills and the coastal Caribbean areas; although the two processes of migration not necessarily may have happen during the same glacial period (i.e. e. LGM). Furthermore, the colonization of Corn Island by *A. callidryas*, most probably occurred during the LGM conditions, since not much genetic separation was suggested by the haplotype networks. After the LGM, Nicaragua and middle Honduras became a
much harder area to migrate through, leading to the distributional patterns that we see today (Figure 12). In Figure 12 we also hypothesized the current occasional migration of individuals through the Caribbean from Costa Rica/Nicaragua to the north east of Honduras, based on the haplotype relatedness found (Figure 10).

Figure 12. Hypothesized colonization and dispersal of Agalychnis callidryas through Mesoamerica. Expected current barriers to dispersal are indicated.

Figure 13. Hypothesized colonization and dispersal of Agalychnis lemur through Mesoamerica. Expected current barriers to dispersal are indicated.
For *A. lemur*, similar to *A. callidryas*, the colonization of Central America from South America may have happened earlier than LIG but the spreading through lower Central America occurred during glacial times and by movements across hills on mainly the Caribbean versant (Figure 13). Dispersal to the Pacific versant (according to historical records), is expected to have happened also during glacial periods and by migration through the hills surrounding the Talamanca cordillera rather than crossing it. No dispersal is expected to take place near the coast on either Pacific or Caribbean versants. Finally, for *A. moreletii* the long term suitable areas were located in the Pacific, where its ancestor may have arrived earlier than the LIG from the south to probably El Salvador and Guatemala. Such ancestor may have given rise to the *A. moreletii* and its closely related taxon *A. annae* (currently only distributed in lower Central America).

*Agalychnis annae* is a Caribbean taxon, therefore a switch from Caribbean to Pacific distribution may have occurred, which, based on the environmental modeling obtained for both species (Figure 3 and S2), most probably took place under LIG-like conditions (Figure 14). Such ancestor that went from Caribbean to Pacific then moved along the Pacific of Nicaragua to El Salvador and into Guatemala. During glaciations these frogs
may have come down to lower altitudes in the mountains (although still not to lowlands), and spread further north to Mexico, and probably through the small hills to the Caribbean versant (either through the area that today is the border Guatemala – Mexico, or in the east through the convergence Guatemala – El Salvador – Honduras). Furthermore, after colonization of the Caribbean versant, the area nearby the lake Izabal (near our sampled site Gesto) in Guatemala has served as refuge for this species and probably has been the place from which frogs have gone out and spread northwards to Belize (Figure 14).

![Time of colonization or dispersal](image)

**Figure 15. Hypothesized colonization and dispersal of Dendropsophus ebraccatus through Mesoamerica. Expected current barriers to dispersal are indicated.**

*Dendropsophus ebraccatus* is the only species under analysis and present in Mesoamerica that belongs to the *D. leucophylatus* group; therefore we do not analyze its colonization and dispersal pattern in conjunction with the rest of species of this genus. Based on the information obtained from haplotype networks and the environmental modelling, the founder population of this species (which is also present in most of Colombia and the Amazonia), entered Panama during a LIG-like period and followed a mainly Caribbean route through the country, colonized Costa Rica and migrate far north to Honduras (Figure 15), not necessarily at the same point in time, but definitely the migration may have been facilitated by LIG-like conditions. During
glacial periods the colonization of the Pacific versant in Costa Rica, by Caribbean migrants was possible through the north limit of the Cordillera de Talamanca. Probably the colonization of the Southern area (Osa Peninsula) was done by Panamanian migrants rather than the north-south dispersal of the Costa Rican individuals that were colonizing the north-pacific areas. Currently, western Panama, most of Nicaragua, El Salvador and Southern Guatemala are a barrier to dispersal for this species (Figure 15).

For the rest of species from the genus *Dendropsophus* the hypothesized colonization and dispersal processes are presented in one model. We based this model in our observations of environmental modelling, haplotype networks and literature about the relationships between the three species, *D. microcephalus*, *D. phlebodes* and *D. robertmertensi*, particularly the hypothesis discussed by Duellman (2001) on their origin in Mesoamerica. Therefore, Figure 16 presents a model in which an ancestor of the three species arrived in lower Central America (i.e. Panama and southern Costa Rica) during a period with similar conditions to those in LIG. Such ancestor was predicted by Duellman (2001) to be a *D. microcephalus*-like species. The dispersal into middle and northern Costa Rica was done by two routes, one through the Caribbean and
the other one through the Pacific, this way giving rise to the Caribbean-restricted *D. phlebodes* and on the Pacific leading to dispersal of a *D. microcephalus* form far north from Costa Rica to Nicaragua. The diversified *D. phlebodes*, disperse further into Nicaragua most likely during LGM-like periods, but not necessarily the LGM or the same period of the first colonization of the ancestor that gave rise to the species.

The movement of the Pacific population (*D. microcephalus*-like form) into Nicaragua most likely took place during a LGM-like period. Such migration again followed two routes one through the Caribbean lowlands and the other one through the Pacific lowlands. The latter migrants most likely gave rise to the currently known *D. robertmertensi*, which occurs from El Salvador northwards to Mexico, in the only area where *D. microcephalus* does not occur. On the other hand, the Caribbean migrants moved northwards through the lowlands of Nicaragua and into most of Honduras up to northern Guatemala and Belize, which constitute the currently recognized *D. microcephalus*. Moreover, the movement of the *D. microcephalus*-like ancestor that moved north from Nicaragua, even though it was facilitated by LGM-like conditions, it was probably not completely stopped during LIG-like climatic conditions. The model presented here assumed *D. microcephalus* as one species.

**mtDNA variation: Phylogenetic analysis and population genetics**

We calculated descriptive genetic statistics for every species within the two genera, *Agalychnis* and *Dendropsophus*. Rather than by sample sites, we divided the populations within each species following their haplotypic relatedness (Figure 10 – 11), and additionally for *Agalychnis* the level of connectivity known to exist between the populations was taken into account (Chapter 2).

Our sampled size for *Agalychnis* consisted of 753 bp fragment of the mitochondrial gene Cytb sequenced for 412 individuals. In *Dendropsophus* for the same gene we sequenced 735 bp for 432 individuals. We found 270 polymorphic sites in *Agalychnis* and 284 in *Dendropsophus*. Number of haplotypes was also similar with 94 total haplotypes in *Agalychnis* and 101 in *Dendropsophus* (Table 2). Species level haplotype diversity was similar between the two genera, with species showing high and low values in both genera. *Agalychnis moreletii* (Hon), *D. ebraccatus* (Pan), *D. robertmertensi*, showed the lowest values, but did not show any particular similarity between the
populations (i.e. geographical location). Overall the *Dendropsophus* species showed lower values of haplotype diversity compared to *Agalychnis*. At the population level the haplotype diversity was very variable within and between genera and there was not a particular relationship between values observed and geographical location that could coincide in both groups.

Table 2. Intra-specific genetic diversity within the genera Agalychnis and Dendropsophus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Content</th>
<th>n</th>
<th>h</th>
<th>S</th>
<th>Hd</th>
<th>σ (± S.D.)</th>
</tr>
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<tbody>
<tr>
<td>A. callidryas</td>
<td></td>
<td>266</td>
<td>70</td>
<td>151</td>
<td>0.95</td>
<td>0.02490 (± 0.0000013)</td>
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<td>AcPan</td>
<td>Pezag; Pgamb; Pvant</td>
<td>13</td>
<td>8</td>
<td>16</td>
<td>0.91</td>
<td>0.00633 (± 0.00076)</td>
</tr>
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<td>AcCosa</td>
<td>Cosa</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>0.8</td>
<td>0.00212 (± 0.00063)</td>
</tr>
<tr>
<td>AcCQue</td>
<td>Cque</td>
<td>26</td>
<td>5</td>
<td>5</td>
<td>0.81</td>
<td>0.00204 (± 0.00025)</td>
</tr>
<tr>
<td>AcCen</td>
<td>Hjara; Htela; Ndata; Nselva; Nmira; Nwls; Nelen; Ncornis; Nndi; Nmoni; Cmari; Caren</td>
<td>56</td>
<td>14</td>
<td>19</td>
<td>0.56</td>
<td>0.00121 (± 0.00029)</td>
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<tr>
<td>AcCR</td>
<td>Cerarc; Chana; Cvie; Ctor; Nbart</td>
<td>57</td>
<td>24</td>
<td>56</td>
<td>0.919</td>
<td>0.00503 (± 0.00133)</td>
</tr>
<tr>
<td>AcNic</td>
<td>Ndeli; Ndiri; Nmade; Nnomb; Nnomt</td>
<td>46</td>
<td>6</td>
<td>7</td>
<td>0.584</td>
<td>0.00156 (± 0.00021)</td>
</tr>
<tr>
<td>AcCen</td>
<td>Beuve; Bchws; Bhil; Btide; Gesto; Gpete; Hjara; Hmang</td>
<td>62</td>
<td>9</td>
<td>9</td>
<td>0.753</td>
<td>0.00174 (± 0.00110)</td>
</tr>
<tr>
<td>A. lemur</td>
<td></td>
<td>12</td>
<td>7</td>
<td>69</td>
<td>0.909</td>
<td>0.04248 (± 0.00526)</td>
</tr>
<tr>
<td>AlPan</td>
<td>Pmari; Prock</td>
<td>5</td>
<td>3</td>
<td>20</td>
<td>0.7</td>
<td>0.01062 (± 0.00594)</td>
</tr>
<tr>
<td>AlCR</td>
<td>Cerarc</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>0.857</td>
<td>0.00759 (± 0.00127)</td>
</tr>
<tr>
<td>A. moreletii</td>
<td></td>
<td>116</td>
<td>15</td>
<td>31</td>
<td>0.64</td>
<td>0.00863 (± 0.00098)</td>
</tr>
<tr>
<td>AmBel</td>
<td>Beceu; Gesto</td>
<td>84</td>
<td>10</td>
<td>11</td>
<td>0.741</td>
<td>0.00204 (± 0.00019)</td>
</tr>
<tr>
<td>AmGua</td>
<td>Gande; Grio; Guniv; Gzon</td>
<td>19</td>
<td>3</td>
<td>3</td>
<td>0.292</td>
<td>0.00067 (± 0.00031)</td>
</tr>
<tr>
<td>AmHon</td>
<td>Hroda</td>
<td>13</td>
<td>2</td>
<td>1</td>
<td>0.154</td>
<td>0.00020 (± 0.00017)</td>
</tr>
<tr>
<td>D. ebraccatus</td>
<td></td>
<td>70</td>
<td>23</td>
<td>101</td>
<td>0.903</td>
<td>0.034 (± 0.00537)</td>
</tr>
<tr>
<td>DeBel</td>
<td>Bchws; Bceuv</td>
<td>18</td>
<td>4</td>
<td>5</td>
<td>0.471</td>
<td>0.00363 (± 0.00118)</td>
</tr>
<tr>
<td>DeCiev</td>
<td>Cviej</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>0.809</td>
<td>0.00295 (± 0.00066)</td>
</tr>
<tr>
<td>DeCosa</td>
<td>Cosa</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0.800</td>
<td>0.00181 (± 0.00060)</td>
</tr>
<tr>
<td>DeCen</td>
<td>Ccumb; Cerarc; Cque; Nlen; Ndata; Nnomt; Nnomi; Nbart; Nwls</td>
<td>29</td>
<td>10</td>
<td>12</td>
<td>0.748</td>
<td>0.000372 (± 0.000078)</td>
</tr>
<tr>
<td>DePan</td>
<td>Pvant; Pgamb</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>0.181</td>
<td>0.00136 (± 0.00068)</td>
</tr>
<tr>
<td>D. microcephalus</td>
<td></td>
<td>236</td>
<td>68</td>
<td>132</td>
<td>0.933</td>
<td>0.0254 (± 0.00553)</td>
</tr>
<tr>
<td>DmNor</td>
<td>Bchws; Bceuv; Bhil; Btide; Gchin; Gesto; Gpete; Hlagu; Hjara</td>
<td>182</td>
<td>37</td>
<td>45</td>
<td>0.878</td>
<td>0.00488 (± 0.00039)</td>
</tr>
<tr>
<td>DmHon</td>
<td>Hceiba; Htela; Hmang; Hasa; Hco; Hlagu; Hroda; Hjuan; Hsluis; Hjara</td>
<td>102</td>
<td>13</td>
<td>11</td>
<td>0.683</td>
<td>0.00380 (± 0.00046)</td>
</tr>
<tr>
<td>DmCque</td>
<td>Cque</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0.500</td>
<td>0.00136 (± 0.00068)</td>
</tr>
<tr>
<td>DmNic</td>
<td>Nlen; Nnomb; Ndata; Nnruja; Nselva; NwisaNcorn</td>
<td>28</td>
<td>9</td>
<td>12</td>
<td>0.822</td>
<td>0.00423 (± 0.00064)</td>
</tr>
<tr>
<td>DmPan</td>
<td>Pvant; Pcanal; Pgamb</td>
<td>20</td>
<td>7</td>
<td>21</td>
<td>0.810</td>
<td>0.01386 (± 0.00230)</td>
</tr>
<tr>
<td>D. phlebodes</td>
<td></td>
<td>17</td>
<td>8</td>
<td>30</td>
<td>0.722</td>
<td>0.01045 (± 0.00560)</td>
</tr>
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<td>DpNic</td>
<td>Nbart; Nwls</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>1.000</td>
<td>0.00363 (± 0.00105)</td>
</tr>
<tr>
<td>DpCR</td>
<td>Ccar; Ccumb</td>
<td>14</td>
<td>5</td>
<td>27</td>
<td>0.659</td>
<td>0.0147 (± 0.00757)</td>
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<tr>
<td>D. robertmertensi</td>
<td></td>
<td>9</td>
<td>2</td>
<td>5</td>
<td>0.222</td>
<td>0.0068 (± 0.00340)</td>
</tr>
</tbody>
</table>

*Origin of the individuals included in the group

* Number of individuals in the analysis

* Number of haplotypes

* Segregating sites

* Haplotype diversity

* Nucleotide diversity
Nucleotide diversity at the species level was similar between the two genera. With some species showing low values (A. moreletii, D. robertmertensi) while others showed much higher values (A. lemur, D. ebraccatus) (Table 2). There was not a particular relationship between the geographical distribution of the species and their nucleotide diversity values either. Nucleotide diversity within species, at the population level, showed very variable values and there was not any particular geographic region where populations would have specially high or low values.

Phylogenetic trees were obtained from Maximum likelihood and Bayesian inferences for both genera, *Agalychnis* and *Dendropsophus* (Figure 17 – 18). To use the full length for all the sequences and given that no appropriate published sequences were available to be used as outgroups to root the phylogenetic trees we used the midpoint rooting for the phylogenies as implemented in FigTree v1.3.1 and used only our ingroup sequences. Our ML and BI topologies were highly congruent; therefore we present only the Bayesian consensus trees for both genera with the posterior probabilities and non-parametric ML bootstrap values for the shared branches (Figure 17 – 18).

*Figure 17. Phylogenetic tree of the genus Agalychnis. Above branches before the slash are the posterior probability from Bayesian Inference and after slash the bootstrap values from Maximum likelihood analysis.*
Tree topologies in both cases recovered the haplotypic relationships of the different identified groups. Within *Agalychnis* haplotype groups were also recovered. *Agalychnis lemur* was clearly split into two clades one from Panama and one from Costa Rica, which was sister to the rest of taxa. Within the *A. callidryas* species group, *A. moreletii* and *A. spurrelli* formed a clade which was sister to the *A. callidryas* complex. Within *A. callidryas* five well supporter clades were recovered: one from the Osa Peninsula (1) appeared as the sister taxon of the rest of clades. The latter corresponded to (2) the north (including Belize, Guatemala and some populations from western Honduras); (3) Panama; (4) the Pacific of Costa Rica (Cque); (5) the populations from the middle region of Mesoamerica, comprising Caribbean Costa Rica, Nicaragua and part of eastern Honduras (Figure 17).

*Figure 18.* Phylogenetic tree of the genus *Dendropsophus*. Above branches before the slash are the posterior probability from Bayesian Inference and after slash the bootstrap values from Maximum likelihood analysis. The black star is pointing the individual KEN661 (see chapter 4 for details on new country record based on the molecular taxonomic identification of this individual).

Within *Dendropsophus*, the presence of two clades within *D. ebraccatus* was evidenced, with Panama forming a sister clade to the rest of the Mesoamerican populations. The three remaining species, formed a clade all together within which, a clade formed by *D. phlebodes* and *D. robertmertensi*, was sister to the complex of *D.
microcephalus. The latter being clearly split north-south geographically into two clades, one in Panama and the other one from Costa Rica northwards. This northern clade contained a northern group of mainly Caribbean populations, and a sister small clade containing the only Pacific population from Costa Rica (Figure 18).

Discussion

We present here hypotheses aiming at explaining the processes that have originated the diversity of Hylid frogs of the genera *Agalychnis* and *Dendropsophus* in Mesoamerica. Despite the small number of species these genera have in the region they revealed that non-identical patterns of diversity and distribution are present within their groups, and therefore complex processes may have shaped their origin and dispersal patterns, with probably important events for both genera happening at different points in time. Similar results have been found in among closely and non-closely related species of snakes in the northernmost Middle America (Daza et al. 2010; Bryson et al. 2011) and in two of our studied species of frogs in lower Central America (Robertson et al. 2009). So, it would not be surprising that within this region taxa may have responded in different ways to changes in the landscape, compared to the most widely spread patterns found in South America (Elmer et al. 2007). It is also not surprising that the expected complex processes of dispersal, isolation and further genetic differentiation may have lead to the origin of cryptic diversity in need for uncovering. Some signals for that were found in our results (and see also Chapter 2).

Phylogeographic hypothesis

Our phylogeographic hypotheses integrate geospatially explicit information on the region itself and on the frogs, as well as mitochondrial genetic information on different populations within species. Such integration of data allowed the analysis from different perspectives of the history of these frogs and the factors that may have played the key role in their evolution. Climatic change has been an important factor in the evolution of much of tropical diversity in the old and new tropics (Kappelle et al. 1999; Martínez-Meyer et al. 2004; Pennington et al. 2004; Peter B 2004; Hawkins et al. 2006; O’Loughlin et al. 2008; Bryson et al. 2011). Investigation on this topic has mainly been done on the Amazonia in the Neotropics (Santos et al. 2009). In Mesoamerica, the work has been scarce and mainly focused in Panama and to a lesser extent in Costa Rica.
(Crawford 2003; Crawford et al. 2007) and Mexico (Daza et al. 2010; Bryson et al. 2011). Most of the hypotheses proposed to explain the origin of different taxa have come from a taxonomic point of view and from such relationships among taxa (Duellman 2001), but little information has been gathered to compile evidences that allow testing such hypotheses and the proposal of complementary ones. We here studied two genera, which, although are not among the most diverse in the region, are distributed through most of it and allowed a good coverage of the different geomorphological characteristics of the area, different habitats, ecologies and, as shown with the results of different diversity patterns and even of degree of endangerment.

Testing of the hypotheses is clearly the next step in this analysis. Complementary analysis to those of ecological niche modelling presented here for the past and present conditions could be achieved by the inference of the ancestral nodes in our phylogenies. One approach could be, for example, the maximum likelihood framework implemented in the software Phylomapper (Lemmon & Lemmon 2008), which allows the identification of the most ancestral node in the phylogeny given the geographical distribution of the individuals, from which dispersal routes could be inferred and our hypotheses complemented. This approach has been widely used in the last couple of years and is a good way of further integrating geographically explicit hypothesis with genetic data (Lemmon & Lemmon 2008; Chan et al. 2011). Furthermore, the addition of nuclear data would give extra evidence and power to the analysis (Lemmon & Lemmon 2008; Thomé et al. 2010; Chan et al. 2011), either to reveal other alternatives not visible with the mitochondrial data or to clarify uncertainties that may arise due to the nature of the marker we used (i.e. the possibility for introgression, high and fast saturation).

Simulation analysis and estimation of divergence times would be a further step in finding the degree of acceptation we can give to our proposed hypotheses as a likely model of origin and dispersal of Hylid frogs, particularly the two genera under study, through Mesoamerica. The Isolation with migration model (Hey 2010), for example, could allow the estimation of historical demographic genetic parameters of closely related species (i.e. within each of our two genera) quantify gene flow and estimate divergence times between pairs of populations (Hey 2010; Strasburg & Rieseberg 2010) and finally compare patterns among groups. Such analyses could be coupled with further GIS analyses to those presented here, in which the genetic data is interpreted in a geographically explicit context that takes into account the history of the region.
particularly climatic) as well as the ecological requirement of the species, to finally interpret results of any simulation in a realistic context of dispersal capacity of the species, presence of suitable areas across the region at different time periods. We conducted preliminary analyses to visualize the possible connectivity among populations of *A. callidryas* and *A. moreletii* under LGM climatic conditions (Figure S7). Such preliminary results on connectivity for example would agree more with the presence of a corridor between Northern Costa Rica and north east Honduras (Kirby & MacFadden 2005), rather than the presence of little islands spread through the current Honduras and Nicaragua (Coates & Obando 1996), which if were the case, little connectivity should be observed between populations across that Caribbean area. Similar results to these hypothesized here, have been found in widespread viper genera (Castoe et al. 2009).

**Cryptic diversity**

One important point that was clear with this first analysis of the data is the need for a deep review of the taxonomic status of several species. Wide morphological variation was observed during the sampling and later it was corroborated by the genetic variation, which in some cases increased the uncertainty of more than one taxonomic entity being contained within the currently recognized species of both genera. The existence of two different subspecies within *D. microcephalus* (Duellman 2001) that worth checking for the likely elevation to the species level was evidenced in this study. Generally the status of subspecies is not being used within this species, and the named subspecies, *D. m. underwoodi* and *D. m. microcephalus* (Duellman 2001) are treated as synonyms among the popular databases (Frost 2009), so the species is considered one of the most widespread in the region.

The case of *D. ebraccatus* is similar but probably not as clear as the *D. microcephalus*. Although in almost every population of *D. ebraccatus* there are more than one of the dorsal patterns we observed (Figure 11), there is clear division of this species into north and south, with one form confined to Panama and the other one spread from Costa Rica northwards to Nuclear Central America. Furthermore, the presence of a long time barrier which persists in the west of Panama, may suggest long term isolation of the two populations forms (north and south). Similar to this case is that of *A. lemur*, whose genetic distance between Costa Rican and Panamanian populations were considerably
large and would need further revision. Moreover, within *A. moreletii*, there was a clear division Pacific/Caribbean that finds some morphological back up when looking at frogs’ coloration (Figure 10).

Although we have only used mitochondrial DNA data, this molecular marker has been used reliably for species delimitation analysis (Pons et al. 2006; Monaghan et al. 2009); however, we recognized that the confirmation of results by using additional markers would give a clearer picture of the pattern we found with the mitochondrial information. Confirming the taxonomic status of all these species would have clear conservation impacts, since their status as defined by the IUCN 2009 would change radically and that information is crucial if effective conservation strategies want to be put in place in Mesoamerica.

**Materials and methods**

**Data Collection and species sampled**

All the samples included in this study were collected through most of Mesoamerica (Figure S1), using mainly a mouth swabbing technique (Poschadel & Möller 2004). Sampling locality information is shown in Table S1. A total of 844 individuals for eight described species: three focal and one additional species of the genus *Agalychnis* (Phyllomedusinae), and two focal and two additional species of *Dendropsophus* (Hylineae) were included in the analyses. Populations in each species were represented by a minimum of 1 and a maximum of 102 individuals. The amphibian classification followed Frost (2011) and the recognition of subspecies is based on discussions by Duellman 2001 (Table S2). The conservation status of the species is based on the IUCN red list (IUCN 2010) and is as follows (Table S2): two critically endangered (CR) species, and six of currently least concern (LC) for conservation.

**Current Species Distribution Modeling**

Distribution modeling was performed on the basis of the currently recognized taxonomic status of the putative species to estimate their geographical distribution given the current climatic conditions. We gathered species occurrence data from different Museums and collections (Accessed through the Global Biodiversity Information...
Facility, GBIF Data Portal, data.gbif.org, 2011-11-22) and from the literature (Robertson et al. 2009). Eighteen environmental variables at a spatial resolution of 1 km (Hijmans et al. 2005) were used to model current distributions. Environmental variables were chosen on the basis of their relation with amphibians’ biology (Carey & Alexander 2003). Therefore, Species Distribution Models (SDMs) were inferred using the following variables that describe changes in precipitation and temperature through the year: annual mean temperature, mean diurnal range (mean of monthly (max temp - min temp)), isothermality, temperature seasonality, max temperature of warmest month, min temperature of coldest month, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of warmest quarter, mean temperature of coldest quarter, annual precipitation, precipitation of wettest month, precipitation of driest month, precipitation seasonality, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter, precipitation of coldest quarter.

Modeling was performed using the maximum entropy algorithm implemented in Maxent v 3.3.3k. (Phillips et al. 2006). Original raster files of each environmental variable were clipped in ArcGIS to the geographical extent of the study area to create the input ASCI files required by Maxent. Presence data was partitioned into two sets: one training set consisting of 75% of occurrence data used for prediction of the model, and the remaining 25% was the test data used to validate the models and calculate statistics. Modelling was performed using the using default settings in Maxent except for restricting model building to ‘hinge features’, which allows a smoother model (Elith et al. 2011); ‘calculating response curves’, to assess the probability of the species occurring in a particular geographical point given different values of the predictors (environmental variables) (Phillips et al. 2006); ‘performing jackknife to measure variable importance’, and choosing the function of ‘don’t extrapolate’ to avoid bogus projections (Thomé et al. 2010). The resulting model from Maxent attempts at ranking locations, within a given geographical area, according to their suitability for the occurrence of the focal species (Phillips et al. 2006). The ‘minimum training presence’ criterion was used to define the threshold for habitat suitability (Thomé et al. 2010; Webber et al. 2011). The evaluation of the obtained distribution models was based on the calculation of the area under the curve (AUC) of the Receiver Operating Characteristics Curve (ROC), a threshold independent index commonly used to compare model performance (Thuiller et al. 2004; Phillips et al. 2006).
Species Paleodistribution Modeling

Current distribution models obtained per species were projected onto past climatic conditions to model their distribution during two periods in the past: The Last Glacial Maximum (LGM) and the Last Interglacial (LIG). Projection was performed in Maxent v 3.3.3k (Phillips et al. 2006). Paleoclimatic was obtained from WorldClim (www.worldclim.org). For the LGM we used simulations from two different models: the Community Climate System Model (CCSM) (Collins et al. 2004) and the Model for Interdisciplinary Research on Climate (MIROC) (Hasumi & Emori 2004). Projections had the same settings as for the current distribution modelling described in the materials and methods “Current Species Distribution Modeling”. Original raster files were clipped in ArcGIS to the geographical extent of the study area to create the input ASCI files required by Maxent.

Identification of long term high stability areas

High stability areas across the geographical distribution of the species were identified by summing in ArcGIS the modeled distribution on the three periods (layers obtained for current, LGM and LIG). The resulting layer was reclassified to show only the 20% highest values. Areas shown in the final layer after reclassifications indicate regions of high ecological stability, which are predicted to have served as likely refugia for the species through the Pleistocene (last 120 Ky). Based on the existence of these areas, hypothesis of origin and dispersal patterns of the species can be proposed (Figure 12 – 18). For comparison and to test the existence of similar refugia in closely related taxa, paleodistribution were additionally obtained for three species of the subfamily Phyllomedusinae, Agalychnis annae, A. saltator, and Cruziophyla calcarifer (Figure S2-S5 and Table S4).

DNA extraction, Amplification and Sequencing

DNA was extracted with the ISOLATE Genomic DNA Mini Kit (Bioline, London, United Kingdom) from buccal swabs and liver tissue. Molecular data include a fragment of the mitochondrial Cytochrome b, Cytb, with a length of 753 bp for the Agalychnis and 735 bp for the Dendropsophus. Fragments were sequenced using either published
(Moritz et al. 1992) or redesigned primers that typically generated higher yields of PCR products. List of primers and PCR conditions are shown in Table S3.

**Sequence alignment and model testing.**

Sequences were manually checked in the program Geneious (Drummond et al. 2011) and aligned in Clustal X2 (Larkin et al. 2007) followed by minor checking and trimming in MacClade v.4. (Maddison & Maddison 2000). The best model of nucleotide substitution for the data in each group, *Agalychnis* and *Dendropsophus*, was determined using the AIC criterion in JModeltest (Posada 2008), which in both cases was the General Time Reversible plus Gamma (GRT + Γ).

**Haplotype Networks, Phylogenetics and Genetic Diversity analyses**

Median joining haplotype networks were built per species using Network v 4.6.1.0 (Fluxus Technology Ltd) with default settings. We used DNASP v.5.10.01 (Librado & Rozas 2009) to calculate descriptive statistics. Intra-specific subdivisions were based on haplotype clusters. We estimated the number of haplotypes (h), segregating sites (S), haplotype diversity (Hd) and nucleotide diversity (π) (Nei 1987). Pairwise genetic distances between groups were assessed by estimating $F_{ST}$ values.

Both groups, *Agalychnis* and *Dendropsophus* were analyzed with ML methods using RaxML (Stamatakis 2006), and with Bayesian inference (BI) with MrBayes 3.2 (Ronquist et al. 2012). In RaxML 100 bootstrap replicates were run to estimate support values. In MrBayes a total of 2 independent runs of 4 heated chains for 10000000 generations were run sampling every 500 generations and discarding the first 30% of samples from the cold chain as burnin. Convergence of the two runs was checked in Tracer v. 1.4 (Rambaut & Drummond 2007).
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Table S1. Geographic location data and habitat of sampled populations.

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Supporting Information
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<tr>
<td>Santa Bárbara</td>
<td>&quot;Aldea San Luis de Planes&quot;</td>
<td>Natural Lagoon</td>
<td>Urbanism, cattle ranching</td>
</tr>
<tr>
<td>Yojoa</td>
<td>&quot;Los Naranjos Ecoarchaeological Park&quot;</td>
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<td>Tourism</td>
</tr>
<tr>
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<td>&quot;Finca Santa Elena&quot;</td>
<td>Roadside Pond</td>
<td>Cattle, urbanism, agriculture</td>
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<tr>
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<td>&quot;Volcán Mombocho National Reserve&quot;</td>
<td>Artificial Pond</td>
<td>Tourism/observation</td>
</tr>
<tr>
<td>Jinotega</td>
<td>&quot;Datanlí&quot;</td>
<td>Artificial Pond</td>
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<td>Urbanism, cattle, agriculture</td>
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<td>&quot;Selva Negra&quot;</td>
<td>Artificial Pond</td>
<td>Tourism, urbanism, coffee farming</td>
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<tr>
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<td>&quot;Wisconsin&quot;</td>
<td>Roadside Pond</td>
<td>Urbanism, transport, cattle ranching, agriculture</td>
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<td>&quot;Little Corn Island&quot;</td>
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<td>Coclé</td>
<td>&quot;El Valle de Anton&quot;</td>
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<td>Urbanism and transport</td>
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<td>&quot;Canal&quot;</td>
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<td>Transport, agriculture, cattle, urbanism</td>
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<td>&quot;Gamboa&quot;</td>
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<td>Urbanism and transport</td>
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<td>Belize</td>
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<td>Forest Pond</td>
<td>Research / observation</td>
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<td>&quot;Finca Las Cumbreras&quot;</td>
<td>Natural Wetland</td>
<td>Cattle ranching, transport, urbanism</td>
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<td>&quot;Costa Rica Amphibian Research Center - CRARC&quot;</td>
<td>Forest Pond</td>
<td>Research - tourism / observation</td>
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<td>Limón</td>
<td>&quot;Puerto Viejo&quot;</td>
<td>Roadside Pond</td>
<td>Transport</td>
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<td>Puntarenas</td>
<td>&quot;Corcovado Natural Park - Control Point El Tigre&quot;</td>
<td>Forest Pond</td>
<td>Urbanism and transport</td>
</tr>
<tr>
<td>Puntarenas</td>
<td>&quot;Hotel Espadilla&quot;</td>
<td>Natural Wetland</td>
<td>Tourism and urbanism</td>
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</table>

**Dendropsophus ebraccatus**

**B.** Belize

**C.** Cockscomb Basin Wildlife Sanctuary

**R.** Research / observation

**CRARC** Costa Rica Amphibian Research Center
<table>
<thead>
<tr>
<th>Country</th>
<th>District/Province</th>
<th>Location Code</th>
<th>Genetic Code</th>
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</tbody>
</table>

Countries are represented by one capital letter: B: Belize; CR: Costa Rica; G: Guatemala; H: Honduras; N: Nicaragua; P: Panama. Underlined are the district/provinces within each country. Each sampling point is written within quotes.

Dendropsophus phlebodes

Dendropsophus phlebodes

Dendropsophus phlebodes

Dendropsophus robertmertensi

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Dendropsophus phlebodes

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Dendropsophus phlebodes

Dendropsophus phlebodes

Countries are represented by one capital letter: B: Belize; CR: Costa Rica; G: Guatemala; H: Honduras; N: Nicaragua; P: Panama. Underlined are the district/provinces within each country. Each sampling point is written within quotes.
<table>
<thead>
<tr>
<th>Species</th>
<th>IUCN Category</th>
<th>Species Group</th>
<th>Recognized subspecies (?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agalychnis callidryas</td>
<td>Least Concern (LC)</td>
<td>A. callidryas</td>
<td>No</td>
</tr>
<tr>
<td>Agalychnis lemur</td>
<td>Critically endangered (CR)</td>
<td>Unassigned</td>
<td>Could include two species</td>
</tr>
<tr>
<td>Agalychnis moreletii</td>
<td>Critically endangered (CR)</td>
<td>A. callidryas</td>
<td>Could include two species</td>
</tr>
<tr>
<td>Dendropsophus ebraccatus</td>
<td>Least Concern (LC)</td>
<td>D. leucophyllus</td>
<td>2 subspecies: D. m. microcophalus and D. microcephala</td>
</tr>
<tr>
<td>Dendropsophus microcephalus</td>
<td>Least Concern (LC)</td>
<td>D. microcephalus</td>
<td>D. m. microcophalus and D. m. underwood</td>
</tr>
<tr>
<td>Dendropsophus phlebodes</td>
<td>Least Concern (LC)</td>
<td>D. microcephalus</td>
<td>2 subspecies: D. m. microcophalus and D. m. underwood</td>
</tr>
<tr>
<td>Dendropsophus robertmertensi</td>
<td>Least Concern (LC)</td>
<td>D. microcephalus</td>
<td>No</td>
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<tr>
<td>Additional Species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agalychnis annae</td>
<td>Endangered (EN)</td>
<td>A. callidryas</td>
<td>No</td>
</tr>
<tr>
<td>Agalychnis saltator</td>
<td>Least Concern (LC)</td>
<td>A. callidryas</td>
<td>No</td>
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<tr>
<td>Agalychnis spurrelli</td>
<td>Least Concern (LC)</td>
<td>A. callidryas</td>
<td>No</td>
</tr>
<tr>
<td>Cruziohyla calcarifer</td>
<td>Least Concern (LC)</td>
<td>Unassigned</td>
<td>No</td>
</tr>
</tbody>
</table>

Table S2: Conservation and IUCN categories of the species of the genera Agalychnis and Dendropsophus under study.
Table S3. List of primers and conditions for PCR reactions.

<table>
<thead>
<tr>
<th>Pair/Primer name</th>
<th>Primer sequence 5′ – 3′</th>
<th>PCR conditions</th>
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<tr>
<td></td>
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<td>First step</td>
</tr>
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<td></td>
<td></td>
<td>Cycle(s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temp(°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min</td>
</tr>
<tr>
<td>MVZ15a</td>
<td>GAACCTGCCCACRRTACGNA</td>
<td>1 cycle</td>
</tr>
<tr>
<td>MVZ16a</td>
<td>AAATGAGGCGATATCAYTCTGGY</td>
<td>38 cycles</td>
</tr>
<tr>
<td>MVZ12a</td>
<td>TCATTCTGGGCTATGAGGGGG</td>
<td>2min</td>
</tr>
<tr>
<td>MVZ16a</td>
<td>TCATTCTGGGCTATGAGGGGG</td>
<td>1min</td>
</tr>
<tr>
<td>MVZ17a</td>
<td>TCATTCTGGGCTATGAGGGGG</td>
<td>30s</td>
</tr>
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<td>MVZ18a</td>
<td>TCATTCTGGGCTATGAGGGGG</td>
<td>5min</td>
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<tr>
<td>AgaF2b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>1 cycle</td>
</tr>
<tr>
<td>AgaR2b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>35 cycles</td>
</tr>
<tr>
<td>AgaF3b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>1 cycle</td>
</tr>
<tr>
<td>AgaR3b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>35 cycles</td>
</tr>
<tr>
<td>AgaF4b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>1 cycle</td>
</tr>
<tr>
<td>AgaR4b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>35 cycles</td>
</tr>
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<td>AgaF5b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>1 cycle</td>
</tr>
<tr>
<td>AgaR5b</td>
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<td>35 cycles</td>
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<td>AgaF6b</td>
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<td>AgaR6b</td>
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<td>AgaF8b</td>
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<td>AgaR8b</td>
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<td>AgaF9b</td>
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<tr>
<td>AgaR9b</td>
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<tr>
<td>AgaF12b</td>
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<td>1 cycle</td>
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<tr>
<td>AgaR12b</td>
<td>TACGNAAAAACCTACATATCAAG</td>
<td>35 cycles</td>
</tr>
</tbody>
</table>

a Moritz, C 1992.
b This study.
c Agalychnis callidryas (Belize, Honduras, Guatemala).
d Agalychnis callidryas (Nicaragua, Costa Rica).
e Agalychnis callidryas (Panama); Agalychnis lemur (Costa Rica and Panama); Dendropsopus robertmertensi.
f Agalychnis moreletii (Belize, Honduras).
g Agalychnis moreletii (Guatemala).
h Agalychnis spurrelli (Costa Rica).
i Dendropsopus microcephalus (Guatemala)
j Dendropsopus microcephalus (Panama)
k Dendropsopus microcephalus (Costa Rica)
l Dendropsopus ebraccatus (Belize, Honduras, Nicaragua, Costa Rica and Panama)
m Dendropsopus phlebodes (Costa Rica and Panama)
**Table S4.** Statistics for the Species Distribution Models (SDM) obtained in Maxent for additional species of the subfamily Phyllomedusinae.

<table>
<thead>
<tr>
<th>Species</th>
<th>SDM</th>
<th>Threshold</th>
<th>AUC</th>
<th>SD</th>
<th>Best Environmental predictor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agalychnis annae</em></td>
<td>Current</td>
<td>0.204</td>
<td>0.991</td>
<td>0.002</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.204</td>
<td>0.991</td>
<td>0.002</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
<td>0.204</td>
<td>0.991</td>
<td>0.002</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.204</td>
<td>0.991</td>
<td>0.002</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>Agalychnis saltator</em></td>
<td>Current</td>
<td>0.179</td>
<td>0.959</td>
<td>0.018</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.179</td>
<td>0.959</td>
<td>0.018</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
<td>0.179</td>
<td>0.959</td>
<td>0.018</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.179</td>
<td>0.959</td>
<td>0.018</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>Agalychnis spurrelli</em></td>
<td>Current</td>
<td>0.002</td>
<td>0.969</td>
<td>0.021</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.003</td>
<td>0.975</td>
<td>0.017</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
<td>0.003</td>
<td>0.975</td>
<td>0.017</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.003</td>
<td>0.975</td>
<td>0.017</td>
<td>Precipitation of the coldest quarter</td>
</tr>
<tr>
<td><em>Cruziohyla calcarifer</em></td>
<td>Current</td>
<td>0.263</td>
<td>0.978</td>
<td>0.008</td>
<td>Precipitation seasonality</td>
</tr>
<tr>
<td></td>
<td>LIG</td>
<td>0.263</td>
<td>0.978</td>
<td>0.008</td>
<td>Precipitation seasonality</td>
</tr>
<tr>
<td></td>
<td>LGM-MIROC</td>
<td>0.263</td>
<td>0.978</td>
<td>0.008</td>
<td>Precipitation seasonality</td>
</tr>
<tr>
<td></td>
<td>LGM-CCSM</td>
<td>0.263</td>
<td>0.978</td>
<td>0.008</td>
<td>Precipitation seasonality</td>
</tr>
</tbody>
</table>

**Table S5.** Locality data for records in Nicaragua, currently considered outside the known geographic distribution of the species recognized by the IUCN.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Altitude</th>
<th>Reference</th>
<th>Voucher specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agalychnis saltator</em></td>
<td>14.38780</td>
<td>-84.97990</td>
<td>180</td>
<td>(Travers &amp; Townsend 2010)</td>
<td>UF(^a) 156023</td>
</tr>
<tr>
<td>N: Jinotega, Reserva de la Biósfera Bosawas, “Kipla Sait Tasbaika indigenous territory”</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cruziohyla calcarifer</em></td>
<td>11.03742</td>
<td>-83.88536</td>
<td>10</td>
<td>(Sunyer Mac Lennan 2009)</td>
<td>SMF(^b) 87274</td>
</tr>
<tr>
<td>N: Río San Juan, “Caño El Venado, near Dos Bocas de Río Indio”</td>
<td></td>
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<td><em>Dendropsophus phleboides</em></td>
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\(^a\) Museum of the University of Florida, USA  
\(^b\) Senckenberg Museum Frankfurt  
\(^c\) Field number corresponding to the collector: Kristen E. Nicholson,
Figure S1. Mesoamerica showing the sampling effort per species for general analysis (colored circles). Localities are labeled following codes in Table S1.
Figure S2. Modelled distribution of Agalychnis annae for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Pounds et al. 2004).
Figure S3. Modelled distribution of Agalychnis saltator for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Bolaños et al. 2004a).
Figure S4. Modelled distribution of Agalychnis spurrelli for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN (Jungfer et al. 2004).
Figure S5. Modelled distribution of Cruziohyla calcarifer for the Current climatic conditions, and its projections onto the last glacial maximum (LGM), for two models the Community Climate System Model (CCSM) and Model for Interdisciplinary Research on Climate (MIROC), and for the last interglacial (LIG). The known distribution is shown according to the IUCN.
Figure S6. Identified likely Pleistocene refugia (red areas) for additional species of the subfamily Phyllomedusinae in Mesoamerica.
**Figure S7.** Level of Connectivity between sampled populations of *A. callidryas* and *A. moreletii* during the Last Glacial Maximum (LGM) in Mesoamerica.
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Chapter 4

Amphibia, Anura, Hylidae, Hylineae, *Dendropsophus phlebodes* (Stejneger, 1906): Distribution extension

This note is prepared for submission to Check List.
Amphibia, Anura, Hylidae, Hylinae, *Dendropsophus phlebodes* (Steneger, 1906): Distribution extension

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3 Department of Biology, Central Michigan University, USA.
We present a new country record of *Dendropsophus phlebodes* in the locality of Wisconsin, department of Region Autonoma del Atlantico Norte (RAAN) in Nicaragua. This record extends the currently known distribution of the species by about 400 Km north.

Nicaragua is the largest country of Central America covering an area of approximately 148 000 km\(^2\) (Taylor 1963). Historically the country has been divided into three main physiographic regions: (1) The Pacific covering the Pacific coast and lowlands, the volcanic chain and part of the Nicaraguan depression; (2) the central mountains located more towards the northern region and running in an east to west direction; (3) the Atlantic region, dominated by lowlands, more forested and humid than the Pacific and that becomes gradually swamps and natural wetlands as it reaches the coastal areas (Taylor 1963).

The herpetofauna of Nicaragua has been historically poorly surveyed, especially if compared to the surrounding countries of Honduras, and Costa Rica (Wilson et al. 2010). Given that most of amphibian diversity in Mesoamerica is mainly associated to mountainous and humid forests (Wilson et al. 2010), the reason why Nicaragua may be less appealing to herpetological expeditions compared to neighbour countries, is that the territory is mainly dominated by lowlands (Taylor 1963; Wilson et al. 2010).

*Dendropsophus phlebodes* occurs only in the Atlantic region within Nicaragua and from there its distribution extends south along the Caribbean coast through Costa Rica and Panama to the Choco region of Colombia, occupying the Pacific versant only from Central Panama southwards (Solis et al. 2010) (Figure 1). It is one of the four Mesoamerican species that belong to the *D. microcephalus* species group (Duellman 2001). It is a small size anuran, which differs from its close and sympatrically distributed relative *D. microcephalus* by a highly divergent mating call, the presence of darker irregular interconnecting lines across the dorsum and thighs (Figure 1) (Duellman 2001) and by a dark longitudinal line that does not pass the eye in *D. phlebodes* while in *D. microcephalus* extends along the whole flank (Köhler 2011).
During fieldwork conducted on May 2010, in the surrounding areas of the Municipality of Wisconsin in the Department of Region Autonoma del Atlantico Norte in Nicaragua (14.51403 N, -84.18591 W, at elevation of 76m), we recorded a population of *D. phlebodes* occurring sympatrically with *D. microcephalus*. A numerous reproductive aggregation of frogs was found calling at approximately 19:00 hours, in a roadside pond in the vicinity of the town. Human activities nearby included cattle ranching, agriculture, urbanization and transport derived contamination.

Amplectant pairs of what appeared to be *D. phlebodes* males and *D. microcephalus* females were observed. The specimen collected was initially identified as *D. microcephalus*, based on the uncertainty of *D. phlebodes* to occur so far north. Later the taxonomic identity of the specimen was clarified by sequencing of 735 bp of the mitochondrial gene Cytochrome b, *Cytb* and 650 bp of the Cytochrome Oxidase subunit I, COI. Molecular Data was analyzed in conjunction with *D. microcephalus* from the same locality and *D. phlebodes* from other localities (Figure 1). Haplotype networks
were built and the samples were also part of a larger scale phylogenetic analysis (Chapter 3 Figure 18). In all cases confirming the identity of the sample as *D. phlebodes*. The specimen identified with the field number KEN661, was deposited as voucher in the Museo Herpetológico de la UNAN-León (MHUL). This constitutes a new country record for the species in Nicaragua and the extension of its current northern limit of distribution by 400 Km further north.

**Acknowledgments**

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Chapter 5

General Discussion
The amphibian diversity of Mesoamerica is one the largest in the world despite of the small land mass the region encompasses (Campbell 1999; Myers et al. 2000; Wilson et al. 2010). However, the level of endangerment of amphibians in the region is so high that extirpation of whole communities and selective disappearance of species have taken place in different regions (Lips et al. 2003; Lips et al. 2004; Santos-Barrera et al. 2004; Solis et al. 2004; Young et al. 2004; Beebee & Griffiths 2005; Crawford et al. 2010). Therefore is clear the need for effective strategies to improve the conservation of particularly endangered species and the management of non-endangered species.

Conservation should aim at preserving wild biodiversity and to achieve this, it should focus on maintaining the processes that have generated the biodiversity in every region under concern (Frankham 1995; Beebee 2005; Beebee & Griffiths 2005). To maintain those processes is necessary to understand how they operate and identify the critical factors promoting such processes, and this may vary among species and even within species distributed across wide areas. The general objective of my project was to improve our understanding of the processes that have generated and could maintain biodiversity in the Neotropics. I used two genera of Hylid frogs from Mesoamerica as study system: Agalychnis and Dendropsophus.

I carried out analyses from three perspectives: Conservation Genetics, species delimitation and Comparative Phylogeography that resulted in interesting insights on the processes that have shaped the current distribution of species and their genetic variation across Mesoamerica. Moreover the results I obtained can serve as key information for the improvement of the conservation and management plans of these taxa in particular and amphibians in general at the regional scale in Mesoamerica.

Diversity

The initial specific aim of the diversity analysis was to identify cryptic diversity within the genus Agalychnis, especially in A. callidryas, which exhibits wide morphological variation and have been the subject of discussions about whether or not it represents more than one species (Funkhouser 1957; Savage & Heyer 1967) but the question is still unresolved (Duellman 2001). Other than identifying the existence of
probably five distinctive forms within the currently recognize *A. callidryas* it was overall evident that the diversity of these groups of frogs, and probably of many other groups especially those highly diverse in the region (Duellman 2001; Wiens et al. 2005; Wilson et al. 2010), as we know it today is being underestimated (Fouquet et al. 2007). Similarly to *A. callidryas* the potential for being a complex of independent taxonomic entities was suggested for *A. lemur, A. moreletii*, and *D. microcephalus*. The latter corresponded to the historically recognized subspecies (Duellman 2001) that are currently treated as synonyms (Frost 2011), suggesting that deeper taxonomic review is needed and that high potential exists for such entities to be elevated to the species level.

The level of cryptic diversity present in Mesoamerica is unknown, but based on the results obtained for not highly diverse genera in my analyses; it is possible to predict that a high level of cryptic diversity may be present in the region. This would not be surprising given the large numbers of taxa uncovered by molecular in the nearby areas of the Andes and Amazonia (Elmer et al. 2007; Fouquet et al. 2007; Hawkins et al. 2007) and the tropical regions in the old world (Stuart et al. 2006; Pearson et al. 2007), and to a less extent the new species recently described in Mesoamerica on the basis of molecular differences only due to the extremely conserve morphology they exhibit (Sunyer et al. 2010; Mccranie & Townsend 2011; Sunyer et al. 2011), which has made it difficult to identify such high cryptic diversity by traditional taxonomic analyses.

Given the uncertainties and sometimes problematic results that mitochondrial-only analysis for species delimitation may have (Ebach & Holdrege 2005; Vences et al. 2005; Rubinoff et al. 2006; Waugh 2007; Smith et al. 2008), the results here obtained would benefit from the comparison with other types of markers, like nuclear gene sequences, in order to take the results to a further instance of formal changes and also to assess the performance of the used molecular markers.

**Phylogeography**

In the comparative phylogeographic analysis between the two genera *Agalychnis* and *Dendropsophus* the influence of climatic change was evidenced and was predicted to
have influenced the shaping of origin or colonization and dispersal of this species in particular in Mesoamerica. The proposed hypothesis aiming at describing the processes by which the diversity of this frogs is as we currently know it were mainly based on geographic, environmental and mitochondrial genetic data (Chapter 4). Further testing of the proposed hypotheses will allow for the validation of these models or the further proposal of new ones.

As they are, the results and the evidence allowing to propose the hypothesis I present highlighted the potential similarities between these groups of frogs and other taxa in the region (Wuster et al. 2005; Daza et al. 2010; Bryson et al. 2011), particularly it was clear that a variable response may be expected across this taxa to the different historical events that have taken place in Mesoamerica, which agrees with the findings of Robertson et al. (2009) in lower Central America on two of the frogs under study here, *A. callidryas* and *D. ebraccatus*, and it is also in accordance with studies on other vertebrates, like highland rodents, in which distinctive and independent responses to climate change have been found in allopatric species with recent speciation (Sullivan et. al. 2000)

The role of some areas within Central America, particularly along the pacific versant, to have acted as refugia for species during mostly adverse climatic conditions, like glaciations, was revealed and was similar to those areas inferred for three neotropical tree species for which especially the region of El Salvador and Nicaragua in the Pacific was an important refuge during the Pleistocene, as it was the area where the actual San Juan River is located in the political limit between Costa Rica and Nicaragua on the Caribbean versant of the region (Poelchau & Hamrick 2011). Those two areas of refugia found for tree species, were also the main likely refugia reconstructed in our study and both areas, in Pacific and Caribbean versant were used at some point in time by different species of the two genera *Agalychnis* and *Dendropsophus* (Chapter 3 Figure 8-9); however, for the tree species it is considered flexible their tolerance to the Pleistocene frequent climatic changes because of their capacity to occur in seasonally moist and dry forest, with which they may not necessarily be obligated to migrate to refugia to survive (Poelchau & Hamrick 2011), but such scenario is unlikely for moist-forest dependent species, like the frogs under study here, and so in the event of being more permissive with the reconstruction of
refugia, still the most likely areas where frogs have prevailed for long term should be considered to be within the reconstructed refugia (Chapter 3 Figure 8-9)

During the process of data gathering a new country record leading to a considerable extension of the geographical distribution of *D. phlebodes* was reported for Nicaragua (Chapter 4). This is a country with low levels of diversity that not only may have been influenced by the high percentage of lowlands (Taylor 1963) in the country but by the lack of surveys (Sunyer Mac Lennan 2009; Wilson et al. 2010). The finding of a new country record that far north for a common species like *D. phlebodes*, highlights the need for better herpetological surveys in the country and for better surveys of particular taxa across the whole region, because that is the first step towards identifying any patterns or cryptic diversity that otherwise will remain overlooked. Only with more complete surveys and characterizations of the diversity at local and large scales it will be possible to uncover cryptic diversity and also establish the real conservation status of the species.

**Conservation**

To contribute to the preservation in the short term of the amphibian diversity in Mesoamerica, of particularly endangered species, I presented a comparative analysis that used a combined approach between endangered and non-endangered species and integrating ecological modelling and genetic information (Chapter 2). Similar approaches have been adopted for the study and improvement of conservation plans for plants mainly (Ge et al. 1999; Burne et al. 2003; FaGen & NianHe 2005), but no similar studies have been conducted in frogs. In this case the analysis resulted in the suggestion of short-term alternatives for conservation of critically endangered species of *Agalychnis* frogs and the management of non-endangered species at regional scale.

**Further work**

Real outcomes for the conservation of critically endangered species of frogs are expected from this project and to achieve this, the results already available concerning the two critically endangered species, *A. lemur* and *A. moreletii*, will be presented to relevant authorities for the information to be integrated in the ongoing and planned
conservation and management strategies for amphibian preservation at the country levels and at the regional scale.

For the continuation of the phylogeographic analysis and the further investigation of the existence of more than one entity within the putative species under study, nuclear sequence data has been obtained. A ~700 bp of the gene Rag-1 has been sequenced for a subset of the individual used for mitochondrial analyses (Chapter 4). Rag-1 was chosen because it has proved to be an excellent marker for phylogenetic and phylogeographic purposes (Chiari et al. 2004; van der Meijden et al. 2004; van der Meijden et al. 2005; Hugall et al. 2007; van der Meijden et al. 2007). Such data together with the collected morphological information of the species will be incorporated to different complimentary analyses, and new approaches will also be added in order to test the proposed hypotheses. Methodologies of analysis for the improvement of the results will involve genetic simulations, estimation of divergence times, and further exploration of the environmental information, using GIS specific and modelling software.

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