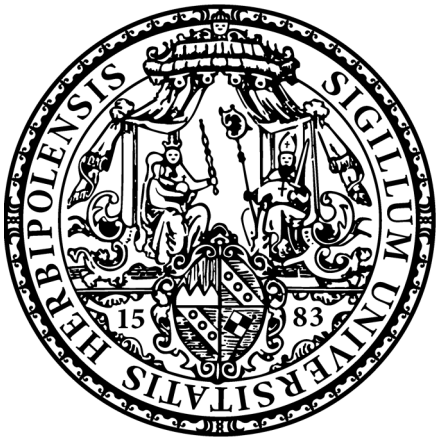

Amphibian diversity along the slope of Mount Kilimanjaro: from species to genes

Diversität von Amphibien im Höhengradienten des Mount Kilimanjaro:
Von Arten zu Genen

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section Integrative Biology

submitted by

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from

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I hereby confirm that my thesis entitled *"Amphibian diversity along the slope of Mount Kilimanjaro: from species to genes"* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 30th September, 2013

Giulia Zancolli

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Summary

1. Since the early nineteenth century describing (and understanding) patterns of distribution of biodiversity across the Earth has represented one of the most significant intellectual challenges to ecologists and biogeographers. Among the most striking patterns of species richness are: the latitudinal and elevational gradients, with peaks in number of species at low latitudes and somewhere at mid altitudes, although other patterns, e.g. declines with increasing elevation, are often observed. Even in highly diverse tropical regions, species richness is not evenly distributed but there are "hotspots" of biodiversity where an exceptional number of species, especially endemics, are concentrated. Unfortunately, such areas are also experiencing dramatic loss of habitat. Among vertebrate taxa, amphibians are facing the most alarming number of extinctions. Habitat destruction, pollution and emergence of infectious diseases such as chytridiomycosis, are causing worldwide population declines. Responses to these drivers can be multidirectional and subtle, i.e. they may not be captured at the species but at the genetic level. Moreover, present patterns of diversity can result from the influence of past geological, climatic and environmental changes.

   In this study, I used a multidisciplinary and multilevel approach to understand how and to which extent the landscape influences amphibian diversity. Mount Kilimanjaro is an exceptional tropical region where the landscape is rapidly evolving due to land use changes; additionally, there is a broad lack of knowledge of its amphibian fauna. During two rainy seasons in 2011, I recorded anurans from the foothills to 3500 m altitude; in addition, I focused on two river frog species and collected tissue samples for genetic analysis and swabs for detection of chytridiomycosis, the deadly disease caused by *Batrachochytrium dendrobatidis* (*Bd*).

2. I analyzed how species richness and composition change with increasing elevation and anthropogenic disturbance. In order to disentangle the observed patterns of species diversity and distribution, I incorporated

inferences from historical biogeography and compared the assemblage of Mt. Kilimanjaro and Mt. Meru (both recent volcanoes) with those of the older Eastern Arc Mountains. Species richness decreased with elevation and locally increased in presence of water bodies, but I did not detect effects of either anthropogenic disturbance or vegetation structure on species richness and composition. Moreover, I found a surprisingly low number of forest species. Historical events seem to underlie the current pattern of species distribution; the young age of Mt. Kilimanjaro and the complex biogeographic processes which occurred in East Africa during the last 20 million years prevented montane forest frogs from colonizing the volcano.

3. I focused on the genetic level of biodiversity and investigated how the landscape, i.e. elevation, topographic relief and land cover, influence genetic variation, population structure and gene flow of two ecologically similar and closely related river frog species, namely *Amietia angolensis* and *Amietia wittei*. I detected greater genetic differentiation among populations in the highland species (*A. wittei*) and higher genetic variation in the lowland species (*A. angolensis*), although genetic diversity was not significantly correlated with elevation. Importantly, human settlements seemed to restrict gene flow in *A. angolensis*, whereas steep slopes were positively correlated with gene flow in *A. wittei*. This results show that even ecologically similar species can respond differently to landscape processes and that the spatial configuration of topographic features combined with species-specific biological attributes can affect dispersal and gene flow in disparate ways.

4. River frogs of the genus *Amietia* seem to be particularly susceptible to chytridiomycosis, showing the highest pathogen load in Kenya and other African countries. In the last study, I collected swab samples from larvae of *A. angolensis* and *A. wittei* for *Bd* detection. Both species resulted *Bd*-positive. The presence of *Bd* on Mt. Kilimanjaro has serious implication. For instance, *Bd* can be transported by footwear of hikers from contaminated water and soil. Tourists visiting Mt. Kilimanjaro may translocate *Bd* zoospores to other areas such as the nearby Eastern Arc Mts. where endemic and vulnerable

species may still be naïve to the fungus and thus suffer of population declines.

5. My study significantly contributed to the knowledge of the amphibian fauna of Mt. Kilimanjaro and of East Africa in general, and it represents a valuable tool for future conservation actions and measures. Finally, it highlights the importance of using a multidisciplinary (i.e. community ecology, historical biogeography, landscape genetics, disease ecology) and multilevel (i.e. community, species, population, gene) approach to disentangle patterns of biodiversity.

Zusammenfassung

1. Seit Ende des 19. Jahrhunderts ist es eine der größten intellektuellen Herausforderungen für Ökologen und Biogeographen, die Verteilungsmuster der Biodiversität auf der Erde zu beschreiben und letztlich zu verstehen. Zu den auffälligsten Mustern des Artenreichtums gehören die Gradienten, die sich in Abhängigkeit von der geographischer Breite und der Höhe über dem Meeresspiegel ergeben. Dabei treten Maxima der Artenzahl in den niederen Breiten und stellenweise in mittleren Höhenregionen auf; es lassen sich aber auch andere Muster beobachten, z.B. eine Abnahme der Artenzahl mit zunehmender Höhe. Selbst in den hochdiversen Tropen sind Arten nicht gleichmäßig verteilt. So gibt es sog. „hotspots" der Biodiversität mit einer außergewöhnlich großen Zahl von Arten (meist Endemiten). Gerade diese Regionen sind es, die heute dramatische Habitatverluste verzeichnen. Unter den Wirbeltieren sind es die Amphibien, die dabei die höchsten Aussterberaten aufweisen. Ihre Populationen gehen weltweit zurück, wofür neben Lebensraumzerstörung auch Umweltverschmutzung und die Ausbreitung von Infektionskrankheiten, z.B. Chytridiomykose, verantwortlich sind. Reaktionen auf solche Faktoren können vielschichtig sein und fast unmerklich bleiben, was bedeutet, dass sie nicht auf Artniveau, sondern nur auf genetischer Ebene erfasst werden können. Hinzu kommt, dass die aktuellen Muster der Biodiversität auf den Einfluss vergangener Veränderungen hinsichtlich Geologie, Klima und Umwelt zurückgeführt werden können.

   In vorliegender Arbeit verwendete ich einen multidisziplinären und mehrstufigen Ansatz um zu verstehen, in welchem Ausmaß die Diversität von Amphibien durch die Landschaft beeinflusst wird. Der Mount Kilimanjaro ist eine außergewöhnliche Tropenregion, deren Landschaftscharakter sich durch Landnutzungsänderungen rapide wandelt. Gleichzeitig ist über die Amphibienfauna dieser Region sehr wenig bekannt. Während zweier Regenzeiten im Jahr 2011 erfasste ich vom Vorland bis in 3 500 Meter Höhe die Froschfauna des Mt. Kilimanjaro. Zusätzlich untersuchte ich zwei

flusslebende Froscharten intensiver, von denen ich Gewebeproben für genetische Analysen und Hautabstriche für den Nachweis von Chytridiomykose nahm, der tödlichen Erkrankung durch *Batrachochytrium dendrobatidis* (*Bd*).

2. Ich habe untersucht wie sich Artenreichtum und Artenzusammensetzung der Amphibienfauna mit zunehmender Höhe und unter anthropogener Störung verändern. Um die beobachteten Muster von Artenvielfalt und Verbreitung zu entflechten, fügte ich Folgerungen aus der historischen Biogeographie ein und verglich das Artenspektrum des Mt. Kilimanjaro und des Mt. Meru (beides jüngere Vulkane) mit den Artenspektren der älteren Berge des Eastern Arc. Der Artenreichtum nahm mit der Höhe ab, stieg aber lokal an, wenn Gewässer verfügbar waren. Einflüsse auf Artenvielfalt und Artenzusammensetzung durch anthropogene Störung oder durch die Vegetationsstruktur konnten nicht nachgewiesen werden. Zudem fand ich eine erstaunlich geringe Zahl an Waldfroscharten. Dem aktuellen Muster der Artenverteilung am Mt. Kilimanjaro scheinen überwiegend historische Ereignisse zu Grunde zu liegen. Das geringe Alter des Mt. Kilimanjaro und die komplexen biogeografischen Prozesse in Ostafrika während der letzten 20 Millionen Jahre verhinderten, dass montane Froscharten den Vulkan besiedeln konnten.

3. Ich habe den Schwerpunkt auf die genetische Ebene der Biodiversität gelegt und untersuchte, welchen Einfluss Landschaftsparameter, d.h. Höhe über dem Meer, topographisches Relief und Landbedeckung, auf die genetische Variation, die Populationsstruktur und den Genfluss zweier ökologisch ähnlicher und nah verwandter Froscharten (*Amietia angolensis* und *Amietia wittei*) haben. Ich konnte eine größere genetische Differenzierung zwischen Populationen der Hochlandart *A. wittei* und höhere genetische Variation bei der Tieflandart *A. angolensis* nachweisen, obwohl die genetische Diversität nicht signifikant mit der Höhe korrelierte. Es ist wichtig zu betonen, dass Siedlungen den Genfluss bei *A. angolensis* zu unterbinden scheinen, während das Vorkommen von Steilhängen positiv mit dem Genfluss von *A. wittei* korrelierte. Die Ergebnisse zeigen, dass selbst ökologisch ähnliche Arten

unterschiedlich auf Landschaftsprozesse reagieren und die räumliche Anordnung topographischer Eigenschaften, kombiniert mit artspezifischen biologischen Merkmalen, Ausbreitungsverhalten und Genfluss unterschiedlich beeinflussen können.

4. Die Flussfrösche der Gattung *Amietia* scheinen besonders empfänglich für Chytridiomykose zu sein und weisen in Kenia und anderen afrikanischen Ländern die höchste Keimbelastung auf. In der letzten Untersuchung sammelte ich Abstrichproben von Kaulquappen von *A. angolensis* und *A. wittei für den Bd*-Nachweis. Beide Arten waren *Bd* positiv. Das Auftreten von *Bd* am Mt. Kilimanjaro hat folgenschwere Auswirkungen. *Bd* aus kontaminiertem Wasser und Bodenmaterial kann z.B. über das Schuhwerk von Wanderern weiterverbreitet werden. Touristen, die den Mt. Kilimanjaro besuchen, können *Bd*-Zoosporen in andere Gebiete wie in die nahegelegenen Berge des Eastern Arc verschleppen, wo endemische und gefährdete Arten dem Pilz noch schutzlos ausgeliefert sein und Populationseinbrüche erleiden könnten.

5. Meine Untersuchung ist ein bedeutender Beitrag zur Kenntnis der Amphibienfauna des Mt. Kilimanjaro und generell der Ostafrikas und stellt ein nützliches Hilfsmittel für zukünftige Erhaltungspläne und Schutzmaßnahmen dar. Letztlich macht die Studie deutlich, wie wichtig ein multidisziplinärer Ansatz (Gemeinschaftsökologie, historische Biogeographie, Landschaftsgenetik, Ökologie von Krankheiten) und ein Ansatz auf mehreren Ebenen (Lebensgemeinschaft, Art, Population, Gene) ist, um die Muster von Biodiversität zu enträtseln.

Chapter 1

Introduction

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1.1 BIODIVERSITY: A HIERARCHICAL CONCEPT

Biological diversity (or simply *Biodiversity*) is the variety of all living organisms on Earth. Although the term is often used to replace the more clearly defined and long established "species diversity" and "species richness", *Biodiversity* is a broader unifying concept, encompassing all forms, levels and combinations of natural variation (Gaston & Spicer 2004). Several definitions have been attempted (e.g. Wilcox 1984; Hawksworth 1996; Larsson 2001; Gaston & Spicer 2004), and even if different in their expression, the hierarchical construct is consistent, and three levels are generally identified: ecological, species and genetic diversity.

Ecological diversity

Ecological diversity encompasses the variety of communities, habitats, ecosystems, ecoregions, provinces and on up to biomes and biogeographic realms. This level of biological diversity is harder to capture and measure because the boundaries of communities and ecosystems are elusive and sometimes changes are gradual and ecosystems are not sharply defined.

Species diversity

Species diversity refers to the variety and abundance of species in a defined unit of study. Such diversity can be measured in many ways depending also on the data available (i.e. incidence or abundance data). The most popular measure used is the Shannon Index which takes into account not only the number of different species but also their abundances, and in practice quantifies the uncertainty in the species identity of an individual that is taken at random from the dataset (Shannon 1948). The most commonly used incidence-based measure is "species richness" which simply corresponds to the count of species in the chosen assemblage (Magurran 2004).

Genetic diversity

Genetic diversity encompasses the components of the genetic coding that structures organisms (nucleotides, genes, chromosomes) and variation in the genetic make-up between individuals within and between populations (Frankham *et al*. 2010). All genetic diversity is originally generated by mutations that change the nucleotides in a sequence of DNA and this allows populations to adapt to changing environments (Frankham *et al*. 2010). Within a species, genetic diversity is commonly described using polymorphism, average heterozygosity and allelic richness. Loss of genetic diversity reduces the ability of populations to evolve, and it is usually associated with inbreeding, small population sizes and isolation. Mutation and migration are the only mechanisms for regaining lost genetic diversity. In fragmented landscapes, the maintenance of genetic diversity depends critically upon gene flow among populations (Frankham *et al*. 2010).

1.2 PATTERNS OF SPECIES DIVERSITY

The geographical distribution of animal species was already object of study in the nineteenth century with Alfred Russel Wallace (1823 – 1913), the "father of biogeography"; yet, understanding why patterns of distribution of biodiversity across the Earth exist constitutes one of the most significant intellectual challenges to nowadays ecologists and biogeographers (Gaston 2000). Most of the known geographic patterns of abundance, distribution and diversity of organisms are along environmental gradients but their fundamental causes are still poorly understood (Brown 2001).

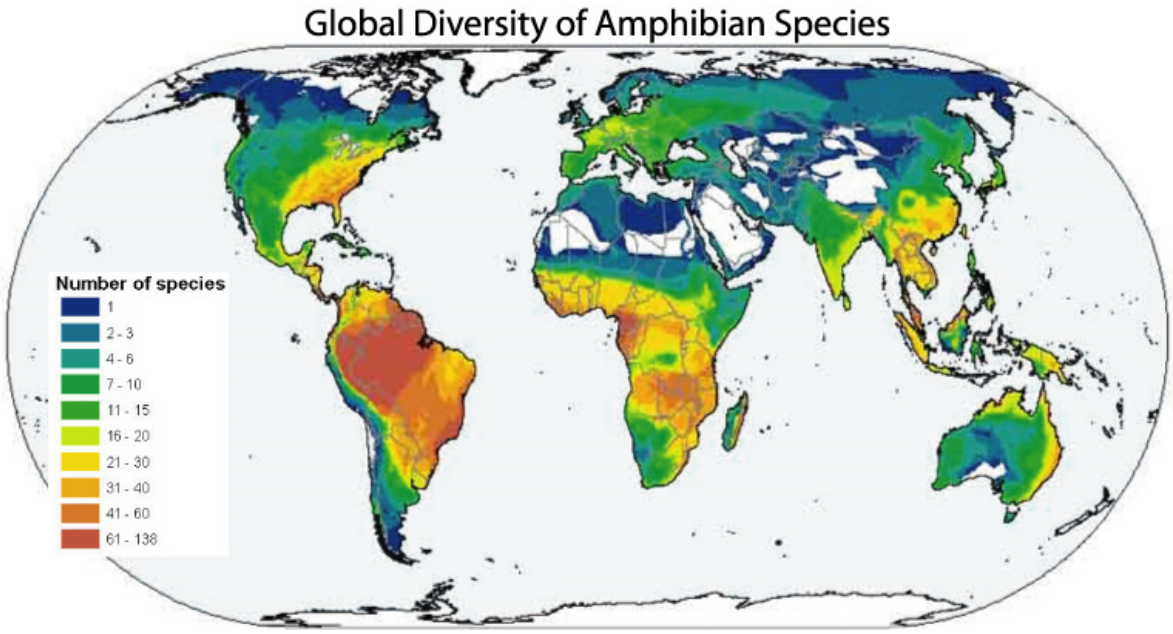


Fig. 1.1 Global distribution of amphibian species. Source: Global Amphibian Assessment.

At a global scale, one of the most striking patterns is the extraordinary variety of life forms in tropical areas compared to temperate regions (Fig. 1.1 for amphibians). Species richness of most taxa increases towards the equator (Stevens 1989; Gaston 1996; Gaston 2000); the strength of this trend does not differ between northern and southern hemispheres (although it is not symmetrical around the equator), nor does it differ between marine and

terrestrial groups, active and passive dispersers, or ectothermic and endothermic taxa (Hillebrand 2004). Yet, a universally accepted explanation for the latitudinal diversity gradient remains elusive (Mittelbach *et al*. 2007). A combination of historical, physical and ecological mechanisms contributes to this pattern and several hypotheses have been formulated. Tropical rainforests are believed to be among the oldest biomes on Earth (Wallace 1878; Fischer 1960; Ricklefs & Schluter 1993; Futuyma 1998; Wiens & Donoghue 2004) and also the largest, leading to higher population numbers, larger species ranges, and lower chances of extinction (Terborgh 1973; Rosenzweig 1995). Climatic stability, stronger biotic interactions and higher temperatures result in increased evolutionary speed (Rohde 1992; Allen *et al*. 2002), faster speciation (Fischer 1960; Schemske 2002) and reduced risk of extinction (Darwin 1859; Wallace 1878; Fischer 1960).

At a smaller scale, landscape features such as mountain ridges, rivers, elevational changes and land cover contribute to shaping the spatial distribution of organisms. Other well-known patterns of biodiversity are indeed along elevational gradients. Many taxa present a peak in species richness at intermediate elevations with hump-shaped distribution (e.g. Heaney 2001; Lomolino, 2001; Sánchez-Cordero 2001; McCain 2004); nonetheless, also monotonic declines with increasing elevation are frequently observed (Chapter 2; see also Patterson *et al*. 1998; Malkmus *et al*. 2002; Chettri *et al*. 2010). Similarly to the latitudinal gradient, there are several hypotheses that try to explain the observed patterns of biodiversity along the elevation (Heaney 2001; Lomolino 2001). Factors such as productivity, habitat complexity, habitat and resource diversity, environmental stress, or competition (Heaney 2001, McCain 2004), but also climatic, biological, geographical and historical factors have been suggested as causes of variation in species richness along elevational gradients (Chapter 2; see also Rosenzweig 1995, Colwell & Lees 2000; Brown 2001; Sanders *et al.* 2003).

1.3 PATTERNS OF GENETIC DIVERSITY

A primary goal of molecular ecologists is to understand spatial distribution of genetic diversity. The most commonly described spatial patterns are clines, isolation by distance (IBD), genetic boundaries (i.e. barriers) to gene flow, metapopulations, or random patterns (Manel *et al*. 2003). Topographic relief, altitudinal clines and habitat fragmentation are generally shown to negatively influence gene flow and genetic diversity (Storfer *et al*. 2010). For instance, elevational gradients can often result in greater genetic variation and gene flow in low altitude populations compare to high populations (Fig. 1.2, Funk *et al*. 2005). Elevational differences can impose an important barrier to dispersal and gene flow because of (i) the energetic costs of moving up steep slopes; (ii) premating barriers including lower survival of dispersers or lower mating success of dispersers as a result of differences in breeding phenology (Howard & Wallace 1985) or in sexually selected traits such as advertisement calls (Narins & Smith 1986; Lüddecke & Sánchez 2002).

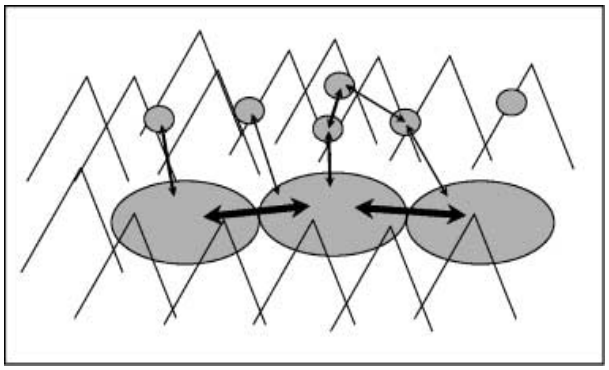


Fig. 1.2 The "valley-mountain" model of population structure proposed by Funk *et al*. (2005) for *Rana luteiventris*. The model consists of three expectations: (i) low elevation populations (large circles) are larger and have higher gene flow (thick arrows); (ii) high elevation populations (small circles) have smaller sizes and little to no gene flow (thinner arrows); and (iii) gene flow is restricted between high and low populations (thin and medium arrows). Source: Fig. 4 in Funk *et al*. (2005).

In chapter 3, I tested whether genetic diversity decrease with elevation and if highland populations are more isolated compared to lowland populations; furthermore, I explored the effects of landscape features and their spatial configuration on genetic variation by means of resistance surfaces and least-cost path analyses (see Adriaensen *et al*. 2003; Spear *et al*. 2010).

1.4 THREATS TO BIODIVERSITY

Although extinctions are a natural part of the evolutionary process, biodiversity is rapidly being depleted at a rate 100 to 1000 times greater than background rate calculated over the eras and, unlike previous mass extinctions, this is mainly due to human activities (Hambler 2013). The International Union for the Conservation of Nature (IUCN) estimates that 22% of known mammals, 32% of amphibians, 14% of birds, 32% of gymnosperms (Vié *et al*. 2009), and 19% of reptiles (Böhm *et al*. 2013) are threatened with extinction. Primary factors contributing to biodiversity loss are habitat destruction and fragmentation (Brooks *et al*. 2002; Fahrig 2003), but also climate change (Williams *et al*. 2007), introduced species (Simberloff 2001; Greenlees *et al*. 2006; Clavero *et al*. 2009) and emerging diseases (Chinchar 2002; Skerratt *et al*. 2007; Frick *et al*. 2010). The underlying mechanisms behind these factors are complex and they may be working synergistically (Kiesecker *et al*. 2001, Blaustein & Kiesecker 2002). Both theoretical and empirical evidences suggest that habitat fragmentation caused by human activities (e.g. settlement, intensive agriculture) have negative effects on population persistence and genetic variability of populations residing in fragmented landscapes (Young & Clarke 2000). Modification, destruction and fragmentation of natural habitats lead animal populations to decrease population size, carrying capacity and migratory rates because of higher isolation. Johansson *et al.* (2005, 2007) showed a general negative trend in the genetic diversity and population size of the common frog (*Rana temporaria*) from low to high agricultural areas, with a reduction of fitness in the latter. However, this pattern was not the rule since the northern region of Sweden showed an opposite trend which can be explained by the latitudinal differences in climate and land uses. It is of extreme importance to consider the spatial configuration of ecological conditions when studying the effects of disturbance on genetic variation and population structure.

1.5 STUDY SYSTEM

1.5.1 Amphibians as model organisms

Amphibians are a unique group of vertebrate containing 7,164 known species (AmphibiaWeb 2013; accessed August 21, 2013), and inhabiting the Earth for over 300 million years. Yet, in the last two decades there have been an alarming number of extinctions and nearly one-third of the world's amphibian species are threatened (Vié *et al*. 2009). Several hypotheses are thought to underlie amphibian declines (Collins & Storfer 2003). Habitat loss, alteration and fragmentation are by far the greatest threats not only to amphibians but to biodiversity of native communities in general (Stuart *et al*. 2008; Vié *et al*. 2009). Besides the general factors contributing to biodiversity loss (see section above), pollution affects around one-fifth (19%) of amphibian species, and these percentages are much higher than those recorded for birds or mammals (Vié *et al*. 2009). The semi-aquatic nature and the permeability of amphibian' skin make them extremely susceptible to chemical contaminants such as pesticides, heavy metals and fertilizers, which can have lethal, sublethal, direct or indirect effects on amphibians (Stuart *et al.* 2008). Emerging infectious diseases are listed among the major threats to global loss of amphibian diversity (Daszak *et al*. 2003; Stuart *et al*. 2008). The two major pathogens are a chytrid fungus, *Batrachochytrium dendrobatidis*, and a group of viruses in the genus *Ranavirus* (Family Iridoviridae) which are responsible for catastrophic population die-offs (Fig. 1.3; Daszak *et al.* 1999; Skerratt *et al*. 2007). Over just the past 30 years, the chytrid fungus has caused decline of at least 200 species of frogs, even in pristine, remote habitats, and for such reason it is responsible for the greatest disease-caused loss of biodiversity in recorded history (Skerratt *et al.* 2007). A detailed section dedicated to *Batrachochytrium dendrobatidis* can be found at pp. 17.

Fig. 1.3 Mountain yellow-legged frogs (*Rana muscosa*) killed by *Batrachochytrium dendrobatidis* in August 2008 at Sixty Lake Basin in the Sierra Nevada mountains, California, USA. Photo © by Vance Vredenburg.

Besides a personal long-standing passion for amphibians, several reasons make them an excellent group for studying the influence of landscape processes on species and genetic diversity. First, amphibians are generally abundant and often the most diverse vertebrate group in many ecosystems, especially in the tropics (Vié *et al*. 2009). Most amphibians have a biphasic life history, with aquatic larvae and terrestrial adults, but a remarkable wide array of different reproductive strategies occurs in this animal group (Duellman & Trueb 1994). Such variety of ecological requirements between life stages within and between species allows researchers to test multiple hypotheses within the same study system. Moreover, amphibians are generally poor dispersers and often highly site philopatric (Rowe *et al*. 2000; Tallmon *et al*. 2000; Funk *et al*. 2005). Low vagility is often attributed to dependence on moist habitats or wetland corridors for dispersal because of desiccation and predation risks associated with terrestrial dispersal (Madison & Farrand 1998). Thus, multiple landscape features related to both aquatic and terrestrial environments are

likely important for explaining patterns of species richness, genetic variation and population structure in amphibians.

Chytridiomycosis

Chytridiomycosis is an emerging infectious disease of amphibians caused by an aquatic fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Daszak *et al*. 1999). *Bd* presence has been documented from nearly sea level on the Caribbean island of Domenica, up to 5,348 m above sea level in the Peruvian Andes (Seimon *et al*. 2006) on all continents where amphibians occur (Fig. 1.4).

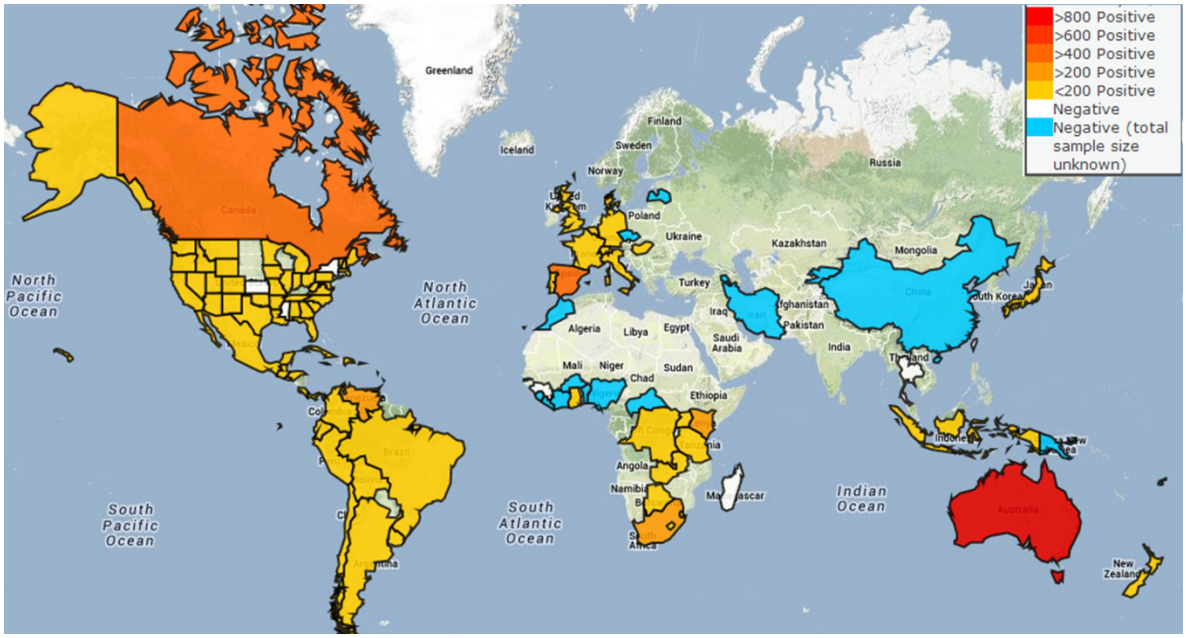


Fig. 1.4 Global distribution of *Batrachochytrium dendrobatidis*. Amphibian tested positive (yellow to red) occur in nearly all continents. Within Africa, positive records have been reported also for Gabon, Cameroon and Nigeria (Doherty-Bone *et al*. 2013; Penner *et al*. 2013). So far, the only tropical areas *Bd*-free are Madagascar and West Africa.

Bd infects the superficial, keratin-containing layers of amphibian skin (Berger *et al*. 1998). In frog tadpoles, only the mouthparts are keratinized and susceptible to *Bd* infection (Berger *et al*. 1998), leading to mouthpart depigmentation (see Fig. 4.2 in chapter 4) and sometimes defects (Rachowicz & Vredenburg 2004). During metamorphosis the skin of the body become increasingly keratinized and the fungal infection is then able to spread over the skin in froglets (and adults) of susceptible species (Marantelli *et al*. 2004; Rachowicz & Vredenburg 2004). In juveniles and adults *Bd* infects and encysts within skin cells particularly on the belly, digits, and pelvic "drink patch" (Berger *et al*. 1998). As infection proceeds the skin becomes much thicker (hyperkeratosis) and sloughs off (Berger *et al*. 1998). Mortality rate and time to death post-exposure depend on a number of factors, and infection intensity appears to be crucial (Vredenburg *et al*. 2010).

Amphibian species are not equally susceptible to the fungus; chytridiomycosis has devastated populations of *Atelopus* species (La Marca *et al*. 2005), whereas others are recurrently reported to be infected but do not show mortality (Longcore *et al*. 2007). For instance, in Africa, species in the genus *Amietia* are found with the highest pathogen load but a lack of mortality (Goldberg *et al*. 2007; Kielgast *et al*. 2010; Conradie *et al*. 2011). We do not know whether *Bd* is a novel pathogen, which spread to new host species and new geographical areas mediated by humans (Berger *et al*. 1999; Daszak *et al*. 1999), or if it is endemic which has become more virulent or to which amphibians have been rendered more sensitive due to environmental changes (Rachowicz *et al*. 2005; Pounds *et al*. 2006). The balance of evidence favors the novel pathogen hypothesis; a putative *Bd* origin has been identified in Africa from where it spread globally via the commercial trade of clawed frogs (*Xenopus* spp.) (Weldon *et al*. 2004; Weldon *et al*. 2007; Soto-Azat *et al*. 2010). Yet, another study found evidence that challenges the "out of Africa" hypothesis (Goka *et al*. 2009).

Given the global threat imposed by *Bd* to amphibian diversity and the lack of records from the area under investigation, I tested two highly

susceptible *Amietia* species for the presence of the pathogen (chapter 4) and discuss possible implications of a positive detection.

1.5.2 The Eastern Afromontane Hotspot of Biodiversity

The Eastern Afromontane Hotspot of biodiversity (Fig. 1.5) encompasses several widely scattered, but biogeographically similar mountain ranges in eastern Africa, from Saudi Arabia and Yemen in the north to Zimbabwe in the south (Mittermeier *et al*., 2004). The main part of the hotspot is made up of three ancient massifs: the Ethiopian Highlands, the Albertine Rift, and the Eastern Arc Mountains along with the Southern Rift. In addition, a number of outlying mountains are part of this hotspot, including the Asir Mountains of southwest Saudi Arabia, the highlands of Yemen, the neogene volcanoes of the Kenyan and Tanzanian Highlands (e.g. Mt. Kenya, Mt. Elgon, Mt. Kilimanjaro, Mt. Meru, and other peaks), and the Chimanimani Highlands of eastern Zimbabwe. The Eastern Afromontane hotspot holds nearly 7.600 species of plants, about 1.300 bird species, nearly 500 mammal species, more than 100 of which are endemic, nearly 350 reptile species and about 230 amphibian species (Mittermeier *et al*., 2004).

Fig. 1.5 The Eastern Afromontane Hotspot of Biodiversity in East Africa.

Eastern African amphibians

The first monograph reporting the results of intensive field surveys for amphibian was published by Barbour & Loveridge (1928). Loveridge (1937) later extended this work providing basic taxonomic and zoogeographic considerations for Eastern African amphibians and from Tanzania in particular. On the basis of the herpetofauna, he divided East Africa into a number of what he termed 'ecological life zones', and made the important observation that 83% of the anurans listed as occurring below an altitude of ca. 300 m were widely distributed, whereas only 48% in the highest zone between 1500 and 3650 m were widespread (Loveridge 1937; Poynton 2007). Loveridge also noted a zonation of climate from the torrid lowlands to the cool highlands which could explain the high amphibian diversity. Associated with this was an altitudinal turnover in species composition, which Poynton (1962) suggested conformed to a tropical—transitional—temperate zonation that is evident over the whole of southern and eastern Africa.

Only recently a conspicuous number of herpetological surveys have been conducted in East Africa, especially in the Eastern Arc and adjoining coastal lowlands (see for instance the special issue of Fieldiana Life and Earth Sciences vol. 4, 2011); despite that, several forests, in particular on the volcanic mountains, have been poorly studied (Howell 1993).

1.5.3 Mount Kilimanjaro

Included in the Eastern Afromontane Hotspot of Biodiversity is Mount Kilimanjaro, a large stratovolcano located 300 km south of the equator (Fig. 1.5) and a UNESCO world heritage site. With its huge altitudinal range, from ca. 700 m to 5895 m, Mt. Kilimanjaro represents the highest peak in Africa and the highest solitary mountain in the world. Several completely different bioclimatic zones encompass the mountainsides (Fig. 1.6): a hot and dry colline zone surrounds the mountain base between 700 and 1000 m, a submontane belt characterized by banana and coffee plantations (so called "homegardens")

between 1000 and 1800 m, montane tropical rain forests in the lower part and cloud forests at higher altitudes between 1800 and 3100 m, subalpine heathlands (*Erica* bushes) up to 3900 m, alpine cushion vegetation up to 4500 m, followed by the upper alpine and nival zone. The topo of Kibo, the main summit, is covered with glaciers.

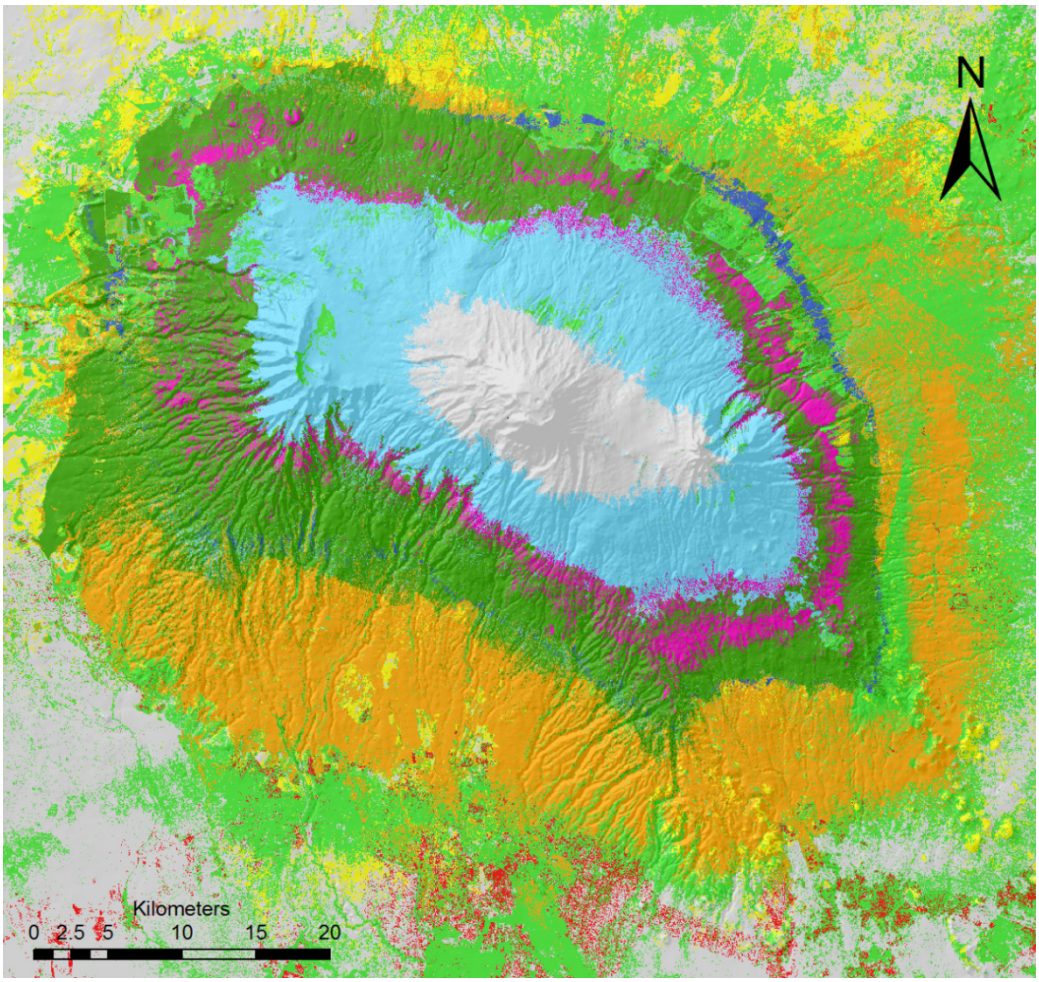


Fig. 1.6 Mount Kilimanjaro, Tanzania. The colline zone is composed by a mosaic of cropland (light green), human settlements (red), natural savanna and grassland (yellow); the submontane zone is dominated by homegardens (orange); the montane belt is characterized by rainforest (green), disturbed forest (pink) and reforestation patches (blue); above is the subalpine zone dominated by *Erica* bushes (light blue). In grey is unclassified land cover. Original land cover surface was produced by T. Appelhans & T. Nauss; background hillshade surface was derived from a DEM produced by J.A. Ong'injo, C. Lambrechts & A. Hemp.

Due to a drier climate, and thereby increased fire frequency, ca. 150 km² (representing 10% of all the forest cover on Mt. Kilimanjaro) of cloud forest were lost during the last decades (Hemp & Beck 2001). In addition, illegal logging and grazing of cattle have altered forest composition below 2500 m elevation (Lambrechts *et al*. 2002). At the foothills, over 40% of savanna grassland and dry forests were converted into cultivated fields and settlements. The parallel occurrence of natural, disturbed and extensively or intensively managed habitat types within a wide altitudinal range provides unique research opportunities to obtain a better understanding of the spatial distribution and influence of elevation and anthropogenic disturbance on biodiversity.

1.6 THE FOCUS OF MY WORK

The very aim of my doctoral thesis was to understand how and to which extent the landscape influences amphibian diversity. I embraced the hierarchical concept of biodiversity by analyzing patterns of (i) anuran assemblage composition across East African mountains (chapter 2), (ii) species richness and distribution along elevational and disturbance gradients on Mt. Kilimanjaro (chapter 2), (iii) genetic diversity and population connectivity (chapter 3). Finally, because of the great threat to amphibian diversity imposed by the fungus *Batrachochytrium dendrobatidis*, I tested some specimen for detection of this pathogen (chapter 4).

Chapter 2

Mount Kilimanjaro is an exceptional tropical region where there is a broad lack of knowledge of its amphibian fauna and where the landscape is rapidly evolving due to land use changes. Here, I address the following main questions:

1. Does species richness decrease with elevation?
2. How are species distributed along the elevation?

3. Does anthropogenic disturbance affect species richness and composition?

In order to understand the observed patterns of species diversity and distribution, I incorporated inferences from historical biogeography and compared the assemblage of Mt. Kilimanjaro with those in the Eastern Arc Mountains and Mt. Meru. Based on species distributions, in particular of forest-dwelling frogs, across those mountains and the complex geological and climatic history of East Africa, I inferred the origins of the amphibian fauna of Mt. Kilimanjaro and the causes underlying the biogeographic pattern.

Chapter 3

Here I investigated how the landscape, i.e. elevation, topographic relief and land cover, influence genetic diversity, population structure and gene flow. I selected two ecologically similar and closely related river frog species, namely *Amietia angolensis* (Fig. 1.7) and *Amietia wittei* (see Fig. 4.2), to answer three main questions:

1. Does genetic diversity decrease with elevation?
2. Is gene flow restricted among high elevation populations (*Amietia wittei*) compared to lowland populations (*Amietia angolensis*) (Fig. 1.2)?
3. How does topography, especially the valley-ridge system along the slope of Mt. Kilimanjaro, and land-use changes affect population structure, genetic variation and connectivity?

I addressed these questions using a "landscape genetics" approach, i.e. by incorporating robust, spatially informed data with population genetic data (Storfer *et al*. 2007; Holderegger & Wagner 2008) and I showed the importance of including the spatial configuration of landscape features when studying patterns of genetic variation and inferring barriers to gene flow.

Chapter 4

Among African amphibians, river frogs of the genus *Amietia* seem to be particularly susceptible to chytridiomycosis, showing the highest zoospore loads in Kenya (Kielgast *et al*. 2010), Malawi (Conradie *et al*. 2011), South Africa and Lesotho (Weldon 2005). Positive *Bd* infections also have been reported in Uganda (Viertel *et al*. 2012), yet records from the Udzungwa Mountains in Tanzania have resulted negative (Moyer & Weldon 2006). Here I reported *Bd* presence in specimen of *Amietia angolensis* and *Amietia wittei* from Mt. Kilimanjaro, and contributed to the knowledge of *Bd* distribution in East Africa. I also discussed possible implications of this finding, not directly as a threat for the river frog themselves, but rather for their potential as reservoir and the means by which the fungus can be spread to other forests, e.g. intense tourism.

Fig. 1.7 *Amietia angolensis* juvenile (upper corner) and larvae (below). On the right, a typical site at ca. 1750 m near the forest edge and the National park border. Tadpoles can be found in proximity of the river bank or in stream pool armoured by rocks and gravel.

Chapter 2

Amphibian diversity on the roof of Africa: effects of habitat degradation, altitude and biogeography[1]

Table of Contents

[1] This chapter is under review as: Zancolli G., Steffan-Dewenter I., Rödel M.O. "Amphibian diversity on the roof of Africa: effects of habitat degradation, altitude and biogeography". *Diversity and Distributions.*

2.1 ABSTRACT

Elevational gradients and land use changes can act together as drivers of species richness and composition. Responses to these drivers can be multidirectional, and resulting patterns of diversity might be unexpected or difficult to interpret. Present ecological relationships often reflect a legacy resulting from the influence of past geological, climatic and environmental changes on species dispersal, extinction, and speciation. Here, we investigate amphibian diversity along altitudinal and disturbance gradients on Mount Kilimanjaro, Tanzania, and evaluate how historical events may underlie contemporary distributional patterns. During two rainy seasons in 2011, we recorded anurans at 36 sites from the foothills to 3500 m altitude. A combination of multiple regression, ordination technique and cluster analysis were used to determine the effects of altitudinal changes and habitat modification on species richness and composition. Furthermore, we compared the anuran fauna of Mt. Kilimanjaro with other East African mountains by means of cluster analysis and analyzed patterns of distributions with a special focus on montane forest species. Species richness declined with elevation and species assemblages were distinctly separated between lowland and highland altitudes. Presence of water bodies locally increased species richness, but we did not find significant correlations of species richness with other environmental variables. Mountains clustered into two major groups which reflected latitudinal position and differences in species distributions. This study highlights the importance of considering the geological history of a place especially when assessing the effects of anthropogenic disturbance. The young age of the volcano and the complex biogeographic processes which occurred in East Africa during the last 20 million years prevented montane forest frogs from colonizing Mt. Kilimanjaro. Increasing aridification and land use changes may cause contraction of breeding sites with consequent local extinctions in the near future.

Keywords: Assemblage composition, elevation, East Africa, land use, landscape history, species richness.

2.2 INTRODUCTION

Understanding spatial and temporal distribution patterns of biodiversity is a core objective for ecologists and biogeographers (Gaston, 2000). Montane regions in general and particularly in the wet tropics are hotspots of species richness for many taxa (Myers *et al.*, 2000), with two thirds of all terrestrial species occurring in tropical rainforests (Terborgh, 1992). However, tropical and montane biotas are expected to experience the highest biodiversity losses in the near future (Ricketts *et al*., 2005). In the face of rapid climate and land use changes, understanding patterns of species richness is thus crucial for long-term conservation of biodiversity (Zhang *et al.*, 2012).

Several studies have documented patterns of species richness along elevational gradients for a diverse array of taxa, such as rodents and bats (Sánchez-Cordero, 2001), lizards (Fischer & Lindenmayer, 2005), fishes (Jaramillo-Villa *et al.*, 2010), as well as frogs (Fu *et al.*, 2006). The most common pattern is the hump-shaped distribution, with a peak in richness at intermediate elevations (Brown, 2001; Lomolino, 2001), but also a monotonic decline with increasing elevation has been observed (Patterson *et al.*, 1996). Some studies are the results of decades of systematic surveys and literature data (Heaney, 2001; Rickart, 2001), while others employ direct surveys focusing on pristine habitats (Chettri *et al.*, 2010; Garcia-López *et al.*, 2012). Very few studies analyzed patterns of species richness and composition along elevational and disturbance gradients in parallel (e.g. Thiollay, 1996; Malonza & Veith, 2012).

Amphibians, in particular, are facing global declines and consequent losses in species richness, largely due to habitat destruction, alteration and fragmentation (Stuart *et al.*, 2008). However, the effects of human-induced environmental changes on amphibian diversity are not consistent; some studies reported a decrease of species richness (Pineda *et al.*, 2005) and functional diversity (Ernst *et al.*, 2006), while others revealed no changes (Pearman, 1997), or even an increase of frog diversity in altered habitats (Heang *et al.*, 1996). Amphibian responses to habitat degradation may also be

species-specific (Ernst & Rödel, 2005, 2008) or habitat-dependent (Ofori-Boateng *et al.*, 2013).

While most studies evaluating the consequences of habitat change on amphibians are confined to the New World, few have been conducted on the African continent (Gardner *et al*. 2007). Mount Kilimanjaro is not only the highest mountain in Africa, but also the highest solitary mountain in the world. It has been designated as UNESCO world heritage site and included in the Eastern Afromontane Hotspot of Biodiversity (Mittermeier *et al*., 2004). In the last decades an impressive effort has been made to unveil the herpetofaunal richness of East Africa, especially in the Eastern Arc Mountains of Tanzania (see e.g. special issue on mammalian and herpetological diversity of Tanzanian mountains in Fieldiana Life and Earth Sciences vol. 4, 2011). Still, the diversity of several mountains is unknown, including Mt. Kilimanjaro, despite its general popularity (Howell, 1993).

Humans have continuously inhabited the slopes of Mt. Kilimanjaro for the last 2000 years (Schmidt, 1989), but during the last decades the population multiplied 20 times (Hemp, 2006a). Below the National Park boundaries at ca. 1700 m, the submontane rainforest has been extensively cleared and only few forest remnants remain and are confined to deep and barely accessible gorges. With its huge elevational range (from 700 to 5895 m) and different degrees of habitat conversion, Mt. Kilimanjaro offers a unique opportunity to assess how anthropogenic disturbance and changes in elevation affect the distributions of frog species.

Recent surveys conducted on isolated rainforests of some Tanzanian mountains have revealed an extraordinary diversity of frogs, with many species restricted to few or even one mountain block (e.g., de Sá *et al.*, 2004; Loader *et al.*, 2010). These findings are consistent with studies suggesting that isolation and reduced dispersal can result in greater differentiation and higher endemism with increasing elevation (Brown, 2001). Consequently, we might expect similar findings on Mt. Kilimanjaro, such as the discovery of endemics and/or high diversity in correspondence to the mid elevation forest. However, the "history of place", that is the sequence of geological, climatic and other historical

environmental changes, has influenced past dispersal, extinction, anagenetic differentiation and speciation processes (Brown, 2001). The Eastern Arc is a chain of ancient crystalline mountains initiated 290-180 Mya, whereas Mt. Kilimanjaro and Mt. Meru are recent volcanoes estimated to be ca. one Myr (Griffiths, 1993). Climatic changes during the Quaternary, or even before, caused withdrawal of cool adapted species which, influenced also by topographical features, contracted their ranges to isolated relict patches. This isolation has created both paleoendemics and neoendemics (Wasser & Lovett, 1993). After the last major eruption between 200 000 and 150 000 years ago (Nonnotte *et al.*, 2008), Mt. Kilimanjaro finally cooled and was eventually colonized by plants and animals. Considering the young age of Mt. Kilimanjaro, we might otherwise expect a low diversity of forest specialists and hence a decline in species richness with increasing elevation.

In this study, we describe patterns of amphibian species distribution along an elevational gradient on Mt. Kilimanjaro, and investigate the effects of anthropogenic disturbance and habitat parameters on species richness and composition. Finally, we consider the relative influence of geological and climatic events in light of how current anthropogenic change contributed to the present distributions of amphibians on Mt. Kilimanjaro and compared them among others in the East African mountain chain.

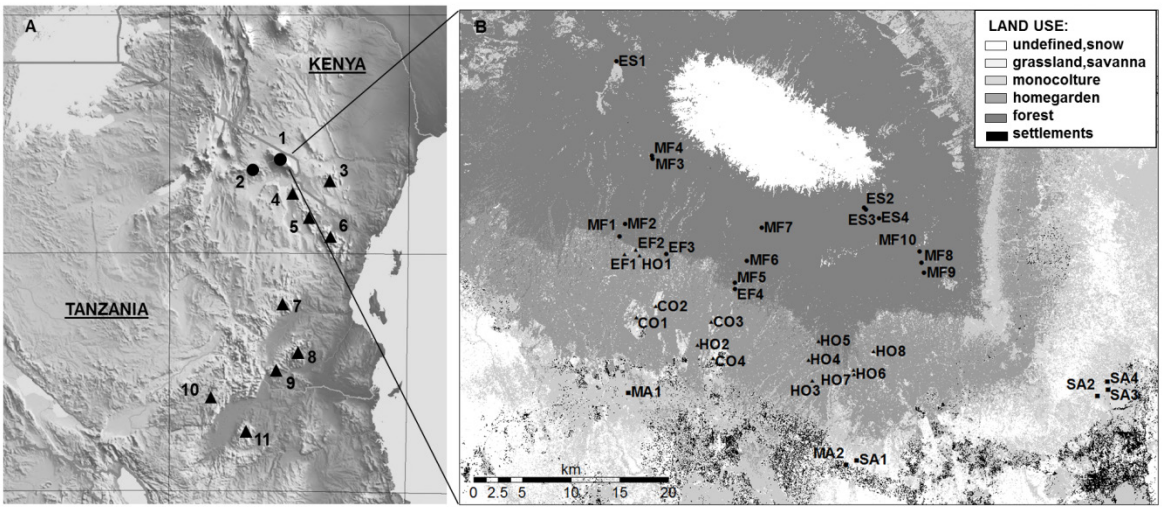


Fig. 2.1 Map of Eastern Africa showing (A) the Eastern Arc Mountains (triangle: 3 = Taita Hills; 4 = North Pare; 5 = South Pare; 6 = West Usambara; 7 = Nguru; 8 = Uluguru; 9 = Maludwe; 10 = Udzungwa; 11 = Mahenge), the volcanoes (circle: 1 = Kilimanjaro; 2 = Meru); and (B) the study sites along the slope of Kilimanjaro (see Table S2.2 for information).

2.3 METHODS

2.3.1 Study area

Mount Kilimanjaro is the remnant of a large Tanzanian volcano, located 300 km south of the Equator on the border with Kenya (2°45' to 3°25'S; 37°00' to 37°43'E). It rises from the savanna plains at 700 m to an iced-clad summit of 5895 m. Because of the wide altitudinal range, it encompasses several climatic zones and vegetation belts: a hot and dry colline zone surrounds the mountain base between 700 and 1000 m. Here, most of the savanna has been converted to monocultures (mainly maize, sunflowers and beans). Between 1100 and 1700 m the submontane zone is dominated by a traditional agroforestry system called "Chagga homegarden". It is a multilayer ecosystem composed by a high tree layer which gives shade to the banana trees and other crop plants beneath (see Hemp, 2006a for detailed description). Above 1700 m, the remainder of the mountain is protected by a National Park. However, illegal logging and grazing are threatening the natural ecosystems and have altered the forest composition below 2500 m (Lambrechts *et al.*, 2002). Between 1700

and 3200 m the montane forest is characterized by the genus *Ocotea* (Lauraceae) in the lower part and by *Podocarpus* (Podocarpaceae) in the cloud zone. Above 3200 m *Erica* bushes (Ericaceae) prevail in the subalpine zone up to 3900 m. The alpine zone is very poor in vegetation and the highest elevations of Kibo peak are covered by glaciers. Mean annual temperature decreases linearly upslope with a lapse rate of 0.56°C per 100 m starting with 23.4°C in Moshi (813 m.; Walter *et al.*, 1975) and decreasing to -7.1°C at the top of Kibo (5794 m; Thompson *et al.*, 2002). Along the southern slope, annual precipitation increases from the foothill (800 – 900 mm) upwards reaching the maximum at ca. 2200 m (3000 mm) and declining towards higher elevations (4000 m; 600 mm); in contrast, the northern slope receives much less annual rainfall (Hemp, 2006b). Therefore, we concentrated on the wettest side of the mountain which presumably offers more suitable habitats for amphibians.

2.3.3 Study design and data collection

Field work was carried out from March to June (long rainy season) and October to November (short rains) in 2011. The 2011 wet season was abnormal in that there were few rains during April. Some sites, which are known to be usually flooded (GZ, unpublished data), were dry and we found mummified tadpoles. We established 36 plots (Fig. 2.1) covering an altitudinal range from 800 m to 3500 m along the southern slope of the mountain. Further surveys covered areas up to 4000 m, however, above 3500 m we did not find any amphibians. During diurnal and nocturnal random walks, we used a combination of visual (VES) and acoustic (AES) encounter surveys to search for frogs in all microhabitats (Rödel & Ernst, 2004). All visits were randomly distributed during the sampling periods and all sites have been visited at least three times. Because of the difficulty in encountering animals, we adopted further sampling techniques, including installing drift-fences and pitfall-traps, and checking water bodies in proximity of the sampling sites by dip-netting. These opportunistic techniques were intended to compile a more comprehensive species list of the study area. Captured animals were identified, sexed,

measured and released, with the exception that the first individual of each species and specimens difficult to identify in the field were euthanized in a chlorobutanol solution and preserved in 75% ethanol. Vouchers are deposited at the Museum für Naturkunde, Berlin (ZMB; see Appendix S2.2). For species identification we used Channing & Howell (2006) and Harper *et al.* (2010); herein, we followed the taxonomy by Frost (2013).

We characterized all sampling sites with the following environmental data: altitude (recorded with a Garmin® GPSMAP 62S), presence of potential lentic or lotic breeding sites, vegetation structure and regime of disturbance. The vegetation was determined at three strata (canopy cover, shrubs and understory) in four density levels: open (4), predominately open (3), predominantly closed (2) and closed (1). We defined the intensity of anthropogenic disturbance as follows: highly disturbed (monoculture; 1), moderately disturbed (homegarden; 2), semi-natural (forest-edge; 3) and natural (savanna and forest; 4). Vegetation density and anthropogenic disturbance were treated as ordinal variables.

2.3.3 Data analysis

We assessed inventory completeness by using a suite of incidence-based estimators, namely ICE, Chao 2, Bootstrap and Jacknife 1 with the program EstimateS 8.20 (Colwell, 2005). Because of the low number of observations and the differences in sampling effort among sites, we used presence-absence data and calculated standardized species richness with rarefaction analysis. We thus used the number of species calculated for each site at the third visit after 1000 permutations without replacement. Data from opportunistic searches were excluded as these were not standardized and did not add any additional record.

To identify assemblage similarities and potential species turnovers along the elevation, we implemented agglomerative hierarchical clustering based on Bray-Curtis (Sørensen) dissimilarity index for binary data. We tested all the

environmental variables for correlation with species richness with backward stepwise multiple regressions. We ran two models: in the first we included all variables, whereas in the second we excluded elevation to eliminate its effect in revealing patterns of species richness along the disturbance gradient.

Patterns of assemblage composition were investigated with Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis (Sørensen) dissimilarity index. In order to determine which habitat parameters contribute most to the observed configuration, we fitted regression models with the ordination scores for each axes as dependent variables and the environmental variables as predictors (Ludwig & Reynolds, 1988). Another way to indirectly analyze gradients is to fit a smooth surface that models how the environmental variable changes over the ordination graph (*ordisurf* command, vegan R package). This method allows the graphical detection of changes in species composition related to changes of the environmental variable under investigation. We subsequently fitted the significant variables from the regression model to the ordination plot.

Finally, we analyzed species richness and distribution of anurans on the two volcanoes (Mt. Kilimanjaro and Mt. Meru) and the Eastern Arc Mountains. We selected nine mountain blocks, namely Taita Hills, North and South Pare, West Usambara, Nguru, Uluguru, Malundwe, Udzungwa Southern Scarp and Mahenge, covering almost the entire North-South gradient of the Eastern Arc chain (Fig. 2.1; references in Appendix S2.1 in Supporting Information). We compiled a presence-absence matrix in which we excluded taxa lacking a scientific name (e.g. *Nectophrynoides* sp. nov. or sp.) and we built a dendrogram based on shared species. We then grouped species into three geographical sets: lowland = species mainly distributed along the coast but with westerly terminations; inland = species that do not occur at the sea level but are widespread in the Tanzanian Highlands and inner parts of the African continent; and montane = species that occur only in mountainous area, hence with patchy, discontinuous distributions. Because of the restricted distribution of the montane species and their almost exclusive occurrence in moist montane forests, we hypothesize that the "montane" category will contain more

vulnerable and specialized taxa, whereas lowland and widespread species are more likely to tolerate habitat disturbance. Based on the relative frequencies of these categories and the similarity among mountains, we inferred whether the amphibian assemblage on Mt. Kilimanjaro is influenced by current degradation, environment correlates such as elevation, or the "history of place".

Analyses were run with the statistical package STATISTICA 10 (StatSoft, 2011) and the software R (R Development Core Team, 2012).

2.4 RESULTS

2.4.1 Amphibian diversity

We recorded a total of 21 anuran species belonging to ten families (Appendix S2.2). Mean species number per site was 2.14 ± 2.39 (range 0-12). Despite multiple visits, we failed to encounter any amphibians at six sites. In 20 sites (55%) we found only one or two species, whereas in one site we recorded 12 species (> 50% of total observed species richness). The ICE and Jacknife 1 estimators gave the lowest estimates of completeness ranging from 50 to 100%, whereas Chao 2 and Bootstrap estimated a completeness of 73-100% for each site. For the entire study region the completeness was about 80% (Table S2.1), indicating that our survey should not be considered exhaustive as the discovery of additional species through further surveys is likely in different years and/or different seasons.

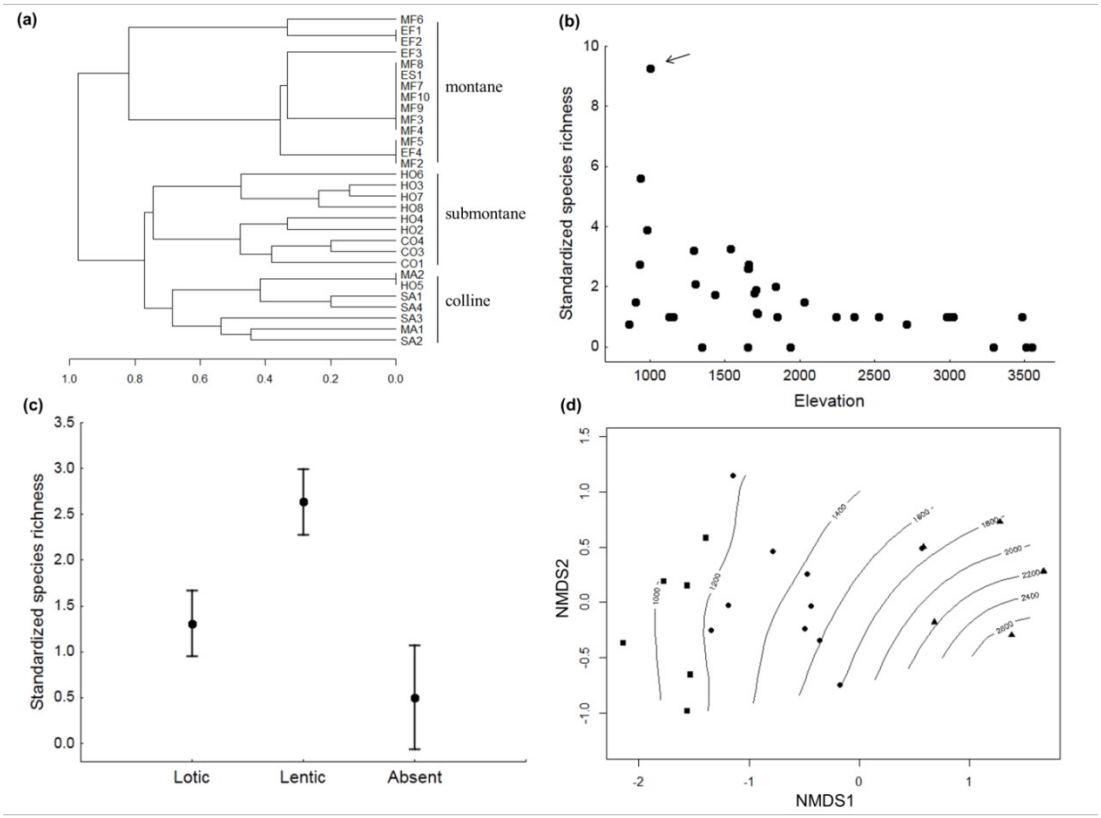


Fig. 2.2 Species richness and composition in relation to the significant environmental variables. (a) Dendrogram based on Sørensen dissimilarity index showing the altitudinal zonation of species assemblages; (b) regression of standardized species richness against elevation (the outlier is pointed by an arrow); (c) standardized species richness of sites grouped by presence of lotic, lentic or absence of water bodies; (d) non-metric multidimensional scaling plot based on the composition of frog species in colline (square), submontane (circle) and montane (triangle) sites. Arrangement of the sites along the altitudinal gradient is highlighted by the fitted surface obtained with the command *ordisurf* in R.

2.4.2 Species distribution along elevation

Cluster analysis highlighted the differences in species composition along an elevational gradient, with clearly distinct assemblages between lowland and high elevation. For instance, the montane sites constituted a well separated unit (Fig. 2.2a). The further subdivision of this cluster was due to the presence of *Strongylopus fuelleborni* Nieden, 1911 in four sites (MF6, MF5, MF2 and EF4). However, only one other species occurs in the montane forest, thus the subdivision did not reflect real differences in assemblage composition but merely presence of both or only one of the two species. The lowland cluster split

into two subgroups which almost perfectly fitted the corresponding altitudinal zones (colline *versus* submontane), except for HO5 which clustered with the colline group. Thus, we identified two species turnovers: the first at ca. 1100 m (colline - submontane transition) and the second at ca. 1700 m (submontane - montane transition).

Table 2.1 Results from the General Regression Models with backward stepwise selection. The Beta coefficients for the explanatory variables (regression coefficients obtained if all the variables are standardized to a mean of zero and a standard deviation of one, so that the relative contribution of each independent variable is comparable), the F-tests and the R^2 for the whole models are reported. The response variable of Mod.1 (a = all samples, B = outlier excluded) and Mod.2 (elevation excluded) is species richness, whereas in NMD1 and NMDS2 the dependent variables are the ordination scores on the first and second axes respectively.

	Mod.1a		Mod.1b		Mod.2		NMDS1		NMDS2	
Elevation	-0.553	***	-0.648	***	-		0.555	***	-0.153	
Water lotic	0.207		0.314	*	-0.009		0.287	**	-0.345	
Water lentic	0.353	*	0.367	**	0.494	**	-0.087		-0.456	*
Disturbance	0.125		-0.115		0.222		0.011		-0.045	
Canopy	0.085		-0.018		-0.049		-0.195		-0.249	
Shrubs	0.186		-0.027		0.279		-0.247	*	0.008	
Understory	0.118		-0.262		0.319		0.220		0.146	
F-test	8.53		13.10		5.12		21.89		5.14	
R^2	0.44	***	0.56	***	0.24	*	0.77	***	0.27	*

*** $p<0.001$, ** $p<0.01$, * $p<0.05$

2.4.3 Influence of environmental variables on species richness and composition

Multiple regressions of environmental variables against standardized species richness revealed a strong effect of elevation and presence of water (Table 2.1; Fig. 2.2b and 2.2c). The outlier in Fig. 2.2b is a maize field with the highest species richness observed. If this site was excluded, the R^2 of the whole model increased from 0.44 to 0.56. When elevation was excluded among the predictor variables, the model explained just 24% of the total variance and only

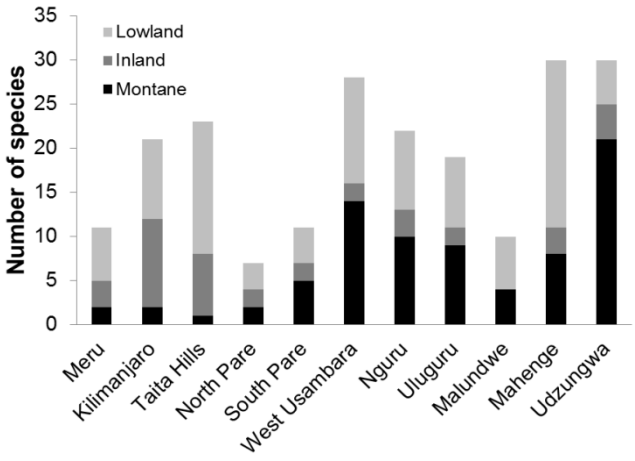


Fig. 2.3 Number of anuran species on each mountain block and proportion of biogeographic categories. Mountains are ordered following the latitudinal gradient from northwest to south and accordingly to Figure 2 in Burgess *et al.* (2007) for comparison purpose.

presence of open water bodies remained significant (Table 2.1). The effects of vegetation structure and anthropogenic disturbance were not significant in either model.

Elevation, presence of water and shrub density were significant predictors for site ordination on the first NMDS axis, whereas ordination on NMDS2 was only correlated with presence of water (Table 2.1). When fitting the elevation as a smooth surface on the ordination plot, it was evident that the spatial arrangement of sites followed the elevational gradient (Fig. 2.2d).

2.4.4 Biogeography of East African anurans

A total of 93 species, excluding species with taxonomic uncertainty, were considered in our final presence-absence matrix (Appendix S2.1). The average species richness per mountain block was 19.3 ± 8.4, with the lowest in the North Pare (7 species) and the highest in the Udzungwa southern scarp and Mahenge (30 species; Fig. 2.3). Overall, anuran assemblages were predominantly composed by lowland and montane species whereas the inland category represented only about 20% ($F = 8.21$, $p < 0.01$; Fig. S2.1). The dendrogram from the cluster analysis depicted two major groups which reflected the latitudinal position of the mountains (Fig. 2.4).

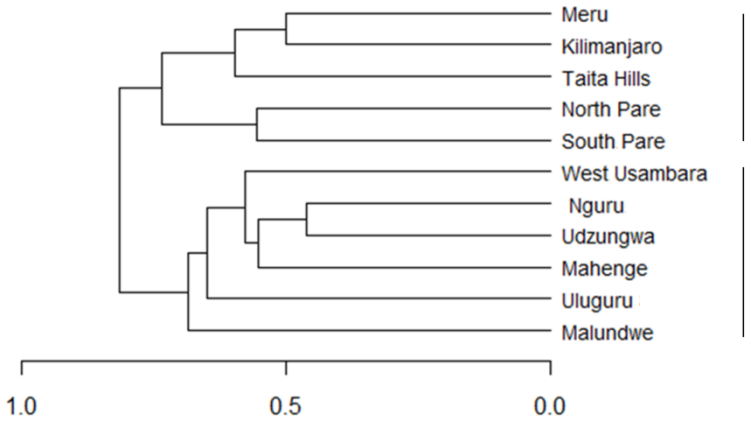


Fig. 2.4 Dendrogram based on Sørensen dissimilarity index showing the similarity across East African mountains and the major clustering into Northern and Southern groups.

The "northern group" was further divided into two clusters: the two volcanoes together with the Taita Hills (the northernmost block of the Eastern Arc) and the Pare Mountains. In the "southern group" the latitudinal gradient was less evident and no major subdivision emerged.

The difference between the two major clusters was supported by pairwise Student's t-tests between group means (relative frequencies and total number of species) of the biogeographic sets (Table S2.2). The relative frequency of inland species was higher in the northern fauna than in the southern group (Fig. 2.5); conversely, the montane species represented only 21% of the northern fauna against 47% in the southern group. The lowland species constituted ca. 50% of the assemblages in both groups (Fig. 2.5). Also in terms of total species richness there was a marked difference between the two groups: the number of montane species was almost four times higher in the south than in the north (9 *vs*. 34 species), the inland species were twice as high in the north than in the south (14 *vs*. 7), whereas the lowlands had about the same number of species across the two groups (21 *vs*. 27).

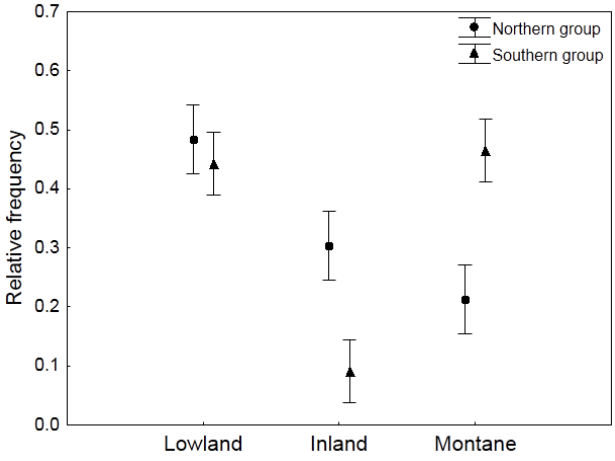


Fig. 2.5 Relative frequencies of lowland, inland and montane species for the Northern and Southern groups (see Fig. 2.4).

2.5 DISCUSSION

2.5.1 Altitudinal distribution of amphibian diversity

In mountainous areas, elevation is certainly a primary factor influencing species diversity and distributions. On the southern slope of Mount Kilimanjaro, anuran species richness decreases with increasing elevation. Though we assume that somewhat further surveys, especially along the northern slope, would have increased total species richness, we do not believe that this would have greatly altered the results of this study, other than possibly tightening the negative correlation of anuran richness with elevation. A similar trend has also been observed on other East African mountains (Ngalason & Mkonyi, 2011; Malonza & Veith, 2012), as well as on isolated mountains in other tropical regions (e.g. Mount Kinabalu in Southeast Asia; Malkmus *et al.*, 2002).

Species composition along the southern slope of Mt. Kilimanjaro can be clearly divided into lowland and highland assemblages. The presence of taxonomic turnovers with distinct lowland and highland anuran fauna in East Africa is well-known (Loveridge, 1937; Poynton, 1962; Poynton & Boycott, 1996; Poynton, 2003). The altitudinal range at which the turnover occurs is not uniform among areas: on the Udzungwa Scarp species composition shifts at ca. 1000 m (Menegon *et al.*, 2011), in the Mahenge below 1000 m (Menegon & Salvidio, 2005), in Uluguru at ca. 1500 m (Ngalason & Mkonyi, 2011) and on Mt. Kilimanjaro at ca. 1700 m (this study). On the Usambara and Uluguru Mts. frost has been recorded at elevations as low as 1500 m, whereas on Mt. Kilimanjaro, due to the "Massenerhebungseffekt" (mass elevation effect; Brockmann-Jerosch, 1919), frost occurs from 2700 m upwards (Hemp, 2006b). Consequently, in the Eastern Arc Mts. the same vegetation occurs at lower elevation when compared to Mt. Kilimanjaro, and thus the upland fauna may reach lower elevations in forested areas, as elsewhere in eastern Africa (Poynton 1999).

Species assemblages on Mt. Kilimanjaro clustered in conformity with the colline, submontane and montane zones, and such subdivision seems to agree with the tropical – transitional – temperate zonation suggested by Poynton

(1962, 2003) over southern and eastern Africa. The colline fauna of Mt. Kilimanjaro is dominated by savanna specialists adapted to hot and dry conditions. This faunal set does not occur above 1100 m where only species that can tolerate cooler temperatures occur. Finally, the firm turnover from lowland to montane fauna is at ca. 1700 m where the National Park boundaries delimit the montane forest. Here, the Afrotemperate fauna (sensu Poynton, 2000) is represented by two species, namely *Amietia wittei* (Angel, 1924) and *Strongylopus fuelleborni*, which replace the tropical fauna.

2.5.2 Effects of land use changes on amphibian richness and composition

The long-term presence of humans on the fertile slope of Mt. Kilimanjaro has greatly modified the habitat, for instance by reducing the natural vegetation and creating new water sources (e.g. water canals and dams). On one hand, these habitat alterations might have negatively affected forest-dependent species but, on the other, environmental heterogeneity and new potential breeding sites might have attracted tolerant species able to reproduce in a wide array of water bodies. Thus, responses to habitat alteration can be more complicated than a simple decline of diversity with increasing of disturbance (see Vera y Conde & Rocha, 2006). In our study, amphibian richness and composition were significantly correlated with elevation and presence of lentic waters. This result is not surprising since most of the recorded species rely on ephemeral ponds for reproduction (see Appendix S2.2). Except for a feebly significant correlation of shrub density with the NMDS1 scores (very likely because of the collinearity with elevation), neither the vegetation structure nor the anthropogenic disturbance were significantly correlated with both species richness and composition. A possible explanation for a lack of disturbance signal may be the limitation of our survey, as we have likely missed some species. Moreover, the absence of pristine forest sites within the submontane zone likely biased the analysis and did not allow for a direct comparison of natural *vs.* disturbed sites in the same altitudinal range. Nevertheless, all species

recorded in this study are considered generalist and somewhat tolerant to habitat degradation (i.e. to changes in vegetation structure). Noteworthy is the site in a bare maize field where we recorded an astonishing high diversity of frogs. Apparently some scattered trees, bushes and rocks as well as few ponds offer enough habitats to sustain 75% of the colline anuran fauna found therein. Even so, the long-term survival of frogs living in altered ecosystems is not certain and may be further threatened by an increasing level of land use.

2.5.3 Biogeography of Eastern African amphibians

Eastern and western African tropical rainforests were once more or less continuous; as climatic conditions changed, the eastern forests became fragmented and finally isolated on scattered mountains surrounded by dry and hot savannas (Lovett, 1993). Thus, the biological histories of these forest islands are linked to each other so that species diversity and composition of one mountain can be better understood when related to the assemblages of other montane forests. In this study, we analyzed assemblage similarities between Mt. Kilimanjaro, the nearby Mt. Meru and the Eastern Arc, and considered the geographical and evolutionary processes underlying the current pattern of amphibian diversity. Based on shared species, Mt. Meru was the most similar to Mt. Kilimanjaro; the two volcanoes clustered together with Taita Hills, North and South Pare Mountains into a "northern group". The other mountains of the Eastern Arc clustered in a separate "southern group" (Fig. 2.4). In addition to similarities based on shared species assemblages, we also investigated relationships based on the geographical ranges of species. We found marked differences among mountains and especially between the southern and northern groups (Fig. 2.5). The northern mountains were characterized by a lowland, widespread fauna with few species restricted to the montane forests; by contrast, the central and southern blocks of the Eastern Arc were dominated by a forest-dependent fauna. Figure 2.3 emphasizes the progressive increase of montane forest species from the northern Tanzanian volcanoes towards the central and southern fragments of the Eastern Arc Mts. Other taxonomic groups

(vertebrates and trees) exhibit a similar trend: if we consider Figure 2 in Burgess *et al*. (2007), the distributions of endemic and near endemic species are almost identical to our result for the montane set, therefore suggesting a biogeographic pattern common to a wide range of taxa. The proximate reasons for this pattern remain obscure as we do not know the exact timing and nature of the last connections among forests. There is, however, evidence that during the Miocene (20-10 Mya) until the Last Glacial Maximum (ca. 12 000 Ya), cool and dry periods alternated with warmer and wetter conditions determined connections and isolations of the forests (Lovett, 1993). The resilience of the Eastern Arc mountain system to such cyclical events is believed to have resulted in high levels of endemism, both by allowing the survival of relic species (Lovett *et al.*, 2005) and promoting speciation (Fjeldså & Bowie, 2008). Evidence supporting inter- and intraspecific diversification events in the Eastern Arc are available for frogs (Blackburn & Measey, 2009), chameleons (Matthee *et al.*, 2004) and birds (Fjeldså & Bowie, 2008; Voelker *et al.*, 2010). During the glacial periods, forest species with higher dispersal abilities were still able to migrate across the Eastern Arc, as supported by their occurrence on several mountain blocks (e.g *Arthroleptis affinis* Ahl, 1939). Despite these few species in common among areas, our study showed a marked distinction between northern and southern assemblages (see also Menegon *et al.*, 2011). A substantial divergence between lineages across the Eastern Arc has also been observed in avian clades (summarized by Kahindo *et al.*, 2007 and Fjeldså & Bowie, 2008), small mammals (Carleton & Stanley, 2005; Stanley & Olson, 2005), chameleons (Tolley *et al.*, 2011), and frogs (Blackburn & Measey, 2009; Lawson, 2010). Even though the exact position of the north-south break slightly varies, these studies show population differentiation across the Eastern Arc with a marked distinction into northern and central-southern lineages. It is thus reasonable to recognize the broad, flat plain between the Usambara Mountains and the central blocks as a dispersal barrier even during favorable climatic conditions.

Blackburn & Measey (2009) hypothesized a migration model through the coastal forests followed by a gradual throwback to higher elevations as the forest receded with increasing aridification. This model would explain the

clustering of the West Usambara with the southern group instead of the northernmost mountains, and the gradual decline of montane anuran species towards northwest. The Usambara Mts. are closer to the Indian Ocean than the other northern blocks and thus connections with the coastal forests might have facilitated the dispersal to and from the central and southern Eastern Arc. Factors like temperature, precipitation, forest size or competition might have prevented frogs from migrating further northward. Although ancient migration routes between the Albertine Rift, Kenyan Highlands and Eastern Arc may have existed (Lovett, 1993), the cool and dry conditions and the establishment of a dry corridor between the highlands and the northernmost Eastern Arc Mountains might have prevented dispersal of forest fauna (Bowie *et al*. 2005; Schick *et al*. 2005; Tolley *et al*. 2011). Evidence of such isolation can be found in the current distribution of amphibians and reptiles with very few species occurring in both the Eastern Arc and the Kenyan Highlands (Howell, 1993; Lötters *et al.*, 2006; Wagner *et al.*, 2008). On the contrary, the occurrence of forest species with southern terminations (e.g. southwards in Malawi and Zambia) suggests the existence of connections between the central Eastern Arc and the central and southern African forests (Howell, 1993).

2.5.4 Why is the forest so quiet?

Incorporating historical biogeography can help understanding the assembly of ecological communities and inferring patterns and timing of colonization (Wiens, 2012). For instance, by using historical biogeography we can infer if a region has higher diversity simply because it has been inhabited longer (e.g. Stephens & Wiens, 2004). On Mt. Kilimanjaro, the amphibian fauna within the montane forest is particularly species poor and none of the genera endemic to the Eastern Arc occurs either here or on Mt. Meru. Moreover, we never encountered caecilians, earthworm-like amphibians (order Gymnophiona) that occur in wet tropical regions including the montane forests of the Eastern Arc (Harper *et al.*, 2010). The absence of caecilians might be due to

incompleteness of our survey; however, they have not been reported also on Mt. Meru or in the Kenyan Highlands (Lötters *et al.*, 2006).

The two montane frogs occurring on the volcanoes do not have origins in the Eastern Arc (van der Meijden *et al.*, 2005). The genus *Amietia* is mainly distributed in central and southern Africa, with *Amietia wittei* occurring in montane areas in Kenya and east Democratic Republic of Congo, whereas *Strongylopus* is centered in southern Africa with *Strongylopus fuelleborni* extending northward across the Eastern Arc to the volcanoes (Frost, 2013). Both species are mainly associated with streams in montane forests and alpine grasslands and, from our own observations, may tolerate a certain degree of habitat disturbance. The taxonomy and phylogeny of these genera are unresolved and future studies are needed, hence we do not have estimates of divergence times and population radiations. However, based on the biogeographic scenario discussed above, we speculate that they probably migrated through riverine habitats more recently than the dispersal events that took place across the Eastern Arc. The ancient forest fauna of the older mountain chain thus never colonized the slope of Mt. Kilimanjaro. During the last glacial periods, when Mt. Kilimanjaro was finally "available", the few forest species on the nearby mountains may have been already too isolated to be able to cross the hostile matrix which separates them from the volcanic mountain.

2.6 SUPPORTING INFORMATION

Appendix S2.1 List of species included in the analysis. In the Nguru South (Menegon *et al.*, 2008) many specimens were newly discovered and lacking of scientific name, consequently the number of montane species for this mountain would be even higher. The sources of data used in the analysis are given at the end of the table and in bibliography for full reference.

Species	Meru*	Kilimanjaro	Taita Hills†	North Pare§	South Pare§	West Usambara§	Nguru South¶	Uluguru South‡	Malundwe**	Mahenge††	Udzungwa Southern Scarp§§
Arthroleptidae											
Arthroleptis affinis						x	x	x	x		x
Arthroleptis anotis					x						
Arthroleptis fichika				x	x	x					
Arthroleptis lonnbergi						x				x	
Arthroleptis reichei										x	x
Arthroleptis stenodactylus		x			x	x		x	x		x
Arthroleptis tanneri						x					
Arthroleptis xenodactyloides			x		x	x	x	x	x	x	x
Arthroleptis xenodactylus							x				
Leptopelis barbouri											x
Leptopelis bocagii		x									
Leptopelis concolor			x								
Leptopelis flavomaculatus						x	x			x	x
Leptopelis parkeri					x	x	x	x			x
Leptopelis uluguruensis							x		x	x	x
Leptopelis vermiculatus						x	x			x	x
Brevicipitidae											
Breviceps fichus						x					
Breviceps mossambicus								x		x	
Callulina dawida			x								
Callulina kisiwamsitu						x					
Callulina kreffti								x			
Callulina laphami				x							
Callulina shengena					x						
Callulina stanleyi					x						
Probreviceps loveridgei								x			
Probreviceps macrodactylus							x		x		x
Probreviceps rungwensis										x	x
Probreviceps uluguruensis								x			
Spelaeophryne methneri								x		x	x

Bufonidae												
Amietophrynus brauni						x	x					x
Amietophrynus garmani		x	x									
Amietophrynus gutturalis	x	x	x		x	x	x	x		x		
Amietophrynus maculatus										x		
Amietophrynus xeros			x									
Mertensophryne loveridgei										x		
Mertensophryne taitiana			x									
Nectophrynoides laevis								x				
Nectophrynoides poyntoni												x
Nectophrynoides tornieri									x	x		x
Nectophrynoides vestergaardi						x						
Nectophrynoides viviparus								x				x
Nectophrynoides wendyae												x
Schismaderma carens							x					
Hemisotidae												
Hemisus marmoratus	x	x	x							x		
Hyperoliidae												
Afrixalus fornasini						x	x		x	x		
Afrixalus morerei												x
Afrixalus septentrionalis		x										
Afrixalus stuhlmanni							x		x			
Afrixalus uluguruensis						x	x			x		x
Hyperolius argus						x						
Hyperolius kihangensis												x
Hyperolius mitchelli				x		x	x			x		x
Hyperolius nasutus	x									x		
Hyperolius parkeri						x						
Hyperolius puncticulatus						x		x	x	x		x
Hyperolius pusillus						x						
Hyperolius spinigularis							x					x
Hyperolius tanneri						x						
Hyperolius tuberilinguis			x							x		
Hyperolius viridiflavus	x	x	x	x	x							
Kassina maculata						x						
Kassina senegalensis	x	x	x					x		x		
Phlyctimantis keithae												x
Microhylidae												
Phrynomantis bifasciatus		x	x							x		
Petropedetidae												
Petropedetes yakusini												x
Phrynobatrachidae												
Phrynobatrachus acridoides		x								x		

Phrynobatrachus bullans		x									
Phrynobatrachus keniensis	x										
Phrynobatrachus krefftii						x					
Phrynobatrachus natalensis		x		x				x		x	
Phrynobatrachus parvulus							x				x
Phrynobatrachus rungwensis											x
Phrynobatrachus scheffleri			x								
Phrynobatrachus uzungwensis							x	x		x	x
Pipidae											
Xenopus borealis			x								
Xenopus muelleri	x									x	
Xenopus petersii							x				
Xenopus victorianus		x				x					
Ptychadenidae											
Hildebrandtia macrotympanum			x								
Ptychadena anchietae		x	x	x	x	x	x			x	
Ptychadena mascareniensis	x	x	x								
Ptychadena mossambica			x								
Ptychadena oxyrhynchus								x	x		
Pyxicephalidae											
Amietia angolensis	x	x	x	x	x	x	x	x		x	x
Amietia wittei	x	x									
Cacosternum plimptoni		x									
Pyxicephalus adspersus			x							x	
Strongylopus fuelleborni	x	x				x		x			x
Tomopterna cryptotis			x								
Tomopterna tuberculosa		x									
Rhacophoridae											
Chiromantis kelleri			x								
Chiromantis petersii		x	x								
Chiromantis xerampelina							x			x	
Total	**11**	**21**	**23**	**7**	**11**	**28**	**22**	**19**	**10**	**30**	**30**

*Razzetti & Msuya, 2002; Lötters *et al.*, 2006

†Malonza & Veith, 2012

§Loader *et al.*, 2011

¶Menegon *et al.*, 2008

‡Ngalason & Mkonyi, 2011

**Lawson & Collett, 2011

††Menegon & Salvidio, 2005

§§Menegon *et al.*, 2011

Appendix S2.2 List of species recorded on the southern slope of Mt. Kilimanjaro. Reproductive modes are simplified and referred to the behaviors observed during the field work. When two modalities are observed for one species, letters are reported in order of observed frequency.

Species	Reproductive mode*	Habitat type**	Number of sites	Bioclimatic zone
Arthroleptidae				
Arthroleptis stenodactylus	C	CO/HO	5	submontane
Leptopelis bocagii	A[1]	SA	1	colline
Bufonidae				
Amietophrynus garmani	A	SA	1	colline
Amietophrynus gutturalis	A,B	SA/MA/CO/HO	12	colline,submontane
Hemisotidae				
Hemisus marmoratus	A[1]	SA/MA	3	colline
Hyperoliidae				
Afrixalus septentrionalis	A[2]	MA	1	colline
Hyperolius viridiflavus	A	MA/HO	4	colline,submontane
Kassina senegalensis	A	SA/MA	2	colline
Microhylidae				
Phrynomantis bifasciatus	A	MA	1	colline
Phrynobatrachidae				
Phrynobatrachus acridoides	A	SA	1	colline
Phrynobatrachus bullans	A	SA/MA	2	colline
Phrynobatrachus natalensis	A	MA	1	colline
Pipidae				
Xenopus victorianus	A,B?	HO	1	submontane
Ptychadenidae				
Ptychadena anchietae	A	SA/MA/CO	6	colline,submontane
Ptychadena mascareniensis	A	SA/MA/CO/HO	7	colline,submontane
Pyxicephalidae				
Amietia angolensis	B,A	CO/HO/FE	8	submontane
Amietia wittei	B,A	EF/MF/ES	11	montane
Cacosternum plimptoni	A	MA	1	colline
Strongylopus fuelleborni	A,B	EF/MF	6	montane
Tomopterna tuberculosa	A	SA	2	colline
Rhacophoridae				
Chiromantis petersii	A	SA/MA	2	colline

*Reproductive modalities: A = eggs and tadpoles in lentic waters, with the exception of: ([1]) eggs terrestrial and ([2]) eggs on plants above the water; B = eggs and tadpoles in lotic waters; C = eggs terrestrial (direct development).

**Abbreviated letters represent different habitat types: MA = maize, SA = savanna, CO = coffee plantation, HO = homegarden, EF = forest edge, MF = montane forest, ES = *Erica* shrubs.

Table S2.1 List of study sites on Mt. Kilimanjaro with relative information. Number of species includes the observations made during the opportunistic surveys and (**) indicates in which sites species encountered exclusively during opportunistic surveys have been excluded from the analysis. Completeness = (number of observed species / number of estimated species) x 100. Minimum - maximum values are shown. Species richness estimators for incidence data were: ICE, Chao2, Bootstrap and Jackknife1.

					Breeding sites		
Sites	**N. of species**	**Elevation (m a.s.l.)**	**Habitat type***	**Disturbance level****	**Lentic**	**Lotic**	**Completeness (%)**
ma2	1	860	MA	4	1	0	57-100
sa1	2	905	SA	1	1	0	57-76
sa4	3	929	SA	1	1	0	80-100
sa3	8	936	SA	1	1	0	74-90
sa2	6	980	SA	1	1	0	67-91
ma1	12	1003	MA	4	1	0	78-89
co4**	3	1127	CO	4	0	0	94-100
ho2	1	1154	HO	3	0	0	94-100
co1	4	1289	CO	4	1	0	82-100
co3	3	1300	CO	4	0	1	50-88
co2	0	1347	CO	4	0	0	-
ho4	2	1433	HO	3	1	0	62-100
ho3	4	1537	HO	3	1	0	82-100
ho1	0	1649	HO	3	0	0	-
ho7	3	1651	HO	3	0	1	94-100
ho8	3	1658	HO	3	1	0	79-100
ho6	3	1659	HO	3	0	1	97-100
ef2	2	1692	EF	2	0	1	96-100
ef3	2	1705	EF	2	0	1	67-100
ef1	2	1710	EF	2	1	0	67-100
ef4	2	1718	EF	2	1	0	64-100
mf5	2	1837	MF	1	0	1	94-100
ho5	1	1850	HO	3	0	0	60-100
mf1	0	1936	MF	1	0	0	-
mf2	2	2027	MF	1	0	1	64-100
mf6	1	2241	MF	1	0	1	94-100
mf10	1	2359	MF	1	0	1	100
mf9	1	2527	MF	1	0	1	94-100
mf8	1	2712	MF	1	1	0	57-100
mf3	1	2978	MF	1	0	1	100
mf7	1	3012	MF	1	1	0	100
mf4	1	3023	MF	1	1	0	100
es4	0	3289	ES	1	0	1	-
es1	1	3479	ES	1	0	1	99-100
es3	0	3511	ES	1	0	1	-
es2	0	3548	ES	1	0	1	-
Landscape	**21**						**71-86**

* Abbreviated letters represent different habitat types: MA = maize, SA = savanna, CO = coffee

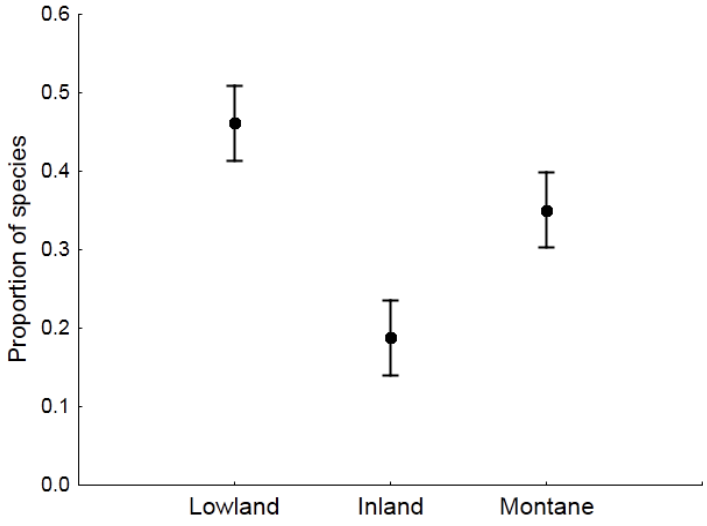
plantation, HO = homegarden, EF = forest edge, MF = montane forest, ES = *Erica* shrubs.

** See Methods for detailed explanations.

Table S2.2 P-values from pairwise comparisons (Student's t-tests) using number of species (upper diagonal) and proportions (lower diagonal) between Northern group (North), Southern group (South), Lowland, Inland and Montane sets. Significant values are indicated by bold type. In brackets are also reported the mean number of species (in columns) and mean proportions (in rows).

Proportion of species				Mean number of species					
				Lowland (8.73)		Inland (3.45)		Montane (7.09)	
				North (7.4)	South (9.83)	North (4.8)	South (2.33)	North (2.4)	South (11)
Lowland	(0.46)	North	(0.49)	-	0.441	0.361		0.058	
		South	(0.44)	0.657	-		**0.006**		0.721
Inland	(0.19)	North	(0.30)	**0.034**		-	0.149	0.203	
		South	(0.09)		**0.001**	**0.002**	-		**0.005**
Montane	(0.35)	North	(0.21)	**0.016**		0.324		-	**0.011**
		South	(0.47)		0.804		**0.000**	**0.022**	-

Fig. S2.1 Mean proportions of species in the lowland, inland and montane categories across the mountains.



Chapter 3

Comparative landscape genetics of two river frog species along an altitudinal gradient on Mount Kilimanjaro[1]

Table of Contents

[1] This chapter is under review as: Zancolli G., Rödel M.O., Steffan-Dewenter I., Storfer A. "Comparative landscape genetics of two river frog species along an altitudinal gradient on Mount Kilimanjaro". *Molecular Ecology*.

3.1 ABSTRACT

In topographically complex landscapes, habitat fragmentation and altitudinal variation strongly affect the spatial distribution of genetic variation via reduction in dispersal and gene flow among terrestrial animal species. Several studies on pond-breeding amphibians from temperate regions report a negative correlation of genetic diversity with increasing elevation, and a higher degree of genetic differentiation between high elevation populations compared to lowland populations. In this study, we investigated how topography and land cover affect genetic connectivity of two stream-dwelling tropical frogs inhabiting the slopes of Mount Kilimanjaro in Tanzania. We used AFLP markers to compare patterns of genetic variation and dispersal among the low altitude species, *Amietia angolensis*, with the high altitude, *Amietia wittei*. We detected greater genetic differentiation among sampling localities in the highland species and higher genetic variation in the lowland species, although genetic diversity was not significantly correlated with elevation. Least-cost path analyses revealed that both species tend to maximize stream-based movements. The intense network of water canals in the lowlands seems to enhance dispersal in *A. angolensis*, whereas human settlements restricted gene flow. In *A. wittei*, the best least-cost path models included steep slopes that were surprisingly positively correlated with gene flow. In contrast to the lowland species, *A. wittei* appears to have less restricted dispersal across rough topography. Our results show that even ecologically similar species can respond differently to landscape processes and that the spatial configuration of topographic features combined with species-specific biological attributes can affect dispersal and gene flow in disparate ways.

Keywords: AFLP, anthropogenic disturbance, dispersal, gene flow, genetic structure, topography.

3.2 INTRODUCTION

Understanding the effects of structural connectivity on functional connectivity is a central goal in landscape genetics (Holderegger & Wagner 2006), and it is crucial for conservation of biological diversity (Semlitsch 2002). That is, the heterogeneity of landscape features has critical influence on the spatial distribution of genetic variation (Manel *et al*., 2003; Holderegger & Wagner 2006; Storfer *et al*. 2007). Landscape characteristics such as mountain ridges, rivers, and habitat fragmentation have generally been shown to limit gene flow in several terrestrial species (see Storfer *et al*. 2010 for examples).

In the last several years, researchers have investigated the effects of elevational gradients and topographic relief on genetic connectivity and population structure, with particular focus on species with poor dispersal capabilities, such as amphibians (Funk *et al*. 2005; Spear *et al*. 2005; Giordano *et al*. 2007; Murphy *et al*. 2010). It is expected that changes in elevation among sites reduce gene flow for two reasons. First, dispersal might be restricted from low to high elevation populations simply because of the energetic costs of moving up steep slopes. Second, even if dispersal is not restricted, premating barriers can restrict gene flow between low and high elevation populations. For example, altitude explained differences in flowering time and consequent genetic divergence of snowbed herbs (Hirao & Kudo 2008). In amphibians, elevational gradients were associated with increasing genetic divergence in Columbia spotted frogs (*Rana luteiventris*) in western Montana and Idaho, USA (Funk *et al*. 2005). A "valley-mountain" model consisting of three expectations was then proposed: (i) high gene flow between low altitude populations; (ii) little gene flow between high altitude populations, and (iii) low gene flow between high and low altitude populations. These predictions were later supported by Giordano *et al*. (2007) for high and low altitude populations of the long-toed salamander (*Ambystoma macrodactylum*). Both studies also reported a significant decline of genetic variation with increasing elevation. On the contrary, Measey *et al*. (2007) found high levels of gene flow in a direct-developing leaf-litter frog (*Arthroleptis xenodactyloides*) among populations on different blocks of the Taita Hills (Kenya) and they proposed a dispersal model

based on a combination of passive downhill and active uphill movements. Similarly, Zhan *et al*. (2009) showed high genetic connectivity among populations of the Chinese wood frog (*Rana chensinensis*) over large geographical distances in the Tsinling-Daba Mountain region in Northern China.

Thus, it appears that topographic relief can greatly reduce dispersal and gene flow in site philopatric pond breeders compared to amphibians with other reproductive strategies. Moreover, patterns of genetic variation and gene flow can vary among latitudes. Janzen (1967, p. 234) proposed that mountain passes in the tropics should be more effective "physiological" barriers to dispersal relative to temperate zones. However, Ghalambor *et al*. (2006) later highlighted the lack of data supporting the prediction of limited dispersal across elevational gradients in tropical areas relative to temperate ones. Clearly more studies are needed, in particular from tropical regions where the interactions between landscape features and microevolutionary processes are still poorly known (Storfer *et al*. 2010).

In tropical Africa, Mount Kilimanjaro (Tanzania) is the highest free-standing mountain, and it is shaped topographically by a series of parallel deep valleys and ridges that result in formation of multiple drainage systems (Fig. 3.1). Rising from a hot and dry savanna at ca. 700 m to an iced-clad summit at 5895 m, this large volcano encompasses several different ecosystems and climatic conditions over relatively short geographic distances. The African river frogs of the genus *Amietia* cover a large part of Mt. Kilimanjaro altitudinal range. The common river frog, *Amietia angolensis* (Bocage, 1866) is a widespread generalist species, ranging from upland areas in Ethiopia, southwards through eastern and southern Africa (Channing & Howell 2006). The tadpoles grow to a large size and have a prolonged development that may last up to two years until metamorphosis (Channing 2004). On Mt. Kilimanjaro, common river frogs can be easily found in proximity of water in a wide array of habitat types, from grassland, to banana forests, coffee plantations and house gardens, and they occur from ca. 1000 to 1700 m where the lower borders of the Kilimanjaro National Park delimit the montane rainforest.

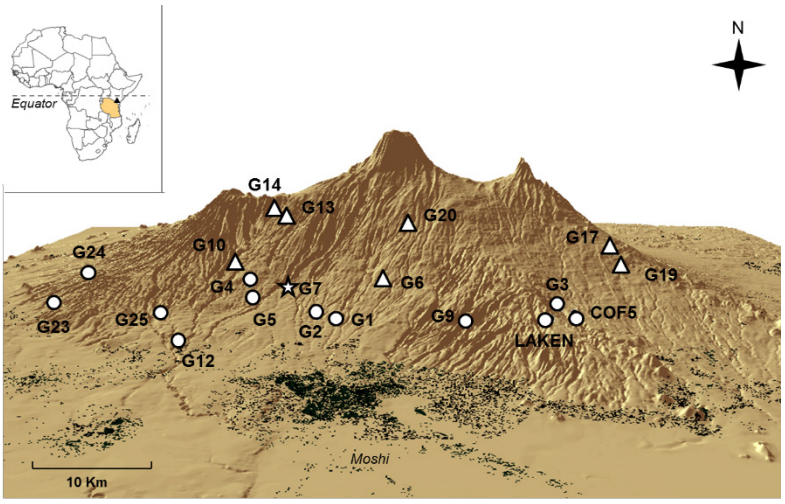


Fig. 3.1 Location of the study area and sampling sites for *Amietia angolensis* (circles) and *A. wittei* (triangles) along the southern slope of Mount Kilimanjaro in Tanzania. Site G7, where both species and hybrid individuals were sampled, is indicated with a star. The hillshade 3D model of Mt. Kilimanjaro was developed in ArcScene 10 (ESRI) from a DEM with 30 m resolution.

Within the National Park, *A. angolensis* is replaced by the Molo frog, *Amietia wittei* (Angel, 1924), which inhabits altitudes ranging from 1700m to 3500 m. This Afromontane species has a limited, patchy distribution encompassing the central highlands of Kenya and northern Tanzania, including Mt. Meru (Lötters *et al*. 2004). Currently, there are no studies on the ecology of this species. In contrast to the common river frog, *A. wittei* is more secretive and difficult to observe; it breeds in streams, creeks and, at higher elevations, in spring pools. During the day, tadpoles hide under stones or in the leaf pack at the bottom of the water bodies and at night they become more active, probably for feeding. The river frogs of the genus *Amietia* are part of a complex of cryptic species and the systematics is currently being reviewed (A. Channing, pers. comm.); hence, we do not know the phylogenetic relationship between the two species occurring on Mt. Kilimanjaro and the others in the genus. Nevertheless, the occurrence of two ecologically similar species at different altitudinal ranges offers the unique opportunity to understand how elevational gradients, topography and different habitat types affect genetic variation and connectivity.

In the present study, we used amplified fragment length polymorphisms (AFLPs) to address the following questions: (1) Does genetic diversity decrease with elevation? (2) Is gene flow restricted among high elevation populations (*A. wittei*) compared to lowland populations (*A. angolensis*)? (3) How does topography, especially the valley-ridge system along Mt. Kilimanjaro, and land cover affect population structure, genetic variation and connectivity? We hypothesize that the valley-mountain model (Funk *et al*. 2005) applies to our lowland versus upland species. Under this model, we predict the montane species to be more genetically differentiated as a consequence of relatively restricted gene flow as compared to the lowland species. Alternatively, the deep valleys and mountain ridges that run parallel along the southern slope of Mt. Kilimanjaro might decrease connectivity in the lowland species, resulting in a higher population subdivision. Moreover, the higher degree of anthropogenic disturbance in the lowlands might also result in reduced *A. angolensis* genetic variation and comparatively higher gene flow among *A. wittei* populations across the relatively undisturbed, continuous forested habitat within the National Park.

3.3 MATERIALS AND METHODS

3.3.1 Study area

Mt. Kilimanjaro is the remnant of a stratovolcano located in Tanzania (2°45' to 3°25'S; 37°00' to 37°43'E). It has been designated as UNESCO world heritage site and it is included in the Eastern Afromontane Hotspot of Biodiversity (Mittermeier *et al*. 2004). Below the National Park boundaries, the slope is heavily populated, with densities varying from 500 to 1000 people per km^2 (FAO 1986; Timberlake 1986). The original vegetation has been almost entirely converted into croplands (maize and sunflowers) at lower elevations, while different degrees of agroforestry systems called "home gardens", with banana and coffee trees as main crops, are found between 1000 and 1700 m. Despite the protection of the National Park, the montane rainforest is regularly exposed

to illegal logging, grazing, small-scale farming and fires (Lambrechts *et al.* 2002). Moreover, the high number of tourists attempting to climb the mountain (35 000 climbers per year; Peaty 2012) is an important source of disturbance and pollution of these vulnerable ecosystems.

3.3.2 Sampling and DNA extraction

We searched for potential *A. angolensis* and *A. wittei* breeding sites from April to June (long rainy season) and October to November (short rains) in 2011. Geographic position was recorded with a Garmin® GPSMAP 62S. Tissue samples were collected by tail clipping (<0.5 cm) from tadpoles at different developmental stages in order to reduce sampling siblings. Only sites where we collected a minimum of ten samples were kept. In total, we sampled 20 sites: 12 for *A. angolensis*, seven for *A. wittei* and one site, G7, where both species were present (Fig. 3.1).

Total genomic DNA was extracted using the Roche High Pure PCR Template Preparation Kit following the manufacturer's instructions, except for an extra step after the digestion by proteinase K aiming to discard residual pigments.

3.3.3 Genotyping and AFLP scoring

AFLP markers were obtained using a modified version of Vos *et al*. (1995) (Appendix S3.1, Supporting Information). After an initial screening, we selected six primer combinations. Fragments were separated in an ABI 3130xl automatic capillary sequencer (Applied Biosystems) with an internal GeneScan 500 ROX size standard. Bin positions and fluorescent intensity of any peaks occurring between 50 and 500 bp and higher than 50 relative fluorescent units (rfu) were determined using GeneMapper 4.1 (Applied Biosystems) and adjusted manually following the semi-automated scoring procedure described in Whitlock *et al*. (2008). Electropherograms with the sum of all peaks particularly

lower than the median fingerprint intensity were removed and coded as missing value for that primer combination. Individuals with two or more missing combinations were excluded from further analysis. Final binary matrices were generated with AFLPscore (Whitlock *et al*. 2008). All fingerprints were normalized and filtered using a locus selection threshold of 130 rfu and a phenotype-calling threshold of 100 rfu. All rejected loci were removed and the peak intensity matrices were rerun so that normalization was based only on the retain loci. To assess reliability of our AFLP markers, mismatch error rate was calculated using randomly replicate samples. Markers with numerous mismatches were discarded. We also removed loci with band frequencies ≤ 3/*N*, which maybe spurious peaks or contamination, and ≥ (1 – 3/*N*) which may be null alleles (Lynch & Milligan 1994).

3.3.4 Population genetic analysis

Because *Amietia* species, especially at the larval stage, are cryptic and difficult to identify, we used the program NewHybrids 1.1 beta (Anderson & Thompson 2002) which computes the posterior distribution that individuals fall into different categories, namely pure, F_1, F_2 and backcrosses. In all sites there was exclusively one species except in G7 where we found both species and backcrossed individuals. Non-pure samples were excluded from the data set.

Summary statistics of genetic diversity for the two species and for each sample site were calculated with AFLP-SURV 1.0 (Vekemans *et al*. 2002), using a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999). We chose this method because it gave the most accurate results in a study using simulated and real AFLP data (Bonin *et al*. 2007). To test the hypothesis that genetic diversity decreases with elevation, regression analyses of expected heterozygosity (*Hj*) and proportion of polymorphic loci at 5% level (%P) against elevation were performed in the R environment (R Core Team 2012). For each response variable, we ran two analyses: first, we tested each species separately (within-species analysis), then we ran a global analysis including both species but fitting linear-mixed effect models (*lme* function) with

species as random effect. We also compared the mean values of genetic diversity estimates between the two species. Allele frequencies were used to calculate F_{ST} following Lynch & Milligan (1994). F_{ST} has been commonly used to estimate levels of genetic differentiation among populations and therefore is useful for comparison with other landscape genetic studies. In contrast to frequency-based approaches such as F_{ST}, we also estimated Φ_{PT} via an analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) implemented in GenAlEx 6.5 (Peakall & Smouse 2012). A pairwise individual-by-individual genetic distance matrix was generated following the method of Huff *et al.* (1993) based on shared band presence or absence and used as input matrix in AMOVA. A band-based method is preferred with dominant markers such as AFLP because it does not require additional assumptions (e.g., Hardy-Weinberg equilibrium) or information about population genotypic structure (e.g., F_{IS}) to estimate allele frequencies; however, Φ_{PT} considerably overestimates differentiation (Bonin *et al*. 2007).

We estimated genetic structure with the Bayesian clustering method implemented in the program STRUCTURE 2.3.4 (Pritchard *et al*. 2000; Falush *et al*. 2007) under a model assuming admixture and correlated allele frequencies. Ten independent runs with a burn-in period of 100 000 replications and 1 000 000 Markov chain Monte Carlo (MCMC) iterations were performed for a number of populations $K = 1 – 6$. To assess the most likely value of K, we used the method described in Evanno *et al*. (2005).

3.3.5 Least-cost path analysis

To test the influence of different landscape features on population structure, we developed ten different potential least-cost paths in ArcGIS 10 (ESRI) for the two species separately. Least-cost analysis was performed with the "path distance" (which already corrects for topography) and "cost path" functions. The first model was a straight-line (Euclidean) distance between sites as a null model, which would be expected if there was no landscape influence on gene flow but merely isolation by distance (IBD; Wright 1942). The first least-cost path (LCP)

was a modification of the null model, in which we calculated topographically corrected straight-lines among all site pairs using a 30 x 30 m digital elevation model (DEM, produced by J. A. Ong'injo, C. Lambrechts and A. Hemp). All further resistance surfaces were calculated from the DEM. Since *Amietia* species are associated with water (Channing & Howell 2006), we expected gene flow to be positively correlated with stream networks. Because an exhaustive stream surface was not available for the Kilimanjaro region, we delineated stream networks using the "flow accumulation" tool in ArcGIS 10 (ESRI) and applied a threshold value of 500. Stream surface was divided into stream (low cost) and upland (high cost) categories and two alternative cost ratios (1:2 and 1:10) were tested.

The next LCPs represent hypotheses that gene flow is enhanced when minimizing slope, roughness (rough) and compound topographic index (cti). The roughness index expresses the amount of elevation changes between adjacent cells, with low values indicating more flat areas (Riley *et al.* 1999). Cti is a steady-state wetness index which is function of both slope and upstream contributing area per unit width orthogonal to the flow direction. High cti values represent drainage depressions; hence, to test whether frogs appear to preferentially move through wet areas, we inverted the raster values so that high values correspond to dry areas such as crests and ridges. These indices were calculated with the Geomorphometric and Gradient Metrics toolbox in ArcGIS 10 (ESRI). We did not assign cost values to these continuous surfaces, instead we stretched the raster data to a specified range (0 – 10) and used them directly into the least-cost analysis. The last four models were based on multiplied effects of topography and stream network. We created multivariate resistance surfaces by combining slope and roughness with stream 1:2 and 1:10 surfaces. The rationale behind a multivariate approach is that we hypothesized river frogs disperse along riverine habitats but, at the same time, try to avoid steep slopes, or crests and ridges (i.e. high roughness) when crossing terrestrial areas. Since some routes were very unlikely (e.g. passing through the glacier on top of Kibo), we assigned to all resistance surfaces a cost value of 5 000 to the cells above 1800 m for *A. angolensis* and those above 3500 m for *A. wittei*. We

chose these constraints based on the observed altitudinal limits of the two species.

For each resistance surface, we measured route lengths (i.e. topographic distances); in addition, we calculated the weighted average of the other landscape variables (i.e. cti, slope and roughness) along each route (Spear *et al*. 2010).To test whether genetic differentiation was affected by land cover, we calculated the proportion of each cover type along the routes by dividing the length of the path crossing a certain habitat type by the total length of the route. The land cover surface (Appelhans & Nauss, unpubl.) consisted of eight discrete habitat types, including cropland, home gardens, settlement, *Erica* shrubs, forest, disturbed forest, reforestation and grassland. We did not develop LCPs based on habitat types because we lacked the empirical data necessary to parameterize accurately cost assignments when either species moves through specific cover types. Instead, we included the proportions of land cover as explanatory variables in the regression models (see Statistical Analyses below).

To better understand habitat connectivity and the overall pattern of topographical features, we compared the landscape variables among areas of occurrence of *A. angolensis* and *A. wittei*. For each species separately, we dissolved all LCP polylines into a polygon which represents the area potentially available to the species for movement (Trumbo *et al*. 2013). To assess whether the two areas were significantly different, we used two approaches. First, we compared the mean values of the landscape features between the two areas. For categorical variables (habitat type and stream network), we used the program Fragstats 4.1 (McGarical *et al*. 2012) to calculate the "area-weighted mean patch radius of gyration" (or correlation length). This measure represents the average traversability of the landscape for an organism confined within a habitat patch, and thus it provides a measure of landscape connectivity (Keitt *et al*. 1997). Specifically, the correlation length gives the average distance from a random starting point and moving in a random direction before encountering the habitat patch boundaries or without leaving the stream network. For continuous variables, we calculated the mean and standard

deviation within the polygon by means of the zonal statistics tool in ArcGIS 10 (ESRI). As we did not have the raw data, we judged the significance of differences using both the "standard" and the "overlap" methods (Schenker & Gentleman 2001). The standard method rejects the null hypothesis that the difference between the two areas is zero (i.e. the two areas are similar for a certain landscape feature) if the nominal 95% confidence interval does not contain zero. In contrast, the more conservative overlap method rejects the null hypothesis if the two associated 95% confidence intervals do not overlap. Although Schenker & Gentleman (2001) do not suggest the use of such approaches for formal significant testing, they are convenient for a qualitative assessment especially when only means and standard deviations are available (Trumbo *et al*. 2013).

Second, we used the Hot Spot analysis tool in ArcGIS 10 (ESRI) to identify statistically significant spatial clusters of either high or low feature values. This tool calculates the Getis-Ord General G statistic which tests the null hypothesis that topographic variables exhibit a spatially random pattern and creates a new output layer with local z-scores and *p*-values. At a confidence level of 95%, z-score values below -1.96 and above 1.96 will be statistically significant; thus resulting in the rejection of the hypothesis of complete spatial randomness. Positive z-scores indicate that cells with high values are surrounded by high values (hot spot), whereas negative z-scores indicate clustering of low values (cold spot). We thus identified which areas on the mountainside are characterized by high or low values of topographic features and where there is complete spatial randomness (i.e. *p*-values > 0.05).

3.3.6 Statistical analyses

The correlations between *A. angolensis* and *A. wittei* gene flow (estimated by F_{ST} and Φ_{PT}) and landscape features were assessed using two methods. First, we employed a new mixed modelling approach (van Strien *et al*. 2012). Methods like linear regression or partial Mantel tests are most often applied in landscape genetic studies (Storfer *et al*. 2010); however, they do not take into account the dependency of pairwise genetic distances (Yang 2004). Clarke *et al.* (2002) described the maximum-likelihood population-effects (MLPE) model, in which the covariate structure is tailored for the specific dependency between values in a distance matrix. We followed the procedure described in van Strien *et al*. (2012), in which the authors used Clarke's MLPE method to fit the regression models between genetic distances and a set of landscape variables. Briefly, we first centered all explanatory variables (i.e. topographic distances and landscape variables) around their mean, then we fitted MLPE models with restricted maximum likelihood (REML) estimation with a modification to account for multiple memberships (i.e. each pairwise distance was associated with two demes). The mixed models were fitted with the *lmer* function in the R package *lme4* (Bates *et al*. 2011) and each model consisted of a genetic distance as response variable, the explanatory variables as fixed effects and the individual deme (which was standard) as random effect. Because we were interested in which landscape features best explained genetic variation, we calculated all possible combinations of the explanatory variables for each LCP for a total of 10 240 models (both species). To select the best LCP models we applied a suite of criteria and measures of fit. Although some authors did not recommend Akaike's Information Criterion (AIC) calculated from REML in linear mixed models with different fixed effects (Verbeke & Molenberghs 2000; Orelien & Edwards 2008), Gurka (2006) demonstrated that information criteria such as AIC, AICc, CAIC and BIC can be employed for REML mixed model selection and that, in many cases, the criteria actually performed better in choosing the proper set of fixed effects under REML compared to when using the maximum likelihood (ML) estimation method. We thus used AICc and BIC to select the best models, and we employed measures of the fit such as log-likelihood

(logLik) and deviance for the REML criterion (REMLdev) to validate selection based on the information criteria.

To compare with the MLPE results, we also used the BIOENV procedure (Clarke & Ainsworth 1993) in the R package *vegan*. BIOENV tests all possible combinations of independent variables to find the best subset using a Spearman rank correlation (ρ_W). The function calculates Euclidean distances for all possible subsets of scaled environmental variables and finds the maximum (rank) correlation with the genetic distance matrix. Although BIOENV is an exploratory procedure, it has been successfully applied in other landscape genetic studies (e.g. Spear *et al*. 2005; Trumbo *et al*. 2013) to corroborate the most-supported models from other statistical analysis (e.g. multiple linear regression on distance matrices; Smouse *et al*. 1986). For both MLPE and BIOENV analyses, we selected the best model for each LCP and reported the top two LCPs explaining most of the genetic differentiation.

3.4 RESULTS

3.4.1 AFLP analysis

In total, we collected 502 samples from 20 sites. After AFLP data refinement, 301 samples of *A. angolensis* and 180 of *A. wittei* were positively scored and analyzed. The six primer combinations produced 165 polymorphic AFLP markers in *A. angolensis* and 235 loci in *A. wittei*. Final mismatch error rate was 3.45 %, hence within the typical range for AFLPs (Bonin *et al*. 2007).

Table 3.1. Summary information for *Amietia angolensis* (*Aa*) and *A. wittei* (*Aw*) study sites, including elevation, sample size collected at site (*N*), number of polymorphic loci (No. P loci), proportion of polymorphic loci at the 5% level expressed as percentage (%P), expected heterozygosity under Hardy-Weinberg genotypic proportions (or Nei's gene diversity, *Hj*) with standard error (SE), and number of loci occurring exclusively in that site.

Species	Site	Elevation	*N*	No. P loci	% P	*Hj*	SE(*Hj*)	Private bands
Aa	G12	1097	11	119	72.1	0.253	0.015	0
	G1	1270	28	118	71.5	0.270	0.015	0
	G25	1276	36	122	73.9	0.272	0.015	0
	G23	1306	20	116	70.3	0.234	0.014	0
	G2	1321	24	118	71.5	0.266	0.015	0
	G9	1416	15	114	69.1	0.263	0.012	0
	G5	1532	20	122	73.9	0.223	0.014	0
	COF5	1658	25	119	72.1	0.266	0.015	0
	LAKEN	1658	18	116	70.3	0.268	0.015	0
	G3	1679	35	120	72.7	0.263	0.014	0
	G4	1692	34	127	77	0.287	0.015	0
	G7	1705	16	148	89.7	0.343	0.015	24
	G24	1708	19	119	72.1	0.258	0.015	0
Aw	G7	1705	14	176	74.9	0.294	0.012	20
	G6	1992	26	145	61.7	0.234	0.013	1
	G10	2027	20	153	65.1	0.229	0.012	0
	G19	2359	35	174	74	0.255	0.012	6
	G17	2527	19	159	67.7	0.228	0.012	1
	G14	2978	18	160	68.1	0.261	0.012	0
	G20	3012	26	152	64.7	0.242	0.012	1
	G13	3023	22	166	70.6	0.268	0.012	0

3.4.2 Genetic diversity

Genetic diversity was generally low in both species (Table 3.1). Even though we identified and discarded putative hybrids from the dataset, G7, the site with both species and the hybrids, had the highest genetic diversity and an exceptional number of private bands (20-24). With New Hybrids we detected backcrosses, and it could be that individuals assigned in the pure species category may not be completely pure but admixed individuals derived from consecutive intercrossing.

Table 3.2 Summary statistics over all *N* populations for *Amietia angolensis* (*Aa*) and *A. wittei* (*Aw*), including total number of AFLP markers from six primer combinations, overall number of polymorphic loci and total gene diversity (*Ht*), average genetic diversity (%P and *Hj*) and differentiation (F_{ST} and Φ_{PT}).

Species	*N*	Tot. loci	Tot. P	*Ht*	Mean % P		Mean *Hj* ± SD		Mean *Fst* ± *SD*		Mean Φ_{PT} ± SD	
Aa	13	351	165	0.295	72.2	± 2.07	0.260	± 0.02	0.086	± 0.04	0.123	± 0.06
Aw	8	395	235	0.285	67.4	± 4.07	0.245	± 0.02	0.106	± 0.04	0.170	± 0.06
U-test					*		n.s.		*		**	

All statistical analyses were calculated excluding site G7 where hybridization occurs.
Differences in mean estimates were calculated with Mann-Whitney *U*-tests, and the asterisks below each genetic estimate represent the significant level (** p < 0.01, * p < 0.05, n.s. = non-significant).

To avoid bias and spurious results, we consequently excluded G7 from all statistical analyses. Expected heterozygosity (*Hj*) and proportion of polymorphic loci (%P) were similar across sites for *A. angolensis*, except for G5 and G4 with the lowest and highest *Hj* values respectively. *Hj* values across *A. wittei* sites were similar to the common river frog whereas %P was significantly lower (Tables 3.1 and 3.2).

Contrary to *A. angolensis* where no private bands were detected, in *A. wittei* three sites showed one private band each and one site (G19) had six private loci.

Although genetic diversity was slightly higher in the lowland species, we did not find any significant correlation of heterozygosity and percentage of polymorphism with

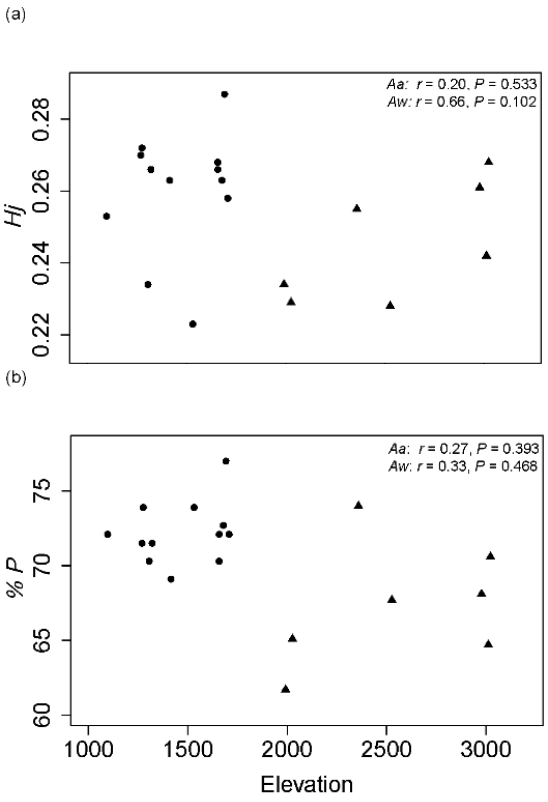


Fig. 3.2 Regression of (a) expected heterozygosity (*Hj*) and (b) proportion of polymorphic loci (%P) against elevation for *Amietia angolensis* (Aa, circle) and *A. wittei* (Aw, triangle). None of the two species exhibited a significant correlation of genetic diversity with increasing elevation.

elevation (Fig. 3.2). Gene diversity estimates showed a positive relationship with increasing elevation when species were analyzed separately, especially in *A. wittei* (r = 0.66) but the correlations where not statistically significant (Fig. 3.2). We also tested the relationship of *Hj* and %P with elevation in a global analysis with linear mixed-effect models but the regressions were again not statistically significant (*Hj*: F = 0.65, p = 0.432; %P: F = 0.37, p = 0.55).

3.4.3 Population structure

The overall level of genetic differentiation significantly differed between the two species (Table 3.2; Table S3.1 and S3.2, Supporting Information). Average F_{ST} values estimated across all loci and sample sites were 0.086 in *A. angolensis* and 0.106 in *A. wittei* (U = 905, p = 0.03). As expected, estimates of Φ_{PT} were higher, with mean values of 0.123 in *A. angolensis* and 0.170 in *A. wittei* (U = 1003, p = 0.002). Higher levels of genetic differentiation in the highland species suggest l ower gene flow among populations and confirm higher degree of isolation compared to the lowland species. However, pairwise F_{ST} values between some sites were quite low considering their Euclidean distances. For example, G14 and G20 are approximately 13.5 km apart and the pairwise F_{ST} was 0.048. Notably, G13 and G19 are 30.2 km apart and their F_{ST} value was 0.098. Pairwise F_{ST} values among populations of the lowland species were even lower among sites which are the same or even more far apart, for examples between G25 and G3 (approximately 30 km, F_{ST} = 0.075) and between G23 and G9 (~ 40 km, F_{ST} = 0.084).

The Bayesian clustering method in STRUCTURE detected K = 2 as the correct number of clusters in *A. angolensis*. However, the two clusters did not represent any spatially significant grouping of individuals and no samples from any specific sampling sites were strongly

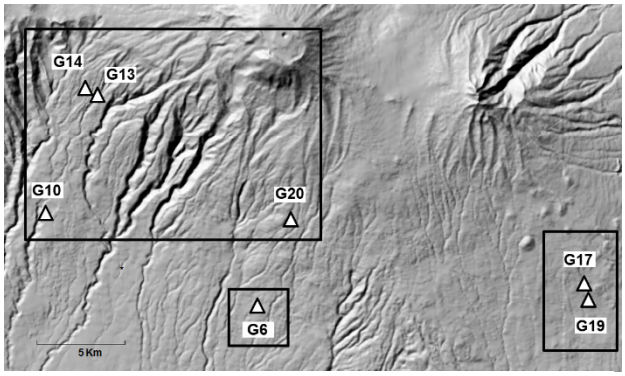


Fig. 3.3 Genetic groups identified in STRUCTURE in the highland species *Amietia wittei*.

assigned. Since the second-order rate of change in log likelihood (Δ*K*) cannot find the best *K* if *K* = 1 (Evanno *et al*. 2005), and there was no clear biological interpretation for the assignments (which were roughly symmetric to all populations), we consequently considered the sampling sites as part of one panmictic population. On the contrary, in *A. wittei* the clustering method identified a total of three populations (Fig. 3.3).

3.4.4 Landscape genetic analysis

Overall, all selection criteria employed in the MLPE method for detecting the best models among all possible combinations were consistent; that is, models with lowest AICc and BIC had also the highest logLik and the lowest REMLdev (Table 3.3).

Table 3.3 Results from MLPE regression analyses calculated by following the procedure in van Strien *et al*. (2012) for *Amietia angolensis* (*Aa*) and *A. wittei* (*Aw*) with the first two best least-cost paths (LCP) and the variables included in each model for each genetic distance (F_{ST} and Φ_{PT}).

Species	Genetic distance	LCP	Variables	AICc	AICc weight	BIC	logLik	REML dev
Aa	F_{ST}	Stream 1:10	settlement (+)	-269.31	0.91	-261.21	139.0	-278.0
		Slope/Stream 1:10	settlement (+)	-266.45	0.97	-258.34	137.6	-275.1
	Φ_{PT}	Stream 1:10	distance (+)	-235.47	0.63	-227.37	122.1	-244.1
		Stream 1:2	distance (+)	-232.93	0.82	-224.83	120.8	-241.6
Aw	F_{ST}	Slope/Stream 1:10	rough (-)	-74.79	0.86	-73.11	42.6	-85.3
		Stream 1:10	rough (-)	-73.04	0.84	-71.36	41.8	-83.5
	Φ_{PT}	Stream 1:10	rough (-)	-61.49	0.81	-59.81	36.0	-72.0
		Rough/Stream 1:10	slope (-)	-57.29	0.11	-55.62	33.9	-67.8

For model selection we used a suite of criteria and measures of fit including AICc, BIC, log-likelihood (logLik) and deviance for the REML criterion (REMLdev). AICc weights represent the values of the corrected Akaike weights.

The null IBD model was never selected as the best model, suggesting that the landscape variables included in our study were important in explaining patterns of genetic differentiation. In *A. angolensis* the least-cost path (LCP) maximizing stream-based movements (Stream 1:10) had the highest support in all analyses (Tables 3.3 and 3.4, Fig. 3.4). Other models with high support included LCPs that maximize movement along streams while minimizing slope. Proportion of settlement and topographic distance were steadily included along the most-supported LCPs revealing a very strong positive relationship of these variables with genetic distances (Tables 3.3 and 3.4).

Table 3.4 Results from the BIOENV procedure for *Amietia angolensis* (*Aa*) and *A. wittei* (*Aw*); for each genetic distance (F_{ST} and Φ_{PT}) the first two best least-cost paths (LCP) are reported with the subset of environmental variables selected by the maximum rank correlation (ρ_W).

Species	genetic distance	LCP	ρ_w	Variables
Aa	F_{ST}	**Stream 1:10**	0.3255	distance
				settlement
		Slope/Stream 1:10	0.3095	distance
				settlement
	Φ_{PT}	**Stream 1:10**	0.4876	distance
				settlement
		Slope/Stream 1:10	0.4515	distance
				settlement
Aw	F_{ST}	**Rough/Stream 1:2**	0.5331	cti
				slope
				erica
		Stream 1:2	0.486	cti
				rough
	Φ_{PT}	**Rough**	0.5334	distance
				slope
				forest
				reforestation
		Rough/Stream 1:2	0.4878	cti
				erica

In *A. wittei* such a strong signal by specific landscape features was less conspicuous and more than one LCP were supported by the two statistical approaches (Tables 3.3 and 3.4).

With the MLPE method, the most-supported LCPs were that maximize movements along streams alone or combined with slope and roughness. In all models the cost ratio of the stream resistance surfaces was 1:10 similarly to *A. angolensis* (Table 3.3). However, in *A. wittei* the variables supported along the best LCPs were roughness and slope, both negatively correlated with genetic distances. The models with the highest rank-correlation coefficients (ρ_W) in the BIOENV procedure were that minimize rough areas, maximize movements along streams (cost ratio 1:2) and a combination of both (Table 3.4). The variables selected in the best models were several and included cti, slope, rough, distance and some habitat types (i.e. *Erica* shrubs, forest and reforestation). However, some of the landscape variables were selected only once suggesting a minor influence or spurious results. Single scatterplots and correlations of genetic distances with these variables revealed non-significant relationships with cti and forest, positive correlation with reforestation and distance, whereas slope, rough and *Erica* shrubs were significantly negatively associated with genetic distances (Table S3.3 Supporting Information).

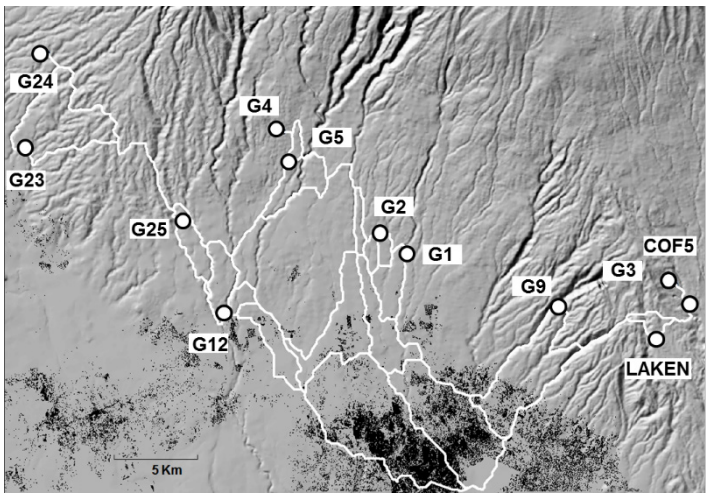


Fig. 3.4 Least-cost paths calculated for the lowland species, *Amietia angolensis,* using a resistance surface that maximizes stream-based movements with a cost ratio 1:10. Background is a shaded relief map with the land cover for settlements (black areas).

3.4.5 Landscape feature distribution across the study area

Overall, landscape variables were not uniformly distributed across the areas of occurrence of the two species (Table 3.5). As expected, human-modified ecosystems were significantly predominant in the lowland, whereas the area occupied by *A. wittei* was characterized by more or less continuous natural forest. Average values of topographic variables were not significantly different between the two areas; mean values of cti were similar, whereas roughness and slope were higher in the area occupied by *A. wittei*, although non-significant (Table 3.5).

Table 3.5 Comparison of landscape variables between areas of occurrence of *Amietia angolensis* (*Aa*) and *A. wittei* (*Aw*). Categorical variables are calculated as correlation lengths (or "area-weighted mean patch radius of gyration", Keitt *et al*. 1997) and standard deviations; continuous variables are expressed as mean values and standard deviations. Significant differences among areas were estimated by examining whether 95% confidence intervals contained zero ("standard" method) or overlapped ("overlap" method, Schenker & Gentleman 2001).

Landscape variable	*Aa*		*Aw*	
	Mean	±SD	Mean	±SD
stream network	3260.05	919.07	3111.59	916.10
cropland	6428.11 *	91.81	189.50	24.76
home gardens	9360.05 *	187.71	3544.67	109.68
settlement	274.35 *	34.08	121.39	28.86
Erica			4396.48 *	76.77
forest	1076.39	44.69	7569.28 *	141.86
disturbed forest	41.90	14.02	1416.31 *	72.58
reforestation	90.50	28.93	103.72	32.46
grassland	184.34	26.66	148.47	31.33
cti	7.76	2.16	7.00	2.01
rough	1.65	0.86	2.30	0.89
slope	7.69	7.71	13.19	9.70

* The variable in the respective area is significantly higher than value from the other area at alpha = 0.05 for both standard and overlap methods. No variables were significant for only one of the two methods.

Even though topographic features were quantitatively similar, they differed in terms of spatial distribution. The Hot Spot analysis revealed that low and high values of cti, roughness and slope were not randomly distributed but clustered in well-defined areas across the mountainside (Fig. 3.5). Specifically, low values of cti were clustered at higher elevations and in the eastern side at lower elevations suggesting low capacity of these areas to retain water which instead concentrates at the foothills (Fig. 3.5a). High values of roughness were spatially clustered at higher elevations, especially on the western side (Fig. 3.5b), and similar spatial patterns were observed for the slope (Fig. 3.5c).

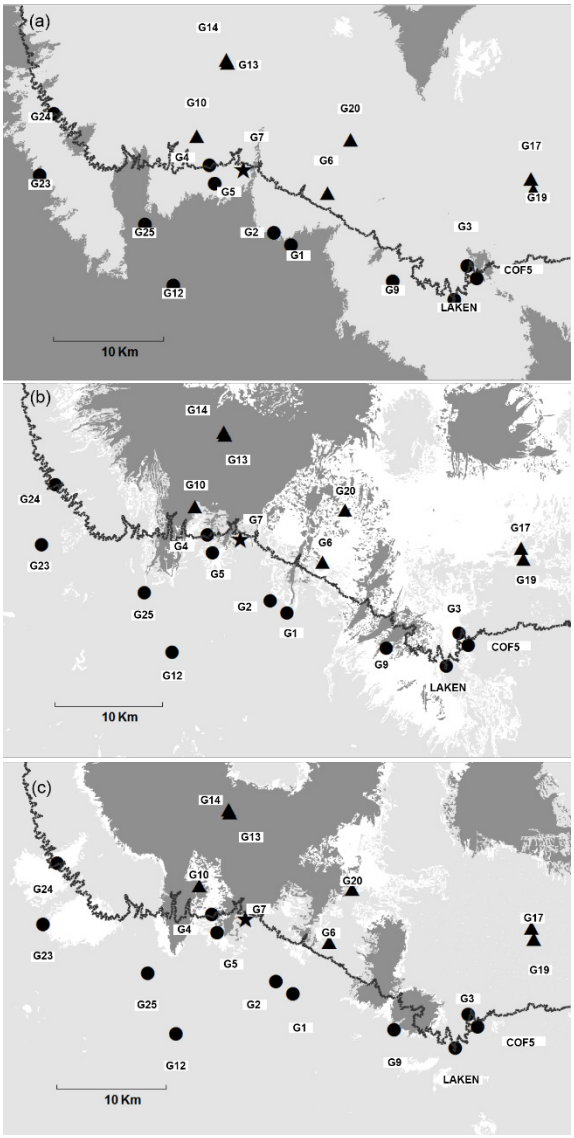


Fig. 3.5 Surfaces resulting from the HotSpot Analysis for (a) the compound topographic index (cti), (b) roughness and (c) slope. For each topographic variable, we identified statistically significant cluster of high (dark grey) and low values (light grey), whereas non-significant areas (white) exhibit a spatially random pattern (i.e. high and low values are randomly distributed; see text for details). To ease comparison between the areas occupied by the two species, the contour of 1700 m is outlined, which approximately corresponds to the National Park boundaries and the upper and lower elevation limits of the lowland and highland species.

3.5 DISCUSSION

Individual dispersal and population genetic structure can result from a combination of physical (e.g. slope, rivers, mountain ridges) and environmental (e.g. land cover, anthropogenic disturbance) attributes, as well as species-specific life-history traits (e.g. movement behavior, reproductive strategies); as a consequence, separating landscape and biological attributes can be extremely challenging (Savage *et al.* 2010). Our results highlight the advantages of using a landscape genetics approach to test for the influence of landscape features on gene flow, especially when there is no information about habitat use of the organisms of interest. Additionally, by using a comparative approach, we found significant differences between ecologically similar species in the response to the landscape that can be attributed to the spatial distribution of landscape variables and differences in dispersal and phenology of each species.

3.5.1 Does genetic diversity decrease with elevation?

Our data show that genetic diversity, as estimated by expected heterozygosity and proportion of polymorphic AFLP markers, does not decrease with increasing elevation in the two study species. Nonetheless, polymorphism (but not heterozygosity) was significantly higher in the lowland *A. angolensis* relative to the upland *A. wittei*, consistent with previous studies that showed higher genetic variation in low altitude populations (Funk *et al*. 2005; Giordano *et al.* 2007). Although these studies analyzed populations of a single species throughout a wide altitudinal range and our study compared two species, we believe our results are comparable with the others. *Amietia* species are morphologically and ecologically similar and hybrids of the two species were found in site G7, suggesting a close phylogenetic relationship. The lower polymorphism and the lack of an elevational pattern of genetic variation across *A. wittei* sites might be due to the low sample sizes. Nonetheless, it appears as though neither of the two species is strongly affected by altitudinal

change as we did not find a significant relationship of genetic diversity with elevation in *A. angolensis* either.

3.5.2 Is gene flow restricted among high elevation populations compare to lowland populations?

Overall, the degree of genetic differentiation among the lowland *A. angolensis* was lower than the highland *A. wittei* populations, although genetic subdivision of the latter species could have been overestimated due to the low number of populations sampled. Mean values of F_{ST} and Φ_{PT} were significantly lower in *A. angolensis* suggesting higher gene flow in this species. Bayesian clustering analysis revealed a lack of population structure suggesting the possibility of panmixia among our sampling sites. However, STRUCTURE may not accurately infer the correct number of populations (*K*) if genetic structure was generally low (Safner *et al.* 2011).

Several factors can contribute to enhance gene flow in this species. *A. angolensis* is a generalist species able to successfully reproduce in a wide array of water bodies (Channing & Howell 2006). As shown by the Hot Spot analysis, lowland areas have much higher water retention capacity than higher elevation areas. The glacier on top of Kibo ensures a continuous water supply to the lowland where a great variety of water bodies provide suitable habitats and potential breeding sites. In addition, the intense network of natural and man-made water canals offers ways for dispersal as we frequently observed one or few tadpoles in portion of canals unsuitable for breeding (e.g. fast flowing water). During field work, we had a high probability of detecting *A. angolensis* near water bodies (G. Zancolli, unpubl.). Moreover, we observed the simultaneous presence of *A. angolensis* tadpoles at different developmental stages during the sampling period. Thus, the common river frog appears not to have seasonal reproductive strategies contrary to most temperate anuran species. Considering that *A. angolensis* has a very prolonged larval stage (Channing 2004), we believe tadpoles passively disperse, especially when floods occur during rainy seasons. After metamorphosis, however, our data

suggest frogs actively move upstream or downstream along canals and streams.

Amietia wittei showed lower levels of gene flow than *A. angolensis*, consistent with the valley-mountain model proposed by Funk *et al*. (2005) for the Columbia spotted frog (*Rana luteiventris*); in both studies, high elevation populations show greater subdivision relative to low elevation populations. However, genetic differentiation in *A. wittei* as estimated by F_{ST} was lower than that among high elevation populations of *R. luteiventris*. F_{ST} values were also lower that those estimated among high elevation populations of another temperate amphibian, the long-toed salamander (Giordano *et al*. 2007). Generally, estimates of F_{ST} calculated from AFLP are much higher than from microsatellites which have a higher mutation rate than AFLP (Curtis & Taylor 2003; Gaudeul *et al*. 2004); therefore, the level of genetic differentiation in *A.wittei* appears even lower compared to high populations of other temperate species.

We expected higher levels of genetic connectivity among *A. wittei* populations because the species largely occupies a continuous natural area. As such, it appears that factors other than human disturbance influence gene flow in this frog species. Streams and other lotic water bodies are fast flowing at high elevations on Mt. Kilimanjaro, and the high concentration of steep slopes, crests and ridges tends to limit the abundance of potential breeding sites. Field observations support this result, with far fewer animals detected than the lowland species with similar effort (G. Zancolli, pers. obs.). Breeding in this species seemed much more seasonal than its lowland congener, which may contribute to higher degrees of reproductive isolation due to more limited recruitment. It is also possible that this species may have more specific habitat requirements than *A. angolensis*. Further studies that focus on breeding phenology of this species could help elucidate which environmental or climatic variables influence breeding timing and, in turn, the observed pattern of genetic structure.

3.5.3 How do topography and land-use change affect connectivity?

Least-cost path analysis revealed several insights into landscape variables that affect genetic connectivity of the two study species. In *A. angolensis* all statistical approaches yielded almost identical results, with LCPs maximizing stream-based movements as the best models. The second most supported LCPs combined streams and slope suggesting that common river frogs, when moving overland, tend to avoid steep slopes. Additionally, the presence of human settlements appears to restrict gene flow. By following water courses, frogs will eventually cross Moshi, the main town at the foothills of Mt. Kilimanjaro, or the surrounding settlements. A reasonable scenario is that tadpoles may be moved passively downstream during floods into the settlement area where the high mortality rate would reduce dispersal (and hence gene flow). When looking at stream-based LCPs in Figure 3.4, many possible routes, especially the paths connecting the central populations with the eastern ones, cross the urban area. For instance, the route connecting G1 and G3 crosses areas with intense settlements and the two populations are moderately differentiated (F_{ST} = 0.103; see Fig. 3.1 and 3.4). However, the path between G1 and G23 does not cross urban patches and the two populations are genetically more similar (F_{ST} = 0.063) even if they are farther away.

In *A. wittei,* a greater number of LCPs were selected as best models. Similarly to the lowland species, *A. wittei* prefers movement along streams and across gentle slopes. The inclusion of topographic variables in the best models indicates that topography plays an important role in shaping genetic differentiation in this species. Within the National Park, water bodies are not connected by a canal network as in the lowland, and rivers are isolated and separated by parallel ridges. Thus, dispersal may not be limited to stream corridors but it may occur overland by adult individuals. Such a scenario would explain the negative correlation of roughness and slope with genetic distance, and the results of the Hot Spot analysis contributed to understand this relationship. Although landscape variables at high and low elevations are not significantly different in terms of mean values, steep slopes are clustered at high altitude. Consequently, the montane species is more likely to encounter steep

slopes when leaving a breeding site, resulting in higher exposure to rough areas during dispersal. Notably, Bayesian clustering analysis grouped together G10, G13, G14 and G20 which are located in an area characterized by high values of slope and roughness (see Fig. 3.3 and 3.5), suggesting that the parallel valleys and ridges found there do not act as barrier to gene flow. A strong association of stream amphibian species with steep gradients is also clearly established in other studies (Corn & Bury 1989; Diller & Wallace 1999; Adams & Bury 2002; Spear & Storfer 2008).

The use of two statistical methods for detecting the influence of various landscape variables on gene flow yielded similar results with no major discrepancies. However, the BIOENV procedure included some variables which were not selected by the MLPE analysis. Individual analyses of genetic distances with these variables revealed some non-significant relationships; hence, any inference about the influence of the landscape variables selected by the BIOENV on gene flow is limited. The low number of sampled populations in *A. wittei* may also explain the inclusion of non-significant variables in the best models. Further, BIOENV has been implemented as an exploratory procedure for an independent analysis of consistency, and it has the drawbacks of lacking for significant testing and calculation of partial correlation coefficients. Although it has been implemented in other landscape genetics studies, BIOENV often selected different best models and variables than multiple regressions (e.g. Spear *et al*. 2005; Trumbo *et al*. 2013).

3.5.4 Conclusions

Based on a climatic-physiological model, Janzen (1967) hypothesized that mountain passes are greater barriers to organismal dispersal in the tropics than in temperate regions; furthermore, he predicted that tropical organisms should have 1) reduced dispersal across elevational gradients and 2) restricted altitudinal ranges compare to species in temperate regions. Strong support for the second hypothesis comes from studies conducted on a wide array of taxa that show narrower elevational distributions among tropical species than those

of temperate species (see references in Ghalambor *et al*. 2006; McCain 2009). On the contrary, few studies investigating patterns of gene flow along elevational gradients are available for tropical regions to test the first part of Janzen's hypothesis (Ghalambor *et al*. 2006; Storfer *et al*. 2010).

We tested the influence of altitude and topography on genetic variability and connectivity of two tropical species of river frog and found low to moderate genetic differentiation suggesting that topographic relief does not reduce gene flow in these species. Similar results have been observed in another generalist breeding amphibian, the Chinese wood frog (Zhan *et al*. 2009), in a direct-developing tropical leaf-litter frog (Measey *et al*. 2007), and in the stream-breeding coastal tailed frog (Spear & Storfer 2008). Pond-breeding amphibians, however, are generally poor dispersers and highly site philopatric as shown for the natterjack toad (Rowe *et al*. 2000), long-toed salamander (Tallmon *et al*. 2000; Giordano *et al*. 2007), Columbia spotted frog (Funk *et al*. 2005; Murphy *et al*. 2010), blotched tiger salamander (Spear *et al*. 2005), and southern long-toed salamander (Savage *et al*. 2010). Thus, life-history traits such as reproductive strategies might exert a stronger structuring force than latitudinal variation in amphibians. Nonetheless, caution must be taken when making such a generalization; most population and landscape genetics studies differ in spatial scale investigated, molecular markers used, genetic differentiation estimates and analytical approaches. Moreover, landscape features do not have a homogeneous impact on individuals of either similar species (this study) or even within the same species in different parts of its geographic range (Short Bull *et al*. 2011; Trumbo *et al*. 2013). Our work highlights the need for further landscape genetics studies of tropical species along elevational gradients to further test Janzen's hypotheses.

3.6 SUPPORTING INFORMATION

Appendix S3.1 AFLP protocol including PCR conditions.

For each sample, 5 µL DNA (concentration 20-100 ng/µl) were digested with 5 U *Eco*RI (NEB) and 5 U *Mse*I (NEB) in 25 µL total volume of 1 x buffer 4 (NEB) and 1 x BSA (NEB) for 2 h at 37°C, followed by 20 min at 65°C for enzyme inactivation. A solution of *Eco*RI adaptor (5 pmol), *Mse*I adaptor (50 pmol), 1 U of T4 DNA ligase (Invitrogen) and 5 µL of T4 buffer was added and samples incubated overnight at 16°C. Pre-selective amplifications were performed using 5 µL of the ligated DNA in final volumes of 20 µL containing 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.2 µM pre-selective primers, 1 x PCR buffer and 0.5 U of *Taq* polymerase (Invitrogen). Cycling conditions were: initial incubation for 2 m at 72°C, 25 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 m, followed by a final elongation at 72°C for 2 m and 60°C for 30 m. For selective amplifications we used 3 µL of 10 x diluted pre-amplified product, 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.15 µM *Eco*RI and 0.2 µM of *Mse*I selective primers, 1 x PCR buffer II and 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 10 µL. EcoRI primers were labelled with FAM and HEX (Metabion). Cycling conditions were: 95°C for 8 min for *Taq* activation, 10 cycles touchdown of 94°C for 20 s, 66°C for 30 s (decreasing 1°C per cycle), 72°C for 2 min, followed by 23 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 2 m and ending with elongation for 2 m at 72°C and 30 m at 60°C.

Table S3.1 Pairwise F_{ST} (upper diagonal) and Φ_{PT} (lower diagonal) values between *Amietia angolensis* populations.

F_{ST} / Φ_{PT}	COF5	G1	G12	G2	G23	G24	G25	G3	G4	G5	G9	LAKEN
COF5	-	0.093	0.155	0.090	0.122	0.142	0.096	0.035	0.088	0.196	0.072	0.040
G1	0.143	-	0.053	0.043	0.064	0.067	0.046	0.103	0.025	0.074	0.043	0.080
G12	0.197	0.059	-	0.100	0.092	0.076	0.064	0.142	0.063	0.075	0.087	0.126
G2	0.140	0.076	0.115	-	0.059	0.086	0.026	0.081	0.039	0.156	0.054	0.084
G23	0.187	0.115	0.132	0.082	-	0.033	0.033	0.114	0.074	0.154	0.084	0.125
G24	0.208	0.105	0.086	0.126	0.055	-	0.051	0.126	0.069	0.130	0.091	0.123
G25	0.145	0.065	0.048	0.043	0.046	0.062	-	0.075	0.032	0.136	0.067	0.090
G3	0.042	0.157	0.172	0.126	0.172	0.185	0.123	-	0.087	0.209	0.049	0.024
G4	0.141	0.040	0.071	0.075	0.112	0.096	0.053	0.144	-	0.071	0.073	0.079
G5	0.245	0.078	0.114	0.176	0.184	0.149	0.131	0.250	0.048	-	0.137	0.164
G9	0.091	0.089	0.116	0.082	0.149	0.154	0.089	0.063	0.118	0.200	-	0.037
LAKEN	0.065	0.145	0.183	0.157	0.219	0.213	0.155	0.047	0.152	0.241	0.058	-

Table S3.2 Pairwise F_{ST} (upper diagonal) and Φ_{PT} (lower diagonal) values between *Amietia wittei* populations.

F_{ST} / Φ_{PT}	G10	G13	G14	G17	G19	G20	G6
G10	-	0.071	0.061	0.154	0.126	0.085	0.137
G13	0.122	-	0.012	0.124	0.098	0.075	0.114
G14	0.113	0.024	-	0.116	0.106	0.048	0.106
G17	0.247	0.181	0.182	-	0.060	0.139	0.167
G19	0.204	0.159	0.178	0.100	-	0.129	0.150
G20	0.140	0.134	0.109	0.209	0.182	-	0.148
G6	0.225	0.176	0.169	0.255	0.229	0.229	-

Table S3.3 Pearson's correlation coefficients between genetic distances (F_{ST} and Φ_{PT}) and landscape variables selected in the best BIOENV models for *Amietia wittei* (see Table 3.4 and text). Simple linear regressions were run for each variable (bold values for $p < 0.05$).

LCP	Variable	F_{ST}	Φ_{PT}
Rough/Stream 1:2	cti	0.001	0.012
	slope	**-0.558**	-
	erica	**-0.675**	**-0.680**
Stream 1:2	cti	0.126	-
	rough	**-0.442**	-
Rough	distance	-	**0.515**
	slope	-	**-0.620**
	forest	-	0.145
	reforestation	-	0.518

Fig. S3.1 Boxplot showing the differences in (a) expected heterozygosity (or Nei's gene diversity, *Hj*), and (b) proportion of polymorphic loci at the 5% level expressed as percentage (%P) between *Amietia angolensis* (*Aa*) and *Amietia wittei* (*Aw*).

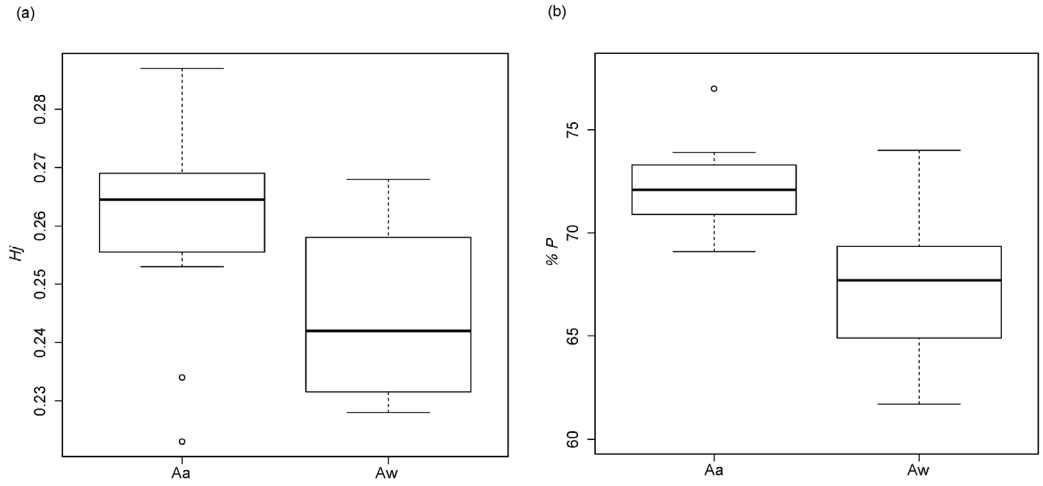
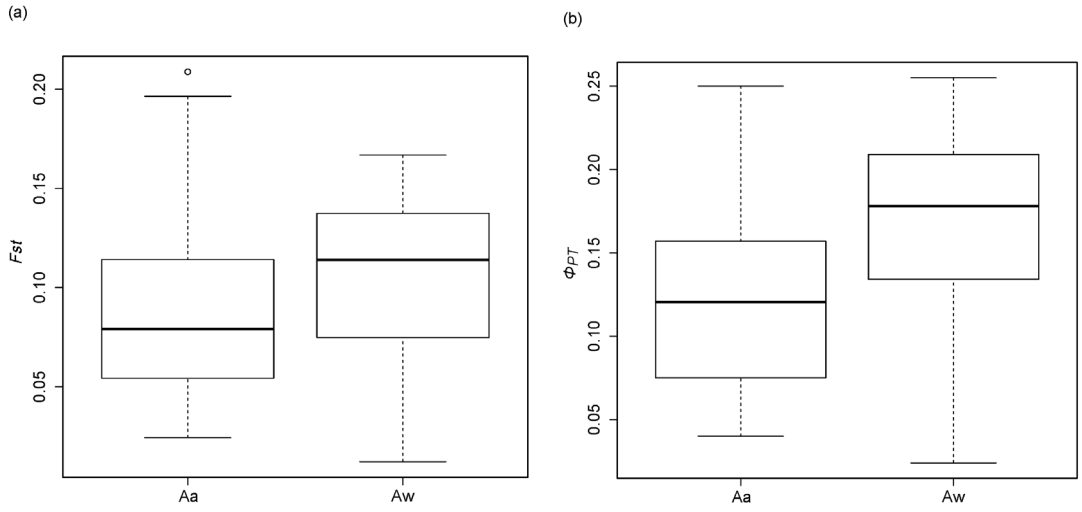


Fig. S3.2 Boxplot showing the differences in (a) F_{ST} and (b) Φ_{PT} between populations of *Amietia angolensis* (*Aa*) and *Amietia wittei* (*Aw*).



Chapter 4

Detection of *Batrachochytrium dendrobatidis* in river frogs (genus *Amietia*) on Mount Kilimanjaro, Tanzania[1]

Table of Contents

[1] This chapter is accepted as: Zancolli G., Storfer A., Rödel M.O. "Detection of *Batrachochytrium dendrobatidis* in river frogs (genus *Amietia*) on Mount Kilimanjaro, Tanzania". *Herpetological Review* (in press).

4.1 INTRODUCTION

Fig. 4.1 Mount Kilimanjaro, Tanzania, where *Batrachochytrium dendrobatidis* (*Bd*) was detected. Records of *Bd* infections from the Eastern Arc Mountains are still poor (www.bd-maps.net, accessed 16 April 2013).

Within Africa, the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been reported from southern, eastern and central parts of the continent, whereas it is currently undetected in West Africa (Penner et al. 2013; see citation therein). Although *Bd* has been found in some African species without aquatic larval stages (Bell et al. 2011; Conradie et al. 2011a; Gower et al. 2012), the majority of species affected by the fungus are from high altitude and inhabit mostly flowing streams (Murray and Skerratt 2012). River frogs of the genus *Amietia* seem to be particularly susceptible, showing the highest zoospore loads known among African frogs in Kenya (Kielgast et al. 2010), Malawi (Conradie et al. 2011a), South Africa and Lesotho (Weldon 2005). *Bd* infection also has been detected in Uganda (Viertel et al. 2012), yet records from the Udzungwa Mountains in Tanzania have not detected *Bd* (Moyer and Weldon 2006). Here, we report the occurrence of *Bd* in *Amietia angolensis* and *Amietia wittei* from Mt. Kilimanjaro, northern Tanzania.

4.2 METHODS

4.2.1 Study area

Mount Kilimanjaro is the remnant of a large volcano located on the border with Kenya (3.07583°S, 37.35333°E; Fig. 4.1). It rises from a hot and dry savanna plain at 700 m to an iced-clad summit of 5895 m, thereby encompassing several different bioclimatic zones. At 1700 m, the borders of the National Park delimit the montane forest, whereas the lower part of the slope has been almost entirely converted into a traditional agroforestry system (mainly banana and coffee trees) and monocultures (mainly maize, sunflowers and beans). *Amietia angolensis* inhabits the lowland (1000 - 1700 m), whereas *A. wittei* occurs from 1700 m to 3500 m on the Shira Plateau (G.Z. unpubl. data).

4.2.2 Sampling and analysis

Samples for *Bd* analysis were collected in 2011 (from March to June – long rains; October and November – short rains) during surveys of *Amietia* tadpoles for other research purposes. Since we observed tadpoles with jaw sheaths and tooth rows completely colorless (Fig. 4.2d), we gently brushed the mouthparts with common cotton swabs and stored them dry in safe-lock tubes. Diagnostic analysis was performed in October 2012 at the Amphibian Disease Diagnostic Center at Washington State University (Pullman, WA, USA). Fungal DNA was extracted using DNeasy extraction kits (Qiagen). Detection and quantification of *Bd* was performed using standards (1000, 100, 10, 1 and 0.1) from serial dilutions by means of RT-qPCR according to Boyle et al. (2004).

4.2 RESULTS

Although sample size was low (N = 17), we detected *Bd* in at least 40% of the specimens for both species (Table 4.1). Overall, the *Bd* infection levels appeared low in individual samples (< 5 zoospore equivalents). We believe these results do not reflect realistic levels of pathogen load but they are very likely due to a combination of technical factors. First, this non-lethal technique for detecting *Bd* on tadpoles is less effective than histological diagnosis (Retallick et al. 2006). In their research, the authors scraped the tadpoles' mouthparts with wooden toothpicks whereas we used swabs which are less invasive (i.e., they are less likely to damage mouthparts and influence the ability of tadpoles to feed). However, swabs may not collect *Bd* DNA as effectively as toothpicks, which regularly break pieces of labial teeth (Retallick et al. 2006). Second, during field work, samples were stored in a freezer to avoid high temperatures, but the power supply at the field station was irregular resulting in frequent black outs and consequent thawing of the freezer's contents. High temperatures and long storage periods can result in reduced recovery of *Bd* DNA from swabs (van Sluys et al. 2008). These factors likely induced to degradation of DNA in our samples; still, we were able to detect *Bd*, suggesting that the pathogen load may have been high originally.

Table 4.1 *Batrachochytrium dendrobatidis* infection data for *Amietia angolensis* and *Amietia wittei* from Mount Kilimanjaro, Tanzania. All samples are from larvae. Because of the extremely low amount of *Bd* DNA, it was not possible to determine *Bd* presence with absolute certainty for some samples. Uncertain results are reported as suspect positive (susp.pos.) or negative (susp.neg.).

Species	Dates	Localities	Coordinates	Elevation
A. angolensis	18-10-11	Waramu river	3.1959°S 37.2555°E	1532
A. angolensis	18-10-11	Nkuu	3.1808°S 37.2511°E	1692
A. angolensis and *A. wittei*	29-10-11	Umbwe route	3.1845°S 37.2789°E	1705
A. wittei	05-05-11	Mweka route	3.2038°S 37.3485°E	1992
A. wittei	26-10-11	Marangu route	3.2018°S 37.5175°E	2359
A. wittei	01-11-11	Machame huts	3.0967°S 37.2667°E	3023
A. wittei	03-11-11	Machame huts	3.0940°S 37.2657°E	2978

4.3 DISCUSSION

This is the first record of *Bd*-positive *Amietia* from Tanzania. All specimens presenting depigmentation of keratinized mouthparts were *Bd*-positive; however, the sample size was too low for a statistically significant correlation between oral deformity and *Bd* presence. During the whole field season, we observed larval deformities with similar gross morphology at other sites but we never encountered dead or moribund adults of *Amietia* nor of other species occurring in the area.

(continued from previous page)

Water bodies and Habitat	N *Bd*-positive samples	N samples	Zoospore equivalents (min-max)	Mouthparts
stream cultivated area	0	1		normal
creek cultivated area	4 (2 susp.neg.)	5	0.001-2.621	depigmented
stream disturbed forest	0	1		normal
creek montane forest	0	1		normal
stream montane forest	1 (susp.pos.)	1	0.087	depigmented
creek moorland	2 (1 susp.neg.)	5	0.004-1.526	depigmented
stream moorland	3 (1 susp.pos.)	3	0.014-1.656	depigmented

Similar observations of a general lack of mortality among *Bd* infected animals have been reported in other studies from Africa, including Kenya (Kielgast et al. 2010), Uganda (Goldberg et al. 2007) and South Africa (Conradie et al. 2011b). The high prevalence of *Bd* and apparent low virulence may suggest that the pathogen is enzootic within those regions, thereby providing support for the "out of Africa" hypothesis (Weldon et al. 2004). However, a lack of connection between *Bd* presence and population decline has been reported elsewhere, for instance in eastern North America (Longcore et al. 2007). The question about the origin of *Bd* still remains open.

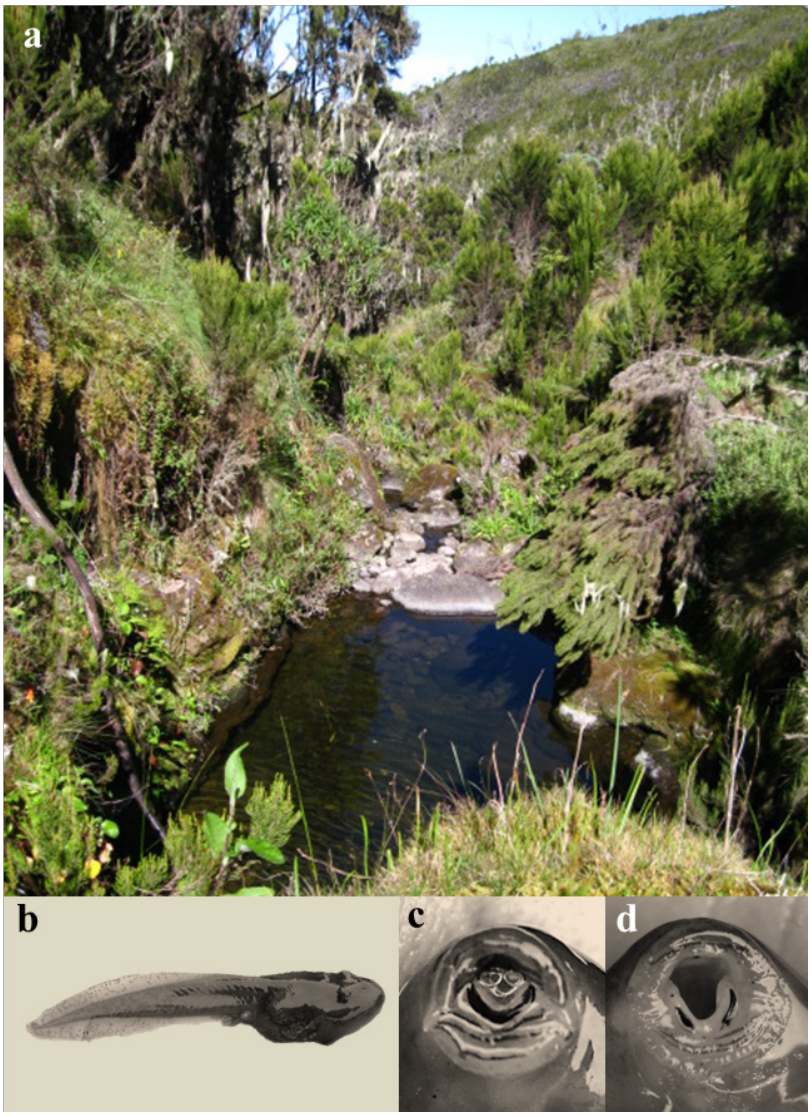


Fig. 4.2 Typical creek within the subalpine zone at ca. 3000 m, Kilimanjaro National Park, Tanzania; a: breeding site of *Amietia wittei* close to the Machame Huts campsite where porters collect water; b: larvae of *Amietia wittei*; c: oral disc with unchanged morphology; d: mouthparts with depigmentation of jaw sheaths and labial teeth.

Kielgast et al. (2010) suggested a taxonomic basis for variation in apparent *Bd* susceptibility among host species, and the genus *Amietia*, in particular, showed the highest pathogen load and prevalence in their study. Other studies have also provided support for aquatic life histories contributing to *Bd* occurrence patterns (e.g., Bancroft et al. 2011; Olson et al. 2013). During our surveys, we regularly observed tadpoles of both species in the water. *Amietia angolensis* adults are semi-aquatic and active throughout the year (Channing and Howell 2006); the tadpoles have a prolonged development and may take

up to two years to complete metamorphosis (Channing 2004). Thus, river frogs can act as reservoirs for *Bd* and facilitate disease persistence in the system. Moreover, *Amietia* species cover most of the elevational range of Mt. Kilimanjaro and this might allow *Bd* to survive during unfavorable conditions within spatially disparate microhabitats (e.g., by persisting at higher elevations during summer and at lower elevations during winter).

Local dispersal of *Bd* may be facilitated by humans. Kilimanjaro National Park is tourist attraction with over 35,000 climbers a year (Peaty 2012), and each climber has at least three porters to conform with park regulations. The first campsites encountered along the climbing routes are usually allocated at the upper ridge of the forest (ca. 3000 m) and close to ponds or streams which are used by *A. wittei* for breeding (G.Z., unpubl. data). We observed porters collecting water (e.g., for cooking and human consumption) at the same water bodies where tadpoles tested *Bd*-positive. Porters are able to walk through the mountain for long distances within a day, and this may translocate zoospores from one site to another. Below the National Park borders, the slope is characterized by an extensive network of water canals built by the local human population for irrigation, and by which *Bd* may disperse, or river frogs or other transmission vectors may spread the pathogen (Morgan et al. 2007; Johnson and Speare 2005).

Our results showed the presence of *Bd* infection in river frog populations on Mt. Kilimanjaro. Considering the high susceptibility of *Amietia* species and their potential to serve both as *Bd* reservoirs and vectors to nearby areas (especially the lowland *A. angolensis*), it would be beneficial to intensify surveys for *Bd* detection in the East African highlands and in particular in the Eastern Arc Mountains. The few data available to date are from the southern Udzungwa Mountains, where *Bd* has been detected in 12 species (Moyer and Weldon 2005; www.Bd-maps.net, accessed 16 April 2013), and from the northern East Usambara Mountains with negative records dated 2006 (www.Bd-maps.net). This hotspot of biodiversity harbors an outstanding number of endemic species (Burgess et al. 2007), which may still be naïve to the fungus and thus suffer of population declines if the infection reaches those forests.

Chapter 5

Discussion

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Within my doctoral thesis I investigated the influence of landscape processes on biological diversity at different levels, from communities to species, populations and genes. The animal group involved in the study system, anurans and river frogs in particular, revealed singular, yet unexpected patterns and shed light on the historical and contemporary processes underlying current spatial distribution of biodiversity.

5.1 AMPHIBIAN DIVERSITY – A HISTORICAL PERSPECTIVE

Biodiversity is not uniformly distributed across the Earth but there are areas where exceptional concentrations of species are found in relatively restricted area (Myers *et al*. 2000; Wiens 2012). In order to understand the origins of species-rich regions and the spatial distribution of species richness in general, it can be crucial to consider the climatic, geological and other historical environmental events that interested that region in the past and that could have contributed in shaping the current patterns (Brown 2001). For instance, a region may be more diverse simply because it has been inhabited longer (e.g. Stephens & Wiens 2004). In chapter 2, I incorporated historical biogeography to understand the assembly of ecological communities and inferring patterns and timing of colonization (Wiens 2012). By comparing the assemblage composition of anurans found on Mt. Kilimanjaro with those on other East African mountains I found that Mt. Kilimanjaro and Mt. Meru lack of true forest specialists and species endemic of the Eastern Arc Mts. (such as the many endemic dwarf toads, *Nectophrynoides*, or various microhylid genera). This pattern seems to be driven primarily by the geological time of these mountains: The Eastern Arc is a chain of ancient crystalline mountains initiated 290-180 Mya, whereas Mt. Kilimanjaro and Mt. Meru are recent volcanoes estimated to be ca. one Myr (Griffiths 1993). Climatic changes during the Quaternary, or even before, caused withdrawal of cool adapted species which, influenced also by topographical features, contracted their ranges to isolated relict patches. When the volcanoes finally cooled down and were "available", the few forest species on the nearby mountains may have been already too isolated to be

able to cross the hostile matrix which separates them from the volcanic mountains. Climatic, geological and topographic processes are thus primary drivers of the spatial distribution of species richness and distribution of Eastern African amphibians. The observed patterns of species distribution along the slope of Mt. Kilimanjaro, that is a drastic decline in species richness with increasing elevation and a lack of signal with increasing anthropogenic disturbance, are also best understood in the light of historical biogeography.

5.2 ALTITUDINAL GRADIENT OF SPECIES DIVERSITY

The amphibian fauna of East Africa and Tanzania in particular, is well-known to have a larger number of lowland species and a less conspicuous, yet extremely diverse, cool-adapted forest fauna (Poynton 1962, 1999, 2003; Poynton & Boycott 1996). This pattern is not surprising as the Tanzanian landscape is dominated by expanse savanna where herds of large mammals attract tourists from all over the world. Within this hot and dry matrix a number of scattered and isolated mountains offer cool and moist climate to what was once a more or less continuously distributed rainforest across tropical Africa (Lovett 1993). Such forest relicts harbor an incredible and unique fauna, with some species confined on one single mountain block (e.g. many species in the genus *Nectophrynoides*).

Along the southern slope of Mt. Kilimanjaro the species distribution reflects the altitudinal turnover observed across Tanzania with the transition from tropical to temperate fauna. Species richness sharply declines with increasing elevation and a surprisingly low number of species are found in the montane forest. The historical biogeographic analysis gave an insight into the processes underlying this pattern. Because of the young age of Mt. Kilimanjaro, the moist forest fauna, already retracted on the Eastern Arc Mountains, was not able to cross and colonize the volcano. Only those species with high dispersal abilities (e.g. *Amietia* spp., see chapter 3) were able to reach the moist rainforest. Furthermore, the montane forest on Mt. Kilimanjaro encompasses

altitudes much higher than that of the Eastern Arc Mountains, consequently some species may have failed to reach or survive at such elevations.

5.3 SPECIES DIVERSITY AND HUMAN DISTURBANCE

Historical biogeography also gave an insight into the current pattern of species richness and composition in relation to environmental changes. Historical processes underlying the lack of vulnerable forest species likely biased the outcome of the disturbance analysis. That is, I was not able to detect effects of either anthropogenic disturbance or vegetation structure on species richness and composition (see Table 2.1) because the species pool of Mt. Kilimanjaro and surrounding area is mainly composed by somewhat tolerant, widespread species. Nevertheless, I cannot rule out that the long-standing presence of human settlements and the loss of natural habitat did not have a negative impact on the amphibian fauna. For the last 2000 years humans have gradually altered the original vegetation insomuch as the lower montane rainforest is nowadays almost entirely converted into croplands; therefore, we cannot know if local extinctions took place in the past. Nevertheless, before making any generalization on organismal responses to habitat degradation and land use change, it is important to include historical biogeographic analyses given that current patterns of biodiversity may be the result of historical and not contemporary or recent events.

5.4 GENETIC DIVERSITY

Terrestrial animal species and amphibians in particular, are expected to have limited genetic connectivity in topographically complex landscapes and a decrease of genetic diversity with increasing elevation as a consequence of restricted gene flow among high elevation populations and smaller population sizes. This "valley-mountain" model was proposed for the Columbia spotted frog, *Rana luteiventris* (Funk *et al.* 2005), and subsequently confirmed in another temperate pond-breeding amphibian (Giordano *et al*. 2007). Two important observations arise regarding this model: first, it seems to work well for seasonal pond-breeding amphibians but less likely for species with different reproductive strategies, as a study of the more generalist wood frog *Rana chensinensis* showed high levels of gene flow across a large mountainous region in northern China. Second, it was tested almost exclusively on temperate species. In chapter 3 I tested the valley-mountain model on a pair of sister taxa of tropical stream dwelling frogs along the southern slope of Mt. Kilimanjaro and I found contradicting results. In agreement with the model, high elevation populations (*Amietia wittei*) were more differentiated than the lowland counterpart (*Amietia angolensis*); however, the overall F_{ST} values of *A. wittei* were much lower compared to that of the Columbia spotted frog in Funk *et al*. (2005), suggesting that *A. wittei* populations are more connected than expected by the mountain-valley model. Moreover, genetic variation, even though higher in the lowland species, did not decrease with increasing elevation. Finally, Janzen (1967) proposed that tropical organisms should have reduced dispersal along elevational gradients compared to temperate species; yet, researchers have not confirmed this hypothesis so far. My study did not support Janzen's prediction; the level of gene flow in the two studied species was overall high to moderate, suggesting that dispersal is not limited by changes in elevation in these tropical species.

5.5 CURRENT THREATS TO AMPHIBIAN DIVERSITY ON MT. KILIMANJARO AND THE EASTERN ARC MOUNTAINS

Across the study area I found the highest species diversity in proximity of few water pools surrounded by a vast maize field; as such, this result is somehow alarming. During the main rainy season, some depressions are filled by rains and become the main attraction of the local anuran fauna; that is, the presence of water offers sites for reproduction. The colline fauna (see chapter 2) is indeed prevalently composed by pond-breeding species characterized by an extremely short reproductive season. Thus, the survival of the local assemblage relies almost entirely on these ephemeral water bodies. The increasing demand for food and the ongoing intensification of land conversion into monocultures could result in reclaiming those areas to gain few squared meters for crops, with the dramatic consequence of extinction of the local fauna. In concert with habitat loss, climate change contributes to threatening the biodiversity of Mt. Kilimanjaro. For instance, the 2011 rainy season was exceptionally dry; at the foothill, swamps which are regularly used by savanna specialists for reproduction were almost completely dry, and in some sites I found mummified tadpoles (Fig. 5.1). Also in the forest the water level of some creeks was particularly low for the season, and this may have compromised the reproductive success of the local species. The 2011 may have been an exceptional year; yet, 85% of Kibo's ice cover has disappeared in the last century, making Mt. Kilimanjaro a worldwide recognized symbol of global climate change (Thompson *et al*. 2009). Increasing temperature, decreasing precipitation and air humidity are superimposed processes driving the current shrinking of the glacier (Thompson *et al*. 2009). Moreover, enhancement of fires, forest destruction, land use intensification, soil erosion and decline of water resources are ongoing environmental changes undermining the overall Kilimanjaro's ecosystems and biodiversity (Agrawala *et al*. 2003).

A direct evidence of the negative impact of anthropogenic disturbance on natural animal populations was the detection of restricted gene flow among

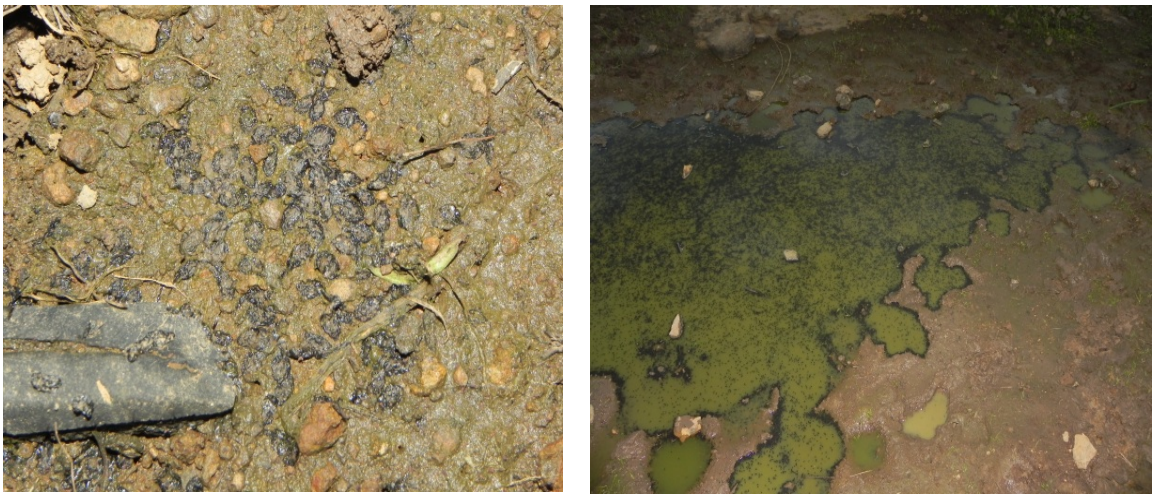

Fig. 5.1 Mummified anuran larvae (left) and drying puddle with early stage larvae (right) found in a maize field (ma1, see chapter 2) during the long rainy season in 2011. Arising temperature and reduction of precipitations especially are a serious threat to tropical amphibians, in particular species that reproduce in ephemeral water bodies.

A. angolensis populations across human settlements (chapter 3). As explained in the introduction, two mechanisms are essential for generating and regaining lost genetic diversity, namely mutation and migration (i.e. gene flow). While mutations typically occur at a very low rate (Franham *et al*. 2010), gene flow has immediate effect where connectivity among populations is not compromise. In my study system, human settlements seemed to reduce gene flow among populations of the common river frog. Considering that *A. angolensis* is a tolerant, generalist, highly mobile widespread species, we might likewise expect similar or even more deleterious effects on less mobile and more vulnerable anuran species.

Another important finding of this study which rises concern is the detection of the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), in *A. angolensis* and *A. wittei* tadpoles. We do not know whether other species are also affected by chytridiomycosis, but considering that *Bd* has the highest growth rate at temperature between 17° and 25° C (Johnson & Speare 2003) and a low virulence already at 22°C (Andre *et al*. 2008), we can exclude the possibility of infection of the species-rich lowland fauna. Although I did not observed moribund or dead frogs during the surveys in 2011, the presence of *Bd* on Mt. Kilimanjaro has important implication and may represent a threat to

susceptible and endangered species in nearby areas. As I discussed in chapter 4, Mt. Kilimanjaro is a highly touristic attraction with thousands of tourists attempting to climb the mountain every year. Campsites along the touristic routes are usually close to pools or creeks for water supply, the very same sites were I detected *Bd* presence. The chytrid fungus is known to survive without a host for long periods (Johnson & Speare 2003), and besides its own movement or by the activity of infected amphibians, it may be transported by other vectors such as footwear of hikers from contaminated water and soil. Tourists visiting Mt. Kilimanjaro may translocate *Bd* zoospores to other areas such as the nearby Eastern Arc Mts. where endemic and vulnerable species may still be naïve to the fungus and thus suffer of population declines.

This study significantly contributed to the knowledge of the amphibian fauna of Mt. Kilimanjaro and of East Africa in general, and it represents a valuable tool for future conservation actions and measures. For instance, local authorities may inform farmers and estate managers about the importance of maintaining water pools and swamps in their properties. Likewise, the Kilimanjaro National Park officers and park rangers could inform tourists on the importance of taking necessary precautions for limiting the spread of the deadly chytrid fungus and avoid contributing to the general global loss of biodiversity and of amphibians in particular.

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List of publications

- **Zancolli G**., Rödel M.O., Steffan-Dewenter I. & Storfer A. (in review) Comparative landscape genetics of two river frog species along an altitudinal gradient on Mount Kilimanjaro. *Molecular Ecology*.
- **Zancolli G.**, Storfer A. & Rödel M.O. (in press) Detection of *Batrachochytrium dendrobatidis* in river frogs (genus *Amietia*) on Mount Kilimanjaro, Tanzania. *Herpetological Review*.
- **Zancolli G.**, Steffan-Dewenter I. & Rödel M.O. (in review) Amphibian diversity on the roof of Africa: unveiling the effects of habitat degradation, altitude and biogeography. *Diversity and Distributions*.
- Angelini C., Sotgiu G., **Zancolli G.**, Giacoma C. & Bovero S (in prep.) Sex, food and dimorphism: insights from *Euproctus platycephalus*. *Amphibia-Reptilia*.
- Sotgiu G., **Zancolli G.**, Bovero S., Angelini C. & Giacoma C. (2008) Analisi della dieta di *Euproctus platycephalus* ("Analysis of *Euproctus platycephalus*' diet"). *Herpetologia Sardiniae* (ed. Corti C.). Societas Herpetologica Italica / Edizioni Belvedere, Latina, "Le Scienze" (8).

Declaration of Authorship

Chapter 2: Zancolli G, Steffan-Dewenter I, Rödel M-O "Amphibian diversity on the roof of Africa: unveiling the effects of habitat degradation, altitude and biogeography". *Diversity and Distributions* (under review).

participated in	author initials (*responsibility decreasing from left to right*)		
study design	GZ	MOR	ISD
data collection	GZ		
data analysis and interpretation	GZ	ISD	
manuscript writing	GZ	MOR	ISD

Chapter 3: Zancolli G, Rödel M-O, Steffan-Dewenter I, Storfer A "Comparative landscape genetics of two river frog species along an altitudinal gradient on Mount Kilimanjaro". *Molecular Ecology* (under review).

participated in	author initials (*responsibility decreasing from left to right*)		
study design	GZ	MOR	ISD
data collection	GZ		
data analysis and interpretation	GZ	AS	
manuscript writing	GZ	AS	

Chapter 4: Zancolli G, Storfer A, Rödel M-O "Detection of *Batrachochytrium dendrobatidis* in river frogs (genus *Amietia*) on Mount Kilimanjaro, Tanzania. *Herpetological Review* (in press).

participated in	author initials (*responsibility decreasing from left to right*)		
study design	GZ	MOR	
data collection	GZ	AS	
data analysis and interpretation	GZ		
manuscript writing	GZ	AS	MOR

I confirm that I have obtained permission from both the publishers and the co-authors for legal second publication.

I also confirm my primary supervisor's acceptance.

Würzburg, 30th September, 2013

Giulia Zancolli

Curriculum Vitae

Education

2010 – 2013	Doctoral studies at the University of Würzburg (Germany). PhD thesis: "*Amphibian diversity along the slope of Mount Kilimanjaro: from species to genes*"
2007 – 2009	Master degree in "Conservation and Animal Biodiversity" at the University of Turin (Italy) with first-class honors. MSc thesis: "*Vulnerability and Conservation of Pelobates fuscus insubricus in Piedmont*"
2003 – 2007	Bachelor degree in "Natural Sciences" at the University of Turin (Italy) with first-class honors. BSc thesis: "*Analysis of the diet of Sardinian brook salamander Euproctus platycephalus*"

Research experience and training

Jun 2013	Summer School in Bioinformatics. EMBL-EBI Wellcome Trust Center, Hinxton, Cambridge (UK)
Jan - Feb 2013	European Course on Comparative Genomics. Ecole Normale Supérieure de Lyon, Lyon (France)
Aug – Nov 2012	Research stay at the Storfer lab, Washington State University. Pullman (WA, USA)
May 2012	GIS workshop. DFG Funded Research Group Kilimanjaro, University of Würzburg (Germany)
May - Aug 2008	Molecular lab technician for the project "Molecular ecology of the elephant seals (genus *Mirounga*) using microsatellites as molecular markers" in collaboration with

	Dr. Filippo Galimberti (ESRG, Elephant Seals Research Group). Laboratory of Molecular Biology, Universidad Autonoma de Baja California, Ensenada (Mexico)
May - Aug 2007	Eco-volunteer in ACE (American Conservation Experience). Collaboration in environmental and conservation projects in the National Parks, national forests and wilderness area of the Western United States of America. Flagstaff (AZ, USA)

Technical skills

- Survey techniques for amphibians
- Molecular techniques for fragment analysis (microsatellites and AFLP)
- Real-Time PCR (qPCR)
- Skeletochronology (laboratory technique for age determination in amphibians)

Computer skills

- Spatial Ecology packages (e.g. Geospatial Modelling Environment, Fragstats)
- ESRI ArcGIS Desktop
- GeneMapper
- Population genetics packages (e.g. STRUCTURE, BAPS, GenAlex, Arlequin, GenePop)
- Genome scan analysis (e.g. Bayescan, matSAM)
- R Environment

Other skills and competences

- Experience in setting up and managing a molecular laboratory
- Organization and management of lab and field work either independently or in a team
- Good communication and collaboration in an international environment

Conferences and Symposia

- Hinxton (UK), 10 June 2013. Poster contest at the Summer School in Bioinformatics
- Vancouver (Canada), 8-14 August 2012. VII World Congress of Herpetology
- Luxembourg, 25-29 September 2011. 16th European Congress of Herpetology
- Oristano (Italy), 1-5 October 2008. VII National Congress of the "Societas Herpetologica Italica"

Grants and Fellowships

- Travel grant "DAAD PROMOS" from the University of Würzburg Graduate Schools (UWGS) for attendance at the "Joint EMBL-EBI Wellcome Trust Summer School in Bioinformatics" in Cambridge (UK), 2013
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- DFG Excellence Initiative Fellowship (Graduate School of Life Sciences, University of Würzburg), April 2010 – September 2013

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