Evolutionary history of Southern Arabian faunal elements with a special focus on habitat fragmentation of two model organisms, *Reissita simonyi* (REBEL, 1899; Lepidoptera: Zygaenidae) and *Hyla savignyi* (AUDOUIN, 1827; Amphibia: Hylidae)

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In memoriam Univ.-Prof. Dr. Clas M. Naumann zu Königsbrück
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Habitatfragmentierung wird als eine der Hauptursachen für die genetische Isolierung von Populationen angesehen. Natürliche Fragmentierung von Habitaten kann durch klimatische Schwankungen und deren Konsequenzen (z. B. Desertifikation), wie auch durch Naturkatastrophen (z. B. Buschfeuer oder Überschwemmungen) hervorgerufen werden. In letzter Zeit wurde Habitatfragmentierung durch anthropogene Prozesse verstärkt. Das Wissen über Fragmentierung und Populationsdifferenzierung als auch die Verbreitung der Tierarten ist für den südarabischen Raum sehr begrenzt. Die beiden ausgewählten faunistischen Elemente waren *Reissita simonyi* (Lepidoptera: Zygaenidae) und *Hyla savignyi* (Amphibia: Hylidae). Diese wurden aufgrund spezieller ökologischer Charakteristika ausgewählt, die sie vermutlich anfällig für Habitatfragmentierung machen. Darüber hinaus zeigen beide Arten ein unterschiedliches Dispersionsvermögen, welches ein essentieller Faktor für die Aufrechterhaltung von Genfluß auch über längere Distanzen ist. Während *Reissita simonyi* ein tagaktives, flugfähiges Widderchen ist, welches vermutlich weitere Distanzen zurücklegen kann als *H. savignyi*, wird bei Letzterem angenommen, dass es ähnlich anderer Amphibien eine hohe Standorttreue zeigt und im Allgemeinen nur wenige Kilometer migriert.

Die Ergebnisse zeigen, dass beide Arten Heterozygotiedefizite und hohe Inzuchtkoeffizienten aufweisen. Außerdem indizieren die hohen gefunden F_{ST}-Werte zwischen Populationspaaren einen eingeschränkten Genfluß zwischen den Habitatfragmenten bei *H. savignyi*. Darüber hinaus kann in beiden Arten eine signifikante Korrelation zwischen genetischer Differenzierung und geographischer Distanz („isolation by distance“) gefunden werden. Ferner konnte in beiden Arten eine signifikante Korrelation von genetischer Differenzierung und Vertikaldistanzen ermittelt werden. Dies und die starke Strukturierung der Populationen (hohe F_{ST}-Werte) bestätigten die zuvor geäußerte Annahme, dass *H. savignyi* über eine geringe Dispersionsfähigkeit verfügt und die untersuchten Populationen bereits stark voneinander differenziert sind. Jedoch zeigte sich in *H. savignyi* auch, dass nicht nur die oben genannten Effekte die genetische Struktur beeinflussten, sondern auch das Auftreten von Genfluß über größere geographische Distanzen die Populationsstruktur dieser Art nachhaltig beeinflusst hat. Darüber hinaus offenbarte die Populationsstruktur von *H. savignyi* eine klare Untergliederung in drei Gruppen, die eine Nord-Südausrichtung aufweisen. Innerhalb dieser Gruppen kann keine zunehmende

genetische Differenzierung mit zunehmender geographischer Distanz festgestellt werden, was auf eine stärkere Vernetzung innerhalb dieser Gruppen hinweist.

In *R. simonyi* kann die Unterteilung in zwei Subspezies genetisch bestätigt werden. Die genetische Analyse zeigt eine deutliche Unterteilung in zwei Gruppen, die sich mit beiden Subspezies deckt. Darüber hinaus wird diese klare Zweiteilung durch einen höheren genetischen Differenzierungsgrad unterstützt. Innerhalb der Subspezies zeigen sich durchschnittlich geringere genetische Differenzierungen, was auf eine höhere Verwandtschaft innerhalb der Subspezies hinweist. Insgesamt zeigt *R. simonyi* einen deutlich geringeren Grad an genetischer Differenzierung als *H. savignyi*. Dies lässt den Schluss zu, dass die Populationen von *R. simonyi* genetisch stärker vernetzt sind, wenn auch hohe Inzuchtkoeffizienten und Heterozygotiedefizite gefunden wurden, welche auf Nullallele zurückgeführt werden. Ein Grund für diese geringe genetische Differenzierung ist sicherlich die Flugfähigkeit von *R. simonyi*, die dazu führt, dass größere geographische Distanzen zurückgelegt werden können. Darüber hinaus scheint die Futterpflanze in ausreichender Häufigkeit vorzukommen, so dass eine starke Isolierung von *R. simonyi*-Populationen vermieden wird. Nicht zuletzt legen die Daten nahe, dass Individuen von *R. simonyi* leicht von einem Berg zum nächsten gelangen können („top-hopping“), so dass eine starke genetische Differenzierung vermieden wird.

Habitat fragmentation is assumed to be one of the major factors for genetic separation of populations. Natural fragmentation of habitats may be caused by climatic changes and their consequences (*e. g.* desertification), as well as by natural disasters like bush fires or inundations. Recently, habitat fragmentation increased due to human impact. Only little is known about habitat fragmentation and population differentiation as well as distributional ranges of faunal elements in Southern Arabia. Therefore, detailed studies for this geographical area were still lacking. The two faunal elements chosen for this study were *Reissita simonyi* (Lepidoptera: Zygaenidae) and *Hyla savignyi* (Amphibia: Hylidae). These species have special ecological features, which make them presumable sensitive to habitat fragmentation. Moreover, both species have different dispersion abilities, which is a crucial factor to maintain gene flow also over higher distances between populations. *Reissita simonyi* is a flying diurnal moth, which is assumed to migrate longer distances than *Hyla savignyi*. Amphibians often demonstrate high pond fidelity and generally migrate only a few kilometers, although long-distance migration was found in a closely related species, *H. arborea*.

Results indicate for both species heterozygosity deficiencies and high inbreeding coefficients. Furthermore, high F_{ST} values between population pairs in *H. savignyi* indicate restricted gene flow between patches. Moreover, in both species a significant correlation of genetic differentiation and geographical distance (isolation by distance) is found. Besides, in both species a significant correlation between altitude and genetic differentiation is present.

Thus, in *Hyla savignyi* population structure is strongly formed by geographical distance and high genetic differentiation in general (F_{ST}), which is consistent with the assumption of low dispersion ability. However, it is also demonstrated that not only the aforementioned effects shaped the genetic structure of *H. savignyi* populations, but also by long distance gene flow can be detected. The population structure of *H. savignyi* show a clear substructure into three major groups, which display a North to South extension. Within these groups, no isolation by distance effects can be observed. This indicates a higher connectivity within than among groups.

In *R. simonyi*, the division in two subspecies can be confirmed with genetic data. The genetic analysis reveals a significant separation of two groups, which are identical with

the subspecies. This clear pattern is supported by a greater genetic differentiation between groups in comparison to within-group differentiation. Thus, within subspecies, the genetic differentiation is lower, which indicates a higher connectivity within subspecies. In total, the degree of genetic differentiation is much lower in *R. simonyi* than in *H. savignyi*. Hence, it is concluded that populations of *R. simonyi* are more genetically tied than populations of *H. savignyi*, besides high inbreeding coefficients and heterozygosity deficiencies, which are assumed to be based on null alleles. One explanation for this low genetic differentiation is surely the higher dispersion ability caused by the ability to fly and therefore, the potential to migrate over larger geographical distances. A second possible explanation is that the larval food plants occur with a sufficient frequency and therefore a strong isolation of populations of *R. simonyi* is avoided. Finally, the data support the well known phenomenon “top-hopping” in Lepidoptera, which is the ability to migrate from one hill to another very easily. A combination of all three possibilities is the most likely explanation for the low genetic differentiation found in *R. simonyi*.

General Introduction

1.1. Preamble

Population genetics aims to understand the population dynamics within and among closely related species with all its aspects (HARTL, 1999), like *e. g.* gene flow, genetic differentiation, isolation of population, and extinction-recolonization events. Moreover, it deals with the variety of causes for genetic differentiation like habitat fragmentation and destruction, genetic drift, effective population size changes, or sex-biased dispersal to name just a few.

The field of population genetics rapidly progressed through the last two decades. This is partly due to the improvements in molecular biology. The development of the polymerase chain reaction (PCR; SAIKI *et al*., 1988) and the following discovery of highly polymorphic genetic markers like AFLPs (VOS *et al*., 1995; MÜLLER & WOLFENBARGER, 1998; BLEARS *et al*., 1998) and microsatellites (SCHLÖTTERER & TAUTZ, 1992) enabled scientists to study genetic polymorphisms below the species level. Simultaneously, progresses in the theoretical background (WEIR & COCKERHAM, 1984; WEIR, 1990; SLATKIN, 1995; ROUSSET, 1997; DYER & NASON, 2004) and the development of freely available software packages (RAYMOND & ROUSSET, 1995; PRITCHARD *et al*., 2000; DYER & NASON, 2004; PIRY *et al*., 2004; EXCOFFIER *et al*., 2005; to name just a few) allow addressing a variety of questions.

Besides these technical improvements, another aspect moved population genetics into the focus of scientific research. The increasing awareness for anthropologenic changes of the environment in public and politics supported this flowering field of research and *vice versa* (AVISE, 1989; BROOKS *et al*., 1992). Therefore, population genetics plays a major role to examine the consequences of anthropological influences on natural systems. Knowledge of population genetic data provides the basis for wildlife management and conservation genetic projects. A series of comparable studies may lead to an increasing knowledge about general patterns of population dynamics and draw the attention to dissimilarities in different systems.

Therefore, a population genetic approach to analyze two dissimilar faunal elements in Southern Arabia is of particular interest to acquire population genetic data from a remote area, where hardly any published data can be found. Additionally, the project represents a logical consequence of the research of Prof. Naumann, who worked intensively on the morphology, ecology and distribution of *R. simonyi*. Similar, *H. savignyi* has been studied in detail by researchers. However, genetic population genetic data for both species have been lacking so far. The selection of a moth and a frog concurrently allows the examination of the consequences of habitat fragmentation on two different systems.

1.2 Study area

The Arabian Peninsula has an interesting geological history which started some 60 million years ago with the seperation of the Arabian landmass from the African plate along the line of the Red Sea and the Gulf of Aden by tectonic drift effects (THOMPSON, 2000). By the same time, the Arabian plate began to move north-eastwards and collided with the Eurasian plate about 15 million years ago. The collision resulted in the formation of the Zagros Mountain in Iran and other mountain systems in Eurasia. First, the Red Sea started as a chain of lakes in the deepest parts of the valley. By that time several connections were left between the African Plate and the Arabian Peninsula along the escarpment. The rifting process continued and a connection to the Mediterranean Sea developed. At the same time, the African and Arabian plates were still connected through a land bridge near Djibouti (40 million years ago). 5 million years ago, a second phase of drift began and the Isthmus of Suez rose, cutting the Red Sea off from the Mediterranean. By this time, the Gulf of Aden and the Straits of Mandab began to sink. These processes allowed the Red Sea and the Indian Ocean to form a continuous waterway. Simultaneously, there was a rise in sea level of the escarpment and of adjacent land masses, which pushed the mountains of Yemen and the Asir Mountains up to their present heights. In addition to this, high rainfalls between 3 and 1 million years ago shaped the Arabian landform in an enduring way. Great and powerful rivers created the land east of the Red Sea Mountains. Today, the deep river beds between the mountains give evidence of these processes. One of the greatest wadis (river beds) created in that time is the Wadi Hadramout in Southern Yemen (THOMPSON, 2000).

These geographical circumstances imply that a biological colonization of Arabia was complicated by these drift processes. Arabian populations became separated from the African continent and within the Arabian Peninsula. On the other hand, a colonization of certain parts of Asia was possible from that point on. As a consequence, this led to genetic drift effects, population (and genetic) isolation, speciation processes and differences in the genetic variation of many floral and faunal elements.

Therefore, a highly specialized fauna and flora of peculiar interest to the biogeographer and to evolutionary biologists inhabits nowadays Southern Arabia. The fauna of southern Arabia consists mainly of Afrotropical elements and a few Palaearctic faunal elements.

1.3 Faunal elements and their ecology

1.3.1 Reissita simonyi (REBEL, 1899)

The portion of endemisms is relatively high in Southern Arabia (*e. g.* 10 % of the butterfly fauna; LARSEN, 1984), probably due the special geological history of the Arabian Peninsula. Distribution and population patterns of these endemic forms are still scarcely studied. One of these endemics was chosen for this study: *Reissita simonyi* (Lepidoptera: Zygaenidae; REBEL, 1899) has interesting features, which made it of suitable for a population genetic analysis. First, it shows two distinct and disjunctive subspecies with special morphological features. *Reissita simonyi simonyi* is distributed along the southern coast (Oman, eastern governorates of Yemen; large, dark blue sheen, both sexes monomorphic) and *Reissita simonyi yemenicola* along the western escarpment from the Hejjaz to Taiz (this subspecies is considerably smaller, the forewing with greenish blue sheen, males are dimorphic, females monomorphic). The distributional gap between these two subspecies was assumed to be about 700 km (NAUMANN & EDELMANN, 1984).

R. simonyi is an ecologically highly specialized moth. Like all other zygaenid species studied so far, it is able to biosynthesize cyanoglucosides in its body tissue (DAVIES & NAHRSTEDT, 1979, 1982, 1985; NIEHUIS, 2005). During an enzymatic process, cyanoglucosides are transformed to toxic hydrogen cyanides (JONES *et al*., 1962; WITTHOHN & NAUMANN, 1984). These toxic cyanides possibly are repellents against

predators (NAUMANN *et al*., 1999), whereas all zygaenid species themselves are resistant against cyanides. Interestingly, the cyanoglucosides discovered in zygaenids (*i. e*. linamarin & lotaustralin) can also be found in certain plant families, and it has been proven that the cyanoglucosides can partly be plant-derived (NAHRSTEDT, 1988). The plant family Celastraceae, to which the unique larval food plants (*Maytenus*) of *R. simonyi* belong, is one of these. Therefore, *R. simonyi* and its larval host-plants present an assumable co-evolutive system and thus are linked in their evolutionary history. A further effect of this close relation is the dependence of *R. simonyi* of its larval food plant and particularly of its distribution. Therefore, *R. simonyi* may be especially sensitive to habitat fragmentation and destruction.

A precise picture of this co-evolutive system will be given elsewhere, because a population genetic study of *Maytenus senegalensis* was simultaneously carried out by a team in Berlin/ Regensburg (MEISTER & OBERPRIELER, pers. communication).

1.3.2 Hyla savignyi (AUDOUIN, 1827)

Recently, population genetic studies of amphibian species have shown increasing evidence that this group is highly vulnerable to habitat fragmentation and destruction (ROWE *et al*., 1998; NEWMAN & SQUIRE, 2001; ANDERSON *et al*., 2004). One major reason for this sensitivity is the relative low mobility in comparison to other species. For example, for *Hyla arborea* - the sister taxon of *H. savignyi* - migration of single individuals rarely exceeds 4 km (STUMPEL & HAHNEKAMP, 1986; FOG, 1993). On the other hand, mark-recapture methods revealed that individuals of *H. arborea* are exceptionally able to migrate about 12.6 km per year (STUMPEL & HANEKAMP, 1986). Furthermore, amphibians often display high pond fidelity (ANDERSON *et al*., 2004). This can become a critical issue, when ponds are often disturbed or even completely destroyed, because this will reduce small population sizes or even cause the extinction of populations and will decrease connectivity between populations. Like many other amphibians, *H. savignyi* additionally is stenoecious regarding water quality and other characteristics of water ponds. Thus, *H. savignyi* is mostly found in shallow water places with clear water and some vegetation around.

All of the mentioned factors are assumed to apply to *H. savignyi* in Southern Arabia. Besides, the situation in Yemen is even more complicated, since populations might not only be isolated by linear geographical distances, but also by altitude differences, because of the mountainous character of the highlands in N-Yemen. Additionally, Southern Yemen represents a desert area and therefore, water ponds are surrounded by habitat conditions, which clearly limit migration of *H. savignyi*.

1.4 Structure and aims of the present thesis

This thesis seeks to investigate population genetic parameters of two faunal elements in Southern Arabia in order to clarify the current situation of these species and to test hypotheses outlined in detail below. To achieve this goal, it was of particular interest to clarify the actual distribution ranges of these species and it was necessary to carefully sample along these distribution ranges. A set of microsatellites has to be developed.

The present thesis is divided into eight chapters. Generally, each chapter is subdivided in Abstract, Introduction, Material and Methods, Results, Discussion, and partially into additional subchapters. Sometimes result and discussion chapters were unified when this appeared useful. The order of the chapters displays a chronological series of analyses and later chapters generally refer to earlier ones in one way or the other. In summary, these eight chapters deal with the following issues:

Chapter 2 and 3 present the actual distribution ranges and study area of both species and of an additional third one, *Lasiommata felix* (Lepidoptera: Nymphalidae: Satyrinae; WARNECKE, 1929). For *L. felix*, samples were taken, but a population genetic analysis was cancelled due to time constraints. However, tissue samples for further genetic analyses are available in the tissue collection of the ZFMK. The aim of these two chapters was to provide detailed and actual distribution maps of the studied species. This is of particular interest for population genetic analyses, which often seek to study gene flow, isolation by distance, and differentiation of populations. Therefore, we aimed to collect complementary information on the distribution of *H. savignyi* and *R. simonyi*. Besides, we intended to collect as many samples as possible along the distribution ranges for the subsequent population genetic analyses.

Chapter 4 discusses potential cross-utility of a microsatellite set developed for *H. arborea*, which is tested for other *Hyla* species in this species complex. Mainly, potential advantages and pitfalls are discussed. The main issues here are to test, whether cross-amplified loci show size homoplasy, ascertainment bias, and hidden polymorphism; these are problems, which are rarely touched in the literature so far.

Chapter 5 presents the development of a microsatellite set for *R. simonyi*. In addition, potential cross-amplification for *Zygaena* species is discussed.

In chapters 6 and 7, the population genetic analyses for *R. simonyi* and *H. savignyi* are presented. Both chapters are dealing with similar questions with a focus on habitat fragmentation and its consequences, like connectivity, isolation by distance, inbreeding effects, and differentiation of populations.

Chapter 8 can be seen as a general discussion of the results of chapter 2-6. A focal point is the discussion of the results of the two population genetic analyses of *R. simonyi* and *H. savignyi*. Finally, controversial issues are discussed and an outlook is given for supplementary studies.

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Distribution of the Yellow-lemon Tree Frog, *Hyla savignyi* (AUDOUIN, 1827) in southern Arabia: updates and extensions of previous records

2.1 Abstract

The distribution of the yellow-lemon tree frog *Hyla savignyi* in south-western Arabia is presented. This isolated distribution area is limited to the western escarpment along the Red Sea. All sites were found between 1.400 and 2.800 m. The northernmost occurrence of *Hyla savignyi* was near to Wadi Amaq in Saudi Arabia (21°21´N, 40°17´E); the southernmost near to Mawah (14°13.266´N/ 44°23.614´E) in Yemen.

Key words. Arabian Peninsula, Yemen, Saudi Arabia, biogeography, distribution pattern, vertical distribution.

2.2 Introduction

Hyla savignyi has first been described by AUDOUIN from Syria in 1827 (DUELLMAN, 1977). BOULENGER (1882) assigned this species to *Hyla arborea* as *Hyla arborea* var. *savignyi*, and NIEDEN (1924) subsequently gave it subspecies rank, i.e. *Hyla arborea savignyi*. Nowadays, *Hyla savignyi* is again generally accepted as a distinct species (Fig. 1; SCHNEIDER & NEVO, 1972; SCHNEIDER, 1974; BRZOSKA & SCHNEIDER, 1982; SCHNEIDER et al., 1984; FROST, 1985). In addition to morphological characters, *Hyla savignyi* differs from other *Hyla* species by its distinct mating calls which are in–between *H. meridionalis* and *H. arborea* in all parameters (SCHNEIDER & NEVO, 1972; BALLETTO *et al.*, 1985). The distribution of *H. savignyi* extends from Turkey in the west to Georgia, Armenia, Azerbaijan und Iran in the east and southeast, and to Israel, Palestine and Jordan in the south. In Turkey, both *Hyla savignyi* and *H. arborea* occur: *H. arborea* is confined to the west, and *H. savignyi* to the southeast and the east. Both species meet at the Mediterranean coast near Anamur (see *e. g.* KAYA & SIMMONS, 1999; SCHNEIDER, 2000, 2001).

Fig. 1. Yellow–lemon Tree Frog, *Hyla savignyi*, from a water place near the German Embassy in Sana´a, Yemen. Photo: C. KLÜTSCH.

A first record from southern Arabia was given by PARKER (1938) who collected some specimens from Abha in the Asir Mountains of Saudi Arabia. A few years later, PARKER (1941) found the species also near Sana´a in Yemen.
This isolated occurrence of the species in southwest Arabia was subsequently confirmed by others, including SCHMIDT (1953); BALLETTO *et al*. (1985); SCHÄTTI & GASPERETTI (1994); and SCHÜTTE (1986). Based on these literature records and new own observations which were made during several field trips to Yemen (June/July 2001, September/October 2001, March/April 2002, and June/July 2002), the vertical and horizontal distribution of the species will be described here.

2.3 Results and discussion

The distribution of *H. savignyi* in southern Arabia is limited to the western escarpment along the Red Sea. The northernmost locality is Wadi Amaq in Saudi Arabia (21°21´N, 40°17´E) in Saudi Arabia; the southernmost locality is located near Mawah (14°13´N, 44°23´E) in Yemen. The occurrence is limited to high altitudes and extends from approx. 1400 to 2800 m above the sea level. Most sites are found between 2200 and 2400 (Fig. 2).

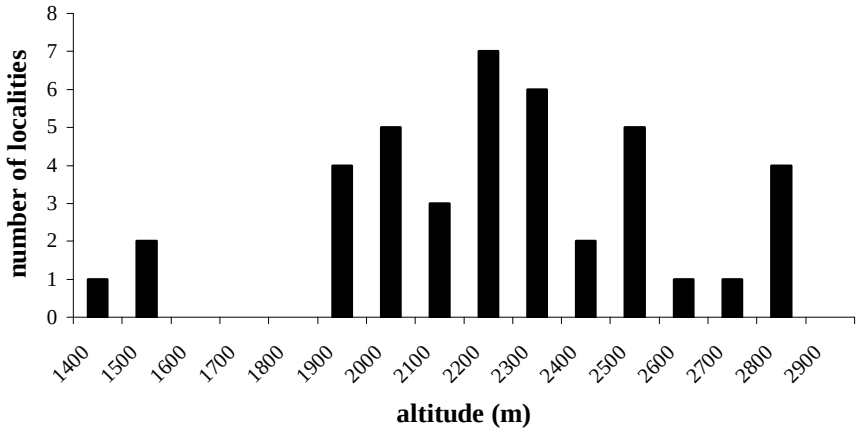

Fig. 2. Vertical distribution of *Hyla savignyi* in Southern Arabia.

The highest locality is in Ad Dogma, Al Haima (15°12´N, 43°58´E; 2845 m) in Yemen, the lowest is Wadi Thareira in Saudi Arabia with an altitude of 1440 m. In Yemen, all localities are found above 2150 m, whereas in Saudi Arabia three localities are found between 1400 and 1600 m. Suitable habitats are found in Yemen also below 1500 m, but *H. savignyi* is not present there. Especially in the eastern part of Yemen (along the Indian Ocean) water places with dense vegetation and clear water are found, but *H. savignyi* could not be found. The absence of *H. savignyi* in the south of Yemen and Oman may be explained by two reasons: Either species never arrived in this area or habitat conditions inhibit a permanent colonization. Due to the presence of permanent water places but absent yellow–lemon tree frogs we speculate that extant climate condition inhabit the establishment of permanent reproducing populations of *H. savignyi* in the lowlands of Southern Yemen and Oman. In mountainous areas temperatures are lower on average than in coastal areas and lowlands. Furthermore, rain falls are higher in mountainous areas, since mountainous barriers serve as “rain catchers”. Probably due to these facts, *H. savignyi* is only found in mountainous areas in Southern Arabia (Fig. 3).

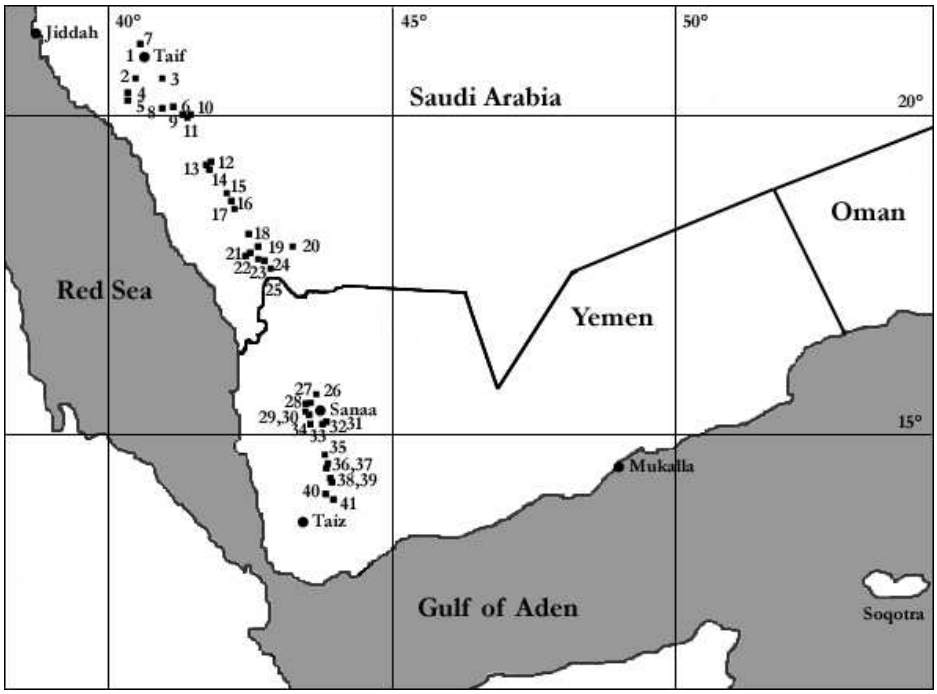

Fig. 3. Distribution of *Hyla savignyi* (AUDOUIN, 1927) in Southern Arabia. Locality numbers correspond to numbers in appendix, List of records; some localities are summarized under one number.

Usually, *H. savignyi* prefers perennial, deep water places (BALLETTO *et al.*, 1985). Breeding seasons extends from mid–December to mid–January in Palestine (SCHNEIDER & NEVO, 1972), but from mid–February to late March in Yemen (SCHÜTTE, 1986). In June 2001, we observed a population of *H. savignyi* in a water pool near Dhamar which exhibited breeding activities, including copulation and spawning.

We found *H. savignyi* only in small pools or tanks near pumping rooms in agricultural areas. These pools are surrounded by dense vegetation, and are never deeper than 1–1.5 m; some are even shallower. These small water bodies often dry out naturally, but water is also extensively used for irrigation during the reproduction season, thus imposing a significant threat to populations (SCHÜTTE, 1986). Moreover, chemicals used in household and industry as well as insecticides, molluscicides, and pesticides cause the destruction of suitable water habitats with natural vegetation (see also SCHÄTTI & GASPERETTI, 1994).

The population of *H. savignyi* in southwest Arabia is likely to be a relict population. During the last glacial series, a branch of tree frogs *Hyla* has probably extended its

distribution range from Europe into the Middle East and Asia, and splitted up into *Hyla arborea* and *H. savignyi*. *H. arborea* is mainly found in Central Europe. About 5000–6000 years ago, when a period of aridization began in the Middle East and the climate became hotter and drier (THOMPSON, 2000), populations were separated and evolved independently like populations in southern Arabia. It is nowadays isolated and has no connection to other distribution areas such as in the north of the Arabian Peninsula.

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Appendix 2.1: List of records

Abbreviations: ZFMK = Alexander Koenig Research Institute and Museum of Zoology; CAS = California Academy of Science; BM(NH) = British Museum (Natural History); IZUG = Instituto di Zoologia Unicersita di Genova; MZUF = Museo Zoologico de "La Specola" Universita di Firenze; MHNG = Muséum d´Histoire naturelle of Geneva.

Saudi Arabia

1 Taif, 21°16´N 40°25´E, 1500 m, 1989, SCHÄTTI & GASPERETTI (1994), MHNG; **2** Wadi Hubaykah, 21°10´N 40°20´E, early 1980, BALLETTO *et al.* (1985), BMNH; **3** Wadi Thareira, 21°09´N 40°44´E, 1440 m, 27.V.1979, BALLETTO *et al.* (1985), IZUG; **4** Wadi Wajj, 21°08´N 40°14´E, 2000 m, II.1980, BALLETTO *et al.* (1985), IZUG; **5** 11 specimens, Jebbel Dakka, 21°07´N 40°14´E, 2000 m, 22.VI.1962, BALLETTO *et al.* (1985), BMNH; **6** Wadi Shumruq, 20°29´N 41°20´E, 1500 m, 27.V.1979, BALLETTO *et al.* (1985), IZUG; **7** Wadi Amaq, 21°21´N 40°17´E, 2000 m, 23.VIII.1974, BALLETTO *et al.* (1985), CAS; **8** Barahara, 20°21´N 41°15´E, 1900 m, 11.IX.1973, BALLETTO *et al.* (1985), CAS; **9** near Bani Sar, 20°06´N 41°26´E, 1./2.VIII.1984, BALLETTO *et al.* (1985), BMNH; **10** 2 specimens, Bani Sar, 20°05´N 41°26´E, 2130 m, 27.V.1979, 10.VIII.1980, 14.VIII.1980, BALLETTO *et al.* (1985), IZUG; **11** Al Bahah, 20°01´N 41°27´E, 2300 m, 1989, SCHÄTTI & GASPERETTI (1994), MHNG; **12** Wadi Mahra, 19°38´N 41°54´E, 1910 m, 21.IV.1977, 29.VI.1977, 15.IX.1977, IX.1978, BALLETTO *et al.* (1985), H. WACHTEL, CAS, BMNH; **13** Wadi Ahger, near Wadi Mahra, 20.III.1980, BALLETTO *et al.* (1985), BMNH, [not shown in map]; **14** 5 specimens, Al Alayyah, 19°37´N 41°57´E, 2000 m, 1.IV.1980, BALLETTO *et al.* (1985), IZUG; **15** Al Khadra, 19°19´N 42°05´E, 2800 m, 17.VI.1983, BALLETTO *et al.* (1985), IZUG; **16** An Nimas, 19°07´N 42°08´E, 2000 m, 22.IV.1977, BALLETTO *et al.* (1985), BMNH (?); **17** 14 specimens, Bani Mashoor, 19°00´N 42°09´E, 2300 m, 6.VI.1979, BALLETTO *et al.* (1985), IZUG; **18** Dahna Shalal, 18°55´N 42°12´E, 2300 m, 5.IV.1984, BALLETTO *et al.* (1985), BMNH; **19** Wadi Mahalla, 18°19´N 42°35´E, 1900 m, 17.IV.1981, BALLETTO *et al.* (1985), BMNH; **20** Hijla, 18°18´N 43°28´E, 1900 m, 6.VII.1977, 17.VI.1979, 25.X.1978, BALLETTO *et al.* (1985), BMNH & IZUG; **21** 3 specimens, Al Mukadda, 18°14´N 42°25´E, 2500 m, 1.XII.1981, BALLETTO *et al.* (1985), BMNH; **22** "Waterfall Wadi",18°14´N 42°2´E, 2500 m, 22.IV.1977, BALLETTO *et al.* (1985), BMNH; **23** 3 ♂,

2 ♀, Abha, Asir, 18°13´N 42°30´E, 2120 m, 12.VI.1938, 12.IV.1976, PARKER (1938); BALLETTO *et al.* (1985), BMNH; **24** Abha, Wadi Abha, 18°13´N 42°30´E, 12.VIII.1977, BALLETTO *et al.* (1985), BMNH; **25** Wadi Jeman, 18°02´N 42°45´E, 2200 m, 4.IV.1984, BALLETTO *et al.* (1985), BMNH.

Yemen

26 19 toe-tips, N Amran, 15°43´N 43°58´E, 2232 m, 10.VII.2002, KLÜTSCH, Tissue collection, ZFMK; **26** near Amran, 15°38´N 43°50´E, 2300 m, 18.XI.1984, BALLETTO *et al.* (1985), MZUF; **27** 6 toe-tips, Thula, 15°35´N 43°53´E, 17.IV.2002, NASHER, Tissue collection, ZFMK; **28** 28 toe-tips, Habiba, 15°33´N 43°52´E, 2.VII.2001, KLÜTSCH, Tissue collection, ZFMK; **29a** ca. 200 individuals observed, 3 km W Shibam, artificial waterhole, 15°30´N 43°53´E, 2500 m, 22.III.1985, SCHÜTTE (1986); **29b** 5 toe-tips, Shibam, 15°30´N 43°53´E, 2500 m, 29.VI.2001, NASHER, Tissue collection, ZFMK; **29c** 28 toe-tips, West Kawkaban, Wadi Annaim, 15°30´N 43°53´E, ca. 2200 m, 9.VII.2002, KLÜTSCH, Tissue collection, ZFMK; **30** 30 toe-tips, Lulah, 25 km N Sana´a, 15°30´N 43°56´E, ca. 2200 m, 9.VII.2002, KLÜTSCH, Tissue collection, ZFMK; **31** Sana´a, 15°21´N 44°12´E, 2400 m, 1951, SCHMIDT (1953); **31a** 36 toe-tips, Sana´a, near German embassy, 15°18´N 44°12´E, 2295 m, 30.VI.2001, 12.IX.2001, 17./18.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **31b** 2 specimens, Sana´a, 15°18´N 44°12´E, ca. 2300 m, VIII.1980, SCHÜTTE (1986), ZFMK; **32** Migyal al Asad, 15°17´N 44°21´E, 2500 m, 18.II.1938, PARKER, 1941, BMNH [not shown on map]; **33** Migyal al Alaf, 15°14´N 44°13´E, 2300 m, 24.II.1938, BMNH, PARKER, 1941, [not shown on map]; **34a** 34 toe-tips, Ad Dogma, Al Haima, 15°12´N 43°58´E, 2845 m, 21.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **34b** 13 km S Sana´a, 2250 m, 24.II.1938, PARKER, Scott expedition, [not shown on map]; **34c** 14 km SE Sana´a, 2250 m, 18.II.1938, PARKER, Scott expedition, [not shown on map]; **34d** ca. 300 individuals observed, 31 km SW Sana´a, Street Sana´a–Hodeida, 2700 m, 23.II.1985, SCHÜTTE, 1986, [not shown on map]; **34e** 8 km W Ma´abar, 14°48´N 44°12´E, 2400 m, no exact date, SCHMIDT, 1953, [not shown on map]; **35** 30 toe-tips, Ma´abar, 20 km N Dhamar, 14°48´N 44°17´E, 7.VII.2002, KLÜTSCH, Tissue collection, ZFMK; **36** 30 toe-tips, 20 km N Dhamar, Rhusabr, 14°33´N 44°21´E, 7.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **37** 25 toe-tips, Dhamar, 14°32´N 4°21´E, 2812 m, 17.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **37** 4 toe-tips, Dhamar, next to checkpoint, 14°32´N 44°21´E, 7.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **38a** 26 toe-tips, between Dhamar and

Yarim, small waterhole, 14°26´N 44°24´E, 2813 m, 18.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **38b** 1 specimen, 130 S Sana´a, VIII.1980, ERDELEN, ZFMK [not shown on map]; **39** 30 toe-tips, Dhihisub, 14°26´N 44°25´E, 2533 m, 7.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **40** 25 toe-tips, Yarim, 14°16´N 44°15´E, 2197 m, 18.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **41a** 30 toe-tips, S Yarim, Mawah, 14°13´N 44°23´E, 2613 m, 6.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **41b** Rada, 155 km SE Sana´a; could be also Raidah N of Sana´a at 2000 m, 1980, Zoologische Staatssammlung Munich, SCHÜTTE (1986).

Extended distribution patterns of the Arabian burnet moth *Reissita simonyi* (Lepidoptera: Zygaenidae; REBEL, 1899) and the Arabian wall brown *Lasiommata felix* (Lepidoptera: Nymphalidae: Satyrinae; WARNECKE, 1929) in Southern Arabia

3.1 Abstract

The extended distribution patterns of two faunal elements of Yemen, *Lasiommata felix* (WARNECKE, 1929) and *Reissita simonyi* (REBEL, 1899) are presented. Both species are endemic to the Arabian Peninsula and information about distribution patterns has been rather fragmentary so far. The chapter summarizes the results of several field trips to Southern Arabia in 2001/ 2002 and reviews additional published evidence on the distribution patterns. Numerous new localities for both studied species from Yemen are offered. Currently, *Reissita simonyi* is known from Al Hada, vic. Ta´if, Asir in Saudi Arabia to Province Dhofar, Jabal Samhan, N of Juffa in Oman. *Lasiommata felix* is distributed from Ta´if, Saudi Arabia to the Jaffah area, E of Taiz/ Yemen in Southern Arabia. *L. felix* seems to be limited to the western escarpment along the Red Sea, whereas *R. simonyi* shows a division into two subspecies: *R. simonyi yemenicola*, which also occurs along the mountainous areas along the Red Sea and *R. simonyi simonyi*, which is distributed along the Indian Ocean. Furthermore, a brief description of morphological characteristics and phylogenetic relationships of these species is given in this context.

Keywords. *Reissita simonyi*, *Lasiommata felix*, distribution pattern, endemism, Southern Arabia, Yemen.

3.2 Introduction

3.2.1 General Introduction

The Arabian Peninsula is of particular interest for evolutionary biologists, since it represent a biogeographical link between Africa, Europe and Asia. As a consequence, the flora and fauna of the Arabian Peninsula contain many taxa, which have evolutionary connections to ancestors from all of these areas. Especially the butterfly fauna of Southern Arabia raised great interest among scientists (REBEL, 1907; GABRIEL, 1954; LARSEN, 1977, 1979, 1980, 1982, 1983, 1984, 1987; PITTAWAY, 1979, 1981; WILTSHIRE, 1980, 1982, 1983, 1986, 1990; HACKER, 1999; HACKER, *et al.*, 2001). This enormous interest was also raised by the relatively high degree of endemism in Lepidoptera in this region. LARSEN (1984) pointed out that about 10 % of the butterfly fauna in this area is endemic to Arabia. Moreover, he emphasized that about 20 species show distinct Arabian subspecies. Our work concentrated on two different species, which differ in habitat requirements, distribution patterns and evolutionary origin. The species examined in this paper are *Reissita simonyi*, REBEL (1899) (Zygaenidae, Lepidoptera) and *Lasiommata felix*, WARNECKE (1929) (Satyrinae, Lepidoptera). *L. felix* has an assumed Palaearctic, *R. simonyi* an assumed Afrotropical origin, but both are endemic to Southern Arabia. *R. simonyi* has the extraordinary feature of its division in two subspecies: *R. simonyi simonyi* and *Reissita simonyi yemenicola*. The distribution ranges of the two subspecies seem to be allopatric. The main goal of this work was to provide actual distribution maps of *Reissita simonyi* and especially for *Lasiommata felix*, where no detailed distribution map has been available so far. In this context, a review of already published records and statements was also carried out to clarify distribution ranges of these two species.

3.2.2 Description of R. simonyi

In this context, only a brief summary about the differences between the two subspecies of *R. simonyi* is given. Detailed descriptions as well as drawings are available in NAUMANN & EDELMANN (1984). The division in two subspecies is based on the following morphological characters:

1. Sexual dimorphism

A striking feature for division is the unusual sexual dimorphism of males of *R. s. yemenicola*. One morph (*f. simonyi*) is similar, although not identical to the blackish-blue female, while the second one (*f. sylviae*) is completely red and resembles the imagines of the Palaearctic genus *Zygaena* FABRICIUS, 1775 whereas in *R. s. simonyi* males are always blackish-blue.

2. Abdominal cingulation

R. s. yemenicola shows a reduction in the abdominal cingulation in comparison *to R. s. simonyi*. Moreover, it seems that *R. simonyi yemenicola* has a slightly smaller body size than *R. s. simonyi* and the mid-line interruption of the red patagia appears to be somewhat wider than in *R. s. simonyi* (NAUMANN & EDELMANN, 1984). However, so far no detailed examination for the supposed different body size has been done.

3.2.3 Biology and Ecology

The larvae of *Reissita simonyi* are limited to the food plants of the genus *Maytenus* (Celastraceae): *M. senegalensis* and *M. dhofarensis*. The larvae exclusively feed on leaves of these taxa (Fig. 1).

Fig. 1: *Maytenus senegalensis*; the larval food plant of *R. simonyi*; photo was taken from Tur-Al-Baha, Jabal Araph.

Therefore, the distribution of *R. simonyi* is strictly connected to the distribution of *Maytenus* (Celastraceae). Usually, the plants are relatively small caused by intensive grazing of goads and sheep (Fig. 2).

Fig. 2: *Maytenus senegalensis*; illustrating a small-sized specimen which is mostly found due to over-grazing. Photo was taken from Ras Fartak.

Occasionally, where strong rocks are present and the locality is protected, *Maytenus* bushes can reach 4 m in height. At the beginning of the rainy season, *Maytenus* shrubs develop fresh shoots after the first rain. Since the first instar larvae are dependent on these soft-leaved fresh shoots, the flight activity of the imagines appears to be limited to the arid periods before the rainy seasons. The first generation is limited to March; the second generation lasts from end of June till mid of July. The third and last generation continues from end of September till early October. The flight period is strictly correlated with these seasons. This corresponds to observations on other Zygaeninae feeding on Celastraceae (e. g. *Orna*, *Epiorna*, *Epizygaenella*). A further ecological adaptation is the daily flight activity of *R. simonyi* which is limited to the hottest period of the day from 11 to 14:30. NAUMANN & EDELMANN (1984) suggested that this behavior is important to safe energy, because imagines do not feed and drink during the entire imaginal phase. This behavioral specialization is mirrored by an extreme reduction of the proboscis. The larvae display further adaptations: the serrate setae are probably used to serve as crystallization points for condensation of water to supply additional water for the larvae. The precipitation is transported in form of fogs or humid air from the coast to the mountains.

Lasiommata felix usually occurs along walls or vertical cliffs in mountainous areas. The movement is always up and down along these vertical features. LARSEN (1982) interpreted this behavior as protection behavior against unwanted dispersal since this species is often found in windy areas. Contrariwise, this vertical movement could also be part of the patrolling behavior, which is shown especially by males of *L. felix*. Nowadays the terraced agricultural regions used for cultivation replaced natural rocky hillsides in many regions (Fig. 3).

Fig. 3: A typical place to find *Lasiommata felix* near Bani Mawhab/ Bait Muzaret. The artificial walls seem to be a suitable habitat for *L. felix*.

LARSEN (1982) suggested that the food plant must be a common grass species. The vegetation, *e. g.* at Wadi Dhar, a classical place where *L. felix* is commonly found is described in DUBAIE *et al*. (1993; and literature within).

3.2.4 Distribution

In the western part of the Arabian Peninsula an escarpment of mountains separates the lowland of Yemen and Saudi Arabia from the Central-Arabian plateau. The distribution of *Reissita simonyi yemenicola* is strongly connected to this western escarpment. *R. s. yemenicola* generally occurs in high altitudes from 1500 m to 2900 m, but can also be found down to 400 m sea level. The second subspecies, *R. s. simonyi* occurs on both sides of the Yemeni-Omani barrier along the Indian Ocean among 350-900 m above sea level.

Lasiommata felix is commonly found in the mountainous area of Saudi Arabia and Yemen. Usually, it can be seen in high altitudes around 2200 m (LARSEN, 1982; own observations). Similar to *R. s. yemenicola*, it seems to have a strong connection to the western escarpment, but no localities are known along the coast of the Indian Ocean.

3.2.5 Remarks

Reissita simonyi was described by REBEL in 1899 from a single specimen from Ras Fartak in SE Yemen. In 1907, he gave detailed description of the habitus and provided morphological data. SCOTT & BRITTON (1942) collected a few specimens in 1938. In 1959, TREMEWAN established the new genus *Reissita* based on differences in wing venation and genital morphology. Moreover, TREMEWAN (1959) divided *Reissita simonyi* in two subspecies, *Reissita simonyi yemenicola* and *Reissita simonyi simonyi*. *Reissita simonyi yemenicola* is distributed in the mountainous areas along the Red Sea where as *Reissita simonyi simonyi* is distributed at both sides of the Yemeni-Omani border along the Indian Ocean. Since *Reissita simonyi yemenicola* features dimorphic males, TREMEWAN described another species for the northern populations, *Reissita sylviae*. In this case, *Reissita sylviae* and *Reissita simonyi yemenicola* shared one distributional area. In 1984, NAUMANN & EDELMANN refused the species status of *Reissita sylviae* based on several facts: First of all, only males of *R. sylviae* are observed. No single red female of this species was observed ever. In other words, no reddish forms of females are known. Additionally, the two different forms of males do not vary in their behavior, period of activity or flight. Despite their different color both forms do not differ in morphological characters. Furthermore, red males mate with dark females. Moreover, there is only one type of larvae found which turn out red and black males. This leads to another aspect; both forms seem not to diverge in their ecological requirements. They are fed on the same food plant and have the same life cycles. All of these facts support *R. sylviae* to be conspecific with *R. simonyi*. NAUMANN & EDELMANN (1984) came to the conclusion that *Reissita sylviae* is a junior synonym for *Reissita simonyi yemenicola*. Furthermore, they suggested to use the name *sylviae* for the red males (forma *sylviae*) to distinguish the two morphs of males in *Reissita simonyi yemenicola* (black morph should be named forma *simonyi*). The separation of two subspecies was still maintained because of the allopatric occurrence of *Reissita simonyi yemenicola* in the north and *Reissita simonyi simonyi* in the southeast. In addition, the

dimorphic males only occur in the subspecies *R. s. yemenicola*, not in *R. s. simonyi*. These two aspects in combination with fore mentioned morphological differences (wing pattern, abdominal cingulation) support the discrimination of two subspecies. In 1980, WILTSHIRE recorded seven specimens from Oman, which had been collected by THOMAS in 1930. Two years later WILTSHIRE (1982) published one of the first records from Saudi Arabia, collected by TALHOUK.

Given the isolated distribution of *L. felix* and *R. simonyi* in Southern Arabia the evolutionary history is of particular interest. Possible origins of *R. simonyi* could be Africa with the Zygaenini genera *Orna*, *Epiorna*, *Neurosymploca*, *Zutulba*, and *Praezygaena*. Moreover, there could be a phylogenetic relationship to the genus *Epizygaenella* in the oriental region and to the genus *Zygaena* in the Palaearctic (NAUMANN, 1977, 1990). In this case, *Reissita* would be the sister group of *Epizygaenella* and *Zygaena* (NAUMANN, 1990, 1999) or to *Zygaena* alone (NAUMANN, 1977). Based on the male pheromone system (first described by KAMES, 1980) which is present in the genera *Reissita*, *Praezygaena*, *Epizygaenella* and *Zygaena* and the similar spindle shaped cocoons with a silk cushion of the genera mentioned above (no data available for *Praezygaena*) it is assumed that *Reissita* is closely related to these three genera (NAUMANN & EDELMANN, 1984). Ongoing molecular approaches (NIEHUIS, 2005) may clarify the phylogenetic position of the monotypic genus *Reissita*.

Lasiommata felix was described by WARNECKE in 1929. SCOTT & BRITTON published in 1942 one specific locality (Jabal Jihaf), where *L. felix* was found in Yemen. This place must be about 90 miles north from Aden, near Dhala. Unfortunately, we were unable to identify this collection site in an actual map, but GABRIEL (1954) examined the collections of SCOTT & BRITTON (1942) and wrote that they collected 14 ♂♂ and 5 ♀♀ in September/ October 1937 at this site. SCOTT & BRITTON (1942) mentioned another place, but did not mentioned the English or Latin name, they circumscribed the butterfly (*e. g.* brown Satyrines). The locality named is Jabal Al Kohl (25 km N of Sana´a, around 3000 m high). Since the locality is situated near Sana´a, a region where we frequently found *Lasiommata felix* in our field trips, it seems plausible to check this locality in further studies. However, since SCOTT & BRITTON (1942) did not mentioned *Lasiommata felix* or the English name wall brown in detail, we refrained to include the named locality in the list of records and distribution map.

L. felix seems to have a close phylogenetic relationship to the Ethiopian species, *L. maderakal* GUÉRIN-MENÉVILLE, 1849 and *L. menava*, MOORE, 1865 of Iran and Afghanistan (LARSEN, 1982). HEYDEMANN (1954) pointed out that it should be handled as a subspecies of *L. meneva*. In 1956, HIGGINS & WILTSHIRE critically examined *L. felix* and *L. meneva*. They found that *L. felix* shows more narrowed androconia in comparison to *L. meneva*, the hind wing margin is more strongly scalloped and eye-spots on the wings are surrounded by an orange ring in comparison to *L. meneva* (see also LARSEN, 1982; BOZANO, 1999). They treated both as specifically distinct. As LARSEN (1982) pointed out all three mentioned species ought to have species status. *L. felix* does not appear closely related to *L. maera* in Europe and the Middle East based on genital morphology (LARSEN, 1982; *L. maera* has three to four small teeth at the tip of the penis; this feature is not found in the other three species). This would suggest that some Palaearctic species found in Arabia and East Africa have ancestors from the Himalaya/ Iranian region (LARSEN, 1984).
In addition, BOZANO (1999) examined morphological variation within *L. felix* (see there and LARSEN, 1984 for pictures) and mainly found variation in wing color between populations from different localities.

3.3 Material and methods

The observed data presented in this work were carried out in four field trips (June/ July and September/ October 2001; March/ April, and June / July 2002). Additionally, data from NAUMANN & EDELMANN (1984) was included and further completed by literature records as well as personal communications. The different localities visited are listed under list of records. GPS data were recorded with GARMIN GPS 12. For *R. simonyi*, usually larvae were knocked off from shrubs using a stick and a box to catch them. For *L. felix*, adults were caught with a standard butterfly net. Specimens were stored in 99 % pure ethanol.

3.4 Results

During literature research, it became obvious that there is quite great confusion about the names of the two subspecies of *R. simonyi* and their distribution. According to statements made above, there are a few clarifications to be made:

- NAUMANN & EDELMANN, 1984, page 487: It is written under point 6. 1. at the end of first line *R. simonyi*. This is incorrect. It should be read as *R. sylviae*.
- NAUMANN & EDELMANN, 1984, page 491: in the description for forma *sylviae* is written "Forewing spots much more pronounced than in f. *sylviae*"; this should be read as "Forewing spots much more pronounced than in f. *simonyi*".
- WILTSHIRE, 1980, page 190: "A subspecies with a black abdomen, instead of red as in the Dhofar-Hadhramaut form, was described by TREMEWAN (1959) from the Yemen." So far as we know, no red form of *Reissita simonyi* is found in the area from Dhofar to Hadhramaut. TREMEWAN (1959) mainly distinguished between the two subspecies because of the different expressed abdominal cingulation. He mentioned also that the red form is exclusively found along the Red Sea.
- WILTSHIRE, 1982, page 276: WILTSHIRE wrote for a specimen found in Asir (Saudi Arabia) *R. simonyi simonyi*. Since TREMEWAN (1959) described the highland populations as *R. simonyi yemenicola* this specimen is given below in the list of records under *R. s. yemenicola*.
- WILTSHIRE, 1990, page 101: He did not name the subspecies *simonyi* particularly. He named *R. simonyi* on the one hand and *R. s. yemenicola* on the other. This might just be an oversight, but correctly one should read under *R. simonyi*, *R. simonyi simonyi* and *R. simonyi yemenicola*. In the first paragraph (under *R. simonyi*) he describes the distribution area of *R. s. simonyi* (Ras Fartak and South Oman) and uses the term "the typical subspecies", but did not mention the subspecies (*R. simonyi simonyi*) specifically, which may lead to confusion with the named subspecies *R. s. yemenicola*, which follows in the second paragraph.
- HACKER, 1999, page 34: It is written that *Reissita simonyi simonyi* is identical with *R. sylviae*. NAUMANN & EDELMANN (1984, page 488) clearly pointed out that *R. sylviae* is a subjective junior synonym of *R. simonyi yemenicola*. Therefore, it should be read *R. simonyi yemenicola* is equal to *R. sylviae*. This synonym cannot be used for the subspecies *R. simonyi simonyi*. However, it would be correct to write that *R. sylviae* is conspecific with *R. simonyi*, because it does not form a species on its own.

Results further display extended distribution patterns for both chosen species, but especially for *Reissita simonyi* the results are very promising. *Reissita simonyi* has a so far known distribution extended from Al Hada, vic. Taif, Asir in Saudi Arabia to Province Dhofar, Jabal Samhan, N of Juffa (17°12.01´N/ 54°56.16´E) in Oman (Fig. 4).

Moreover, it was possible to find populations within a distribution gap known in the past (NAUMANN & EDELMANN, 1984).

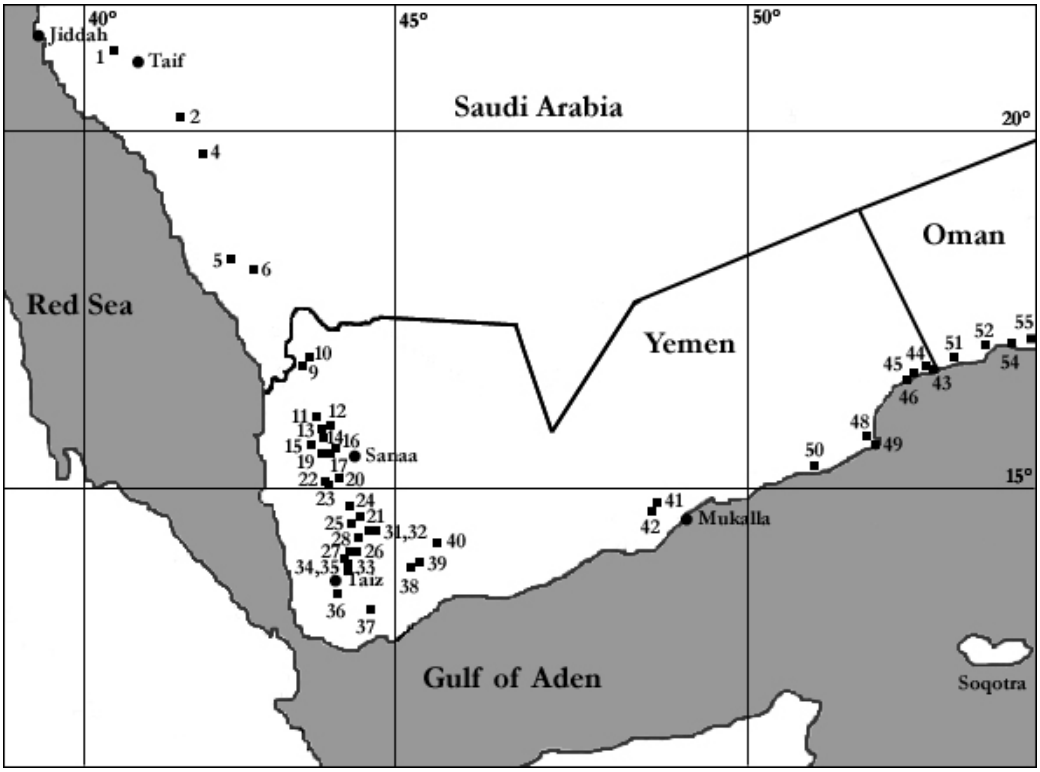

Fig. 4: Distribution of *Reissita simonyi yemenicola* (along the Red Sea) and *Reissita simonyi simonyi* (along the Indian Ocean at both sides of the Yemeni-Omani border). Interesting new localities were found south-east Taiz (**37** Jabal Araph/ **38** Jaffah/ **39** E Labus/ **40** S Al Bayda) as well as right in the middle (**41** Korseban/ **42** Mola Matar/ **50** Seyhout) of the so far known gap.

Lasiommata felix is distributed from Taif, Saudi Arabia (21°16´N 40°24´E) to the Jaffah area, E of Taiz/ Yemen (13°47´N 45°11´E) in Southern Arabia (Fig. 5).

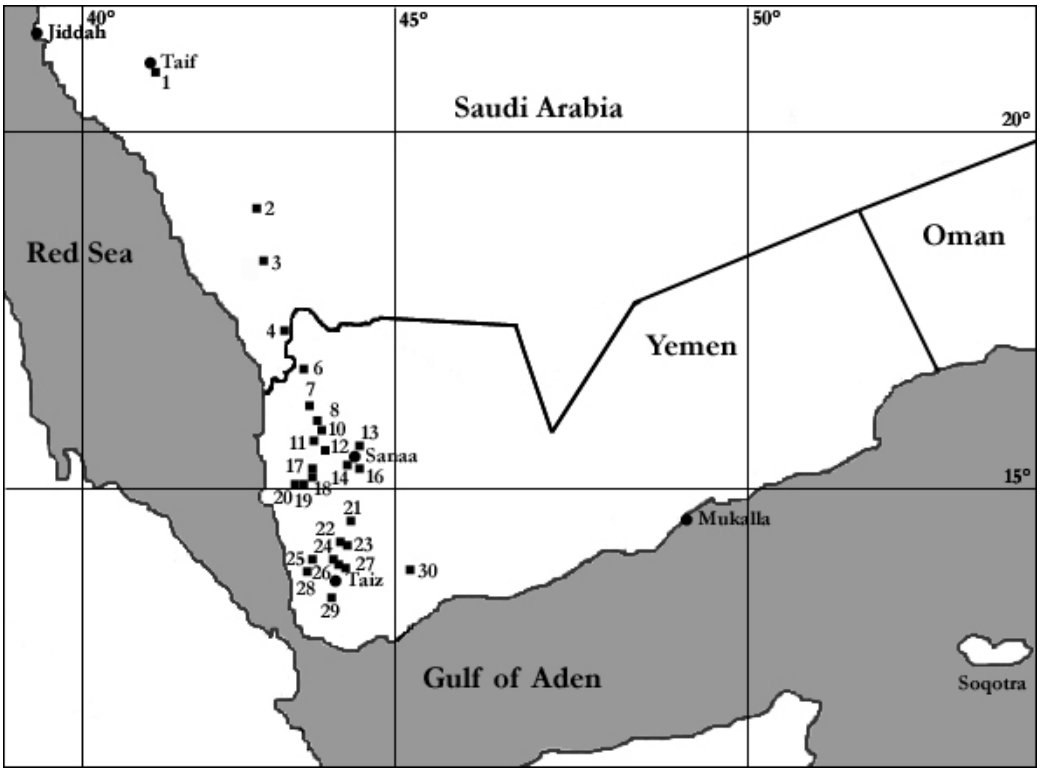

Fig. 5: Distribution of *Lasiommata felix*; the distribution is limited to the western escarpment along the Red Sea.

It looks like it is strictly limited to the western escarpment along the Red Sea. So far, no localities could be found in SE Yemen along the Indian Ocean.

3.5 Discussion

For *R. simonyi*, the predominant opinion was that both subspecies have allopatric distribution patterns. Our results suggest that there could be a connection between both subspecies (*R. simonyi yemenicola* and *R. simonyi simonyi*). Although there are still distribution gaps between Jabal Araph and Jaffah area – Mola Matar of 390 km and between Mola Matar/ Korseban – Seyhout of 260 km, there could be a meeting point between these subpopulations in the area somewhere between Jabal Araph/ Jaffah and Mola Matar. Moreover, at a locality named Jabal Urays (13°32´N/ 45°55´E), which lies exactly in between Jabal Araph/ Jaffah and Mola Matar, the larval food plant of *R. simonyi* could be found, but in spring 2002, *R. simonyi* was not found; this locality should be checked again in further expeditions, since the habitat has all features important to *Reissita simonyi*: an altitude about 1700 m, the larval food plant *Maytenus*

senegalensis and the exposition to the coast, where foggy clouds transport dew to plants and larvae. If there is a connection between both subspecies, it would be of special interest how and why the subspecies barrier is abided. In addition, if there would be a hybridization zone between the two subspecies, these subspecies could eventually exchange genetic material. The division into two subspecies could still be warranted; if the exchange of genetic material would be strictly limited to the hybridization zone (*e. g*. hybrid individuals are infertile). However, results of the genetic analysis support the divergence into two subspecies (KLÜTSCH *et al*., chapter 7).

The fact that *R. s. yemenicola* as well as *L. felix* are strictly limited to the mountainous area along the Red Sea suggests specific characteristics of the mountainous areas in Northwestern Yemen/ Saudi Arabia to be responsible for this restricted distribution. Probable features could be the higher altitude in combination with higher precipitation in mountainous areas in Northwestern Yemen/ Saudi Arabia. It is also possible that a restricted distribution range of the food plant limits the actual distribution of *L. felix*. Further studies could concentrate on examination of possible food plants in order to identify the food plants of *L. felix* in connection to the question which floristic composition is preferred by *L. felix*. Furthermore, the analysis of abiotic factors as potentially limiting reasons is suggested. Finally, it is highly likely to find more localities for both species in Saudi Arabia, since the restricted number of records might be more probably caused by the limited access as well as low research activities in this area rather by restriction of the distribution area itself.

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Appendix

Appendix 3.1: List of records

Reissita simonyi (REBEL, 1899)

Zygaena simonyi REBEL, 1899: 359-361.

List of Records

Abbreviations: ZFMK = Alexander Koenig Research Institute and Museum of Zoology, CMN = private collection of Clas M. NAUMANN, BMNH = British Museum of Natural History, NHMW = Vienna Natural History Museum. Record numbers correspond to numbers given in maps. Some records are not given in map, because coordinates and/ or geographical positions were not sufficiently known. Information given in bold is not given in original publication, but is complementary information from different sources.

***Reissita simonyi yemenicola* (TREMEWAN, 1959):**

= *Reissita sylviae* TREMEWAN, 1959: 213-217 (syn. nov., NAUMANN & EDELMANN, 1984).

Saudi Arabia

1 larvae, Asir, Al Hada, vic. Ta´if, 1200 m, PITTAWAY; **2** 1 ♂, Asir, An-Namaah, **20°14´N 41°16´E**, 2100 m, 11.IX.1983, BÜTTIKER; **3** 1 specimen, Asir, Wadi Jurah, vic. Jizan, 500 m, 1.XII.1981, TALHOUK [not shown in map]; **4** larvae, Asir, Mikhwa, **19°90´N 41°60´E,** PITTAWAY; **5** larvae, 1 ♀, 1 ♂, Asir, Muhayil, **18°16´N 41°80´E**, 75 km NW Abha, 400 m, 7.XI.1982, PITTAWAY; **6** larvae, Asir, Abha, **18°13´N 42°29´E, ca. 2500 m,** PITTAWAY; **7** larvae, Asir, vic. Mifah, PITTAWAY [not shown in map]; **8** larvae, Asir, Al Foqa, PITTAWAY [not shown in map].

Yemen

9a larvae, Province Sadah, J. Razah, vic. Zerra´a, 2050-2100 m, 11.VI.2001, KLÜTSCH & NAUMANN, CMN; **9b** larvae, Province Sa´ada, Jabal Razah, 16°50´E 43°18´N, 2300 m, 12.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **10a** larvae, Province Sa´ada, Zera´a (Jabal Razah), 16°52´E 43°20´N, 2300 m, 11.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **10b** larvae, Province Sadah, J. Razah, 200 m below Summut, **16°52´E 43°20´N,** 2150 m, 12.VI.2001, KLÜTSCH & NAUMANN, CMN;

11 larvae, Province Hajjah, N Hajjah, 15°55.975´N 43°32.689´E, 1521 m, 26.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **12a** larvae (e. l. 9.IX.1982 1 ♀), Province Hajjah, 7 km W Kohlan, road to Hajjah, **15°44´N 43°42´E,** 9.-10.V.1982, NAUMANN, CMN; **12b** 1 specimen, Province Hajjah, between Kohlan and Wadi Shares (way to Hajjah), 1500 m, B. TURLIN (personal communication); **13a** larvae, Province Manakhah, **15°42.734´N 43°35.638´E,** 1987 m, 27.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **13b** larvae, 2820 m, 27.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **13c** larvae, Province Kohlan, Bait Zarefat, **15°42.74´N 43°35.63´E,** 1987 m, KLÜTSCH, Tissue collection, ZFMK; **13d** larvae, 1-2 km from Bait Zarefat, 14.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **14** larvae, Province Hajjah, Hajjah, **15°41´N 43°36´E,** 2600 m, 14.VI.2001**,** KLÜTSCH & NAUMANN, CMN, Tissue collection, ZFMK; **15a** larvae (1 ♀ e. l., 22.VII.1982), Province Al Machwit, Al Hijrah, Jabal Haiadi, **15°40´N 43°29´E,** 1800 m, 31.V.1982, NAUMANN, CMN; **15b** larvae, 8.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **16** larvae (2 ♂♂, 1 ♀ e. l.), Province Al Mahwit, 1-5 km NNW Al Mahwit, Jabal Radman, **15°32´N 43°49´E,** 2300 m, 8. + 25.VII.1982, NAUMANN, CMN; **17a** eggs, first instar larvae, imagines observed, Shamat (between At Tawilah and Shibam), **15°29´N 43°44´E,** 1900 m, 1.VI.1982, NAUMANN, CMN; **17b** eggs, larvae, Province Al Machwit, 5,5 km W At Tawilah, Beni Khaiat, **15°29´N 43°44´E,** 1.VI.1982, NAUMANN, CMN; **18** larvae (e. l. 1 ♀, VII. 1982), Province Al Machwit, Bait Turki, Jabal Haiadi, 1700 m, 31.V.1982, NAUMANN, CMN [not shown in map]; **19a** Imago, 1 ♂, Al Rujum (between Al Mahwit and At Tawilah), **15°29´N 43°40´E,** 1800 m, leg. B. TURLIN ?, coll. C. NAUMANN; **19b** larvae, Ar Rajun, Road Shibam-Mahwit, **15°28´N 43°50´E,** 8.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **19c** larvae, Al Mahwit, **15°29´N 43°33´E,** 2100 m, 8.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **19d** larvae, 14.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **20** 2 ♂♂, 1 ♀, Province Manakhah, Jabal Al Khamis, Al Haima, **15°09´N 43°56´E,** 2700 m, 30.V.1982, NAUMANN, CMN; **21a** larvae, Province Al Qubba, J. Masnah area, 5 km S Al Qubba, **14°36´N 44°12´E,** 2300 m, 5.VI.2001, NAUMANN, CMN; **21b** ♂♂, ♀ ♀ (forma *sylviae*), Province Dhamar, Jabal Masnah area, 5 km SW Al Qubba, 14°36´54´N 44°12´38´E, 2200-2400 m, 5.III.1980, EDELMANN & NAUMANN, CMN; **21c** larvae, 4.VI.2001, NAUMANN, Tissue collection, ZFMK [not shown in map]; **22** larvae, Province Menakhah, vic. Hajjarah,

15°04´N 43°42´E, 2500 m, 26.X.2001, NAUMANN, CMN; **23a** larvae, Al Hudaib/ Menakhah, 15°02.641´N 43°45.132´E, 2818 m, 26.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **23b** Province Sana´a, Jabal al Hotep (S Menakhah), 15°02´N 43°38´E, 2800 m (more localities than written), 26.II.2000, HACKER *et al.*; **24** larvae, Province Dhamar, Jabal-as Sharq, 14°42.54´N 44°09.41´E, 2300 m, 4.VI.2001, NAUMANN, CMN; **25a** imagines, Jabal Masnah, 30 km SW of Ma´abar, **14°35´N 44°08´E,** 9.III.1938, SCOTT & BRITTON; **25b** eggs, larvae, pupae, imagines, Jabal Masnah area, 2 km N Al Qubba, 2300-2400 m, 3-7.III.1980, EDELMANN & NAUMANN, CMN, **25c** 7 ♂♂, ♀♀ (forma *sylviae*), 30.VI.1980, DECKERT, CMN; **25d** larvae, VIII.1981 (e. l. 1 ♂, 1 ♂, 4.-8.X.1980), DECKERT; **25e** larvae, 1 ♂, 6-7.VI.1982 (e.l. July 1982), NAUMANN, CMN; **25e** imago, 26.III.1999, NAUMANN, CMN; **25f** larvae, 3.VI.2001, NAUMANN, CMN; **25g** larvae, 3.VI.2001, NAUMANN, CMN; **25h** larvae, 5.VI.2001, NAUMANN, CMN; **25i** larvae, 18.X.2001, NAUMANN, CMN; **26a** larvae, Province Ibb, 4 km S Ibb, **13°58´N 44°10´E, 1600 m,** 2-4.VI.1982, NAUMANN, CMN: **26b** larvae, 2100-2200 m, 16.X.2001, NAUMANN, CMN; **27** larvae, Al Manswra, 10 km W Ibb and W Jiblah, **13°58´N 44°08´E,** 2200 m, 16.X.2001, NAUMANN, CMN; **28** Eggs, larvae, pupae, ♂♂, ♀♀ (forma *sylviae*), Province Ibb, Jabal Badaan, **14°00´N 44°10´E**, 2400 m, 16-20.V.1982, LARSEN, CMN; **29** Larvae, ♂♂, ♀♀ (forma *sylviae*), Province Ibb, Sumarah Pass, south-western slope, 2900-3000 m, 3-6.VI.1982, NAUMANN, CMN [not shown in map]; **30** larvae, Sumarah, 4 km S Al Hosn, **14°16´N 44°10´E**, 2700 m, 17.X.2001, NAUMANN, CMN [not shown in map]; **31** larvae, Province Yarim, Yarim, 14°16.111´N 44°15.958´E, 2197 m, 18.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **32** larvae, 13 km W Yarim (Al Irian road), vill. Al Thahatein, **14°17´N 44°19´E,** 2850 m, 17.X.2001, NAUMANN, CMN; **33** larvae, pupae, Al Jablah, 13°55.348´N 44°05.513´E, 2311 m, 19.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **34** larvae, Province Taiz, Dhi Al Sefal (= Dhi Sufal), 25 km N Taiz, **13°51´N 44°06´E,** 1950-2050 m, 16.X.2001, NAUMANN, CMN; **35** larvae, Province Taiz, Al Alarifal, 13°49.852´N 44°06.089´E, 2396 m, 20.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **36a** larvae, Province Taiz, Jabal Sabir, 13°31.917´N 44°00.962´E, 2569 m, KLÜTSCH, Tissue collection, ZFMK; **36b** larvae, vic. Hatab, 15.X.2001, NAUMANN, CMN; larvae, Province Taiz, Jabal Sabr, vill. Mahzaf, 2500-2600 m, 15.X.2001, NAUMANN, CMN; **37** larvae, Govenerate Lahaj, next village Tur-Al-Baha, Jabal Araph, 13°06´N 44°14´E,

1330 m, 22./ 25.VII.2002, 1.VII.2002, KLÜTSCH, Tissue collection, ZFMK; **38** larvae, Province Jaffah, Jabal Manwara, 13°47.280´N 45°11.040´E, 2311 m, 30.III.2002, KLÜTSCH, Tissue collection, ZFMK; **39** larvae, E Labus, 13°54.016´N 45°17.325´E, 2256 m, KLÜTSCH, Tissue collection, ZFMK; **40** larvae, S Al Bayda, **13°59´N 45°34´E,** 2250 m, 30.VI.2002, KLÜTSCH, Tissue collection, ZFMK.

***Reissita simonyi simonyi* (Tremewan, 1959):**

Reissita simonyi simonyi Tremewan, 1959: 213-217.

Yemen

41a larvae, Korseban, 14°49.158´N 48°48.129´E, 1750 m, 15.X.2001, KLÜTSCH, Tissue collection, ZFMK; **41b** larvae, 1950 m, 16.X.2001, KLÜTSCH, Tissue collection, ZFMK; **42** larvae, Mola Matar, 14°47.701´N 48°46.709´E, 1750 m, 16.X.2001, KLÜTSCH, Tissue collection, ZFMK; **43a** imago, Province Al Mahra, 5 km NW of Jadib, **16°38´N 52°57´E,** 600 m, 13.XI.2000, NAUMANN, CMN; **43b** imago, Province Al Mahra, 5 km NNW Jadib, 16°38´N 52°57´E, 600 m, XI.2000, NAUMANN, CMN; **44a** imago, Province Al Mahra, ca. 10 km NNE Al Hawf, 16°39´N 53°02´E, 650-700 m, XI.1999, HEIN & KILIAN, CMN; **44b** imago, 11. & 14.XI.2000, NAUMANN, CMN; **44c** larvae, Province Al Mahra, Al Hawf, 16°38.958´N 52°57.655´E, 800 m, 29./30.IX.2001, KLÜTSCH, Tissue collection, ZFMK; **44d** larvae, Province Al Mahra, near Al Hawf (near a spring), 16°38.290´N 52°56.643´E, 792 m, 1.X.2001, KLÜTSCH, Tissue collection, ZFMK; **45** imago, Province Al Mahra, ca. 5 km NNW Damqut, 16°34´N 52°48´E, 360-400 m, 12.XI.2000, NAUMANN, CMN; **46** larvae, Province Al Mahra, Damqut, 16°33.942´N 52°46.433´E, 792 m, 2./3.X.2001, KLÜTSCH, Tissue collection, ZFMK; **47** imago, ♀ (holotype), Ras Fartak, Wadi, III.1899, SIMONY, NHMW [not shown in map]; **48** larvae, Ras Fartak, 15°50.270´N 52°00.100´E, 966 m, 6.X.2001, KLÜTSCH, Tissue collection, ZFMK; **49** larvae, S-Ras Fartak, 15°37.583´N 52°11.53.7´E, 546 m, 10.X.2001, KLÜTSCH, Tissue collection, ZFMK; **50** imago, 20 km N Seyhout, 15°17.348´N 51°10.902´E, 800 m (769 m), NAUMANN, CMN.

Oman

51a imago, Jabal Al Qamar, 5 km N Rakhyut, Bait Sa´b, 16°46´N 53°20´E, 850-900 m, 6.XI.1997, NAUMANN, CMN; **51b** imago, Jabal Qamar, NAUMANN, CMN; **51c** imago, Jabal Qamar, vic. Arift: Bait Handawb, vic. Shaat, 950 m, 5 + 7.XI.1997, NAUMANN,

CMN; **52a** imago, Province Dhofar, Jabal Qara, 1,5 km NNE Qairoon Hairitti, 17°16´N 54°06´E, 850 m, 2-3.XI.1997, NAUMANN, CMN; **52b** larvae, Province Dhofar, Jabal Qara, 1,5 km SW Qairoon Hairitti, 17°16´N 54°06´E, 850 m, 20.-31.XI.1999/ 2000, NAUMANN & KEIL, CMN, KEIL, Dresden, 4 spec. in Tissue collection ZFMK; **53** 1 ♂, 1 ♀, Province Dhofar, Jabal Qara (N of Salalah), Hatab, 2.IV.1978, Walker, Royal Scottish Museum, Edinburgh [not shown in map]; **54a** imago, Jabal Samhan, 8 km E Tawi Attair, 17°07´N 54°38´E, 750 m, NAUMANN, CMN; **54b** imago, Jabal Samhan, 45 km N Agarhanawt Teyq waterhole, 17°09´N 54°37´E, 800 m, 3.XI.1997, V. POLAK (personal communication), CMN; **55** larvae, Province Dhofar, Jabal Samhan, S-facing rocky slopes of Jabal Samhan along Wadi N of Juffa, 17°12´01´N 54°56`16´E, 455-737 m, 29.IX.2002, MEISTER & OBERPRIELER, Tissue collection, ZFMK; **56a** ♂♂, ♀♀, Province Dhofar, Qara mts., Khyount, 1750 ft. (530 m), 11.XI.1930, B. THOMAS, BMNH [not shown in map]; **56b** 1 specimen, Province Dhofar, Qara mts., Hamirar, 1500 ft. (460 m), 14.XI.1930, B. THOMAS, BMNH [not shown in map]; **56c** 1 specimen, Province Dhofar, Qara mts., Fusul, 1350 ft. (410 m), 15.XI.1930, B. THOMAS, BMNH [not shown in map].

***Lasiommata felix* (WARNECKE) – Arabian Wall Butterfly**

Pararge felix WARNECKE, 1929. Int. ent. Z. 22: 365 (Yemen, Sana´a).

Saudi Arabia

1 1 ♂, Asir, Taif, **21°16´N 40°24´E, ca. 1750 m**, 13.VII.1934; **2** Asir, As-Nimas, **19°11´N 42°19´E, ca. 2400 m** (mentioned in LARSEN, 1983); **3** 7 ♂, 4 ♀, Asir, Suda, **18°16´N 42°22´E, ca. 3000 m**, 9.X.1936; **4** 1 ♂, Province Gizan, Feifa, 17°16´N 43°05´E ca. 1200 m, 22.-23.XII.1936; **5** 1 ♂, Asir, Musaira, 28.XI.1936, PHILBY [not shown in map].

Yemen

6 Province Sadah, Jabal Rhaza, vill. Zerra´a, **16°52´E 43°20´N,** 2050-2100 m, 11.VI.2001, KLÜTSCH & NAUMANN; Tissue collection, ZFMK; **7** 2 spec., Bani Mawhab/ Bait Muzaret, 15°44.180´N 43°40.150´E, 1760 m, 14.06.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **8** 1-3 specimens, Province Sana´a, mountains WSW Amran,

Mada´a, 15°37´N 43°44`E, 3000 m, 29.X.1996, HACKER *et al.*; **9** Wadi Sharas (below Hajjah), **around 900 m high** (locality mentioned in LARSEN, 1983; not shown in map); **10** > 10 specimens, Province Sana´a, mountains WSW Amran, Masaani, 15°36´N 43°50´E, 2900 m, 30.X.1996, HACKER *et al.*; **11** > 10 specimens, Province Sana´a, mountains WSW Amran, Masaani-Gummama, 15°35´N 43°47´E, 3000 m, 30.X.1996, HACKER *et al.*; **12a** 1-3 specimens, Province Al-Mahwit, E Kawkaban, 15°29´N 43°55´E, 2750 m, 31.X.1996, HACKER *et al.*; **12b** 3 spec., Al Mahwit, **15°29´N 43°33´E,** 2100 m, 8.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **13a** Wadi Dhar, 1982, LARSEN; **13b** 2 spec., Wadi Dhar, 15°26.266´N 44°08.133´E, 4./ 7.VI.2001, NAUMANN; Tissue collection, ZFMK; 31 spec., 15.VI.2001, KLÜTSCH & NAUMANN, Tissue collection, ZFMK; **14a** Province Sana´a, Sana´a, **15°21´N 44°12´E,** 1934, RATHJENS & WISSMANN; **14b** 1 ♂, 3 ♀, Province Sana´a, approx. 2400 m, II. 1938, SCOTT & BRITTON; **14c** 2 ♂, 2 ♀, Province Sana´a, Sana´a, approx. 2400 m, X.1937, RATHJENS; **14d** 1 ♂, 2 ♀, Province Sana´a, Sana´a, X.1938, PETRIE; **15a** Province Sana´a, Road Sana´a-Hodeida, 1982, LARSEN [not shown in map]; **15b** 3 ♂, Al Asr (10 km W of Sana´a, around 2500 m high); **15c** 3 ♂, 1 ♀, Hada´a (6 km SW of Sana´a, around 2700 m high), 14.I.1938; **15d** 3 ♀, Ghaiman (15 km SE of Sana´a, around 2200 m high), 18.II.1938; **15e** 3 ♀, Wadi Sabir (S of Taiz, around 2000 m high), 19.XII.1937; **15f** 1 ♂, 1 ♀, Beit Baus (7 km S of Sana´a, 15°16´N 44°11´E, around 2500 m high), 21.I.1938; **16** > 10 specimens, Province Sana´a, mountains SW Sana´a, Jabal Ayban, Bait Na´ama, 15°18´12´´N 44°16´48´´E, 2700-2750 m, 18.IV.1998, 24.II.2000, HACKER *et al.*; **17** > 10 specimens, Province Sana´a, Jabal an Nabi Shu´ayb, S-side 15°16´33´´N 43°59´23´´E, 3000 m, 7.V.1998, HACKER *et al.*; **18** 1 spec., Al Dogma/ Al Haima, 15°12.015´N 43°58.009´E, 2845 m, KLÜTSCH, tissue collection, ZFMK; **19a** 7 + 22 spec., ca. 10 km S of Al Hudaib, 15°04.129´N 43°43.550´E, 2818 m, 27./ 28.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **19b** 3 + 30 spec., Al Hudaib near Menakhah, 15°02.641´N 43°45.132´E, 2818 m, 26./27.VI.2001, 21.VI.2002, KLÜTSCH, Tissue collection, ZFMK; **20a >** 10 specimens, Province Sana´a, 60 km SW Sana´a, Makaban, Naqil Menakhah, (westside), 15°04´N 43°39´E, 1900 m, 2.XI.1996, HACKER *et al.*; **20b** > 10 specimens, Province Sana´a, Jabal Al Hotep, (S Menakhah), 15°02´N 43°38´E, 2800 m, 26.II.2000, HACKER *et al.*; **21a** Jabal Sumarah, **14°16´N**

44°10´E, 1982, LARSEN; **21b** Jabal Sumarah, VI.1982, NAUMANN; **21c** Sumarah Pass, Naqil Sumarah, **around 2800 m high**, mentioned in LARSEN, 1983; **22** Province Ibb, 5 km N Ibb, vill. Al Bahrin, **14°02´N 44°09´E**, 2200 m, 17.X.2001, NAUMANN; **23** Province Ibb, Jabal Bada´an, **14°00´N 44°10´E**, 1982, LARSEN; **24** 16 + 8 spec., Al Udayn, 13°58.537´N 44°05.913´E, 2300 m, 19./ 20.VI.2001, KLÜTSCH, Tissue collection, ZFMK; **25** 4-10 specimens, Province Ibb, 1,5 km W Jiblah, 13°57´N 43°57´E, 2100 m, 7.XI.1996, HACKER *et al.*; **26** 4-10 specimens, Province Ibb, 5 km NE Al Qa´idah, Mahal al Houmeira, 13°45´N 44°10´E, 1800 m, 6.XI.1996, HACKER *et al.*; **27** Province Ibb, 30 km S Ibb, **13°50´N 44°10´E**, 1982, LARSEN; **28a** Suq Al Khamis, **13°44´N 43°45´E**, **2900 m,** 1982, LARSEN; **28b** Suq Al Khamis, near Taiz, 1982, Carden, in: LARSEN, 1982; **29a** Province Taiz, Jabal Sabir, 1982, LARSEN; **29b** 3 + 19 spec., Province Taiz, Jabal Sabir (vill. Mahzaf), 13°31.917´N 44°00.962´E, 2569 m, 21./ 24.VI.2001, KLÜTSCH, Tissue collection, ZFMK; 10 spec., 2.VII.2002, KLÜTSCH, Tissue collection, ZFMK; **29c** Province Taiz, Jabal Sabir, below top, **13°31´N 44°00´E,** 2800 m, NAUMANN; **30** Jaffah area, 30.VI.2002, 13°47´N 45°11´E, ~ 2300 m, KLÜTSCH; **31** Hizyaz, **20 km S of Sana´a, around 2000 m high**, 1982, LARSEN [not shown in map]; **32** Jabal Dawran, **20 km W of Ma´abar, around 2000 m high**, 1982, LARSEN [not shown in map]; **33** Jabal Jihaf, ca. 90 km N of Aden, **around 2000 m high**, 19.-20.IX.1938, SCOTT & BRITTON [not shown in map].

Cross-utility, size homoplasy, and evolution of microsatellites in the genus *Hyla* (Amphibia: Hylidae)

4.1 Abstract

Cross-species amplification of microsatellites was tested in *Hyla savignyi*, *Hyla meridionalis*, and *Hyla sarda* and tended to result in shorter allele sizes in cross-amplified species. Additionally, a highly variable microsatellite locus was selected in order to investigate sources of microsatellite length variation within and among populations in *H. savignyi* and among species. It is found that the main mutational mechanisms contributing to the allelic variation were (1) addition/ deletion of repeats, (2) substitutions and indels in the flanking region, and (3) mutations interrupting or changing the repeat. Size homoplasy can be detected within populations as well as among different populations of *H. savignyi*. Additional mutations in the flanking regions are found; indicating an underestimation of total mutation rate. The most probable explanation for the broad microsatellite length variation within *H. savignyi* is that different mutation rates are present in two main lineages (Syria/ Yemeni *versus* Iraqi lineage).

Keywords. Size homoplasy, *Hyla*, molecular markers, SSR, cross-utility, microsatellites.

4.2 Introduction

Microsatellites are DNA motifs of 1-6 base pairs, which are repeated up to 100 times (TAUTZ, 1993). A special characteristic of microsatellites are high mutation rates varying from 10^{-4} – 10^{-5} probability of mutation per site (HENDERSON & PETES, 1992; JEFFREYS *et al.*, 1998). Therefore, they are widely used in population genetics, evolutionary genetics and forensic studies as well as paternity investigations and disease detection (*e. g.* JARNE & LAGODA, 1996; GUSMÃO *et al.*, 2001; NEFF, 2001; BESSERT & ORTÍ, 2003). Reasons for the versatile applicability of this genetic marker system are the following aspects: 1) they are found in all eukaryotic genomes examined so far (MAKOVA *et al.*, 2000), 2) they are highly polymorphic, 3) are easily amplified from

small tissue samples and 4) are co-dominant. One additional important feature of these markers is that they can often be used to study closely related species (cross-utility). As a consequence, a lot of microsatellites are applied in different species (SCRIBNER *et al.*, 1996; NEFF *et al.*, 1999; CHAMBERS *et al.*, 2004; KIM *et al*., 2004). In most of these studies, the analyses rely on the scoring of product size, not direct sequence data. Therefore, cross-utility runs into the risk that the detected electromorphs/ sizes of fragments are not identical by descent (IBD), but identical in state (IIS). Thus, size homoplasy describes the incident of identical microsatellite product sizes caused by autonomous mutational events. Questions of size homoplasy have been addressed in invertebrate species (ESTOUP *et al.*, 1995; VIARD *et al.*, 1998) as well as mammals (MAKOVA *et al.*, 2000). To our knowledge, size homoplasy has not yet been tested in amphibians. This may be based on the apparent difficulties of cross-amplification in amphibians and reptiles (ROWE *et al.*, 2000; PRIMMER & MERILÄ, 2002). Multiple independent mutational mechanisms are responsible for size homoplasy: Addition or deletion of repeats in the repeat region as well as addition or deletion of bases in the flanking regions and mutations which interrupt or change the repeat section (MAKOVA *et al.*, 2000).

Besides homoplasy, microsatellites used for cross-utility show another well-known phenomenon, ascertainment bias. Ascertainment bias occurs, when there is a significant decrease in repeat length in cross-amplified species, whereas the focal species has the longest repeats. Some authors suggested that ascertainment bias is caused by an artifact in the development process of microsatellites (ELLEGREN *et al.*, 1995; FORBES *et al.*, 1995). Usually microsatellites with long repetitive motifs are preferred in order to yield highly polymorphic markers. Consequently, microsatellite size may be at the upper bound of evolutionary stability in the target DNA pool and smaller amplicons are detected in cross-utility assays more often just by chance. Other authors (AMOS *et al*., 1996; COOPER *et al*., 1998; HUTTER *et al*., 1998; AMOS, 1999; AMOS *et al.*, 2003) suggested that a mutational bias alone or in combination with ascertainment bias might be responsible for the distribution of allele lengths among different species or populations within a species.

In order to test cross-utility, we examined 15 microsatellites developed by ARENS *et al.* (2000) in four species of the genus *Hyla* (*Hyla arborea*, *Hyla savignyi*, *Hyla sarda*, and

Hyla meridionalis) for amplification results and electromorph sizes. In addition, one microsatellite locus was sequenced to identify eventual size homoplasy and hidden polymorphism (additional mutations in the flanking or core regions) among alleles within and between populations. Based on our results, we will discuss controversial hypotheses of allele length distributions among and within species.

4.3 Material and Methods

DNA was extracted using a standard Chelex method (GERKEN *et al.*, 1998). Approximately 10 - 25 mg tissue was incubated in a total volume of 500 µL containing 5 % Chelex solution at 56°C overnight followed by a final denaturation at 95°C for 10 min. The extracted DNA served for cross-utility tests and sequencing. For cross-utility tests, the PCR was carried out in 20 µL containing 2 µL 10 x PCR buffer (without $MgCL_2$; containing 100 mM Tris-HCL, 500 mM KCL, pH 8.3, Sigma), 2.0 µL $MgCl_2$ (25 mM, Sigma), 100 µM of each dNTP (Sigma), 10 nM of each primer and 0.25 U *Taq* polymerase (Sigma). PCR was performed according to the instructions in Arens *et al.* (2000). PCR products were diluted 1 to 5 with dH_2O. 1.5 µL of diluted PCR product was mixed with 0.2 µL GeneScan-500 Size standard (Applied Biosystems), 0.4 µL loading buffer, and 1.5 µL deionized formamide. Samples were denatured at 92°C for 90 s and then immediately cooled on ice. Microsatellites were electrophoresed using an ABI Prism 377 (Applied Biosystems) on a 6% polyacrylamid gel. Digital gel data was collected by ABI Prism GENESCAN ANALYSIS SOFTWARE 3.1.2 (Applied Biosystems) and afterwards analyzed with the GENOTYPER 2.0 SOFTWARE (Applied Biosystems).

One microsatellite locus was chosen to test for size homoplasy and was sequenced. WHA5-22A was selected; because electromorphs displayed different lengths in all populations tested and represented a compound motif (Table 1, Fig. 1; GARZA & FREIMER, 1996), which usually is sensitive for size homoplasy (VIARD *et al.*, 1998). Primers were taken from ARENS *et al.* (2000) to amplify and sequence this locus. PCR was carried out in 50 µL total volume containing 10 x PCR buffer (without $MgCL_2$; containing 100 mM Tris-HCL, 500 mM KCL, pH 8.3, Sigma), 15 – 20 mM $MgCl_2$ (Sigma), 100 µM of each dNTP (Sigma), 10 nM of each primer and 0.02 µL *Taq* polymerase (5U/µL, containing 20 mM Tris-HCL, 100 mM KCL, 01 mM EDTA, 1 mM DTT, 05 % Tween 20, 50 % glycerol; Ampli-*Taq*-Gold, Applied Biosystems). PCR was

carried out following the protocol of Arens *et al.* (2000). Cycle sequencing was carried out in 10 µL containing 4 µL ABI Prism Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems), 10 nM of each primer and 2 µL of 5 x sequencing buffer (Applied Biosystems). Cycle sequencing was started at 94°C for 2 min, followed by 35 thermal cycles (94°C for 15 s, 50°C for 15 s and 60°C for 150 s) and additional 8 thermal cycles at 93°C for 20 s and 60°C for 15 s. Amplification and cycle sequencing were performed in a GeneAmp 2700 thermal cycler (Applied Biosystems).

Additionally, an approx. 750 bp long fragment of the cytb gene was sequenced. Primers for amplification and sequencing were taken from MORITZ *et al.* (1992) (MVZ 15: 5´-GAACTAATGGCCCACACTTTACG-3´ + MVZ 16: 5´-AAATAGGAAGTATCACT CTGGTTTGAT-3´). PCR was carried out in 25 µL containing 2.5 µL 10X PCR buffer (Sigma), 3.5 µL $MgCl_2$ (25 mM, Sigma), 100 mM of each dNTP (Sigma), 0,4 µL of each primer (both 10 pmol/µL), and 0.075 µL *Taq* (5 U/µL, Sigma). After an initial denaturation at 95°C for 2 min, 15 cycles of 30 s at 94°C, 30 s at 55°C (temperature decreased every cycle for 1.0°C until 40°C was reached), 2 min at 72°C were performed, followed by 20 cycles of 30 s at 94°C, 30 s at 40°C and 2 min at 72°C. A final elongation of 10 min at 72°C was added. Cycle sequencing was carried out with the same protocol and in the same way like described above. Cytb sequences are deposited in Genbank under the accession numbers AY960639-AY960679; microsatellite sequences are deposited under accession numbers DQ097310-DQ097331. The original clone of ARENS *et al.* (2000) is deposited under the accession number AJ403996.

Sequences were aligned in BIOEDIT VERSION 7.0.1 (HALL, 1998) and alignment was checked by eye afterwards. The alignment was analyzed by Bayesian inference with the program MRBAYES 3.084 (RONQUIST & HUELSENBECK, 2003). In order to choose an appropriate model for the cytb data set, a series of runs (generations = 1.000.000) including different parameters were performed. The following model parameters were changed in all possible combinations for each run: Nst (1 = constrains all substitution rates to be the same; 2 = allows transitions and transversions to have potentially different rates; 6 = allows all rates to be different), rate heterogeneity (equal, gamma, adgamma, propinv, and invgamma), Omegavar (equal, Ny98, M3), All other parameters were run with default settings. A Bayes factor test was applied (NYLANDER *et al.*, 2004)

on harmonic means of the lnL of each run after trees of the burn-in phase had been removed and the HKY model (Nst = 2, rates = adgamma) was chosen, because the Bayes factor test clearly favored this model over others. The final run was terminated after 5.000.000 generations with four chains (one cold, three hot chains) starting from random trees and relying on the default prior and proposal settings of MrBayes. The burn-in length was set to 50.000 according to the results of the previous runs.

4.4 Results and Discussion

4.4.1 Cross-species amplification

Cross-species amplification results are summarized in appendix 4.2.
Of the 15 primer pairs, 4 show only minimal cross amplification success (WHA1-103, WHA1-29, WHA1-140, and WHA1-9). All remaining primer pairs generally demonstrate amplification across all species, although the Yemeni population does not amplify for WHA1-54, WHA1-60, and WHA1-133. Regarding other lacking products (in Table 1, marked with a *), we assume that further testing with different PCR conditions and/ or more samples may be successful, because usually these deficient PCR products are found in rows, where other populations and/ or species show amplification for primer pairs under investigation. Thus, non-amplification may be artificial in these cases.

4.4.2 Size homoplasy and undetected mutations

In total, 22 microsatellite sequences of WHA5-22A are characterized (Table 2; appendix 4.1). Of these, 12 belong to *H. savignyi* from Yemen covering 6 populations and one ancient sample where a detailed locality description is lacking. For three populations, 2-4 individuals are sequenced. Within Yemeni populations, *H. savignyi* display size homoplasy. The two specimens from S Yarim/Yemen have the same allele length, but differed in sequence (appendix 4.1). Dhamar-Yarim/Yemen is the second population, in which size homoplasy is detected. A specimen of the Dhamar-Yarim/Yemen 4 population show one mutation (an additional C) in the core region as well as a deletion at the end of the sequence and therefore display size homoplasy with two other individuals of the same population (1 and 3). In addition, specimens 1 and 3 of this population have a deletion at position 240 and an insertion at position 244; thus,

also these individuals show size homoplasy to each other. However, it has to be kept in mind that these cases are two out of three within population comparisons in Yemen. Thus, with more comparisons, the detected number may have raised. Among populations, the already mentioned cases are the only detected ones, which demonstrate size homoplasy to other populations with the same length within the Yemeni populations. Within the Iraqi population, all three sequences have different sequences in the flanking region.

Table 2: Results of size-scored and sequenced individuals for microsatellite WHA5-22A.

Species and Population	**Number of individuals scored by size**	**Electromorphs (Sequenced in bold)**	**Kind of repeat and repeat number**	**Number of haplotypes sequenced**
***Hyla meridionalis*, Teneriffe**	6	222 230 232	-	0
***Hyla meridionalis*, La Gomera**	4	222 232 **235**	$CAGT(CAG)_3(CAA)_2$ $CAG(CAA)_4CAG$ $(CAA)_2G(CAG)_5$ $CAGT(CAG)_3(CAA)_2$ $CAG(CAA)_4CAG$ $(CAA)_2C(CAA)_2$ $(CAG)_3$	2
***Hyla meridionalis*, Morocco**	1	**235**	$(CAG)_4(CAA)_2(CAG)_2$ $(CAA)_3CAG(CAA)_4$ $(CAG)_3$	1
***Hyla meridionalis*, France**	4	226 230 **235**	$CAA(CAG)_4(CAA)_2$ $CAG(CAA)_4CAG$ $(CAA)_4(CAG)_3$	1
***Hyla sarda*, Sardinia,**	2	228 234 238	-	0
***Hyla arborea*, Switzerland,**	2	234 **238**	$(CAG)_3(CAA)_4(CAG)_4$ $(CAA)_2(CAG)_6$	1
***Hyla arborea*, NW Spain**	1	236	-	0
***Hyla arborea*, Greece**	2	230	-	0
***Hyla savignyi*, Iraq**	6	232 236 **238** **240** **242**	$(CAA)_2(CAG)_{10}(CAA)_5$ $(CAG)_3$ $(CA)_2(CAG)_6(CAA)_3$ $(CAG)_4(CAA)_2CAG$ $CAA(CAG)_3$ $AC(CAG)_6(CAA)_2$ $(CAG)_4(CAA)_3CAG$ $CAA(CAG)_3$	3
***Hyla savignyi*, Syria**	4	218 **222**	$CAGCAA(CAG)_4$ $(CAA)_4CAG(CAA)_2$ $(CAG)_3$	2
***Hyla savignyi*, Yemen,**	361	209 211 **213** **214**	$CAGCAA(CAG)_4$ $(CAA)_4(CAG)_2CAA$ $(CAG)_3$	12
Total	393			22

In a strict sense, the alleles within the Iraqi population are not identical in size and thus, size homoplasy cannot be studied; however, the alleles are not identical in flanking regions. Within the Syrian population, size homoplasy is clearly detected. Both sequences have the same allele size, but show different sequences. Obviously, including all populations within *H. savignyi*, flanking regions are not identical. Within *H. meridionalis*, the two sequenced alleles from La Gomera have different flanking regions as well and different allele length is due to a mutation in the flanking region and not in the core region. The two other sequenced alleles from *H. meridionalis*, one from S-France and one from Morocco show identical allele sizes, but not identical sequences. A mutation in the core region differentiates the sequences. Furthermore, the two sequenced alleles of *H. arborea* are not identical in flanking regions. Therefore, both - mutations in the flanking regions (including substitutions and deletion/insertions) and in the core region contribute to size homoplasy.

Interestingly, the core region is significantly different from the original sequence (Table 2; appendix 4.1). Mainly, the third base within the repeat (CAA or CAG in the original sequence) changes in the other species or specimens to G or A, respectively, indicating that the third position is highly variable within and among species. Already within *H. arborea* - the species for which the microsatellite set was developed - the core region have changes in the third base, which cannot be accounted for total allele size (allele difference is due to longer core sequence in the original allele). Thus, the substitution rate in the core region is higher than detected only by length difference alone. A similar pattern is found within the Iraqi population. Size differences of alleles are due to additional repeats, but supplementary substitutions occur in the core region as well. Therefore, the substitution rate of this locus is grossly underestimated.

In summary, sequencing of locus WHA5-22A resulted in the detection of size homoplasy within populations and among populations of *H. savignyi*. The three major mutational mechanisms contributing to the allelic variation are (1) addition/ deletion of repeats, (2) substitutions and indels in the flanking region and core region, (3) mutations interrupting or changing the repeat.

4.4.3 Evolution of microsatellite locus WHA5-22A

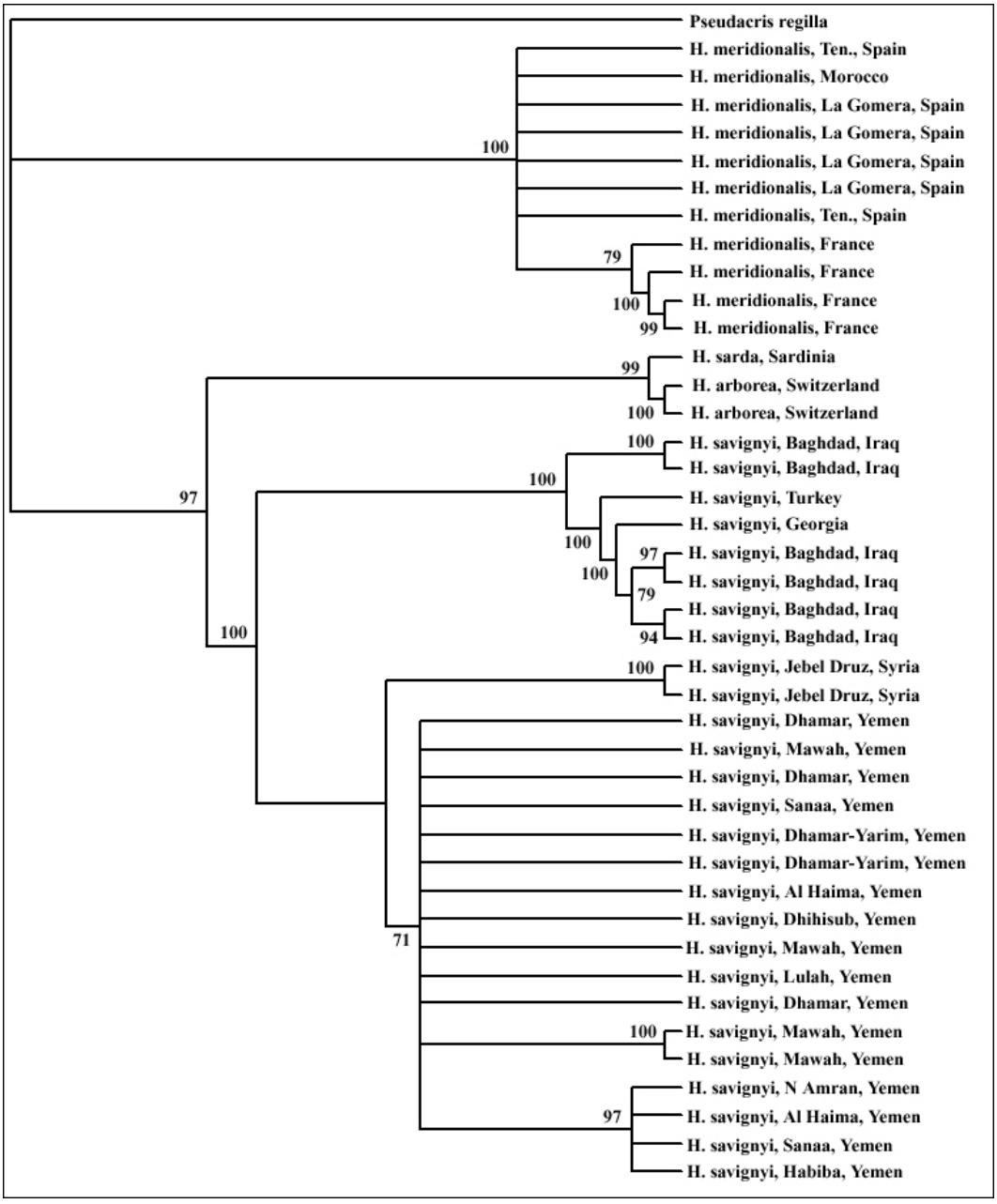

Fig. 2: Bayesian tree of mitochondrial cytb gene from *Hyla* species. Branch support is given on each branch, respectively.

The phylogenetic tree based on cytb sequences shows that *Hyla savignyi* is sister taxon to a monophyletic group composed of *H. arborea* and *H. sarda*; *H. meridionalis* branches off earlier (Fig. 2). *H. sarda* is closely related to *H. arborea*. The sibling

species of *H. arborea*, *H. intermedia* (not available for this study) is distributed in Italy and presumably colonized Sardinia from the mainland. Within *H. savignyi*, the Yemeni population is more closely related to the Syrian than to the Iraqi population.

Besides size homoplasy, locus WHA5-22A has another specific feature. Given that this microsatellite set was developed for *H. arborea*, it is interesting to notice the tendency to shorter repeat motifs compared to *H. arborea* (Fig. 2) is present both in *H. meridionalis* and *H. savignyi*. Going in more detail, it is obvious that within *H. savignyi* the Iraqi population shows longest repeat motifs; one Iraqi specimen has the longest, although altered repeat motif found in this study. The Syrian population shows shorter repeat motifs in comparison to the Iraqi population and the Yemeni populations the shortest in the whole study. Similar tendencies in decrement of allele length appear to be present in the loci WHA1-20, WHA1-67, WHA1-57 (Table 1). The latter two also display the same pattern like WHA5-22A; the decrement in allele length is stronger in *H. savignyi* in comparison to *H. meridionalis*. Finally, WHA1-104 and WHA1-61 have the longest microsatellite lengths in the Iraqi population judging from cross-utility tests.

Contrary to other studies, the cross amplification success generally is good. This may be explained by the fact that *H. savignyi* is closely related to *H. arborea* and has still conserved large sections of the microsatellite flanking regions including the priming sites. According to the cytb tree, *H. meridionalis* appears as a sister group to *H. arborea* and *H. savignyi*, which explains the successful cross amplification. The sample size for *H. meridionalis* is still limited, which can probably explain the low number of observed alleles in this species. In general, the set appears useful for population genetic studies within *H. meridionalis*, *H. savignyi*, and *H. arborea*.

However, the sequenced locus WHA5-22A exposes size homoplasy. Sequencing more copies may even increase the number of homoplastic alleles (VIARD *et al.*, 1998). Moreover, one has to keep in mind that the molecular accessible size homoplasy (MASH, ESTOUP *et al.*, 2002) can always display only a part of the actual present size homoplasy. Thus, total size homoplasy will always be higher than the detectable part. Although the consequences of size homoplasy in population genetic studies are difficult to predict (ROUSSET, 1996), it has to be stressed that due to size homoplasy, population structure may be falsely interpreted. Based on empirical studies, possible effects of size

homoplasy can be a reduced number of alleles in populations, a reduced proportion of heterozygotic individuals and decreased gene diversity (ESTOUP *et al.*, 1995; ANGERS & BERNATCHEZ, 1997; TAYLOR *et al*., 1999; ANGERS *et al.*, 2000; ESTOUP *et al*., 2002).
Nevertheless, ESTOUP *et al*. (2002) also pointed out that size homoplasy may not represent a significant problem in most population genetic studies, because the high variability of microsatellites possibly will often recompense the effects of homoplasious evolution. However, size homoplasy probably affects recently developed assignment tests (CORNUET *et al*., 1999; ESTOUP *et al*., 2002).
ESTOUP *et al*. (2002) also pointed out that size homoplasy is more likely to occur when microsatellites are influenced by strong allele size constraints in combination with high mutation rates and large populations.

Additional sources of polymorphisms are mutations in flanking and core regions. This source of polymorphism is mostly ignored in population genetic studies. However, our spot checks of sequenced flanking regions (appendix 4.1) clearly demonstrate that mutations in flanking regions cannot be ignored in their contribution to (size) polymorphism. Further investigations at a larger scale might best deliver insights in microsatellite evolution and probably initialize the development of more realistic mutational models.

4.4.4 Ascertainment bias or directional evolution?

Reciprocal studies of allele length variation between two species appear to be the most appropriate method to test for ascertainment bias (COOPER, 1998; Amos *et al*., 2003). Reciprocal studies use two microsatellite sets, each developed for one species under investigation (focal species), and test both sets in the focal species and against the other species (cross-amplified species). In this case, it is possible to test whether allele length is significantly longer in the focal species than in the cross-amplified species and consequently directional evolution can be excluded as a cause for asymmetry in the distribution of microsatellite length. Thus, ascertainment bias due to the criteria used in the development of the microsatellite sets would be the most likely alternative explanation. If directional evolution would play a role, microsatellites would show the same mutation bias in both, focal and cross-amplified species. We were unable to design a completely reciprocal study setting since a microsatellite marker system had

been developed just for *H. arborea*. Nevertheless, it seems to be unlikely that the reduction of allele length is solely based on ascertainment bias. Within *H. savignyi*, the shortest microsatellites length (Yemeni populations) as well as the longest microsatellite length (Iraqi population) is detected. Thus, WHA5-22A demonstrates both increment and decrement in *H. savignyi*, which cannot be explained by ascertainment bias alone. Based on the combined results of the phylogenetic analysis and the sequenced microsatellite locus WHA5-22A, mutational bias along biogeographical migration routes appears to be more likely. This would also explain why the Syrian population has an intermediate allele length between the Iraqi and Yemeni population. From the phylogenetic analysis, the Syrian population is also intermediately positioned between the Iraqi and Yemeni populations. A possible explanation for this mutation mechanism in *H. savignyi* could be that mutation rates and mutation bias in two evolutionary lineages (Iraqi *versus* Syrian/ Yemeni lineage) are different. Lineages, in which microsatellites are longer, should have experienced a greater average number of expansion mutations. Causes for this increased number of expansion mutations can be a shift in the equilibrium length distribution in the genome(s) or alternatively a biased mutation process, in which divergence in the average mutation rates in the different lineages occurred (AMOS, 1999). For the former, heterozygote instability could be a possible mechanism to change the mutation rate of microsatellites within a short evolutionary time frame.

Another potential explanation for longer allele lengths in the Iraqi population in comparison to the Syrian/ Yemeni population may be different population sizes. RUBINSZTEIN *et al.* (1996) discussed this issue in human-primate comparisons; humans tend to have generally longer microsatellites as well as larger population sizes and, accordingly, higher rates of microsatellite mutations. Since microsatellites tend to expand in their allele length, the species with larger effective population size and therefore higher mutation rates should show longer microsatellite lengths (HUTTER *et al.*, 1998). Thus, expanded populations should display longer microsatellite lengths. Although this statement is based on species comparisons, the same argument may be applied for the differential allele lengths between the Syrian, Yemeni and Iraqi populations, because the Yemeni population is assumable a glacial relict and additionally the most southern population of *Hyla* in Europe and the Middle East. This indicates that the Yemeni population is a marginal population, which probably had a

low effective population size. Therefore, population size in the Yemeni population may be much smaller than in the Iraqi or Syrian ranges of the species. The Iraqi population could represent an expanded population with longer microsatellites. Of course, this argument may work also for the among species comparison; *H. arborea* probably had the greatest geographical range in comparison to *H. meridionalis* and *H. savignyi*.

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Appendices

Appendix 4.1: Sequences of microsatellite locus WHA5-22A from 22 individuals of *Hyla*. A Complete microsatellite sequence is given for the original clone WHA5-22A (*H. arborea*). Other sequences with nucleotides identical to this one are represented by periods (.). Gaps are represented by -.

```
                                          10        20        30        40
                                    ....|....|....|....|....|....|....|....|
H. arborea, original clone WHA      GTTACAGCAAC-AGCAAATGGCAGCGCATATTCACCGAAT
H. arborea, Switzerland             T.........-.............................
H. meridionalis, La Gomera, Spain   T.........-........T....................
H. meridionalis, La Gomera, Spain   T.........-........T....................
H. meridionalis, S-France           T.........-.............................
H. meridionalis, Morocco            T.........-.............................
H. savignyi, Baghdad, Iraq1         T.........-......................T......
H. savignyi, Baghdad, Iraq2         T.........-......................TT.....
H. savignyi, Baghdad, Iraq3         T.......G..T......................T......
H. savignyi, Syria1                 T.........-...........................A...
H. savignyi, Syria2                 T.........-.............................
H. savignyi, N Amran, Yemen         T.........-.............................
H. savignyi, Lulah, Yemen           T.........-.............................
H. savignyi, Habiba, Yemen1         T.........-.............................
H. savignyi, Habiba, Yemen2         T.........-.............................
H. savignyi, Ad Dogma, Yemen        T.........-.............................
H. savignyi, Dhamar-Yarim, Yemen    T.........-.............................
H. savignyi, Dhamar-Yarim, Yemen    T.........-.............................
H. savignyi, Dhamar-Yarim, Yemen    T.........-.............................
H. savignyi, Dhamar-Yarim, Yemen    T.........-.............................
H. savignyi, S Yarim, Yemen1        T.........-.............................
H. savignyi, S Yarim, Yemen2        T.........-.............................
H. savignyi, Yemen, 1985            T.........-.............................

                                          50        60        70        80
                                    ....|....|....|....|....|....|....|....|
H. arborea, original clone WHA      AC--AGCAGCAG-CAGCAACAACAACAACAACAACAGCAG
H. arborea, Switzerland             ----.-.......-.....G.............G......
H. meridionalis, La Gomera, Sp      --------A...T.....G..G........G.....A..A
H. meridionalis, La Gomera, Sp      --------A...T.....G..G........G.....A..A
H. meridionalis, S-France           --------A...-.....G..G........G.....A..A
H. meridionalis, Morocco            --------A...-.....G..G........G..G..A..A
H. savignyi, Baghdad, Iraq1         ----.-..A..A-.....G..G..G..G..G..G......
H. savignyi, Baghdad, Iraq2         ..AC.-.......-.....G..G..G..............
H. savignyi, Baghdad, Iraq3         ----.C.......-.....G..G..G........G......
H. savignyi, Syria1                 ------------------.-..G.....G..G..G.....A
H. savignyi, Syria2                 ------------------.-..G.....G..G..G.....A
H. savignyi, N Amran, Yemen         ----------------------------...G.........
H. savignyi, Lulah, Yemen           ----------------------------...G.........
H. savignyi, Habiba, Yemen1         ----------------------------...G.........
H. savignyi, Habiba, Yemen2         ----------------------------...G.........
H. savignyi, Ad Dogma, Yemen        ----------------------------...G.........
H. savignyi, Dhamar-Yarim, Yemen    ----------------------------...G.........
H. savignyi, Dhamar-Yarim, Yemen    ----------------------------...G.........
H. savignyi, Dhamar-Yarim, Yemen    ----------------------------...G.........
H. savignyi, Dhamar-Yarim, Yemen    -------------------------.-...G.........
H. savignyi, S Yarim, Yemen1        ----------------------------...G.........
H. savignyi, S Yarim, Yemen2        ----------------------------...G.........
```

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H. savignyi, Yemen, 1985           ----------------------------...G.........

                                                90       100       110       120
                                   ....|....|....|....|....|....|....|....|....|
H. arborea, original clone WHA     CAGCAGCAACACAGCAGCAGCAGCAGGTTATGCAACA
H. arborea, Switzerland            .....A.....G-C-.....................
H. meridionalis, La Gomera, Spain  ..A........A-.....................
H. meridionalis, La Gomera, Spain  ..A........A-C-.A..A..............
H. meridionalis, S-France          ..A........A-C-.A..A..............
H. meridionalis, Morocco           ..A........A-C-.A..A..............
H. savignyi, Baghdad, Iraq1        .....A.....A-C-....A..............
H. savignyi, Baghdad, Iraq2        ..........A-C-....A..............
H. savignyi, Baghdad, Iraq3        .....A.....A-C-....A..............
H. savignyi, Syria1                ..A..A.....G-C-.A..A..............
H. savignyi, Syria2                ..A..A.....G-C-.A..A..............
H. savignyi, N Amran, Yemen        ..A..A.....G-C-....A..............
H. savignyi, Lulah, Yemen          ..A..A.....G-C-....A..............
H. savignyi, Habiba, Yemen1        ..A..A.....G-C-....A..............
H. savignyi, Habiba, Yemen2        ..A..A.....G-C-....A..............
H. savignyi, Ad Dogma, Yemen       ..A..A.....G-C-....A..............
H. savignyi, Dhamar-Yarim, Yemen   ..A..A.....G-C-....A..............
H. savignyi, Dhamar-Yarim, Yemen   ..A..A.....G-C-....A..............
H. savignyi, Dhamar-Yarim, Yemen   ..A..A.....G-C-....A..............
H. savignyi, Dhamar-Yarim, Yemen   ..A..A.....G-C-....A..............
H. savignyi, S Yarim, Yemen1       ..A..A.....G-C-....A..............
H. savignyi, S Yarim, Yemen2       ..A..A.....G-C-....A ..............
H. savignyi, Yemen, 1985           ..A..A.....G-C-....A ..............

                                               130       140       150       160
                                   ....|....|....|....|....|....|....|....|....|
H. arborea, original clone WHA     GCCTATACAGCAACAGCAAATGCAGCAGCAGCCACAACAG
H. arborea, Switzerland            ........................................
H. meridionalis, La Gomera, Spain  ........................................
H. meridionalis, La Gomera, Spain  ........................................
H. meridionalis, S-France          ........................................
H. meridionalis, Morocco           ........................................
H. savignyi, Baghdad, Iraq1        ........................................
H. savignyi, Baghdad, Iraq2        ........................................
H. savignyi, Baghdad, Iraq3        ........................................
H. savignyi, Syria1                ........................................
H. savignyi, Syria2                ........................................
H. savignyi, N Amran, Yemen        ........................................
H. savignyi, Lulah, Yemen          ........................................
H. savignyi, Habiba, Yemen1        ........................................
H. savignyi, Habiba, Yemen2        ........................................
H. savignyi, Ad Dogma, Yemen       ........................................
H. savignyi, Dhamar-Yarim, Yemen   ........................................
H. savignyi, Dhamar-Yarim, Yemen   ........................................
H. savignyi, Dhamar-Yarim, Yemen   ........................................
H. savignyi, Dhamar-Yarim, Yemen   ........................................
H. savignyi, S Yarim, Yemen1       ........................................
H. savignyi, S Yarim, Yemen2       ........................................
H. savignyi, Yemen, 1985           ........................................
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                                        170       180       190       200
                                    ....|....|....|....|....|....|....|....|
H. arborea, original clone WHA      GTAATGCAGCTCCAGTTGCAGCAGCAGCAAGTGGCACAAA
H. arborea, Switzerland             ........................................
H. meridionalis, La Gomera, Spain   ........................................
H. meridionalis, La Gomera, Spain   ........................................
H. meridionalis, S-France           ........................................
H. meridionalis, Morocco            ........................................
H. savignyi, Baghdad, Iraq1         ........................................
H. savignyi, Baghdad, Iraq2         ........................................
H. savignyi, Baghdad, Iraq3         ........................................
H. savignyi, Syria1                 ........................................
H. savignyi, Syria2                 ........................................
H. savignyi, N Amran, Yemen         ........................................
H. savignyi, Lulah, Yemen           ........................................
H. savignyi, Habiba, Yemen1         ........................................
H. savignyi, Habiba, Yemen2         ........................................
H. savignyi, Ad Dogma, Yemen        ........................................
H. savignyi, Dhamar-Yarim, Yemen    ........................................
H. savignyi, Dhamar-Yarim, Yemen    ........................................
H. savignyi, Dhamar-Yarim, Yemen    ........................................
H. savignyi, Dhamar-Yarim, Yemen    ........................................
H. savignyi, S Yarim, Yemen1        ........................................
H. savignyi, S Yarim, Yemen2        ........................................
H. savignyi, Yemen, 1985            ........................................

                                        210       220       230       240
                                    ....|....|....|....|....|....|....|....|
H. arborea, original clone WHA      GTCAGCAGCAACTGCTTTCTACGCAGCCACAGACCCAGTC
H. arborea, Switzerland             ........................................
H. meridionalis, La Gomera, Spain   ..........GA..............................
H. meridionalis, La Gomera, Spain   ..........GA..............................
H. meridionalis, S-France           ..........GA..............................-
H. meridionalis, Morocco            ..........GA..............................-
H. savignyi, Baghdad, Iraq1         ........................................-
H. savignyi, Baghdad, Iraq2         ........................................-
H. savignyi, Baghdad, Iraq3         ........................................
H. savignyi, Syria1                 ........................................
H. savignyi, Syria2                 ........................................
H. savignyi, N Amran, Yemen         ........................................
H. savignyi, Lulah, Yemen           ........................................
H. savignyi, Habiba, Yemen1         ........................................
H. savignyi, Habiba, Yemen2         ........................................
H. savignyi, Ad Dogma, Yemen        ........................................
H. savignyi, Dhamar-Yarim, Yemen    ........................................-
H. savignyi, Dhamar-Yarim, Yemen    ........................................
H. savignyi, Dhamar-Yarim, Yemen    ........................................-
H. savignyi, Dhamar-Yarim, Yemen    ........................................-
H. savignyi, S Yarim, Yemen1        ..................T.....................
H. savignyi, S Yarim, Yemen2        ........................................
H. savignyi, Yemen, 1985            ........................................
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                                          250
                                    ....|....|
H. arborea, original clone WHA      CCT-GATA
H. arborea, Switzerland             ...-....
H. meridionalis, La Gomera, Spain   ...-....
H. meridionalis, La Gomera, Spain   ...-....
H. meridionalis, S-France           ...G....
H. meridionalis, Morocco            ...G....
H. savignyi, Baghdad, Iraq1         ...G....
H. savignyi, Baghdad, Iraq2         ...G....
H. savignyi, Baghdad, Iraq3         ...-....
H. savignyi, Syria1                 ...-....
H. savignyi, Syria2                 ...-....
H. savignyi, N Amran, Yemen         ...-....
H. savignyi, Lulah, Yemen           ...-....
H. savignyi, Habiba, Yemen1         ...-....
H. savignyi, Habiba, Yemen2         ...-....
H. savignyi, Ad Dogma, Yemen        ...-....
H. savignyi, Dhamar-Yarim, Yemen    ...T....
H. savignyi, Dhamar-Yarim, Yemen    ...G....
H. savignyi, Dhamar-Yarim, Yemen    ...G....
H. savignyi, Dhamar-Yarim, Yemen    ...-....
H. savignyi, S Yarim, Yemen1        .T.-....
H. savignyi, S Yarim, Yemen2        ...-....
H. savignyi, Yemen, 1985            ...-....
```

Appendix 4.2: Results of cross-utility tests; numbers give range of detected allele sizes. Locality and number of specimens tested are given in brackets. Abbreviations: m = monomorphic, * = although no product could be obtained in this study, further testing with different PCR conditions might be successful, because primers were tested with only one specimen and / or all other rows have products.

Microsatellite locus	***Hyla arborea* (6 from Switzerland/ 13 from the Netherlands & Croatia; Arens *et al.*, 2001)**	***Hyla savignyi* (Iraq, 6)**	***Hyla savignyi* (Yemen, 361)**	***Hyla savignyi* (Syria, Jebel Druz, 2)**	***Hyla meridionalis* (Morocco, 1)**	***Hyla sarda* (Sardinia, 1)**	***Hyla meridionalis* (Spain, Canary Islands, 4)**	***Hyla meridionalis* (France, 4)**
WHA5-22A	235-241/ 233-239	224-232	209-214	223	235	234-238	222-232	226-230
WHA1- 103	230-244/ 242	-	-	-	-	234	-	214-219
WHA1- 104	264-292/ 270-272	259-270	268-276	270-276	276-278	277-279	278	274-276
WHA5- 201	238-247	260-270	189 m	190	-*	-*	190	191
WHA5- 29	330-336/ 331-333	309	-	-	-	-	-	310
WHA1-140	112-132	-	-	-	-	-	-	104-114
WHA1- 9	126-136	-	-	-	-	-	-	-
WHA1- 20	189-193	157-169	163-169	168	-*	-*	167-169	167-169
WHA1- 25	111-117/ 103-109	97-113	89-95	89-97	95-101	89-95	87-101	101
WHA1- 54	129-145	149	-	115-122	149	142	142-149	149
WHA1- 60	153-169/ 153-157	-*	-	157-171	-*	-*	139	139
WHA1- 61	142-150/ 149	136-160	153-155	142	-*	-*	142	153
WHA1- 67	202-226/ 214-226	176-180	165-180	203	203	211-213	204	207
WHA1- 133	145-157/ 151-159	137-149	-	140	147	149-152	142-147	150, 152
WHA1- 57	262-332/ 278	236-245	224-234	225-234	239-241	234	268-295	-*

Characterization of microsatellite loci for *Reissita simonyi* (REBEL, 1899), (Lepidoptera, Zygaenidae)

5.1 Abstract

The newly developed microsatellite set for *Reissita simonyi* (REBEL, 1899) (Lepidoptera, Zyganidae) is presented. In total, 14 loci turned out to be highly polymorphic. The microsatellite set will be used for a population genetic analysis of this endemic species in Southern Arabia. In focus of this study stands the genetic differentiation and variability of isolated populations, which are assumed to be based on habitat fragmentation due to natural processes and human impact. Moreover, this new microsatellite set showed distinct band patterns in the Palaearctic genus *Zygaena* which seems to be promising for further studies.

Keywords: habitat fragmentation, *Reissita simonyi*, molecular markers, SSR.

5.2 Introduction

Habitat loss, desertification, human impact and over-grazing seem to be the main causes for habitat fragmentation and decline of biodiversity in Arabia (GLOBAL ENVIRONMENT OUTLOOK-1, 1997; MALCOLM & MARKHAM, 2000). The fauna and flora of Southern Arabia consist mainly of Afrotropical and a few Paleartic (Euro-Asiatic) faunal elements, a considerable portion of which is represented by endemic species (*e. g.* 11 endemic bird species; WHEATLEY, 1997). Therefore, the fauna and flora of Southern Arabia can be considered as a remnant of an early migration from these areas. A natural process of desertification led to an isolation of these areas into fragmented habitats (THOMPSON, 2000). Nowadays habitat fragmentation and desertification is rapidly progressing due to anthropogenic influences.

In order to assess the impact of ancient and recent habitat fragmentation on levels of gene flow and genetic differentiation between isolated populations we have chosen *Reissita simonyi* (REBEL, 1899). The diurnal moth *Reissita simonyi* (Lepidoptera,

Zygaenidae) is an endemic species to Southern Arabia (NAUMANN & EDELMANN, 1984). Two probably allopatric forms of *Reissita simonyi* are described: *Reissita simonyi simonyi* and *Reissita simonyi yemenicola* (TREMEWAN, 1959; NAUMANN & EDELMANN, 1984). The larvae of this species exclusively feed on plants of the genus *Maytenus* (Celastracae), containing *M. senegalensis* and *M. dhofarensis*, which are assumed to show similar patchy populations. Therefore, *Reissita simonyi* is probably more sensitive to fragmentation and habitat loss than other faunal elements in this area.

We selected microsatellite markers to study gene flow, the extent of differentiation and genetic diversity between isolated populations and the effects of ancient as well as recent habitat fragmentation. Microsatellite loci offer several advantages over RFLP/ AFLP or allozyme techniques, since they are highly polymorphic, suitable to be amplified from small tissue amounts, and they have co-dominant properties.

5.3 Material and Methods

DNA was extracted from a single larva (Qiagen DNeasy Tissue Kit) and digested with *Hinf I*. DNA fragments were enriched by ligation with an adapter and hybridization to biotin labelled CA probes and subsequently isolated with streptavidin beads. Afterwards, fragments were amplified with PCR (adapter sequence served as primer) and transformed into the vector pCR®2.1-TOPO® (Invitrogen). One hundred positive clones were isolated, of which 54 were sequenced using an ABI Prism Sequencing Kit and an ABI 377 sequencer (Applied Biosystems). About 50 % of the sequences contained long repetitive motifs.

Primers were designed using the software package PRIMER 0.5 (Whitehead Institute for Biomedical Research). Forward primers were labelled with fluorescent dyes (Sigma: 6-FAM; Applied Biosystems: NED and VIC). PCR amplification was carried out in 10 µL containing 10 x PCR buffer (without $MgCL_2$; contains 100 mM Tris-HCL, 500 mM KCL, pH 8.3, Sigma), 1,5 – 2,0 mM $MgCl_2$ (Sigma), 100 µM of each dNTP (Sigma), 500 nM of each primer and 0,5 unit *Taq* polymerase (contains 20 mM Tris-HCL, 100 mM KCL, 0,1 mM EDTA, 1 mM DTT, 0,5 % Tween 20, 50 % glycerol; Sigma). Amplification was performed in an Applied Biosystems GeneAmp 2700 thermal cycler. Following an initial denaturation step of 3 minutes at 94°C, the reactions underwent 35

thermal cycles (94°C for 30 s, 60 s at annealing temperature (appendix 5.1) and 65 s at 72°C) and a final extension time of 20 min at 72°C. Using a 6 % acrylamide gel and an ABI 377 sequencer, products were separated. Results were analysed with GENESCAN 3.1.2 (Applied Biosystems) and only high quality fragments (no stutter bands or stutter bands present, but well-scorable) were taken into account.

Primer pairs were tested on a set of 60 randomly chosen individuals from 20 different populations distributed over Yemen and Oman. Distances between populations vary from 5 to 1000 kilometers.

5.4 Results and discussion

In total, 14 *R. simonyi* microsatellite loci turned out to be polymorphic (appendix 5.1). For each locus, expected and observed heterozygosities were calculated over all populations using POPGEN 32 (YEH & BOYLE, 1997). For almost all loci, observed heterozygosities strongly deviate from expected values. Most of the observed heterozygosities are considerable lower than expected values. This can be a sampling artefact, since we used only 3 specimens per population to test polymorphism or it could suggest a highly fragmented population structure, which has to be examined by the following population genetic analysis.

The developed set of microsatellite loci is highly polymorphic. For this reason, it could be of use for other studies such as paternity investigations. Also three species of the genus *Zygaena* were tested in PCR amplifications and displayed clearly scorable band patterns on agarose gels (Table 2). However, we did not have enough samples to check for polymorphism, but tests were promising for further investigations.

Table 2: Successful amplifications in the genus *Zygaena*, + = distinct band patterns with same PCR conditions like *R. simonyi*, - = no successful amplification, 0 = successful amplification, but slightly different amplification conditions (further information in brackets)

Microsatellite locus	***Zygaena transalpina latina***	***Zygaena transalpina hippocrepidis***	***Zygaena angelicae elegans***
RS-3	+	+	+
RS-4	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-5	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-17	0 (smeary, higher annealing temperature and 1,5 mM $MgCl_2$)	0 (smeary, higher annealing temperature and 1,5mM $MgCl_2$)	0 (smeary, higher annealing temperature and 1,5 mM $MgCl_2$)
RS-19	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-24	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-28	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-31	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-32	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)
RS-33	-	-	-
RS-37	-	-	-
RS-44A	-	-	-
RS-47	0 (smeary, higher annealing temperature and 1,5 mM $MgCl_2$)	0 (smeary, higher annealing temperature and 1,5 mM $MgCl_2$)	0 (smeary, higher annealing temperature and 1,5 mM $MgCl_2$)
RS-50	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)	0 (1-2 °C higher annealing temperatue)

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Appendix

Appendix 5.1: Characterization of *Reissita simonyi* microsatellite markers and their products. M_l = Microsatellite locus; Ppl = Predicted product length (bp); A_t = Annealing temperature; NoA = number of alleles.

M_l	Repeat	Ppl	Primer sequences (forward, reverse)	A_t	No A	Allelic range	Mean H_O/ H_E	Genbank Accesion numbers
RS-3	$(CA)_{11}$	107	5´-TCAGAAGTTCCAAACGAAGAG-3´ 5´-ATCATAGCAAGCTCGAAAAGC-3´	48°C	19	99-124	0.5357 0.9273	AY250755
RS-4	$(GT)_5(GC)_2$ GTGC $(GT)_3$ATGT AT$(GT)_3$AT $(GT)_3$GCG TGCCT $(GT)_5$ATGT $(GCGT)_3$ GA$(GT)_9$	390	5´-AGCTGACTGCCAAACTA-3´ 5´-AGACTGCGACCACTATGA-3´	49°C	7	346-360	0.0769 0.7781	AY250744
RS-5	$(CA)_3$TA $(CA)_9$	286	5´-GAGCGGCCGCCAGTGTGA-3´ 5´-ATAAAGCATGCAAAAGTTCT-3´	50°C	6	244-254	0.0000 0.3189	AY250742
RS-17	$(CA)_9(TA)_2$ $(CA)_3$	173	5´-GTATGGAAACTGCTAACAAAT-3´ 5´-TTCGTAACGTCACACTCACA-3´	52°C	5	138-155	0.2000 0.6136	AY250743
RS-19	$(CA)_8$CTA A $(CA)_9$	281	5´-ATGTAGAATTTTGTGATGTAGAG-3´ 5´-TAGCCTGATATAAAGAGTTCC-3´	49°C	13	233-284	0.1600 0.8098	AY250745
RS-24	$(CA)_2$CG $(CA)_6$CC $(CA)_7$CC $(CA)_6$CGC ATATG $(CA)_4$CGT AAA$(CA)_4$	213	5´-GGAAGCAGGAGTAAAAGCAAATAA-3´ 5´-CCGACTAAGGTGACAATGGTGATA-3´	51°C	7	189-205	0.4000 0.7467	AY250746
RS-28	$(GT)_{11}$	115	5´-TGAAGGAATTTTTAAAGTGACAAC-3´ 5´-ATTCCCCTTAGATTTATGATGATT-3´	48°C	9	106-119	0.4048 0.5306	AY250747
RS-31	$(GTTT)_{14}$	204	5´-GGTCCACCATTCCTTAGAGATA-3´ 5´-ACCAGTAGTTTCGGAGATTAG-3´	47°C	15	147-217	0.2273 0.8946	AY250748
RS-32	$(CA)_6$CTC G$(CA)_7$CG CG$(CA)_5$ CTCGGCG G$(CA)_{18}$	225	5´-GGCAGGGAAAGAAGGAGAGGA-3´ 5´-CAAGTGTAAGTTCGGATGTG-3´	51°C	5	163-169	0.0000 0.6200	AY250749
RS-33	$(CA)_8$	329	5´-GACAACAGCCACATTCATACT-3´ 5´-CCCGAGGAAATCCCGAAAGTT-3´	50°C	22	312-347	0.3878 0.9325	AY250750
RS-37	$(GT)_9$	295	5´-TAGACTGCGTACCAATCGTG-3´ 5´-CTTAAGTCGGCTAGTCAAATC-3´	48°C	7	276-304	0.1316 0.6434	AY250751
RS-44A	$(GAAAT)_3$ GAAT $(GAAAT)_5$	173	5´-TATTACCACCGTTTTTGTTTGTCA-3´ 5´-TATCCTCTTTGTTGCGGCTCAGTA-3´	48°C	9	163-177	0.2632 0.8601	AY250752

Appendix 5.1: continued

RS-47	$(CA)_2CG$ $(CA)_6CC$ $(CA)_7CC$ $(CA)_6$	217	5´-GGAAGCAGGAGTAAAAGCAAATAA-3´ 5´-AAATCCGACTAAGGTGACAATGGT-3´	51°C	14	192-214	0.3810 0.8605	AY250753
RS-50	$(GT)_3GC$ $(GT)_5AC$ $(GT)_4GC$ $(GT)_5AC$ $(GT)_4(GC)_4$ $GY(GT)_{12}$	245	5´-GAGAGTGCGAAATCCATAAT-3´ 5´-ATACGCGCATAACAAACTAC-3´	50°C	9	210-229	0.1500 0.7725	AY250754

Population genetic structure of *Hyla savignyi* (AUDOUIN, 1827; Hylidae; Amphibia) in the highlands of Yemen is shaped by a mixture of isolation by distance, partial limited migration, and long- distance gene flow

6.1 Abstract

Hyla savignyi AUDOUIN, 1827, the yellow-lemon tree frog is found from Turkey down to the Saudi Arabian Peninsula in Yemen and Oman. It belongs to the *Hyla arborea* - group, the common European green tree frog, but is geographically isolated from its closest relatives. Populations of *Hyla savignyi*, like most other amphibians, are highly linked to water places (at least for reproduction). In the highlands of Yemen, suitable water places are extremely scattered and highly disturbed by human impact (*e. g.* insecticides). Therefore, *Hyla savignyi* is probably more sensitive to fragmentation and habitat loss than other faunal elements in this area. Additionally, migration and population structure may also be influenced by altitude differences up to approximately 600 m.

We selected 5 microsatellite markers from ARENS *et al.* (2000) to study population structure, gene flow, the extent of differentiation and genetic diversity between presumably isolated populations. In total, 325 tree frogs from 14 populations were analyzed. F_{ST} values range from 0.0004 to 0.6857, which generally indicate strong subdivision of populations. Over all populations, isolation by distance and isolation by altitude are obvious; although the latter is not significant in partial mantel tests. Population-structure analysis with assignment tests further suggest the existence of genetically different populations, which mainly correspond to the sampled geographic sites. Differences in gene frequencies result in three major clusters. Within clusters, migration is detectable; among clusters migration is measurable to a much lower extent or clearly absent. However, we also find signs of long-distance gene flow between certain populations. The influence of geographical isolation is not surprising, taken into account that *H. savignyi* is an amphibian with low dispersion abilities. In contrast, long-distance gene flow may be based on frogs or tadpoles transported through tank trucks to other ponds. Finally, it appears that migration shows a South to North extension,

indicating that successful migration is supported towards the South and that potential barriers to gene flow towards the North are present.
In summary, the complex population structure of *H. savignyi* in Yemen is shaped by different isolation factors, partial limited migration, and long distance gene flow.

Keywords: habitat fragmentation, *Hyla savignyi*, microsatellites, isolation by distance.

6.2 Introduction

Numbers of publications of population genetic and biogeographical studies of amphibians increased in recent years (ALEXANDRINO *et al*., 2000; LEBLOIS *et al*., 2000; LAMPERT *et al*., 2003; BABIK *et al*., 2004). Mainly, this was due to efforts in conservation genetics, as an attempt to understand the world wide decline of species and loss of genetic variation in amphibians (HOULAHAN *et al*., 2000; REASER, 2000; DAVIDSON *et al*., 2002; ANDERSEN *et al*., 2004). In these studies, special attention has been paid to isolation mechanisms (*e. g.* roads, urbanization, habitat destruction), which were assumed to be responsible for inbreeding effects and impediment of dispersal/ migration (HITCHINGS & BEEBEE, 1997; JOHNSTON & FRID, 2002). Additionally, habitat fragmentation and loss were assumed to play an important role in isolation and extinction of populations (VOS & STUMPEL, 1995; FUNK *et al.*, 2005a).

A first consequence of habitat fragmentation will be a decreased genetic effective population size in habitat patches. If isolation and restricted gene flow continues for a number of generations in small effective populations, a loss of heterozygosity and alleles due to random drift will occur at an increasing rate (HARTL, 1999) which can be measured as an inbreeding effect. Inbreeding can be seen as an indicator of viability of populations (HARTL, 1999). A loss of genetic variation is assumed to have negative effects on the evolutionary potential and fitness of populations and species (ANDERSEN *et al.*, 2004). Genetic variation is thought to maintain adaptability of a population (and species) in a changing environment, although the exact mechanisms for this reciprocal relationship between genetic variation and fitness of a population remain unclear (ANDERSEN *et al*., 2004). Nevertheless, conservation biologists aim to detect inbreeding effects and subsequently develop action plans in order to raise heterozygosity in vulnerable populations. Therefore, detailed studies about genetic differentiation within

and across populations are necessary to detect endangered populations and populations that display properties to serve as source stocks for genetic variability. Moreover, a critical examination of probable isolation factors as well as migration between populations is necessary. Particularly in Southern Arabia these detailed population genetic studies are still lacking despite a highly vulnerable ecosystem and massive human impact.

This is particularly true for amphibian species with an assumable stenoecicous ecological range, like *Hyla savignyi*, which prefers calm, well temperate, non-eutrophicated and shallow ponds. Therefore, amphibian populations often are assembled in a metapopulation spatial structure (ALFORD & RICHARDS, 1999; MARSH & TRENHAM, 2001) corresponding to the geographical distribution of suitable ponds. Additionally, *H. savignyi* is assumed to have low migration abilities of about 4 km comparable to other western Palaearctic tree frog species (FOG, 1993; ANDERSEN *et al.*, 2004). Thus, an interconnected or dense network of water ponds will maintain connectivity of populations and support gene flow. Therefore, the species may suffer the consequences of habitat - area reduction and disturbance in a more sensitive way than many other species. However, at the same time, individuals may disperse over long distances, as found in *H. arborea* (STUMPEL & HANEKAMP, 1986). It was found that *Hyla arborea* individuals are able to migrate distances of 12.6 km per year (mark-recapture methods). Therefore, it may be possible that single individuals display long distance dispersal.

Hyla savignyi was also chosen as a model species, because it has another important property for this kind of studies. The yellow-lemon tree frog (*Hyla savignyi*) inhabits a wide range from Turkey, Azerbaijan and Georgia in the north to Saudi Arabia and Yemen in the south. The Yemeni populations are assumed to be a remnant of a settlement from the last glacial series (10000 to 12000 years ago, THOMPSON, 2000). Due to the long temporal isolation, the population in Yemen and Saudi Arabia can be considered as a closed system without genetic introgression from outside populations. Hence, most of the detected population genetic effects will be a consequence of local changes in the population structure.

Potential hazards for this species are environmental pollution (*e. g.* sewage and different kinds of oils) in combination with chemical substances like insecticides and molluscicides (SCHÄTTI & GASPERETTI, 1994). *Hyla savignyi* is threatened by the fact that connectivity of water ponds may be restricted due to roads and, maybe even more important, due to regular draining of ponds to irrigate agricultural fields. Due to the latter, connectivity of populations may be influenced by periodical extinction (no reproduction possibility) of populations or at least reduction in population size. On the other hand, displacement of tadpoles or eggs may occur, because water from ponds is occasionally transferred (*e. g.* tank trucks; KLÜTSCH, pers. observations) to other ponds or agricultural fields in the surrounding areas. Therefore, connectivity of population and migration between them may be restricted or supported in one or the other way.

The landscape topology of Southern Arabia may represent a supplementary challenge for species with presumable low dispersal abilities (FUNK *et al*., 2005b), like *H. savignyi*. Isolation by distance effects may influence the population structure intensively (GARNIER *et al*., 2004; VIGNIERI, 2005), but the highland structure of Yemen could also separate populations of frogs by altitudinal effects. Recently, altitudinal separation was addressed in a number of studies (STORZ & DUBACH, 2004; DE NAVASCUÉS MELERO, 2005).

In summary, the aim of the present study was to analyze the genetic composition of the Yemeni populations of the yellow-lemon tree frog *H. savignyi*. Focus was put on the following central questions:

(i) What is the population structure and genetic variation among populations?

(ii) Are there possible migration routes and are they related to the connectivity of the water pond system?

(iii) Does isolation by distance (IBD) contribute to the population structure and thus does connectivity of ponds decrease with geographical distance? Additionally, does an altitudinal distance contribute to the genetic differentiation as well and therefore to connectivity of populations?

6.3 Material and Methods

6.3.1 Sampling

The study was conducted in Yemen, Southern Arabia. The landscape is mainly a desert area with partly natural, partly artificial water ponds. Tree frogs were sampled from 14 localities (Fig. 1) over a two year period (2001-2002) in Yemen and from one population from Baghdad as outgroup.

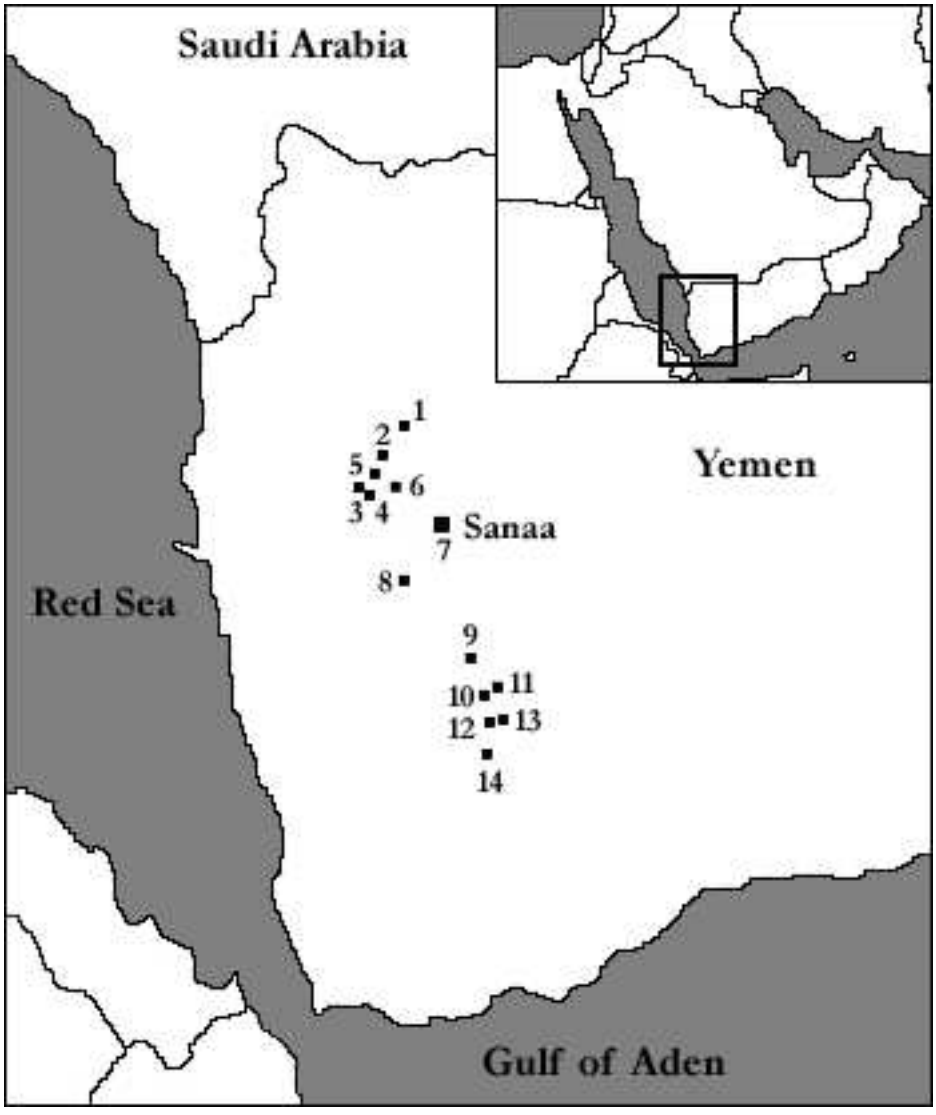

Fig.1: Sampling sites of *H. savignyi* in Yemen. Numbers correspond to those in Table 1.

Toe-tips of 335 specimens were collected. After catching by hand, one toe-tip of the right hind foot was clipped. To avoid re-sampling, individuals with one toe-tip less at the right hind foot were not sampled. Tissue was directly stored in 100 % ethanol and specimens were immediately released at sampling site. Samples are stored in the tissue collection of ZFMK at -20°C. Of these 14 populations, five had sample sizes under or equal to 10; all other sites were sampled with 19-37 samples per site. In Southern Yemen suitable habitats for this frog were found in lower altitudes, but so far, tree frogs have not been found at these sites (KLÜTSCH *et al.*, chapter 2). Therefore, collection has been limited to the northern highlands of Yemen. No localities are known from Oman.

6.3.2 DNA extraction and amplification

Genomic DNA was extracted from toes using a standard Chelex incubation (GERKEN *et al*., 1998). Primers were taken from ARENS *et al.* (2000). These primers were originally developed for *Hyla arborea* and now adapted for *Hyla savignyi* in this study. Five loci (WHA1-20, WHA5-22A, WHA1-104, WHA1-25, WHA1-61), which displayed low to moderate polymorphism, were chosen. Recently, an increasing number of animal studies report cross utility of microsatellites (chapter 4 & 5, and literature within). However, investigators have to be aware that reduced polymorphism in microsatellites in different species can be artificial. There seems to be a tendency for reduced polymorphism and allele length in microsatellites products for species they were not developed for (ascertainment bias; AMOS, 2003). Paying attention to this phenomenon, cross-utility tests were performed to test polymorphisms within *Hyla savignyi* (KLÜTSCH *et al.*, chapter 4). It could be proven that lower polymorphism in the Yemeni population is not caused by ascertainment bias or mutations in primer sites, but represents a special feature of the Yemeni populations.

PCR amplification was carried out in 20 µL containing 10 x PCR buffer (without $MgCL_2$; containing100 mM Tris-HCL, 500 mM KCL, pH 8.3, Sigma), 1,5 – 2,0 mM $MgCl_2$ (Sigma), 100 µM of each dNTP (Sigma), 500 nM of each primer and 0,5 unit *Taq* polymerase (containing 20 mM Tris-HCL, 100 mM KCL, 0,1 mM EDTA, 1 mM DTT, 0,5 % Tween 20, 50 % glycerol; Sigma). Amplification was performed in an Applied Biosystems GeneAmp 2700 thermal cycler. Following an initial denaturation step of 3 minutes at 94°C, the reactions underwent 35 thermal cycles (94°C for 30 s, 60

s at annealing temperature and 65 s at 72°C) and a final extension time of 20 min at 72°C (see ARENS *et al.* (2000) for annealing temperatures). Using a 6 % acrylamide gel and an ABI 377 sequencer, products were separated. Results were analyzed with GENESCAN 3.1.2 (Applied Biosystems) and GENOTYPER (Applied Biosystems).

6.3.3 Statistical data analysis

Statistical analysis was performed with GENEPOP 3.4 (RAYMOND & ROUSSET, 1995). Genotypic linkage disequilibrium (LD = non random association of genotypes occurring at different loci) was tested by the exact probability test. The null hypothesis is that genotypes at one locus are independent from genotypes at all other loci. This test computed unbiased estimates by randomization (10.000 iterations) and the exact probabilities of random association for all contingency tables corresponding to all possible pairs of loci within each population by the Markov-chain method. Moreover, GENEPOP 3.4 (RAYMOND & ROUSSET, 1995) was used to calculate Hardy-Weinberg exact tests, heterozygosity deficiencies, and F_{IS} / F_{ST} values at the population level. HWE was tested at the single locus level using the two tests for heterozygosity excess and heterozygosity deficit. POPGEN32 was used to calculate F_{ST} and F_{IS} for groups of populations and FSTAT (GOUDET, 2001) for the calculation of allelic richness. Small populations were retained for further analyses, because they showed no deviation from Hardy-Weinberg equilibrium or specific linkage disequilibrium. Furthermore, a test for recent bottlenecks was conducted with the program BOTTLENECK (CORNUET & LUIKART, 1996). POPGEN32 (YEH & BOYLE, 1997) and TREEVIEW (PAGE, 1996) was used to calculate Nei's unbiased measures of genetic identity and genetic distance and to construct a population tree. In addition, IBD ON THE WEB (BOHONAK, 2002) was used to perform simple and partial Mantel tests (MANTEL, 1967) to test for correlation between genetic and geographic distance (isolation by distance) and isolation by altitude. Mantel tests were performed with 30.000 permutations. Linear geographic distances were obtained from MICROSOFT ENCARTA WORLD ATLAS (2005). Genetic differentiation was not only tested for correlation with geographical distance, but also for altitude differences. This was done to address the special situation in Southern Arabia, where tree frogs may not only be isolated by linear geographic distances, but also by altitudes, since the sampling localities ranged from 2200 m to 2845 m altitude.

IBD was tested at three hierarchical levels. First, the overall significance was tested including all populations. Second, relationships among clusters were further investigated to identify which combination contributed mostly to the overall significant result. Lastly, significance of IBD was tested within clusters.

6.3.4 Population structure and admixture

Population-structure analysis was performed with a genetic assignment method. Assignment methods use a likelihood approach to assign individuals to populations. Thus, composition of a population can be assessed and relationships as well as structure of populations can be analyzed. Assignment of individual frogs to their most likely population of origin was performed using GENECLASS v.2.0.f (PIRY *et al.*, 2004; downloadable from http://www.ensam.inra.fr/URLB/geneclass/geneclass.html). In GENECLASS2, the Bayesian model (PAETKAU *et al.*, 1995) in combination with the simulation algorithm of CORNUET *et al.*, 1999) was used to assign individuals to populations. Self-assignment of individuals to populations was calculated using the `leave one out´ option, which calculates the reference allele frequencies using all individuals from the reference population except the individual that is being assigned. An assignment threshold for scores was set to 0.05. This means, individuals with an assignment value lower than 0.05 were not assigned to the predefined population or to any population, respectively. The latter case would indicate an origin from an un-sampled population.

However, all three implemented types of individual assignment (Bayesian, frequency-based, and distance methods) were tested and assignment success was similar in Bayesian and frequency-based methods whereas distance methods demonstrated lower assignment success to some extent (results not shown).
Migration between sites was estimated using the “Detection for first generation migrants” option (PAETKAU *et al.*, 2004). As computation criteria, a Bayesian method (RANNALA & MOUNTAIN, 1997) was used in combination with the Monte-Carlo-resampling algorithm of PAETKAU *et al.* (2004). Number of simulated individuals was set to 10.000 with a type I error of 0.01.

6.4 Results

6.4.1 Within population genetic variation

Table 1 summarizes the patterns of genetic variation detected within populations.

Table 1: Summary of allelic variation statistics for all loci. Number of individuals (N_{ind}), expected heterozygosity (H_E), observed heterozygosity (H_O), F_{IS} estimates after WEIR & COCKERHAM (1984) (F_{IS}); Number of polymorphic loci (N_{pol}), number of alleles within population (A), mean number of alleles within population (A_m), A_r allelic richness (calculated in FSTAT (GOUDET, 2001)). Please note that allelic richness is adjusted to the smallest population and therefore Ar has very small values.

Number in map		**N_{ind}**	**H_e**	**H_o**	**F_{IS}**	**N_{pol}**	**A**	**A_m**	**A_r**
Not shown	Baghdad	10	0.696	0.405	0.3688	5	21	4.2	3.4
4	Wadi Annaim	28	0.246	0.219	0.1114	3	12	4.0	1.7
1	N Amran	19	0.229	0.158	0.3173	3	10	3.3	1.6
6	Lulah	30	0.416	0.280	0.3310	5	16	3.2	2.3
8	Ad Dogma/ Al Haima	34	0.448	0.265	0.4257	5	18	3.6	2.4
3	Shibam	5	0.268	0.330	-0.2800	3	8	2.6	1.6
7	Sanaa	37	0.275	0.272	0.0124	4	13	3.2	1.8
5	Habiba	34	0.242	0.252	-0.0406	4	11	2.7	1.7
2	Thula	6	0.209	0.253	-0.2389	3	8	2.6	1.5
12	Between Dhamar-Yarim	27	0.211	0.178	0.1579	3	12	4.0	1.7
13	Dhihisub	33	0.238	0.333	-0.3925	3	12	4.0	1.6
11	Dhamar checkpoint	4	0.429	0.400	0.0769	4	10	2.5	2.0
14	S Yarim/ Mawah	31	0.388	0.278	0.2837	4	16	4.0	2.2
10	Dhamar	7	0.345	0.343	0.0069	3	12	4.0	2.1
9	Maabar	30	0.412	0.275	0.3391	4	18	4.5	2.4
	Total	335							

In total, the number of alleles over all loci varies from 8 to 18 within the Yemeni populations. Including all loci, the number of alleles significantly correlates with sample size (SPEARMAN; $P < 0.01$). However, Baghdad has a high allele number (21 alleles) although only a low sample size (sample size: 10) was obtained. All loci are polymorphic, but locus WHA5-22A has in only 3 populations (Baghdad, Lulah, S Yarim/ Mawah) more than one allele and only in one population (S Yarim/Mawah) the total number of three detected alleles. Altogether, 23 alleles are detected in the Yemeni populations; of these one allele is unique to a single population (WHA5-22A

in population S Yarim/ Mawah; the most southern population), but four alleles are not found in more than two populations. At the locus WHA1-104, one allele is found in the populations of Sana´a and N Amran and two alleles each in Ma´abar and Ad Dogma/ Al Haima. At the locus WHA5-22A, an allele is shared by the populations of S Yarim/ Mawah and Lulah. Genetic variation in terms of mean expected heterozygosity ranges from 0.209 in Thula to 0.448 in Ad Dogma/ Al Haima.

6.4.2 Linkage disequilibrium

Statistical tests for LD are conducted for all pairs of microsatellite loci; only 7 of the 78 possible tests have significant results ($P < 0.001$; Bonferroni correction). However, no considerable tendency regarding linkage of particular loci can be observed. The seven cases are distributed among the populations Ad Dogma (3x), Lulah (3x), and Maabar (1x). Thus, linkage disequilibrium is highly connected to populations rather than to a specific loci combination. Therefore, other causes than a physical linkage of loci is likely to be responsible for these results. Linkage disequilibrium may be caused by admixture of two or more populations differing in allele frequencies, natural selection, or by inbreeding (HARTL, 1999). The most likely explanation for the observed linkage disequilibrium pattern here is inbreeding effects, because the three populations, where LD is observed, have also high F_{IS} values ranging from 0.3310-0.4257.

6.4.3 Hardy Weinberg tests and bottlenecks

Deviations from HWEs are observed in 9 out of 104 tests at the single locus level (data not shown) after applying a Bonferroni correction ($P < 0.0025$). 8 out of 9 cases are caused by heterozygote deficiency. None of the loci exhibited deviations from HWEs in all of the ponds, but all loci have at least once a deviation from HWE without having an accumulation within a particular pond. To test whether this heterozygote deficiency is caused by recent bottlenecks, the program Bottleneck (CORNUET & LUIKART, 1996) was used. The hypothesis of recent bottlenecks is rejected, because the populations (Baghdad, Thula, Dhamar checkpoint, and Shibam), where a shifted mode of allele frequency can be observed, do not correspond to populations where a deviation of HWE can be observed. All of these populations with a shifted mode are small (4-10 individuals).

Thus, we interpret this as an artificial result, which is also pointed out by CORNUET & LUIKART (1996). Small sample sizes may result in wrongly significant results for recent bottlenecks, because allele frequencies are not representatively sampled. However, no recent bottlenecks have been observed in those populations, in which a deviation from HWE is detected. Other explanations for deviation of HWE expectations include the Wahlund effect, non-random mating, the presence of null alleles, or inbreeding effects. The latter is also supported by the results found for the linkage disequilibrium (see chapter 6.4.2).

6.4.4 Population genetic differentiation

Significant genetic differentiation across the 14 Yemeni populations is detected at the population by population level, with pairwise F_{ST} values varying from 0.0004 (Ma´abar-Dhamar) to 0.6867 (N Amran - between Dhamar and Yarim) (appendix 6.1).
Wright (1978) has suggested guidelines for the interpretation of F_{ST} values. Following these guidelines, little to great genetic variation could be observed. A similar picture is drawn by Nei´s (1978) unbiased measure of genetic distance (appendix 6.4), where the greatest genetic distance can be observed either between “N Amran” and “Lulah” to the locality “between Dhamar and Yarim”, with genetic distances of 0.9965 and 0.9965. The dendrogram based on Nei´s genetic distance values (Fig. 2) reveals another interesting feature of the population structure of *Hyla savignyi* in Yemen. Three major groups of populations can be detected (see also Fig. 3). One group includes the three populations from N Amran, Lulah, and Wadi Annaim, which are all situated in the North of the capital city Sana´a (Fig. 3). A second group encompasses the populations from Ad Dogma/ Al Haima, Sana´a, Shibam, Habiba, and Thula, which are situated in the West and North-West of Sana´a and includes Sana´a itself. The only exception is Ad Dogma/ Al Haima, which is placed in the South-West of Sana´a and has largest F_{ST} values to all other populations in this group. The last group includes populations, located South of Sana´a; namely between Dhamar-Yarim, Dhihisub, Dhamar checkpoint, S Yarim/ Mawah, Dhamar, and Ma´abar. Roughly speaking, the three groups correspond to a structure from North to South, but group 1 and 2 partly overlap geographically. Population differentiation within groups varies greatly.

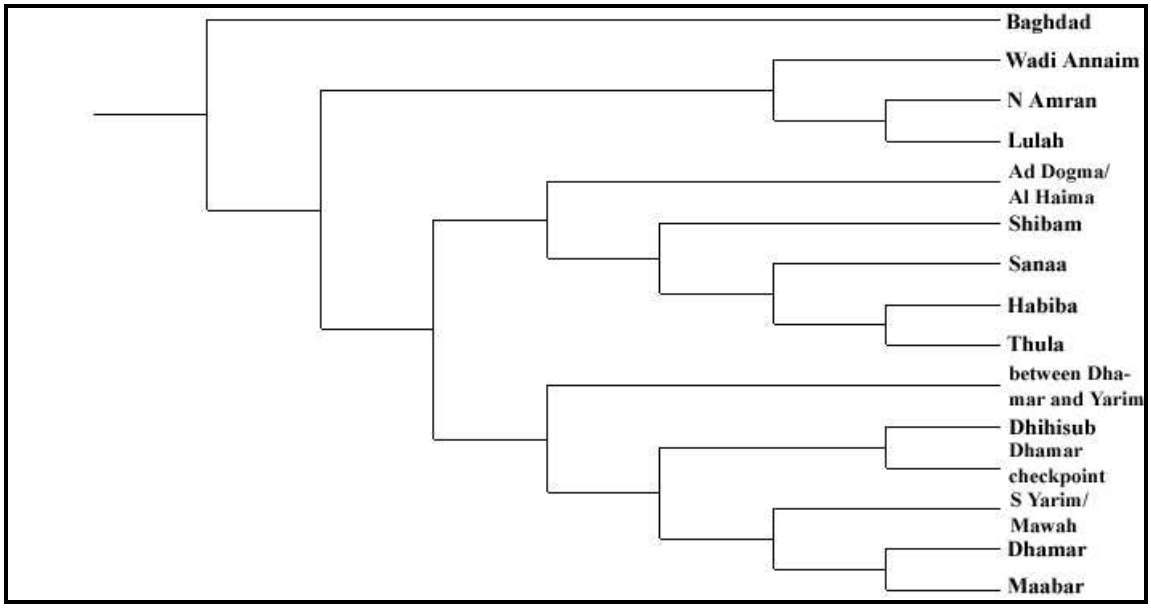

Figure 2: UPGMA dendrogram after NEI (1978), based on genetic distance data (appendix 6.4); branch lengths represent genetic distance; built by POPGEN32.

In group 1, F_{ST} values range from 0.1122 to 0.2708 whereas in group 2, F_{ST} values vary from 0.0126 to 0.2005. Genetic differentiation within group 3 diverges from 0.0004 to 0.3344. F_{IS} for group 1 is equal to 0.2550, for group 2 = 0.0405, and for group 3 = 0.533, indicating a higher inbreeding effect in group 1 in comparison to the other two groups. Subdivision of populations is similar in all groups (1; F_{ST} = 0.1695, 2; F_{ST} = 0.1505, and 3; F_{ST} = 0.1429). Overall, the F_{ST} value is equal to 0.3667, indicating a high differentiation (F_{IS} was 0.0904).

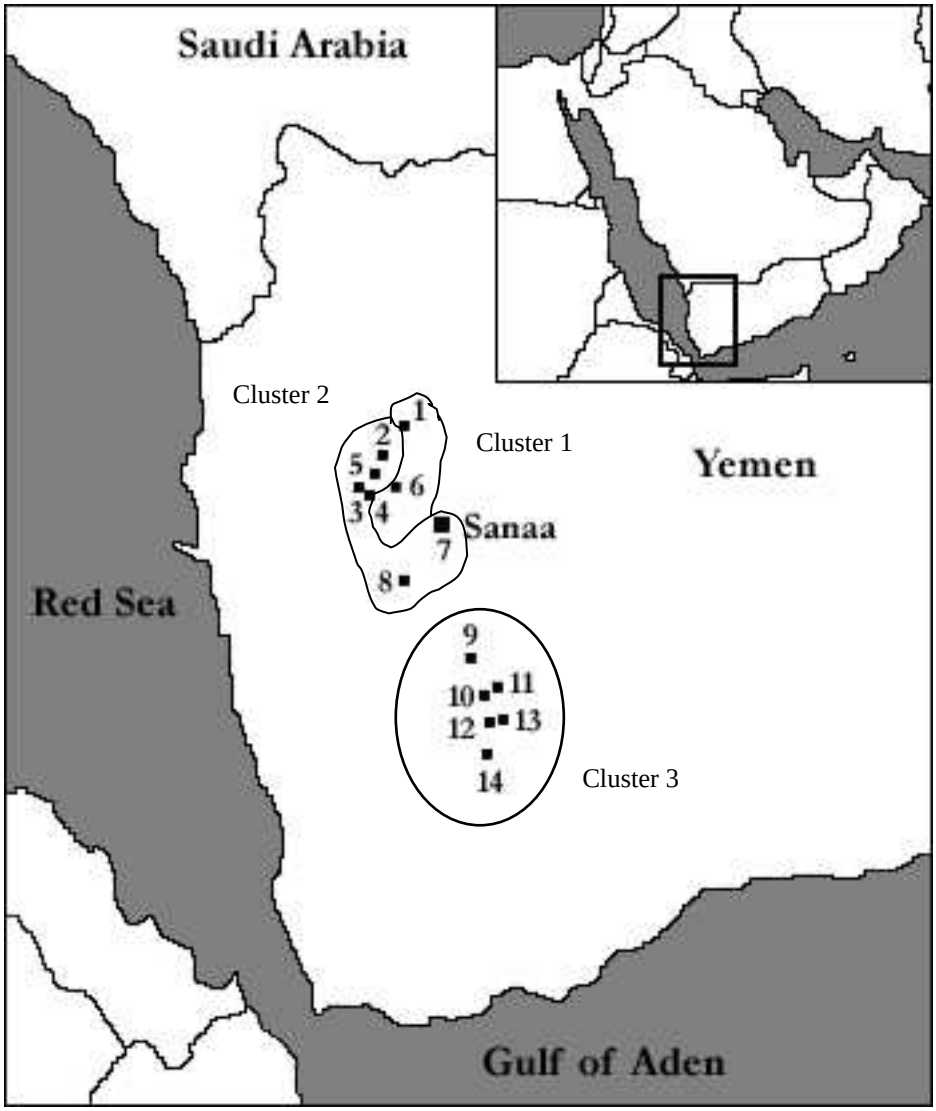

Fig. 3: Geographical position of clusters found in Nei´s distance tree. Numbers correspond to those in Table 1.

6.4.5 Isolation by distance

Including all populations, a significant positive correlation between genetic F_{ST} and geographic distance (km) is detected ($P < 0.009$, Fig. 4).

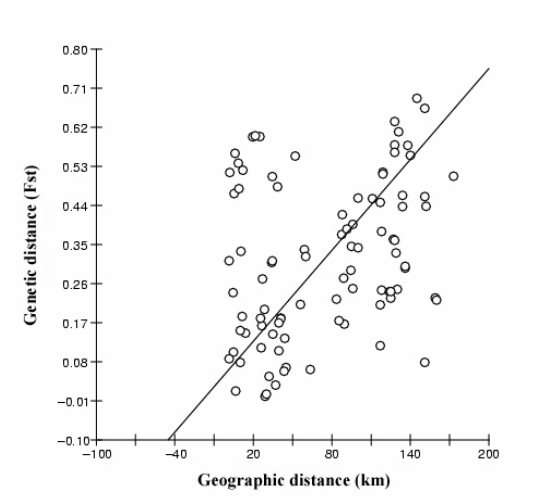

Fig. 4: Relationship between genetic differentiation (F_{ST}) and geographic distance (km) over all populations.

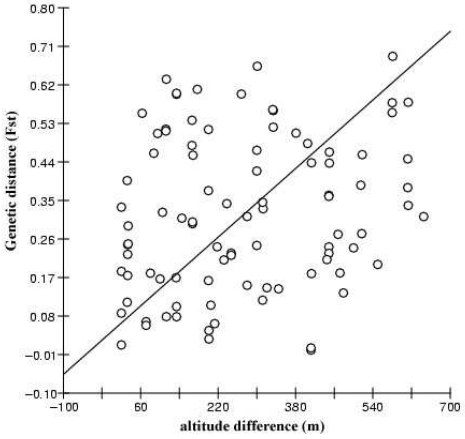

Fig. 5: Relationship between genetic differentiation (F_{ST}) and altitudinal difference (m) over all populations.

A positive correlation between genetic differentiation and altitudinal difference (m) including all populations is also significant ($P < 0.042$, Fig. 5). However, applying partial Mantel tests, the correlation of genetic differentiation and altitude is still present, but not significant. Among clusters, cluster pairs 1 and 3 as well as 2 and 3 show a significant correlation between genetic differentiation and geographical distance. Geographical adjacent/ overlapping areas of clusters 1 and 2 display no significant IBD pattern (Table 2). Geographical and altitudinal distances are given in appendices 6.2 and 6.3.

Table 2: Mantel and partial Mantel test results for isolation by distance and altitude analysis. IBD ON THE WEB (BOHONAK, 2002) was run with 30.000 randomizations. Geographical distance is given in km whereas altitudinal difference is given m.

Genetic distance	1st geographic distance	Indicator matrix	Z	r	p
Among all individuals					
$\mathbf{F_{ST}}$	Geographic distance (km)	no	2430.2777	0.3165	0.0090
$\mathbf{F_{ST}}$	Altitudinal difference (m)	no	8529.9163	0.2043	0.0420
$\mathbf{F_{ST}}$	Geographic distance (km)	Altitudinal difference (m)		0.2865	0.0152
$\mathbf{F_{ST}}$	Altitudinal difference (m)	Geographic distance (km)		0.1500	0.0967
Among pairs of clusters					
Cluster 1 and 2	Geographic distance (km)	no	214.0077	-0.2032	0.8881
	Altitudinal difference (m)	no	2440.3296	0.0124	0.3769
	Geographic distance (km)	Altitudinal difference (m)		-0.2105	0.8781
	Altitudinal difference (m)	Geographic distance (km)		0.0574	0.2740
Cluster 1 and 3	Geographic distance (km)	no	1278.4232	0.8498	0.0040
	Altitudinal difference (m)	no	3903.0069	0.2805	0.0778
	Geographic distance (km)	Altitudinal difference (m)		0.8357	0.0042
	Altitudinal difference (m)	Geographic distance (km)		-0.0141	0.4565
Cluster 2 and 3	Geographic distance (km)	no	1023.5636	0.5416	0.0027
	Altitudinal difference (m)	no	3163.5148	0.2705	0.0394
	Geographic distance (km)	Altitudinal difference (m)		0.5200	0.0033
	Altitudinal difference (m)	Geographic distance (km)		0.2076	0.0692
Within clusters					
Cluster 1					
$\mathbf{F_{ST}}$	Geographic distance (km)	no	11.3052	-0.2907	0.4945
$\mathbf{F_{ST}}$	Altitudinal difference (m)	no	249.9248	0.9688	0.1642
$\mathbf{F_{ST}}$	Geographic distance (km)	Altitudinal difference (m)		1.0000	0.4945
$\mathbf{F_{ST}}$	Altitudinal difference (m)	Geographic distance (km)		1.0000	0.4930
Cluster 2					
$\mathbf{F_{ST}}$	Geographic distance (km)	no	35.0889	0.3360	0.2009
$\mathbf{F_{ST}}$	Altitudinal difference (m)	no	364.2004	0.9386	0.0158

Table 2: continued

F_{ST}	Geographic distance (km)	Altitudinal difference (m)		-0.0397	0.4258
F_{ST}	Altitudinal difference (m)	Geographic distance (km)		0.9306	0.0084
Cluster 3					
F_{ST}	Geographic distance (km)	no	39.3227	-0.4852	0.8663
F_{ST}	Altitudinal difference (m)	no	369.4365	-0.3602	0.9711
F_{ST}	Geographic distance (km)	Altitudinal difference (m)		-0.4238	0.8157
F_{ST}	Altitudinal difference (m)	Geographic distance (km)		-0.2574	0.8518

Regarding isolation by altitude, only cluster pair 2 and 3 shows a tendency for differentiation by altitude. Within groups, none of the correlations are significant with the exception of a significant correlation between genetic differentiation and altitude within cluster 2 ($P < 0.016$), which even becomes more significant in partial Mantel tests ($P < 0.008$). Cluster 2 displays a significant ($P < 0.03$) correlation of expected heterozygosity and altitude indicating that with increasing altitude variability is increasing.

6.4.6 Assignment of individuals and detection of first generation migrants

A total of 198 (59.1 %) individuals are assigned to their sampling population (Table 3). Exclusion of small populations (< 10) only slightly increased total assignment (~ 66 %; results not shown). At the group level, inferred from the dendrogram by Nei´s distances (Fig. 2), individual assignment to group 1 is 97.6 % (appendix 6.7). Only one individual from Ad Dogma/ Al Haima (group 2) is assigned to this group (to population Wadi Annaim). To group 2, 80.2 % of individuals are assigned to this cluster. 19.8 % of individuals sampled from geographic localities in cluster 2, are assigned to group 3. In more detail, 16.4 % are assigned to S Yarim/ Mawah; 1.7 % each to Ma´abar and Dhamar. Concerning group 3, 9.6 % of the individuals are assigned to group 2 instead of group 3. One individual (0.7 %) is of unknown origin and cannot be assigned to any population. Thus, 89.7 % of the individuals sampled in this area are also assigned to this group. The results of assignment tests at the group level clearly support the clusters inferred from Nei´s distance analysis.

Table 3: Summary about detection of first generation migrants. Three analyses were performed with GENECLASS2 (PIRY *et. al*., 2004). Number attached to name of sampled population gives type of analysis, in which it was detected. In the case of repeated detection, the combination of analyses is given. L = L_home/ L_max (1); L=L_home/ L-max_ not home (2); Direct Likelihood L_home (3). -log(L_home) is the likelihood value obtained for the presumed population of origin; -log (L) is the likelihood value of the population, where the individual is most likely originated.

Sampled Population	**-log(L_home)**	**Probability**	**Most likely origin of population**	**-log (L)**
Habiba[1,2,3]	5.445	0.0000	Ad Dogma/ Al Haima	2.204
Lulah[1,2]	1.970	0.0072	Wadi Annaim	2.403
Lulah[1,2]	2.347	0.0024	Wadi Annaim	1.989
Dhihisub[1,2,3]	2.653	0.0016	S Yarim/ Mawah	2.247
Dhamar checkpoint[2]	2.918	0.0060	Dhihisub	1.765
Ad Dogma/ Al Haima[1,2,3]	3.743	0.0000	Dhamar and Yarim	2.922
Sana´a[1,2]	2.044	0.0071	Thula	1.624
Sana´a[1,2]	2.498	0.0024	S Yarim/ Mawah	2.580
S Yarim/ Mawah[1,2,3]	2.520	0.0057	Dhihisub	1.613
S Yarim/ Mawah[1,2,3]	2.510	0.0040	Dhamar and Yarim	3.528
S Yarim/ Mawah[1,2]	2.220	0.0094	Dhamar checkpoint	2.845
S Yarim/ Mawah[1,2]	2.420	0.0054	Dhamar and Yarim	0.790
Dhamar[3]	5.090	0.0064	Thula	4.533
Wadi Annaim[3]	5.727	0.0035	N Amran	5.600
Wadi Annaim[3]	5.031	0.0051	Thula	4.386
Ma´abar[3]	8.261	0.0028	Dhamar checkpoint	7.267
Ma´abar[3]	8.133	0.0028	Thula	5.885

Especially, the isolated character of group 1 is supported by assignment tests. It is clear that migration is highly limited from and into this group, judging from the assignment of only one individual to group 2 and the lacking of assignment to group 3. Between group 2 and 3 miss-assignment to the alternative group is more frequent. Group 2 has the highest assignment to group 3 whereas group 3 shows an assignment to group 2, which is only half as high as *vice versa*.

The composition of populations (assigned individuals in %) is illustrated in appendices 6.5 and 6.6. The structure of three main groups is apparent. Group 1 encompasses three populations, in which every population display an assignment to Lulah and Wadi Annaim to some extent, indicating a closer relationship to each other than to all other populations and possible migration within this group, but not among groups. In group 2 and 3, a similar pattern can be detected. In group 2, assignment to different populations within groups is common. Therefore, it is reasonable to conclude that relationships

within groups are much closer than among them. Migration within groups is less restricted than migration among groups.

This is also supported by the analysis of first generation migrants. Table 5 gives a summary about all three analyses conducted. 2/3 of first generation migrants have their origin in the same group. In contrast, only 1/3 of first generation migrants have their origin in a different group than their sampling group. Moreover, group 1 populations only have first generation migrants within this group. A different picture is drawn among group 2 and 3. Although first generation migrants are more common within groups, a small fraction has their origin in the alternative group.

6.5 Discussion

In summary, population genetic structure of *Hyla savignyi* in the mountainous areas of Yemen appears to be shaped by a combination of isolation by distance and altitudinal distance as well as by restricted gene flow among populations. However, long distance gene flow between populations is detected as well (mainly between group 2 and 3). The genetic structure of the *H. savignyi* populations in Yemen shows a differentiation along a North - South extension in Northern Yemen.

6.5.1 Explanations for strong differentiation of populations and low diversity in populations

Explanations for strong isolation and differentiation of the studied populations are manifold. First, tree frogs are known to be linked to water places at least for reproduction and individuals usually do not migrate more than approx. 4 km (STUMPEL & HANEKAMP, 1986; FOG, 1993; ANDERSEN *et al.*, 2004). Geographic distances between collection localities ranged from 2 km to 173 km in this study, which exceeds the migration radius of individual tree frogs in most cases. Therefore, high differentiation of populations should be interpreted with caution, since we have only little information about connectivity of populations within a range of 5.0 km. Thus, high genetic differentiation may be partly due to large geographic distances. However, high genetic differentiation in this study is comparable to other studies of amphibian species

(*e. g*. ROWE *et al.*, 1998; NEWMAN & SQUIRE, 2001) and support the metapopulation structure found in other amphibians (ALFORD & RICHARDS, 1999; MARSH & TRENHAM, 2001).

Second, special attention has to be drawn to the situation in the Yemeni highlands, where not only geographic distance contributed to genetic differentiation, but also altitudinal distance. Thus, due to this combined effect of altitudinal and geographic distance, populations are additionally isolated.

Third, the Yemeni population can be considered as a peripheral population of a past continuous population extending from Asia Minor and eastern Mediterranean into the Arabian Peninsula. In this case, lower diversity in Yemeni populations than populations connected to the rest of the distribution area, is probably owed to isolation, founder effects, and smaller population sizes (LESICA & ALLENDORF, 1995; HOFFMAN & BLOUIN, 2004). The last point is supported by the fact that the "outgroup" population from Baghdad has a higher allelic richness than Yemeni populations, although only a small number of individuals were analyzed. Further analyses have to be carried out to investigate, whether lower diversity in Yemeni populations are due to founder effects and unstable population dynamics in the past.

6.5.2 Connectivity of populations, asymmetrical gene flow, and long distance gene flow

Assignment tests and Nei´s genetic distance analyses mainly indicate that connectivity is found within populations and if individuals are not assigned to the presumed population, individuals usually came from one of the neighboring ponds within this group. This is also supported by the absent isolation by distance pattern within groups. Therefore, connectivity of populations is given within a geographical range of 4.0 - 27.0 km (group 1), 4.8 - 45.0 km (group 2), and 1.5 – 63.5 km (group 3). Thus, it appears that connectivity between the clusters is differential. The most southern cluster (group 3) has the greatest geographical range in which populations are connected to some extent, whereas the most northern cluster (group 1) has the smallest geographical range. Therefore, it emerged likely that the most southern cluster also has the most connected ponds or the most suitable environmental conditions in comparison to other clusters. Although connectivity of populations strongly corresponds to the three clusters found,

another interesting factor obviously shaped population genetic structure of *H. savignyi*. According to the GENECLASS2 analysis, long distance gene flow occurs among cluster 2 and 3. At first glance, long distance gene flow seems to be contradictive for a tree frog species with presumable low migration ability and in an environment like the Yemeni highlands from first glance. However, other studies (BERRY, 2001; SQUIRE & NEWMAN, 2002) reported high gene flow in some species or populations at least at a small geographical scale. In addition, long distance dispersal may not be always limited to neighboring ponds as some studies (MARSH & TRENHAM, 2001) suggest. For example, with mark-recapture methods, it is found that in the Netherlands, *Hyla arborea* individuals are able to migrate distances of 12.6 km per year (STUMPEL & HANEKAMP, 1986). Therefore, it appears likely that in *H. savignyi*, which is closely related to *H. arborea*, similar migration distances may occur. However, it has to be kept in mind that the environmental conditions are highly dissimilar in the Netherlands and Yemen. In the special case of *H. savignyi*, it would be of interest to further investigate the possibility of human impact on dispersal. As mentioned in the introduction, our own observations suggest that tadpoles or eggs may be transported to other ponds through tank trucks. However, a study testing this hypothesis in particular is still missing.

Obviously, forces shaping the genetic structure in clusters are different regarding isolation by altitude, inbreeding effects and genetic differentiation. For example, in cluster 2 populations are separated by altitude whereas in clusters 1 and 3, no significant correlation with altitude can be detected. Moreover, cluster 1 shows high inbreeding effects, whereas cluster 2 displays outbreeding to some extent and cluster 3 displays both patterns. Therefore, gene flow/ connectivity of populations within and among clusters appear to vary.

Asymmetrical gene flow is detected between group 2 and 3, where gene flow from group 3 to 2 appears to be approx. twice as high as *vice versa*. Group 1 shows only restricted gene flow from group 2. Thus, there is evidence for a directional gene flow from South to North, although gene flow between group 1 and 2 is marginal. Based on the results of the assignment tests, it is reasonable to conclude that cluster 2 displays outbreeding, because the degree of migration from cluster 3 is highest in the whole study. The same explanation may be applied to the mixed situation in cluster 3, where

migration from cluster 2 is detected, but at a much lesser scale. Since the third cluster has the largest geographical extension, it is probable that outbreeded and inbreeded population coexist in this cluster. Thus, population genetic structure is shaped by a complex mixture of isolating factors, partial limited migration, and long distance gene flow.

6.5.3 Isolation factors

Another important result is the detection of different isolating effects. Populations are not simply isolated by isolation by distance, but also isolated by altitudinal distance. This is especially true for group 2, where isolation by distance is the dominant factor in comparison to geographic distance. The overall tendencies for altitude effects is not significant in partial Mantel tests, but this may be a sampling artifact caused by the limited populations at the same longitudes/latitudes and different altitudes. Thus, additional studies should concentrate on a more sophisticated sampling scheme; *e. g.* transect sampling (STORZ & DUBACH, 2004; DE NAVASCUÉS MELERO, 2005) to further investigate the effect of altitude on genetic differentiation. Additionally, one has to keep in mind that altitude does not affect genetic differentiation directly, but is often referred as an ecological factor (STORZ & DUBACH, 2004). Therefore, supplementary ecological studies will be warranted to explore, which ecological factor changes with altitude (*e. g.* temperature) and can explain genetic differentiation along an altitudinal gradient in more detail. However, it has to be kept in mind that no significant correlation between altitude and allelic richness and observed or expected heterozygosity is found. Thus, altitudinal differences appear to contribute to general genetic differentiation rather than give evidence for an ecological differentiation. Complementary, it would be of interest to use GIS-based methods to improve the “real” geographic distance between two sites. Thus, it may be possible to include the way between two sites plus the way down and up a hill in order to refine isolation by distance analysis. Moreover it may be promising to take in probable migration barriers as for example, steepness or mountain ridges (FUNK *et al*., 2005b).

6.5.4 Conclusions

In the present study, we make obvious that an overall significant IBD pattern does not necessarily imply that genetic differentiation is mainly shaped by geographic distance alone in mountainous areas. Instead, we demonstrate that genetic differentiation is additionally formed by migration, which is generally limited to certain groups of populations. Which physical barriers contributed to structured population groups remains to be determined in further studies. Additionally, IBD patterns are quite different when considering mentioned subpopulations, in which IBD is absent, but isolation by altitude is significant in one cluster. This result strongly emphasizes that population structure is formed by a range of factors. We pointed out that a general IBD pattern may be highly influenced by barriers to gene flow rather than a continuous range of genetic differentiation. Overall, population structures within the three main clusters are different from one another, suggesting that evolutionary forces act in different ways within groups. The population-structure analysis in combination with a population tree based on genetic distances demonstrates a valuable combination of methods to investigate population structure more effectively and in particular to uncover barriers to gene flow on the basis of population genetic data.

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Appendices

Appendix 6.1: Population by population pairwise mean F_{ST} values

Population	Shibam	Habiba	Dhamar	Between Dhamar and Yarim	Wadi Annaim	N Amran	Lulah
Shibam	-						
Habiba	0.1022	-					
Dhamar	0.1171	0.2416	-				
Between Dhamar and Yarim	0.3306	0.4373	0.1844	-			
Wadi Annaim	0.5158	0.5600	0.5165	0.6332	-		
N Amran	0.5985	0.5980	0.5783	0.6867	0.2708	-	
Lulah	0.4673	0.5373	0.4470	0.5793	0.2392	0.1122	-
Dhihisub	0.2468	0.2957	0.1460	0.3126	0.6096	0.6635	0.5622
Dhamar checkpoint	0.2449	0.3621	0.0868	0.3344	0.5123	0.5552	0.3800
Thula	0.0784	0.0126	0.2265	0.4631	0.5211	0.6003	0.4785
Ad Dogma/ Al Haima	0.1435	0.1807	0.2241	0.3965	0.3086	0.3383	0.3123
Ma´abar	0.1667	0.2906	0.0004	0.1791	0.4189	0.4556	0.3730
Sana´a	0.1054	0.0668	0.2725	0.4570	0.4830	0.5537	0.5065
S Yarim/ Mawah	0.0786	0.2270	0.0263	0.1629	0.4603	0.5073	0.4380

Popu-lation	Dhi-hisub	Dhamar check-point	Thula	Ad Dogma/ Al Haima	Ma´abar	Sana´a	S Yarim/ Mawah
Shibam							
Habiba							
Dhamar							
Between Dhamar and Yarim							
Wadi Annaim							
N Amran							
Lulah							
Dhih-isub	-						
Dhamar check-point	0.1519	-					
Thula	0.2998	0.3596	-				
Ad Dogma/ Al Haima	0.3457	0.1747	0.1340	-			
Ma´a-bar	0.1695	0.0055	0.2486	0.2119	-		

Appendix 6.1: continued

Sana´a	0.3426	0.3856	0.0583	0.2005	0.3223	-	
S	0.1801	0.0463	0.2220	0.2111	0.0622	0.2417	-
Yarim/ Mawah							

Appendix 6.2: Geographic distances among population pairs

Population	Shibam	Habiba	Dhamar	Between Dhamar and Yarim	Wadi Annaim	N Amran	Lulah
Shibam	-						
Habiba	4.8	-					
Dhamar	117.0	124.0	-				
Between Dhamar and Yarim	129.0	134.0	11.5	-			
Wadi Annaim	2.0	6.0	119.0	128.0	-		
N Amran	25.0	19.5	138.0	145.0	27.0	-	
Lulah	5.3	8.5	117.0	128.0	4.5	26.0	-
Dhihisub	130.0	136.0	14.0	1.5	131.0	151.0	128.0
Dhamar checkpoint	118.0	127.0	1.5	10.5	119.0	140.0	118.0
Thula	10.0	6.5	125.0	134.0	12.0	21.5	9.0
Ad Dogma/ Al Haima	34.9	41.0	83.5	96.0	34.0	59.0	34.5
Ma´abar	89.5	94.5	29.0	41.0	88.0	111.0	87.5
Sana´a	39.5	45.0	89.0	100.0	38.5	52.0	34.5
S Yarim/ Mawah	151.0	159.0	37.0	26.5	151.0	173.0	152.0

Population	Dhihisub	Dhamar checkpoint	Thula	Ad Dogma/ Al Haima	Ma´abar	Sana´a	S Yarim/ Mawah
Shibam							
Habiba							
Dhamar							
Between Dhamar and Yarim							
Wadi Annaim							
N Amran							
Lulah							
Dhihisub	-						
Dhamar checkpoint	10.0	-					
Thula	136.0	128.0	-				
Ad Dogma/ Al Haima	95.0	85.5	44.0	-			
Ma´abar	39.5	30.0	96.0	56.0	-		
Sana´a	100.0	91.5	43.5	28.5	60.0	-	
S Yarim/ Mawah	25.5	32.0	160.0	117.0	63.5	125.0	-

Appendix 6.3: Altitudinal distances among population pairs

Population	Shibam	Habiba	Dhamar	Between Dhamar and Yarim	Wadi Annaim	N Amran	Lulah
Shibam	-						
Habiba	200.0	-					
Dhamar	312.0	513.0	-				
Between Dhamar and Yarim	313.0	134.0	1.0	-			
Wadi Annaim	300.0	100.0	612.0	613.0	-		
N Amran	268.0	68.0	138.0	581.0	32.0	-	
Lulah	300.0	100.0	612.0	613.0	20.0	32.0	-
Dhihisub	33.0	233.0	321.0	280.0	333.0	301.0	333.0
Dhamar checkpoint	300.0	510.0	20.0	20.0	600.0	580.0	612.0
Thula	200.0	20.0	500.0	513.0	100.0	68.0	100.0
Ad Dogma/ Al Haima	345.0	545.0	33.0	32.0	645.0	613.0	645.0
Ma´abar	100.0	100.0	412.0	413.0	200.0	168.0	200.0
Sana´a	205.0	5.0	517.0	518.0	95.0	63.0	95.0
S Yarim/ Mawah	113.0	313.0	201.0	200.0	413.0	381.0	313.0

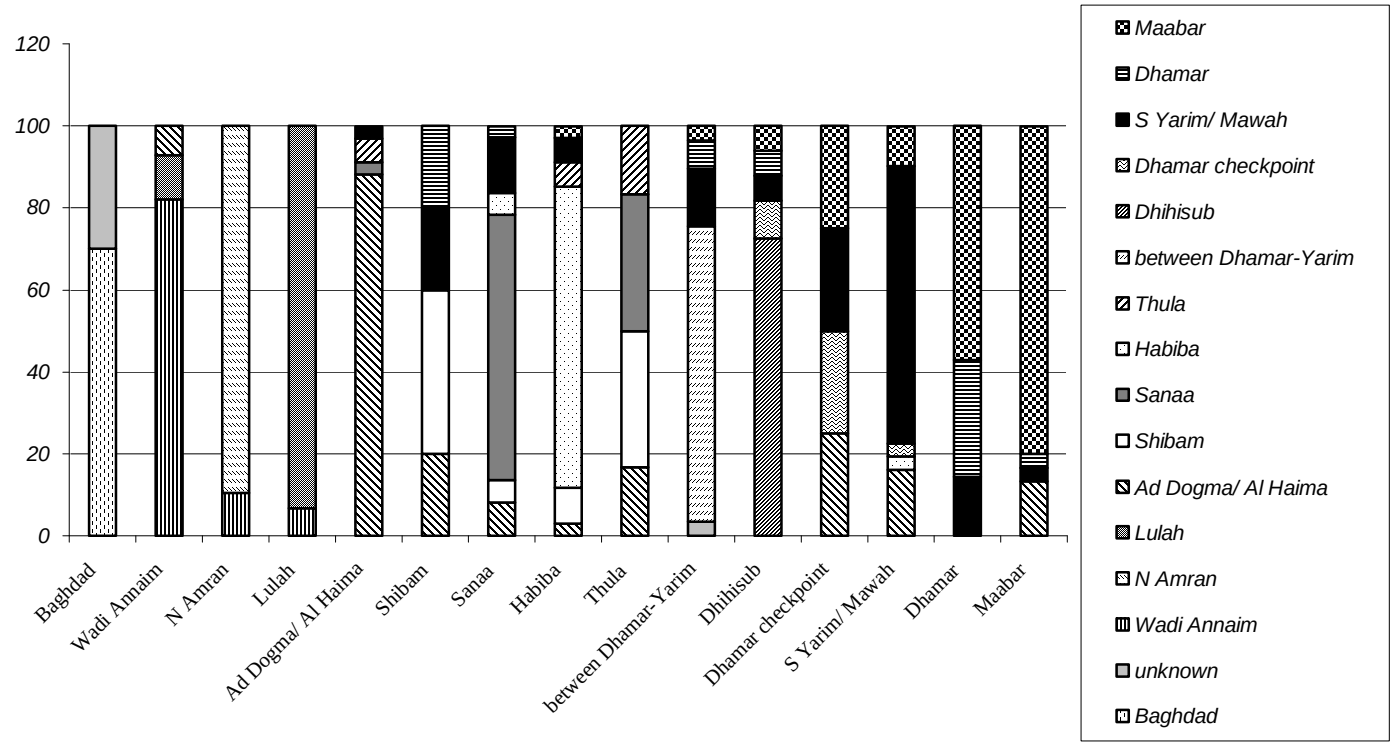
Population	Dhihisub	Dhamar checkpoint	Thula	Ad Dogma/ Al Haima	Ma´abar	Sana´a	S Yarim/ Mawah
Shibam							
Habiba							
Dhamar							
Between Dhamar and Yarim							
Wadi Annaim							
N Amran							
Lulah							
Dhihisub	-						
Dhamar checkpoint	280.0	-					
Thula	233.0	512.0	-				
Ad Dogma/ Al Haima	312.0	33.0	545.0	-			
Ma´abar	133.0	412.0	100.0	445.0	-		
Sana´a	238.0	515.0	5.0	550.0	105.0	-	
S Yarim/ Mawah	80.0	201.0	313.0	232.0	213.0	218.0	-

Appendix 6.4: Nei´s unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal), (Nei, 1978).

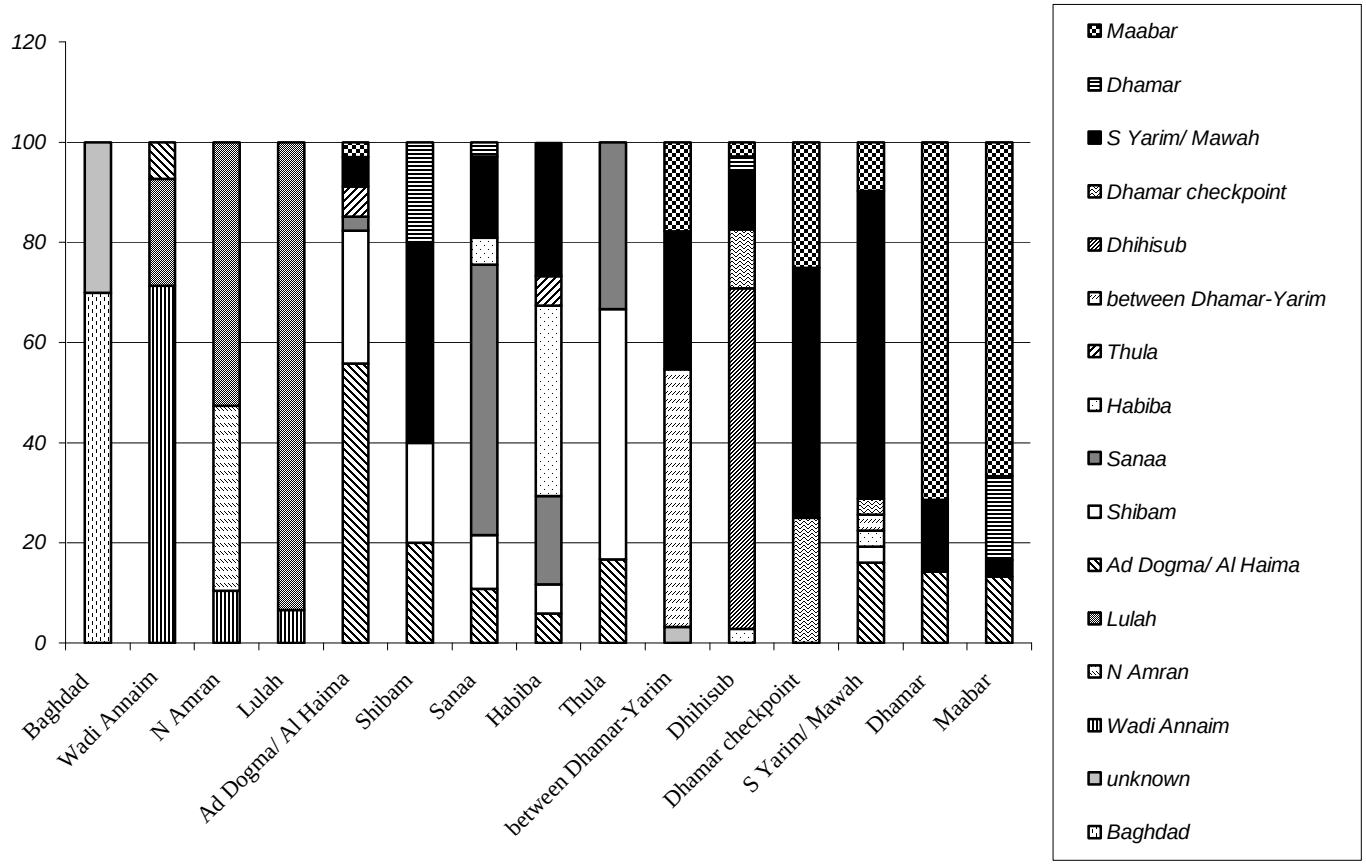
Population	Shibam	Habiba	Dhamar	Between Dhamar and Yarim	Baghdad	Wadi Annaim	N Amran	Lulah
Shibam	-	0.9541	0.9239	0.8461	0.1140	0.6363	0.5090	0.4373
Habiba	0.0470	-	0.8805	0.7647	0.1104	0.5869	0.5353	0.4385
Dhamar	0.0792	0.1272	-	0.9214	0.0961	0.5932	0.4927	0.4483
Between Dhamar and Yarim	0.1672	0.2683	0.0819	-	0.0771	0.4718	0.3692	0.3431
Baghdad	2.1712	2.2035	2.3426	2.5629	-	0.0756	0.0898	0.0755
Wadi Annaim	0.4521	0.5329	0.5222	0.7513	2.5820	-	0.8775	0.8411
N Amran	0.6753	0.6249	0.7079	0.9965	2.4105	0.1307	-	0.9347
Lulah	0.8271	0.8244	0.8024	1.0696	2.5836	0.1731	0.0676	-
Dhihisub	0.1127	0.1418	0.0610	0.1448	2.8205	0.6914	0.9190	0.9563
Dhamar checkpoint	0.2069	0.2458	0.0898	0.1908	2.3317	0.5492	0.6756	0.6470
Thula	0.0307	0.0114	0.1296	0.2697	2.4038	0.4239	0.5848	0.7649
Ad Dogma/ Al Haima	0.1581	0.1188	0.2639	0.4152	2.0591	0.2790	0.3404	0.4409
Ma´abar	0.1611	0.2176	0.0229	0.1038	2.4017	0.4489	0.5548	0.5643
Sana´a	0.0568	0.0277	0.1751	0.3308	2.2644	0.4073	0.5696	0.7642
S Yarim/ Mawah	0.0763	0.1439	0.0369	0.0892	2.0312	0.5210	0.6836	0.7621

Population	Dhihisub	Dhamar checkpoint	Thula	Ad Dogma/ Al Haima	Ma´abar	Sana´a	S Yarim/ Mawah
Shibam	0.8934	0.8131	0.9697	0.8538	0.8512	0.9448	0.9265
Habiba	0.8678	0.7821	0.9887	0.8880	0.8045	0.9727	0.8660
Dhamar	0.9408	0.9141	0.8785	0.7681	0.9773	0.8393	0.9638
Between Dhamar and Yarim	0.8652	0.8263	0.7636	0.6602	0.9014	0.7183	0.9147
Baghdad	0.0596	0.0971	0.0904	0.1276	0.0906	0.1039	0.1312
Wadi Annaim	0.5009	0.5774	0.6545	0.7565	0.6383	0.6654	0.5939
N Amran	0.3989	0.5089	0.5572	0.7115	0.5742	0.5657	0.5048
Lulah	0.3843	0.5236	0.4654	0.6435	0.5688	0.4657	0.4667
Dhihisub	-	0.9426	0.8715	0.7280	0.9068	0.8196	0.9027
Dhamar checkpoint	0.0591	-	0.7583	0.7841	0.9552	0.7195	0.9336
Thula	0.1376	0.2767	-	0.8856	0.7994	0.9692	0.8355
Ad Dogma/ Al Haima	0.3174	0.2432	0.1215	-	0.7863	0.8611	0.7985
Ma´abar	0.0978	0.0459	0.2239	0.2405	-	0.7547	0.9467
Sana´a	0.1989	0.3292	0.0313	0.1496	0.2814	-	0.8423
S Yarim/ Mawah	0.1024	0.0687	0.1797	0.2250	0.0548	0.1717	-

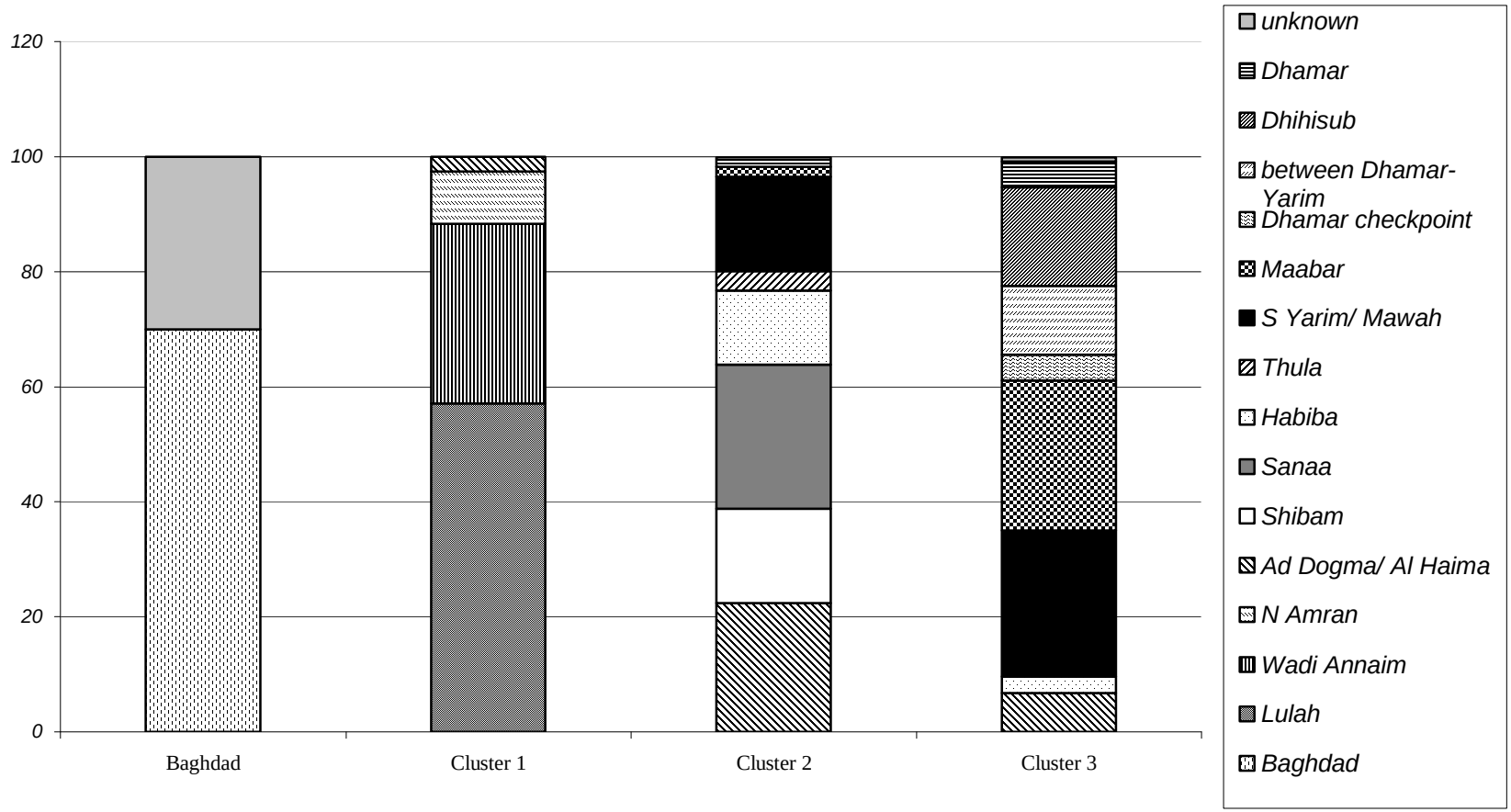
Appendix 6.5: Assignment of specimens including best and second best assignment score of individuals for “home” population. This means that individuals with a second best assignment score to their sampling locality were favored to belong to this population. Thus, assignment results tend to be more conservative regarding assignment to an alternative population and identity of a population tend to be higher. The legend on the right gives explanation for locality identity color. Numbers of individuals assigned to a specific locality are given in percent of the population´s individual number.



Appendix 6.6: Assignment of specimens to populations. The legend on the right gives explanation for locality identity color. Numbers of individuals assigned to a specific locality are given in percent of the population´s individual number.



Appendix 6.7: Summary of assignment tests at the group level inferred from Nei´s distance dendrogram.



The population structure of the burnet moth *Reissita simonyi* (REBEL, 1899; Zygaenidae; Lepidoptera) in the highlands of Yemen is predominantly shaped by landscape topology effects and dispersion abilities rather than habitat fragmentation

7.1 Abstract

Six microsatellite loci were used to infer the population structure of 35 populations (788 individuals) of the burnet moth *Reissita simonyi* (Lepidoptera, Zygaenidae). Within this species, which is endemic to the Arabian Peninsula, two subspecies are recognized: *Reissita simonyi simonyi* and *Reissita simonyi yemenicola*. The latter is distributed along the western escarpment along the Red Sea whereas *R. s. simonyi* is known from an area along the Indian Ocean near the Yemeni-Omani border. Larvae of *R. simonyi* feed on plants of the *Maytenus* species - complex (Celastraceae) and due to the patchy distribution of the food plants *R. simonyi* is not continuously distributed throughout Yemen and Oman.

The genetic differentiation between populations within subspecies is generally lower than between subspecies, indicating a clear differentiation between subspecies. Isolation by distance is found along the sampled range as well as within the subspecies *R. s. yemenicola*. Within *R. s. simonyi*, isolation by distance is not present when under-sampled populations are excluded from analyses. In addition, isolation by altitudinal distance is found along the species range as well as within subspecies. However, no significant correlation between altitude and parameters like allelic richness, inbreeding coefficient, observed heterozygosity or expected heterozygosity is detected. Thus, we conclude that altitude induces genetic differentiation in general but has not a strong impact as an ecological factor.

A further aspect is the analysis of the connectivity of populations within and between subspecies. Between subspecies, we find only few connections, supporting taxonomic distinction. Within subspecies, connectivity of populations is much closer. However, a further subtle structure within *R. s. yemenicola* is detected by both, Nei´s distance (UPGMA tree) and edge distances (network analysis). This structure corresponds to a

specific geographical feature; the mountain chain is bent and therefore, geographic distances may have been increased in this area, considering that individuals are closely linked to the mountain chain. Low genetic differentiation in combination with accumulation of populations found around and above 2000 m further implies that individuals can easily migrate from one mountain to another (top-hopping). We conclude that recent anthropological habitat fragmentation plays a minor role in comparison to differentiation due to geographical and altitudinal distances, landscape topology, and dispersion abilities in shaping population structure of *R. simonyi*.

Keywords: habitat fragmentation, *Reissita simonyi*, microsatellites, isolation by distance, isolation by altitude.

7.2 Introduction

Habitat fragmentation, habitat loss and desertification due to natural climatic changes in combination with human impact isolate natural populations (LANDE, 1988; TEMPLETON *et al.*, 2001). Particularly, desert areas are threatened by the consequences of anthropogenic destructions. Animal as well as plant species suffer from the loss of suitable natural and undisturbed habitats. Conversely, the persistence of species depends on the persistence of local populations, which contribute to the connectivity of populations. Habitat fragmentation hinders regular gene flow and therefore induces the risk of decrement of genetic diversity and increment of inbreeding effects (FRANKHAM, 1995). If the loss of genetic diversity reaches a critical point, extinction of local populations is possible and connectivity of remaining populations may be further diminished.

Butterflies and moths have been widely used as model systems for addressing questions of population genetics with a focus on habitat fragmentation and loss as well as population dynamics (HANSKI *et al*, 1994; HILL *et al*., 1996; NEVE *et al*., 1996; BROOKES *et al*., 1997; LEWIS *et al*., 1997; SUTCLIFFE *et al*., 1997; MEGLÉCZ *et al*., 1998; SACCHERI *et al*., 1998; HARPER *et al.*, 2003; TAKAMI *et al*., 2004). This is partly due to the wide range of background literature on distribution, ecological preferences, and the knowledge about larval food plants, which make them particularly interesting for

ecologists and population genetists. Despite the availability of these data, the majority of studies concentrate on the palaearctic region and on common lepidopteran species. However, it would be of special interest to gain more information on rare species in other geographical areas to complement the already collected data. Therefore, we selected the burnet moth *R. simonyi* (REBEL, 1899; Lepidoptera, Zygaenidae) as a model system. *R. simonyi* is an endemic of the Arabian Peninsula. Two subspecies are described, *R. s. yemenicola* and *R. s. simonyi*. The former is distributed along the western escarpment parallel to the Red Sea whereas the latter is distributed along the Indian Ocean on both sides of the Yemeni-Omani border (Fig. 1; NAUMANN & EDELMANN, 1984; KLÜTSCH *et al.*, chapter 3). In addition, the distributional range of *R. simonyi* is strictly linked to the larval food plants of the genus *Maytenus* (Celastraceae). Like in other lepidopteran species, this dependence on special larval food plants increases the vulnerability of *R. simonyi* populations. The presence of food plants is threatened by over-grazing through goats which prefer fresh shoots. Since the first instars of *R. simonyi* also depend on fresh and soft leaves, populations of *R. simonyi* are assumed to suffer from habitat disturbance and loss. Moreover, the populations of *R. simonyi* may be indirectly endangered by habitat loss through desertification and agricultural land use. Under natural conditions, *Maytenus* species are found in rocky places on hillsides. Nowadays, in order to use as much land for agriculture as possible, terrace cultivation is widely applied. Hence, natural habitats become fragmented or lost by agricultural land use. To understand how landscape and/or ecology shape population structure it is fundamental to describe additional effects of landscape features (*e. g.* mountain chain formation, mountain ridges or river valleys) on the genetic composition of populations. Natural landscape features present potential barriers for dispersal, gene flow, and population differentiation as well as speciation (MANEL *et al.*, 2003). An often neglected factor for isolation in mountainous areas is isolation by altitudinal differences. However, recent studies dealing with this issue discuss the potential impact of altitudinal differences between populations as an additional factor for population differentiation (STORZ & DUBACH, 2004; DE NAVASCUÉS MELERO, 2005; FUNK *et al.*, 2005). Altitudinal differences along hillsides often present ecological gradients. Therefore, altitude may have a particularly strong impact on growth rates and development of larval food plants and consequently on dispersal and gene flow of *R. simonyi*.

We studied patterns of genetic differentiation and genetic diversity across the species´ range (except Saudi Arabia) in Southern Arabia. In particular, we examined the connectivity of populations in order to study whether populations form a network or if populations show signs of isolation (restricted or lacking gene flow) and are assembled in genetically distinct groups. Complementary isolation factors, like isolation by distance and isolation by altitudinal distance as well as isolation by landscape features are studied to address the special characteristics of this geographical area. Finally, a discussion whether habitat fragmentation or landscape topology predominantly shaped population structure is given. Hereby, special attention is drawn to dispersion abilities of *R. simonyi*.

7.3 Material and Methods

7.3.1 Sampling

The study was carried out in Yemen, Southern Arabia. The landscape is mainly a mountainous desert area. Special characteristics of the study site are the high mountains (up to 2818 m in this study), which resulted in altitudinal differences of 11.0 to 1900.0 meters between the populations. Tissue samples were collected from 35 localities (Fig. 1) over a two years period (2001-2003) in Yemen. Of these 35 populations, five had sample sizes under 9; all other sites were sampled with 9 to 83 samples per site. Tissue was directly stored in 100 % ethanol. Samples are stored in the tissue collection of ZFMK at -20°C. A total of 788 tissue samples (mainly larvae, some adults) across the species´ distribution range (except Saudi Arabia) were collected (Fig. 1; appendix 7.1). Geographical distances between populations ranged from 0.5 to 1243.0 km.

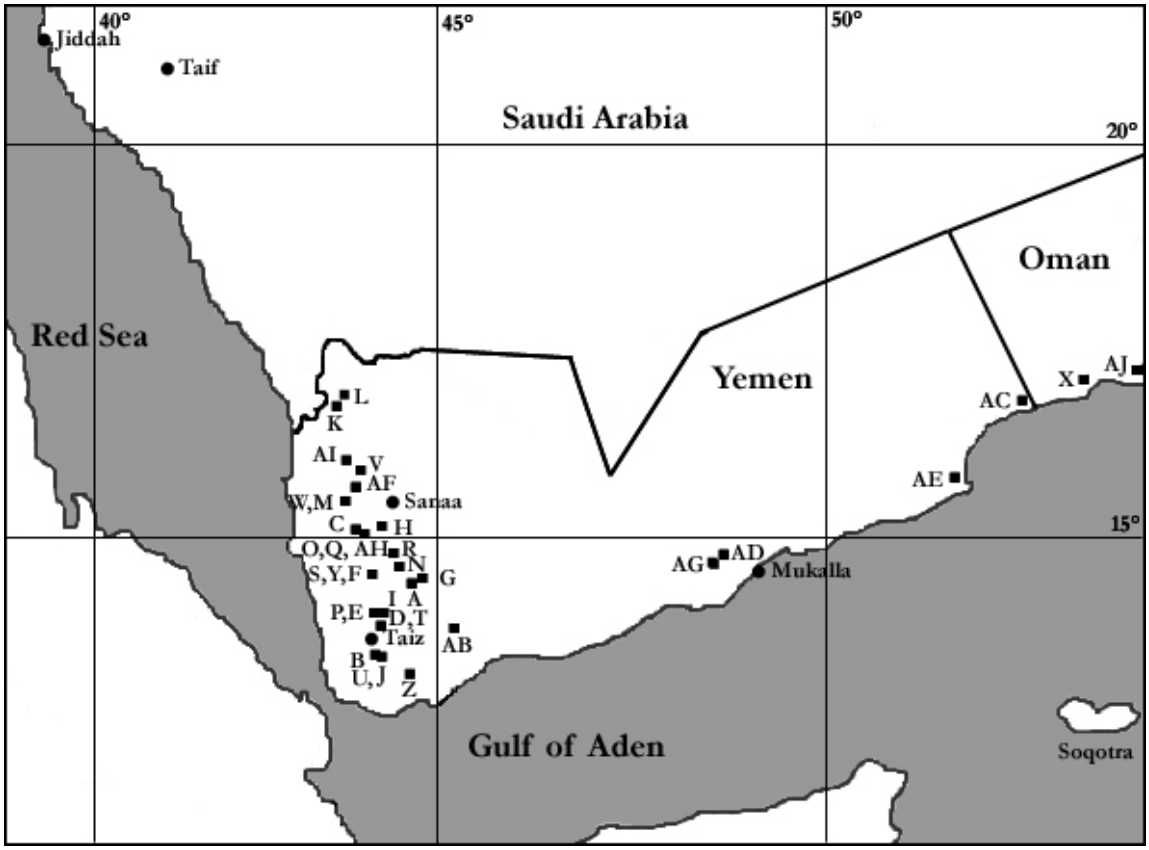


Fig. 1: Overview about collection sites in Yemen and Oman. Some localities are summarized under one square and labeled by two or more letters (separated by a ",") in order to ensure lucidity. Letters equal to those in appendix 7.1 and Fig. 2 + 4 & 5.

7.3.2 DNA extraction and amplification

Genomic DNA was extracted using a standard Chelex protocol (GERKEN *et al*., 1998). Primers of six microsatellite loci (RS-3, RS-4, RS-17, RS-37, RS-47, RS-50) were taken from KLÜTSCH *et al*. (chapter 5). PCR amplification was carried out in 20 µL containing 10 x PCR buffer (without $MgCL_2$; containing100 mM Tris-HCL, 500 mM KCL, pH 8.3, Sigma), 1,5 – 2,0 mM $MgCl_2$ (Sigma), 100 µM of each dNTP (Sigma), 500 nM of each primer and 0,5 unit *Taq* polymerase (containing 20 mM Tris-HCL, 100 mM KCL, 0,1 mM EDTA, 1 mM DTT, 0,5 % Tween 20, 50 % glycerol; Sigma).

Amplification was performed in an Applied Biosystems GeneAmp 2700 thermal cycler. Following an initial denaturation step of 3 minutes at 94°C, the reactions underwent 35 thermal cycles (94°C for 30 s, 60 s at annealing temperature and 65 s at 72°C) and a final extension time of 20 min at 72°C (see KLÜTSCH *et al*. (chapter 5) for annealing temperatures). Products were separated using a 6 % acrylamide gel and an ABI 377 sequencer (Applied Biosystems). Signals were analyzed with GENESCAN 3.1.2 (Applied Biosystems) and GENOTYPER (Applied Biosystems).

7.3.3 Statistical data analysis

Statistical analysis was performed with GENEPOP 3.4 (RAYMOND & ROUSSET, 1995). Genotypic linkage disequilibrium (LD = non random association of genotypes occurring at different loci) was tested by the exact probability test. The null hypothesis is that genotypes at one locus are independent from genotypes at all other loci. This test computed unbiased estimates by randomization (10.000 iterations) and the exact probabilities of random association for all contingency tables corresponding to all possible pairs of loci within each population by the Markov-chain method. Additionally, GENEPOP 3.4 (RAYMOND & ROUSSET, 1995) was used to calculate exact tests on Hardy-Weinberg equilibria, heterozygosity deficiencies, and F_{IS} / F_{ST} values at the population level. HWE was tested at the single locus level using the two tests of heterozygosity excess and heterozygosity deficit. The program POPGEN 32 (YEH & BOYLE, 1997) was used to calculate Nei´s distance and construct a UPGMA tree. FSTAT (GOUDET, 2001) was used for the calculation of allelic richness. Small populations were retained for further analyses, because they displayed no deviation from Hardy-Weinberg equilibrium or specific linkage disequilibrium. Furthermore, a test for recent bottlenecks was conducted with the program BOTTLENECK (CORNUET & LUIKART, 1996) with 5000 replications.

7.3.4 Analysis of population structure using a network approach (DYER & NASON, 2004)

The program POPGRAPH (DYER & NASON, 2004) was used to study population connectivity, population topology and inter-population relationships, the identification of key populations for genetic connectivity, and population-level assignment in order to address the complex mixture of contemporary gene flow and gene flow barriers within and among subspecies of *R. simonyi*. POPGRAPH served also as an illustration of within-population genetic variability and its geographic pattern. POPGRAPH uses a multivariate graph-theoretic approach, which is free of any a priori model of population assembling. Population relationships are analyzed in a geometric space, which encompasses genetic covariance in a multi-dimensional way instead of a classical pairwise fashion. Thus, populations are put into a multi-dimensional context, which allows describing several statistical relationships among populations simultaneously.

7.3.5 Analysis of isolation by distance and isolation by altitudinal distance

In addition, IBD ON THE WEB (BOHONAK, 2002) tested with simple and partial Mantel tests (MANTEL, 1967) if a potential correlation between genetic and geographic distance (isolation by distance) and isolation by altitudinal distance is present. Geographic distances were log - transformed, because distances were not normally distributed. Mantel tests were performed with 30.000 permutations. Linear geographic distances were obtained from MICROSOFT ENCARTA WORLD ATLAS (2005). IBD and isolation by altitudinal distance were tested at three hierarchical levels. First, the overall significance was tested including all populations. Second, relationships within subspecies were further investigated to identify which combination contributed mostly to the overall significant result. Third, significance of IBD was tested within two clusters of *R. s. yemenicola* according to Fig. 2.

7.4 Results and discussion

7.4.1 Linkage disequilibrium

15 of the 337 possible tests have significant results ($P < 0.05$). However, after applying a Bonferroni correction, none of these 15 remains significant ($P< 0.001$). Thus, linkage between pairs of microsatellites is not present.

7.4.2 Hardy Weinberg tests and bottlenecks

Deviations from HWEs are observed in 66 out of 408 tests at the single locus level (data not shown) after applying Bonferroni corrections (P<0.001). All deviations are caused by heterozygote deficiency. An accumulation of loci with heterozygote deficiency can be observed in the following populations: Sumarah/ Al Hosn (5 loci), Province Taiz Jabal Sabr vic. Hatab (6 loci), Province Menakhah/ Hajjarah (4 loci), Province Taiz/ Dhi Al Sefal (5 loci), Al Qubba (4 loci), Sa´ada Zara´a (4 loci), Mitwa/ Menakhah (5 loci), Masnah area/ 5 km SW Al Qubba (5 loci), Wadi Doran (4 loci), and Al Machwit/ Jabal Haiadi (4 loci).

In order to test whether this heterozygote deficiency is caused by recent bottlenecks, the program BOTTLENECK (CORNUET & LUIKART, 1996) was used. A sign of possible recent bottlenecks is found for the populations Province Ibb 4 km S Ibb, Province Taiz vill.Mahzaf, Province Zadaa Jabal Rhaza, OMAN, Masnah area 2 km N Al Qubba, Province Al Mahra Al Hawf, Korseban, and Province Hajjah 1.5 km N Hajjah.

7.4.3 High inbreeding coefficients versus strong connectivity of populations

Extraordinary high inbreeding coefficients are found within populations, which can be interpreted as evidence for isolation of population and poor within-population diversity. However, it is likely that the populations found in BOTTLENECK represent false positive results, because all populations, which appear to be bottlenecked are small populations and therefore, may not sufficiently sampled as suggested by CORNUET & LUIKART (1996). Hence, the hypothesis of recent bottlenecks as a cause for accumulation of heterozygote deficiency in particular populations is rejected. Other explanations for deviation of HWE expectations include the Wahlund effect, non-random mating, or

inbreeding effects. One other explanation is especially likely for lepidopterean studies. MEGLÉCZ *et al.* (2004) reported that in most population genetic studies in Lepidoptera, which used microsatellites as genetic markers, a significant deviation from Hardy-Weinberg equilibrium at many loci is found. The main reason for this departure is a deficit of heterozygotes. These consistent results found in all Lepidopteran studies suggest the frequent presence of null alleles. Therefore, the high inbreeding coefficient found in this study should be interpreted with caution, since the presence of null alleles may have influenced it.

7.4.4 Isolation by distance and isolation by altitudinal distance

A highly significant isolation by distance pattern is found ($p < 0.0001$; appendix 7.2) among all populations (Fig. 2a). A significant correlation between genetic differentiation and altitudinal distance is also present ($p < 0.008$) in partial mantel tests (Fig. 2b). Details about geographical and altitudinal distances between populations are given in appendix 7.4 and 7.5.

Within subspecies, a similar pattern is found within *R. s. yemenicola* ($p< 0.0001$), although the correlation of genetic differentiation and altitudinal difference is not significant anymore in partial mantel tests. In contrast, within *R. s. simonyi* none of the correlations remains significant. If only populations with or more than 9 individuals are analyzed in order to exclude random effects caused by small population size, the picture is slightly different. In partial mantel tests, the correlation of genetic differentiation and altitudinal distance becomes significant or even more significant in all cases (appendix 7.2). Taken the sub-clusters from Fig. 4 into account, the two clusters within *R. s. yemenicola* are tested for isolation by distance and isolation by altitudinal distance. Within clusters, neither isolation by distance nor isolation by altitudinal distance is detected (appendix 7.2).

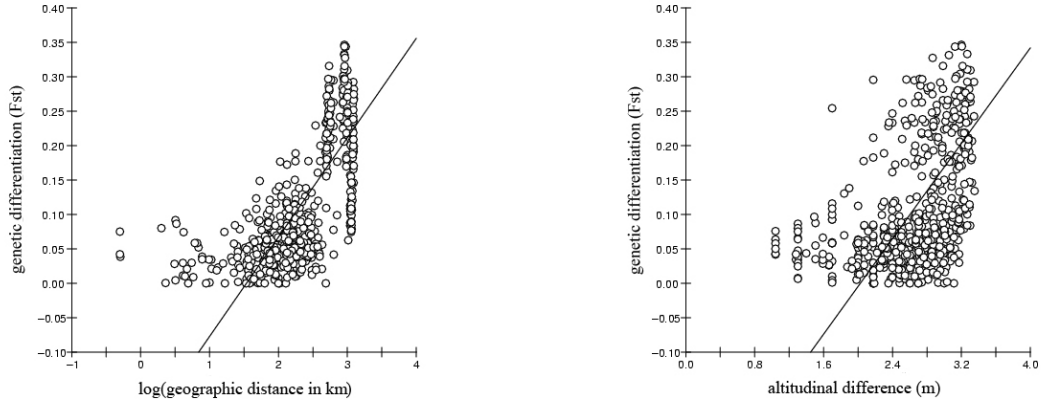


Fig. 2: a) Correlation of genetic differentiation (Fst) and geographic distance (in km); b) correlation of genetic differentiation (Fst) and altitudinal distance (in m).

7.4.5 Differentiation of subspecies using a network approach

The most obvious result of the applied POPGRAPH analysis is the division into two main subgraphs, which coincide with the subspecies *R. s. yemenicola* (larger subgraph) and *R. s. simonyi* (smaller subgraph; Fig. 3). The smaller subgraph representing *R. s. simonyi* contains all populations assumed to belong to this subspecies, namely Mola Matar (AG), Korseban (AD), N Ras –Fartak (AE), Province Al Mahra Al Hawf (AC), OMAN A (X), OMAN B (AJ). The larger subgraph contains all remaining populations belonging to *R. s. yemenicola*. In contrast to the low genetic differentiation within *R. s. yemenicola*, a clear distinction of both subspecies is detected in all analyses (Fig. 3 + 4; F_{ST} values in appendix 7.3). Therefore, the taxonomic subspecies status captures a real distinction among the populations. In addition, populations of *R. s. simonyi* appear to have a slightly lower genetic diversity than populations of *R. s. yemenicola*.

7.4.6 Identification of key populations

The main key populations, which represent connection points between the two subspecies, are Jabal Sabr (U) and Jabal Manwara (AB) followed by Jabal Rhaza (K). Jabal Sabr (U) and Jabal Manwara (AB) can be seen as bridge populations between the two subspecies, because they are the closest populations to the distribution area of *R. s.*

simonyi. In contrast, the third (Jabal Rhaza) belong to one of the most northern populations at the Yemeni-Saudi-Arabian border (Fig. 1). However, only one edge connects Jabal Rhaza with *R. s. simonyi* and the edge distance is extraordinary high (15.13). Especially, population AB (Jabal Manwara) with 9 edges (2 to *R. s. simonyi* and 7 to *R. s. yemenicola*) clearly represents a key population. Due to the high number of edges (7) and low edge distance (mean: 4.46) to populations of *R. s. yemenicola*, Jabal Manwara evidently belongs to this subspecies. However, this population also has connection to *R. s. simonyi*. The low number of edges (2) and the comparatively high edge distances (mean: 8.76) to *R. s. simonyi* suggest that connection of subspecies is weak. In total, only 5 edges connect both subspecies. Jabal Sabr (U) also has two connections to *R. s. simonyi*, but edge distance is high in these cases (mean: 13.99). Thus, the geographically nearest population to *R. s. simonyi*, Jabal Manwara (AB) displays also smallest edge distances to *R. simonyi*. Geographically, both subspecies are separated by a geographical gap of 300 km, which probably explains the weak connection of subspecies.

7.4.7 Genetic connectivity of populations within subspecies

We focused on the genetic connectivity of populations to test whether potential habitat fragmentation or natural barriers to gene flow has led to discontinuous connectivity of populations and isolation of some populations. According to Fig. 4, *R. s. yemenicola* is mainly divided into two sub-clusters, which have low branch lengths; indicating close relationships among populations and clusters. In the POPGRAPH analysis this pattern also appears as a subtle structure (Fig. 5). Regarding edge distances, the mean edge distance within the two clusters is 3.80 (cluster 1) and 4.10 (cluster 2) whereas mean between-cluster distance is 4.90.

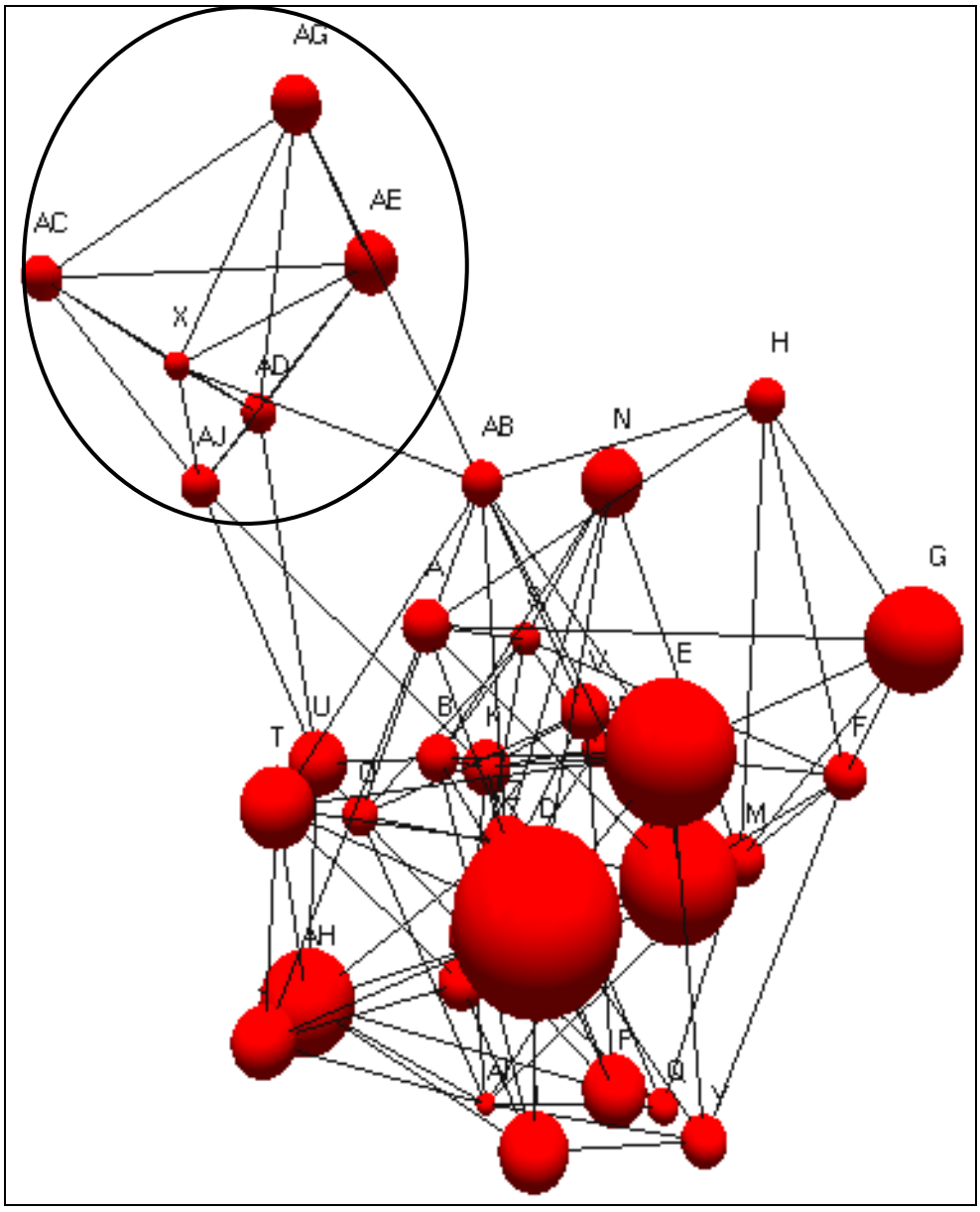


Figure 3: Illustration of POPGRAPH analysis. The two major groups represent the two subspecies, *R. s. yemenicola* (bottom) and *R. s. simonyi* (upper left, black circle). Node size represents within-population variance. Edge lengths represent variance among populations.

Focusing on the number of edges connecting populations as a measurement of connectivity, the populations Mitwa/ Menkhah (10), Hajjarah (9), and Province Jaffah/ Jabal Manwara (9) exhibit the highest number of connections to other populations. Thus, these populations represent highly interacting populations. The lowest number of connections is found in Jabal Sabr/ Mahzaf (4), Sa´ada Zara´a (4), Masnah (4), and Al Hudaib (3). Consequently, these populations appear to be less connected to other populations. Generally, these results are in congruence with results of other authors (CULLENWARD *et al*., 1979; BAUGHMAN *et al*., 1990; PETERSON, 1995) who found only little genetic differentiation among butterfly populations at larger scales among a geographical range from 30 to hundreds of kilometers.

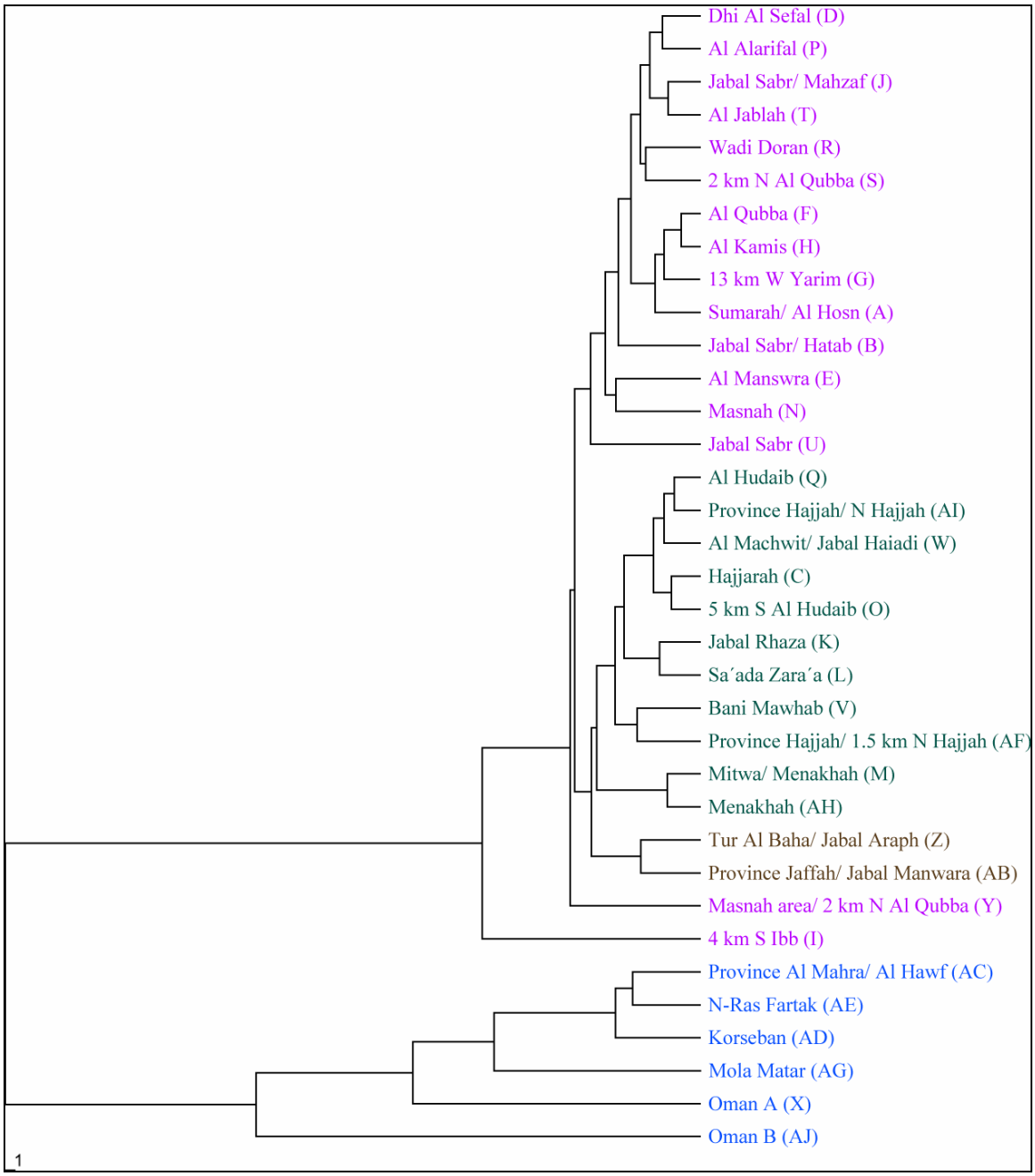


Fig. 4: Nei´s genetic distance tree (UPGMA) based on the genetic distance data in appendix 7.6. Subspecies *R. s. simonyi* is labeled in blue; the two main clusters of *R. s. yemenicola* are labeled in pink and green. The most eastern populations of *R. s. yemenicola* are labeled in brown.

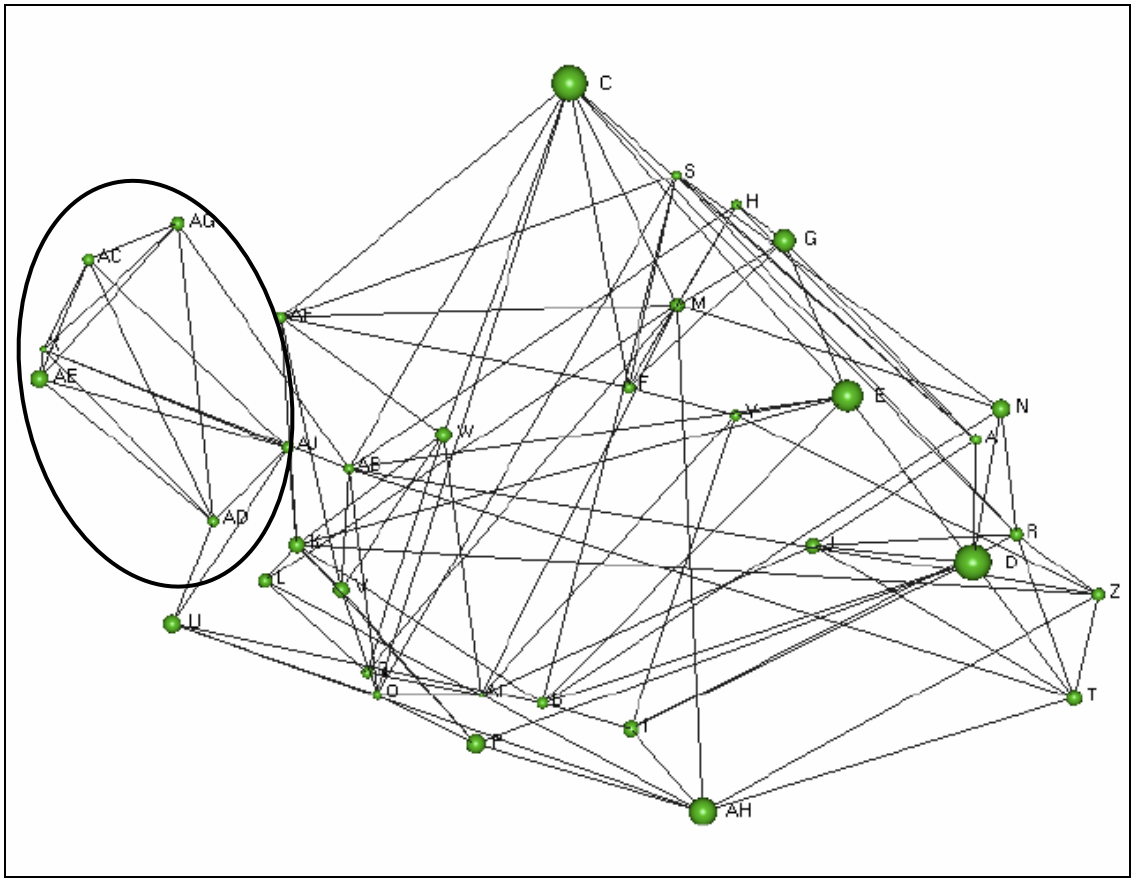


Fig. 5: Additional perspective on the POPGRAPH analysis to illustrate the two sub-clusters. In this graph, the two subtle sub-clusters within *R. s. yemenicola* are clearly visible. Differences to Fig. 4 in node size are only based on different scales, but relations between nodes are the same. The black circle again shows the subspecies *R. s. simonyi*.

7.4.8 Within population variability

The second obvious result is the great range of variability within populations. The populations Dhi Al Sefal (D), Al Manswra (E), and 13 km W of Yarim (G) have highest variability among populations. Although there is a correlation between population size and allele number, these populations remain to be among the most variable ones when calculating allelic richness (appendix 7.1). In total, *R. s. simonyi* has a mean allelic richness of 3.35 whereas *R. s. yemenicola* shows a mean allelic richness of 3.95 indicating a higher variability within *R. s. yemenicola* than in *R. s. simonyi*.

7.4.9 Habitat fragmentation versus influence of landscape topology on genetic structure

The strong genetic connectivity of populations leads to another aspect of this work. Recent habitat fragmentation appears to be a negligible factor on genetic differentiation among populations. The subtle population structure indicates that genetic differentiation generally is low. Additionally, within the two main clusters of *R. s. yemenicola*, isolation by distance and isolation by altitudinal distance are absent. In contrast, among clusters of *R. s. yemenicola* and among both subspecies, the main genetic differentiation appears to be strongly connected to isolation by distance and isolation by altitudinal distance.

Hostile areas are most likely to impede gene flow in butterfly and moth species (MEGLÉCZ *et al.*, 1997; VAN DONGEN *et al.*, 1998; KEYGHOBADI *et al.*, 1999; SCHMITT *et al.*, 2000). Therefore, it is worthwhile to briefly discuss which potential gene flow barrier may have contributed to the division into two sub-clusters within *R. s. yemenicola*. The mountain chain is bent in the area, where both clusters meet. Thus, an additional isolation effect due to larger geographical distances in this area may have contributed to the subtle structure within *R. s. yemenicola* considering that *R. simonyi* is linked to this mountain chain. However, also other factors may have contributed to this result, like a more intensive agricultural land use in the contact zone of both clusters. In this case, the division into two clusters would represent a case of habitat fragmentation or loss.

The study area of *R. s. yemenicola* mainly consists of patches at altitudes of 2000-2800 m which are separated by lower areas with altitudes of 1000-2000 m. Although it cannot be ruled out that the larval food plants occur at lower altitudes as well, they are highly restricted to areas around 2000 m altitude and above in the distributional range of *R. s. yemenicola*. Generally, *Maytenus* is also found in lower altitudes (*e. g.* along the Indian Ocean), but we were not able to find many localities with this plant at low altitudes in the distribution area of *R. s. yemenicola*. This is maybe due to the fact that agricultural land use may be more common at altitudes of 1000-2000 m and thus, the density of food plants may be lower at lower altitudes. Given the general low genetic differentiation in *R. s. yemenicola*, this study indicates that *R. s. yemenicola* is able to move from one mountain to another. A pattern called top-hopping. A consequence of this dispersion ability is that genetic connectivity is high in this species.

Within clusters, no isolation by distance pattern can be detected, which is in congruence with butterfly studies in alpine areas (BRITTEN *et al.*, 1995; PETERSON, 1995). BRITTEN *et al.* (1995) suggested that populations of *Euphydryas editha* in the Great Basin in comparison to those in the Rocky Mountains displayed no isolation by distance pattern because of the patchy distribution of populations. Suitable high-elevation populations are separated by unsuitable habitat patches at lower altitudes like in this study. Thus, genetic differentiation would not follow a simple isolation by linear geographical distance pattern; instead drift effects may have contributed to genetic differentiation within clusters. In this case, isolation by distance and isolation by altitudinal distance would act as gene flow barriers at a larger geographical scale whereas genetic differentiation within clusters of *R. s. yemenicola* is based on the patchy distribution of populations and is caused by other effects like genetic drift. It has to be kept in mind that genetic differentiation generally is low and that these potential barriers to gene flow have acted in a restrained manner.

In summary, the main objective of this study was to analyze the genetic structure and diversity of *R. simonyi* populations in order to examine whether recent habitat fragmentation or landscape topology contributed more to the genetic structure. We find significant regional genetic differentiation as well as an isolation by distance and isolation by altitudinal distance pattern in addition to strong connectivity of populations within subspecies. Additionally, strong connectivity of populations in combination with

accumulation of high-elevation habitats implies that dispersion abilities of *R. simonyi* follow a top-hopping pattern.

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Appendices

Appendix 7.1: Summary of allelic variation statistics for all loci. Number of individuals (N_{ind}), expected heterozygosity (H_E), observed heterozygosity (H_O), F_{IS} estimates after WEIR & COCKERHAM (1984) (F_{IS}); Number of polymorphic loci (N_{pol}), number of alleles within population (A), mean number of alleles within population (A_m), A_r allelic richness (calculated in FSTAT (GOUDET, 2001)). Please note that allelic richness is adjusted to the smallest population.

Letter(s) in map		height (m)	N_{ind}	H_e	H_o	F_{IS}	N_{pol}	A	A_m	A_r	A_{rgp}	Total number of nodes
A	Sumarah/Al Hosn	2700	21	0.733	0.380	0.488	6	43	7.17	3.51	4.06	6
B	Jabal Sabr/Hatab	2550	23	0.741	0.326	0.565	6	44	7.33	3.62	4.23	7
C	Hajjarah	2500	28	0.683	0.437	0.364	6	49	8.17	3.39	3.98	9
D	Dhi Al Sefal	2000	83	0.787	0.443	0.439	6	77	12.83	3.90	4.62	8
E	Al Manswra	2200	29	0.775	0.497	0.364	6	51	8.50	3.72	4.35	8
F	Al Qubba	2300	26	0.719	0.374	0.485	6	45	7.50	3.36	3.87	7
G	13 km W Yarim	2850	10	0.762	0.337	0.573	6	37	6.17	3.62	4.20	5
H	Al Kamis	2700	14	0.718	0.358	0.514	6	37	6.17	3.39	3.93	5
I	4 km S Ibb	1600	10	0.708	0.409	0.438	6	33	5.50	3.44	4.00	5
J	Jabal Sabr/ Mahzaf	2550	9	0.621	0.319	0.501	6	30	5.00	3.22	3.91	4
K	Jabal Rhaza	2300	6	0.606	0.222	0.655	5	23	3.83	3.00	-	7
L	Sa´ada Zara´a	2075	27	0.724	0.316	0.568	6	47	7.83	3.49	4.07	4
M	Mitwa/ Menakhah	2700	75	0.756	0.348	0.541	6	74	12.33	3.70	4.37	10
N	Masnah	2300	45	0.807	0.438	0.460	6	63	10.50	3.93	4.63	4
O	5 km S Al Hudaib	2818	16	0.620	0.487	0.222	6	35	5.83	2.99	3.43	7
P	Al Alarifal	2396	16	0.714	0.392	0.464	6	36	6.00	3.41	3.95	5
Q	Al Hudaib	2818	14	0.671	0.452	0.336	6	38	6.33	3.33	3.87	3
R	Wadi Doran	2500	50	0.735	0.447	0.394	6	49	8.17	3.43	3.94	7
S	2 km N Al Qubba	2350	22	0.675	0.447	0.343	6	36	6.00	3.18	3.64	7
T	Al Jablah	2311	39	0.672	0.437	0.353	6	46	7.67	3.27	3.85	7
U	Jabal Sabr	2569	11	0.656	0.472	0.293	6	29	4.83	3.05	3.46	5
V	Bani Mawhab	2100	10	0.682	0.440	0.373	6	29	4.83	3.17	3.62	6
W	Al Machwit/ Jabal Haiadi	1800	49	0.644	0.402	0.378	6	60	10.00	3.19	3.72	6
X	Oman “A”	850	5	0.738	0.400	0.393	5	18	3.60	-	-	6
Y	Masnah area/ 2 km N Al Qubba	2350	5	0.707	0.517	0.293	6	22	3.67	3.14	-	5
Z	Tur-Al-Baha/ Jabal Araph	1330	19	0.624	0.429	0.318	6	39	6.50	3.07	3.55	6
AB	Province Jaffah/ Jabal Manwara	2311	20	0.717	0.518	0.281	6	40	6.67	3.40	3.92	9
AC	Province Al Mahra/ Al Hawf	800	9	0.704	0.429	0.406	6	33	5.50	3.47	4.04	5
AD	Korseban	1850	5	0.660	0.317	0.550	5	22	3.67	3.15	-	6
AE	N - Ras Fartak	966	14	0.584	0.464	0.213	6	26	4.33	2.75	3.07	5
AF	Province Hajjah/ 1.5 km N Hajjah	2600	7	0.695	0.409	0.433	6	25	4.17	3.39	-	7
AG	Mola Matar	1750	13	0.676	0.407	0.409	6	34	5.67	3.84	3.93	5
AH	Menakhah	2818	35	0.682	0.447	0.348	6	45	7.50	3.22	3.71	7
AI	Province Hajjah/ N Hajjah	1521	21	0.685	0.380	0.452	6	38	6.33	3.24	3.72	8
AJ	Oman “B”	600	2	0.784	0.412	0.380	6	13	2.17	-	-	6
	Total		788									

Appendix 7.2: Mantel and partial Mantel test results for isolation by distance and altitude analysis. IBD ON THE WEB (BOHONAK, 2002) was run with 30.000 randomizations.

Genetic distance	**1st geographic distance**	**Indicator matrix**	**Z**	**r**	**p < =**	**P <= only populations above 9 individuals**
among all individuals						
F_{ST}	log (geographic distance in km)	No	159.1574	0.6634	0.0001	0.0001
F_{ST}	altitudinal difference (m)	No	56891.4683	0.5376	0.0001	0.0005
F_{ST}	log (geographic distance in km)	Altitudinal difference (m)		0.5065	0.0001	0.0001
F_{ST}	altitudinal difference (m)	log (geographic distance in km)		0.2362	0.008	0.002
within *R. s. yemenicola*)						
F_{ST}	log (geographic distance in km)	No	45.8985	0.2678	0.0001	0.002
F_{ST}	altitudinal difference (m)	No	10772.6025	0.1726	0.046	0.015
F_{ST}	log (geographic distance in km)	Altitudinal difference (m)		0.2442	0.0006	0.006
F_{ST}	altitudinal difference (m)	log (geographic distance in km)		0.1311	0.096	0.029
within *R. s. simonyi*)						
F_{ST}	log (geographic distance in km)	No	3.7932	0.1848	0.2576	0.3389
F_{ST}	altitudinal difference (m)	No	1226.4152	-0.114	0.6275	0.3389
F_{ST}	log (geographic distance in km)	Altitudinal difference (m)		0.2344	0.2061	1.0000
F_{ST}	altitudinal difference (m)	log (geographic distance in km)		-0.1855	0.6922	0.0000
within cluster 1 (labeled in pink, Fig. 2)						
F_{ST}	log (geographic distance in km)	No	7.4046	0.0453	0.3128	
F_{ST}	altitudinal difference (m)	No	1736.1081	0.3163	0.0682	
F_{ST}	log (geographic distance in km)	Altitudinal difference (m)		-0.0033	0.4954	
F_{ST}	altitudinal difference (m)	log (geographic distance in km		0.3134	0.0718	
within cluster 2 (labeled in green, Fig. 2)						
F_{ST}	log (geographic distance in km)	no	3.8748	0.0520	0.3523	
F_{ST}	altitudinal difference (m)	no	1155.4294	-0.0702	0.6072	
F_{ST}	log (geographic distance in km)	Altitudinal difference (m)		0.0840	0.2801	
F_{ST}	altitudinal difference (m)	log (geographic distance in km)		-0.0963	0.6825	

Appendix 7.3: F_{ST} values among population pairs

POP	A	B	C	D	E	F	G	H	I	J	K	L
A												
B	0.0523											
C	0.0556	0.1159										
D	0.0205	0.0177	0.0699									
E	0.0516	0.0370	0.0975	0.0229								
F	0.0101	0.0418	0.0483	0.0372	0.0518							
G	-0.0073	0.0070	0.0674	0.0009	0.0053	0.0015						
H	0.0076	0.0104	0.0786	0.0220	0.0393	-0.0155	-0.0309					
I	0.1018	0.0512	0.1433	0.0746	0.0918	0.0590	0.0454	0.0350				
J	0.0182	0.0343	0.0926	0.0225	0.0626	0.0302	0.0350	0.0294	0.1345			
K	0.0701	0.0550	0.0541	0.0439	0.1125	0.0474	0.0190	0.0286	0.1317	0.0663		
L	0.0372	0.0448	0.0463	0.0238	0.0625	0.0367	0.0124	0.0159	0.0994	0.0474	-0.0117	
M	0.0436	0.0584	0.0374	0.0458	0.0637	0.0192	-0.0044	0.0065	0.0505	0.0850	0.0223	0.0275
N	0.0416	0.0353	0.0707	0.0243	0.0303	0.0385	0.0116	0.0263	0.0351	0.0553	0.0640	0.0486
O	0.0559	0.1107	0.0113	0.0666	0.1159	0.0489	0.0875	0.0844	0.1767	0.0747	0.0503	0.0457
P	0.0003	0.0262	0.0467	0.0005	0.0288	0.0051	0.0101	0.0129	0.0775	-0.0107	0.0572	0.0195
Q	0.0360	0.0905	0.0209	0.0470	0.1016	0.0483	0.0549	0.0542	0.1405	0.0525	0.0187	0.0160
R	0.0315	0.0581	0.0657	0.0363	0.0545	0.0282	0.0401	0.0322	0.0644	0.0320	0.0686	0.0383
S	0.0320	0.0354	0.0669	0.0382	0.0695	0.0102	0.0251	0.0154	0.0947	0.0323	0.0595	0.0396
T	0.0550	0.0487	0.1120	0.0387	0.0521	0.0418	0.0423	0.0400	0.1080	0.0061	0.0758	0.0533
U	0.0533	0.0345	0.1304	0.0532	0.0891	0.0378	0.0557	0.0389	0.1159	0.0422	0.1198	0.0879
V	0.0211	0.0953	0.0558	0.0365	0.0635	0.0495	0.0298	0.0675	0.1773	0.0320	0.0407	0.0436
W	0.0662	0.1382	0.0259	0.0811	0.1218	0.0649	0.0840	0.0956	0.1888	0.0871	0.0509	0.0625
X	0.1093	0.0790	0.1217	0.0746	0.1128	0.1078	0.0838	0.1001	0.1571	0.1065	0.0989	0.0895
Y	0.0065	0.0690	0.0826	0.0333	0.0190	0.0044	-0.0211	0.0182	0.0537	0.0473	0.0585	0.0452
Z	0.0811	0.1234	0.0602	0.0741	0.1198	0.0768	0.0872	0.0982	0.1724	0.0913	0.0208	0.0560
AB	0.0620	0.0687	0.0992	0.0358	0.0609	0.0637	0.0443	0.0486	0.1261	0.0551	0.0429	0.0390
AC	0.2115	0.2185	0.2738	0.1862	0.1835	0.2313	0.1789	0.2063	0.2357	0.2781	0.2794	0.2262
AD	0.2117	0.2130	0.2799	0.1827	0.1752	0.2335	0.1758	0.2056	0.2331	0.2860	0.2948	0.2282
AE	0.2769	0.2791	0.3043	0.2354	0.2390	0.2736	0.2623	0.2597	0.2838	0.3464	0.3437	0.2638
AF	0.0391	0.0925	0.0238	0.0514	0.0676	0.0428	0.0099	0.0581	0.1503	0.0999	0.0319	0.0318
AG	0.1902	0.2125	0.2270	0.1614	0.1906	0.2167	0.1804	0.2000	0.2115	0.2474	0.2628	0.2095
AH	0.0833	0.0794	0.0743	0.0716	0.0972	0.0311	0.0351	0.0267	0.0401	0.1149	0.0592	0.0634
AI	0.0399	0.1029	0.0249	0.0582	0.1002	0.0406	0.0503	0.0518	0.1382	0.0680	0.0293	0.0232
AJ	0.2066	0.1910	0.2352	0.1657	0.1710	0.1959	0.1341	0.1823	0.2233	0.2821	0.1993	0.1707

Appendix 7.3: F_{ST} values among population pairs (continued)

POP	M	N	O	P	Q	R	S	T	U	V	W	X
N	0.0362											
O	0.0576	0.0898										
P	0.0281	0.0257	0.0427									
Q	0.0458	0.0642	0.0296	0.0309								
R	0.0528	0.0349	0.0521	0.0130	0.0310							
S	0.0480	0.0584	0.0385	0.0260	0.0583	0.0298						
T	0.0821	0.0579	0.0773	0.0212	0.0731	0.0332	0.0285					
U	0.0755	0.0728	0.0946	0.0491	0.1139	0.0697	0.0503	0.0599				
V	0.0613	0.0680	0.0459	0.0201	0.0394	0.0410	0.0741	0.0616	0.0950			
W	0.0622	0.0974	0.0335	0.0555	0.0163	0.0628	0.0827	0.1016	0.1261	0.0318		
X	0.0842	0.0879	0.1471	0.0768	0.1106	0.1085	0.1049	0.0888	0.1440	0.1551	0.1463	
Y	0.0113	0.0011	0.1004	0.0267	0.0577	0.0090	0.0751	0.0340	0.0668	0.0224	0.0616	0.1330
Z	0.0677	0.0870	0.0423	0.0640	0.0299	0.0519	0.0747	0.0695	0.1487	0.0809	0.0421	0.1257
AB	0.0591	0.0501	0.0901	0.0325	0.0458	0.0340	0.0663	0.0247	0.1167	0.0515	0.0726	0.0626
AC	0.2089	0.1697	0.3094	0.2183	0.2638	0.2182	0.2707	0.2550	0.2767	0.2499	0.2916	0.1085
AD	0.2118	0.1707	0.3157	0.2187	0.2659	0.2174	0.2720	0.2622	0.2590	0.2467	0.2955	0.0776
AE	0.2423	0.2251	0.3330	0.2803	0.3106	0.2384	0.3009	0.2972	0.3441	0.3314	0.3274	0.1775
AF	0.0131	0.0681	0.0469	0.0571	0.0438	0.0626	0.0769	0.1100	0.0966	- 0.0116	0.0188	0.1090
AG	0.1964	0.1554	0.2586	0.1856	0.2243	0.1928	0.2339	0.2383	0.2500	0.2361	0.2543	0.0417
AH	0.0154	0.0615	0.0865	0.0610	0.0801	0.0682	0.0623	0.0834	0.1062	0.1212	0.1066	0.1145
AI	0.0320	0.0745	0.0375	0.0438	- 0.0035	0.0474	0.0740	0.0803	0.0983	0.0377	0.0226	0.1085
AJ	0.1460	0.1205	0.2924	0.1945	0.2426	0.2090	0.2598	0.2518	0.2682	0.2383	0.2661	0.0839

Appendix 7.3: F_{ST} values among population pairs (continued)

POP	Y	Z	AB	AC	AD	AE	AF	AG	AH	AI	AJ
Z	0.0693										
AB	0.0426	0.0296									
AC	0.1809	0.2871	0.2058								
AD	0.1802	0.2969	0.2000	-0.0102							
AE	0.2628	0.2960	0.2416	0.0444	0.0532						
AF	0.0085	0.0953	0.0888	0.2332	0.2346	0.3156					
AG	0.1968	0.2399	0.1775	0.1094	0.0585	0.1607	0.2285				
AH	0.0633	0.0928	0.0895	0.2708	0.2825	0.2926	0.0883	0.2441			
AI	0.0127	0.0526	0.0705	0.2480	0.2620	0.2964	0.0020	0.2336	0.0668		
AJ	0.1874	0.2746	0.2008	0.1184	0.0990	0.2293	0.1995	0.1766	0.2187	0.2090	

Appendix 7.4: Geographic distances among population pairs

POP	A	B	C	D	E	F	G	H	I	J	K	L
A												
B	83.3											
C	103.0	179.0										
D	48.1	36.5	143.0									
E	32.9	50.5	131.0	13.3								
F	38.2	122.0	69.0	87.2	73.7							
G	16.7	88.2	109.0	53.7	42.0	40.4						
H	100.0	182.0	27.4	146.0	133.0	63.1	103.9					
I	34.0	51.8	132.0	16.4	3.2	71.3	38.3	135.0				
J	84.5	10.6	175.0	37.7	50.6	122.0	91.3	182.0	91.9			
K	300.0	379.0	202.0	344.0	332.0	263.0	304.0	199.0	302.0	377.0		
L	302.0	382.0	204.0	347.0	335.0	266.0	306.0	203.0	305.0	380.0	4.9	
M	121.0	196.0	20.6	161.0	150.0	88.8	128.0	39.6	129.0	192.0	184.0	187.0
N	36.8	122.0	72.8	86.6	72.7	4.6	36.8	66.8	36.3	122.0	267.0	269.0
O	99.5	176.0	4.1	141.0	130.0	66.9	107.0	23.4	106.0	173.0	203.0	206.0
P	50.5	33.9	146.0	2.3	15.7	88.1	56.5	149.0	57.0	33.9	347.0	351.0
Q	96.3	204.0	4.7	164.0	126.0	63.8	103.0	25.0	102.0	171.0	206.0	209.0
R	40.4	125.0	65.9	86.7	75.3	3.1	42.9	60.3	42.3	125.0	260.0	263.0
S	34.7	120.0	70.6	80.7	69.9	4.4	38.0	66.5	37.6	119.0	266.0	269.0
T	40.4	45.1	133.0	7.8	6.9	77.1	48.6	139.0	47.8	44.0	336.0	338.0
U	85.7	9.8	175.0	38.9	50.3	123.0	91.4	182.0	91.3	0.5	376.0	380.0
V	171.0	252.0	73.3	214.0	245.0	135.0	174.0	70.8	176.0	249.0	130.0	132.0
W	173.0	250.0	71.3	213.0	178.0	137.0	177.0	76.8	177.0	245.0	132.0	136.0
X	1115.0	1152.0	1137.0	1137.0	1099.0	1105.0	1099.0	1111.0	1100.0	1160.0	1150.0	1146.0
Y	34.4	120.0	68.9	82.8	36.5	3.2	38.1	65.0	37.4	120.0	266.0	269.0
Z	131.0	47.5	225.0	85.9	132.0	169.0	133.0	230.0	133.0	53.1	428.0	431.0
AB	122.0	121.0	213.0	117.0	110.0	144.0	109.0	201.0	110.0	131.0	395.0	397.0
AC	978.0	1011.0	1004.0	998.0	962.0	968.0	962.0	977.0	961.0	1021.0	1030.0	1026.0
AD	503.0	528.0	547.0	518.0	486.0	501.0	487.0	523.0	486.0	537.0	630.0	629.0
AE	868.0	897.0	904.0	886.0	852.0	861.0	853.0	877.0	853.0	905.0	948.0	946.0
AF	169.0	248.0	68.9	210.0	174.0	134.0	174.0	71.3	173.0	244.0	132.0	136.0
AG	498.0	523.0	545.0	513.0	481.0	497.0	483.0	519.0	484.0	533.0	629.0	626.0
AH	98.7	176.0	4.2	138.0	106.0	66.6	106.0	26.1	105.0	172.0	204.0	207.0
AI	197.0	275.0	96.1	236.0	200.0	160.0	201.0	96.5	199.0	271.0	105.0	109.0
AJ	1198.0	1232.0	1224.0	1220.0	1096.0	1189.0	1183.0	1196.0	1184.0	1242.0	1239.0	1235.0

Appendix 7.4: Geographic distances among population pairs (continued)

POP	M	N	O	P	Q	R	S	T	U	V	W	X
N	94.8											
O	22.6	70.3										
P	164.0	88.0	145.0									
Q	25.8	68.5	4.1	140.0								
R	86.6	7.8	63.0	91.1	61.3							
S	90.6	6.6	67.6	85.0	65.2	5.5						
T	152.0	77.1	132.0	10.7	129.0	80.0	74.1					
U	192.0	122.0	175.0	34.7	171.0	125.0	119.0	45.0				
V	59.5	139.0	74.3	219.0	77.3	131.0	136.0	206.0	249.0			
W	52.4	142.0	71.6	215.0	75.3	134.0	139.0	204.0	245.0	21.5		
X	1147.0	1101.0	1133.0	1139.0	1133.0	1106.0	1107.0	1137.0	1161.0	1127.0	1148.0	
Y	91.7	10.0	68.3	84.6	65.8	5.8	0.5	74.5	119.0	136.0	140.0	1107.0
Z	244.0	167.0	226.0	81.9	222.0	172.0	165.0	93.5	53.5	299.0	297.0	1157.0
AB	234.0	139.0	210.0	118.0	208.0	148.0	144.0	119.0	132.0	271.0	279.0	1031.0
AC	1015.0	964.0	1000.0	1000.0	1001.0	970.0	971.0	997.0	1021.0	997.0	1019.0	142.0
AD	563.0	496.0	543.0	519.0	543.0	502.0	502.0	518.0	538.0	560.0	579.0	628.0
AE	916.0	858.0	900.0	888.0	900.0	865.0	866.0	886.0	906.0	904.0	925.0	272.0
AF	54.2	137.0	69.6	214.0	74.4	129.0	134.0	203.0	244.0	8.9	12.7	1133.0
AG	559.0	493.0	540.0	514.0	540.0	499.0	499.0	515.0	533.0	558.0	577.0	634.0
AH	22.6	71.2	3.3	143.0	2.0	63.8	67.8	132.0	172.0	75.1	73.9	1135.0
AI	80.9	163.0	96.9	241.0	99.7	156.0	161.0	231.0	271.0	25.4	27.5	1136.0
AJ	1233.0	1184.0	1219.0	1222.0	1219.0	1190.0	1192.0	1219.0	1243.0	1213.0	1234.0	90.0

Appendix 7.4: Geographic distances among population pairs (continued)

POP	Y	Z	AB	AC	AD	AE	AF	AG	AH	AI	AJ
Z	165.0										
AB	144.0	127.0									
AC	971.0	1015.0	892.0								
AD	502.0	528.0	406.0	487.0							
AE	866.0	897.0	777.0	133.0	369.0						
AF	134.0	295.0	272.0	1006.0	565.0	911.0					
AG	499.0	523.0	403.0	493.0	6.1	372.0	564.0				
AH	67.8	224.0	211.0	1002.0	545.0	900.0	71.9	542.0			
AI	161.0	322.0	296.0	1010.0	578.0	918.0	26.0	576.0	98.5		
AJ	1192.0	1236.0	1112.0	220.0	708.0	345.0	1221.0	712.0	1220.0	1225.0	

Appendix 7.5: Altitudinal distances among sites

POP	A	B	C	D	E	F	G	H	I	J	K	L
A												
B	150.0											
C	200.0	50.0										
D	700.0	550.0	500.0									
E	500.0	350.0	300.0	200.0								
F	400.0	250.0	200.0	300.0	100.0							
G	150.0	300.0	350.0	850.0	650.0	550.0						
H	20.0	150.0	200.0	700.0	500.0	400.0	150.0					
I	1100.0	950.0	900.0	400.0	600.0	700.0	1250.0	1100.0				
J	150.0	20.0	50.0	550.0	350.0	250.0	300.0	150.0	950.0			
K	400.0	250.0	200.0	300.0	100.0	20.0	550.0	400.0	700.0	250.0		
L	625.0	475.0	425.0	75.0	125.0	225.0	775.0	625.0	475.0	475.0	225.0	
M	20.0	150.0	200.0	700.0	500.0	400.0	150.0	20.0	1100.0	150.0	400.0	625.0
N	400.0	250.0	200.0	300.0	100.0	20.0	550.0	400.0	700.0	250.0	20.0	225.0
O	118.0	268.0	318.0	818.0	618.0	518.0	32.0	118.0	1218.0	268.0	518.0	743.0
P	304.0	154.0	104.0	396.0	196.0	96.0	454.0	304.0	769.0	154.0	96.0	321.0
Q	118.0	268.0	318.0	818.0	618.0	518.0	32.0	118.0	1218.0	268.0	518.0	743.0
R	200.0	50.0	20.0	500.0	300.0	200.0	350.0	200.0	900.0	50.0	200.0	425.0
S	350.0	200.0	150.0	350.0	150.0	50.0	500.0	350.0	750.0	200.0	50.0	275.0
T	389.0	239.0	189.0	311.0	111.0	11.0	539.0	389.0	711.0	239.0	11.0	236.0
U	131.0	19.0	69.0	569.0	369.0	269.0	281.0	131.0	969.0	19.0	269.0	494.0
V	600.0	450.0	400.0	100.0	100.0	200.0	750.0	600.0	500.0	450.0	200.0	25.0
W	900.0	750.0	700.0	200.0	400.0	500.0	1050.0	900.0	200.0	750.0	500.0	275.0
X	1850.0	1700.0	1650.0	1150.0	1350.0	1450.0	2000.0	1850.0	750.0	1700.0	1450.0	1225.0
Y	350.0	200.0	150.0	350.0	150.0	50.0	500.0	350.0	750.0	200.0	50.0	275.0
Z	1370.0	1220.0	1170.0	670.0	870.0	970.0	1520.0	1370.0	270.0	1220.0	970.0	745.0
AB	389.0	239.0	189.0	311.0	111.0	11.0	539.0	389.0	711.0	239.0	11.0	236.0
AC	1900.0	1750.0	1700.0	1200.0	1400.0	1500.0	2050.0	1900.0	800.0	1750.0	1500.0	1275.0
AD	850.0	700.0	650.0	150.0	350.0	450.0	1000.0	850.0	250.0	700.0	450.0	225.0
AE	1734.0	1584.0	1534.0	1034.0	1234.0	1334.0	1884.0	1734.0	634.0	1584.0	1334.0	1109.0
AF	100.0	50.0	100.0	600.0	400.0	300.0	250.0	100.0	1000.0	50.0	300.0	525.0
AG	950.0	800.0	750.0	250.0	450.0	550.0	1100.0	950.0	150.0	800.0	550.0	325.0
AH	118.0	268.0	318.0	818.0	618.0	518.0	32.0	118.0	1218.0	268.0	518.0	743.0
AI	1179.0	1029.0	979.0	479.0	679.0	779.0	1329.0	1179.0	79.0	1029.0	779.0	1054.0
AJ	2100.0	1950.0	1900.0	1400.0	1600.0	1700.0	2250.0	2100.0	1000.0	1950.0	1700.0	1475.0

Appendix 7.5: Altitudinal distances among sites (continued)

POP	M	N	O	P	Q	R	S	T	U	V	W	X
N	400.0											
O	118.0	518.0										
P	304.0	96.0	422.0									
Q	118.0	518.0	20.0	422.0								
R	200.0	200.0	318.0	104.0	318.0							
S	350.0	50.0	46.0	46.0	468.0	150.0						
T	389.0	11.0	507.0	85.0	507.0	189.0	39.0					
U	131.0	269.0	249.0	173.0	249.0	69.0	219.0	258.0				
V	600.0	200.0	718.0	296.0	718.0	400.0	250.0	211.0	469.0			
W	900.0	500.0	1018.0	596.0	1018.0	700.0	550.0	511.0	769.0	300.0		
X	1850.0	1450.0	1968.0	1546.0	1968.0	1650.0	1500.0	1461.0	1719.0	1250.0	850.0	
Y	350.0	50.0	468.0	46.0	468.0	150.0	20.0	39.0	219.0	250.0	650.0	1500.0
Z	1370.0	970.0	1488.0	1066.0	1488.0	1170.0	1020.0	981.0	1239.0	770.0	370.0	480.0
AB	389.0	11.0	507.0	85.0	507.0	189.0	39.0	20.0	258.0	211.0	611.0	1461.0
AC	1900.0	1500.0	2018.0	1596.0	2018.0	1700.0	1550.0	1511.0	1769.0	1300.0	900.0	50.0
AD	850.0	450.0	968.0	546.0	968.0	650.0	500.0	461.0	819.0	250.0	150.0	1000.0
AE	1734.0	1334.0	1852.0	1430.0	1852.0	1534.0	1384.0	1345.0	1603.0	1134.0	734.0	116.0
AF	100.0	300.0	218.0	204.0	218.0	100.0	250.0	289.0	31.0	500.0	900.0	1750.0
AG	950.0	550.0	1068.0	646.0	1068.0	750.0	600.0	561.0	819.0	350.0	50.0	900.0
AH	118.0	518.0	20.0	422.0	20.0	318.0	468.0	507.0	249.0	718.0	1118.0	1968.0
AI	1179.0	779.0	1297.0	875.0	1297.0	979.0	829.0	790.0	1048.0	579.0	179.0	671.0
AJ	2100.0	1700.0	2218.0	1796.0	2218.0	1900.0	1750.0	1711.0	1969.0	1500.0	1100.0	250.0

Appendix 7.5: Altitudinal distances among sites (continued)

POP	Y	Z	AB	AC	AD	AE	AF	AG	AH	AI	AJ
Z	1020.0										
AB	39.0	981.0									
AC	1550.0	530.0	1511.0								
AD	500.0	520.0	461.0	1000.0							
AE	1384.0	364.0	1345.0	166.0	884.0						
AF	250.0	1270.0	289.0	1800.0	750.0	1634.0					
AG	600.0	420.0	561.0	950.0	100.0	784.0	750.0				
AH	468.0	1488.0	507.0	2018.0	968.0	1852.0	318.0	1068.0			
AI	829.0	190.0	790.0	721.0	329.0	555.0	1079.0	229.0	1297.0		
AJ	1750.0	730.0	1711.0	200.0	1250.0	366.0	1900.0	1150.0	1218.0	1900.0	

Appendix 7.6: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

POP	A	B	C	D	E	F	G	H	I	J	K	L
A	-	0.7798	0.8153	0.8921	0.7804	0.9183	0.9122	0.8961	0.6193	0.8805	0.7378	0.8356
B	0.2487	-	0.6351	0.8997	0.8243	0.8194	0.8631	0.8839	0.7450	0.8422	0.7651	0.8138
C	0.2042	0.4540	-	0.7689	0.6811	0.8423	0.7598	0.7422	0.5732	0.7366	0.8119	0.8407
D	0.1142	0.1057	0.2628	-	0.8774	0.8436	0.9055	0.8696	0.6693	0.8939	0.8131	0.8920
E	0.2480	0.1932	0.3840	0.1307	-	0.7832	0.8743	0.7884	0.6207	0.7839	0.6146	0.7508
F	0.0852	0.1992	0.1716	0.1701	0.2444	-	0.9012	0.9605	0.7547	0.8602	0.7935	0.8425
G	0.0919	0.1472	0.2747	0.0993	0.1343	0.1041	-	0.9523	0.7214	0.8096	0.8073	0.8597
H	0.1097	0.1234	0.2982	0.1397	0.2378	0.0403	0.0488	-	0.7781	0.8446	0.8090	0.8728
I	0.4792	0.2944	0.5566	0.4015	0.4770	0.2814	0.3266	0.2509	-	0.5945	0.5724	0.6208
J	0.1273	0.1718	0.3058	0.1122	0.2435	0.1506	0.2112	0.1688	0.5201	-	0.7629	0.8115
K	0.3040	0.2678	0.2084	0.2069	0.4867	0.2313	0.2140	0.2120	0.5579	0.2706	-	0.9174
L	0.1796	0.2060	0.1735	0.1143	0.2866	0.1714	0.1511	0.1360	0.4768	0.2089	0.7629	-
M	0.1969	0.2611	0.1357	0.2072	0.3019	0.0957	0.0828	0.0968	0.2682	0.3490	0.1502	0.1386
N	0.2178	0.1942	0.2766	0.1412	0.1778	0.2010	0.1661	0.1983	0.2209	0.2363	0.3013	0.2343
O	0.1943	0.3786	0.0609	0.2315	0.4063	0.1522	0.2832	0.2622	0.6254	0.2342	0.1704	0.1570
P	0.1121	0.1727	0.2574	0.0801	0.1740	0.1133	0.1934	0.1508	0.3918	0.0752	0.3170	0.1758
Q	0.1621	0.3684	0.0995	0.1958	0.4299	0.1923	0.2607	0.2282	0.5722	0.2039	0.1429	0.1081
R	0.1413	0.2428	0.2228	0.1501	0.2314	0.1175	0.2118	0.1571	0.2753	0.1400	0.2705	0.1612
S	0.1414	0.1452	0.2278	0.1449	0.2524	0.0729	0.1502	0.0976	0.3691	0.1326	0.2306	0.1635
T	0.1880	0.1714	0.3540	0.1281	0.1802	0.1425	0.1817	0.1544	0.3965	0.0676	0.2542	0.1867
U	0.2396	0.1771	0.4542	0.2358	0.3626	0.1638	0.2712	0.1800	0.4828	0.1738	0.4112	0.3479
V	0.1696	0.4284	0.2063	0.2043	0.2817	0.2221	0.2241	0.3125	0.8846	0.1920	0.2199	0.2208
W	0.1979	0.4785	0.0770	0.2570	0.4187	0.1824	0.2665	0.3007	0.6860	0.2593	0.1736	0.1829
X	0.6863	0.6800	0.6272	0.6185	0.9758	0.7250	0.7700	0.7747	0.9613	0.8410	0.6182	0.5833
Y	0.2047	0.4687	0.3611	0.3259	0.2696	0.1704	0.1883	0.2505	0.3381	0.2904	0.3684	0.3560
Z	0.2633	0.4357	0.1658	0.2512	0.4286	0.2384	0.2971	0.3184	0.6269	0.2710	0.1205	0.1834
AB	0.2640	0.2818	0.3797	0.1720	0.2501	0.2543	0.2530	0.2271	0.5870	0.1997	0.2163	0.1907
AC	1.4039	1.7050	2.1208	1.4193	1.2899	1.7991	1.3493	1.6414	1.7357	1.9135	1.9558	1.6573
AD	1.6240	1.8891	2.3990	1.6217	1.3382	2.0233	1.5407	1.7330	1.8687	2.2320	2.7672	1.9803
AE	1.6955	1.8884	1.7422	1.6389	1.3973	1.6919	1.6752	1.6301	1.4829	2.0091	1.7816	1.4917
AF	0.2554	0.5013	0.1476	0.3095	0.3784	0.2458	0.2207	0.3419	0.8144	0.4238	0.2354	0.2292
AG	1.0277	1.4501	1.1490	0.9566	1.3096	1.4394	1.2444	1.4195	1.2215	1.3049	1.4520	1.2691
AH	0.3047	0.2974	0.2235	0.2898	0.3901	0.1160	0.1712	0.1307	0.1846	0.3735	0.2181	0.2321
AI	0.1739	0.4160	0.1020	0.2404	0.4243	0.1607	0.2411	0.2202	0.5650	0.2569	0.1662	0.1219
AJ	1.7753	1.9732	1.2778	1.6706	1.6636	1.5187	1.3963	1.5648	1.8700	2.2969	1.1105	1.2956

Appendix 7.6: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) (continued).

POP	M	N	O	P	Q	R	S	T	U	V	W	X
A	0.8213	0.8043	0.8234	0.8940	0.8503	0.8682	0.8681	0.8286	0.7869	0.8440	0.8205	0.5035
B	0.7702	0.8235	0.6848	0.8414	0.6918	0.7844	0.8649	0.8425	0.8377	0.6515	0.6197	0.5066
C	0.8731	0.7583	0.9409	0.7731	0.9053	0.8003	0.7963	0.7019	0.6350	0.8136	0.9259	0.5341
D	0.8129	0.8683	0.7933	0.9230	0.8222	0.8606	0.8651	0.8798	0.7899	0.8152	0.7733	0.5388
E	0.7394	0.8371	0.6661	0.8403	0.6506	0.7934	0.7769	0.8351	0.6959	0.7545	0.6579	0.3769
F	0.9088	0.8179	0.8588	0.8929	0.8250	0.8891	0.9297	0.8672	0.8489	0.8008	0.8332	0.4843
G	0.9206	0.8470	0.7534	0.8241	0.7705	0.8091	0.8606	0.8338	0.7624	0.7992	0.7660	0.4630
H	0.9077	0.8201	0.7693	0.8600	0.7959	0.8546	0.9070	0.8569	0.8353	0.7316	0.7403	0.4609
I	0.7648	0.8018	0.5351	0.6758	0.5643	0.7593	0.6913	0.6727	0.6171	0.4129	0.5036	0.3824
J	0.7054	0.7896	0.7912	0.9276	0.8156	0.8694	0.8758	0.9347	0.8404	0.8253	0.7716	0.4313
K	0.8605	0.7399	0.8433	0.7284	0.8668	0.7630	0.7940	0.7755	0.6629	0.8026	0.8406	0.5389
L	0.8706	0.7911	0.8547	0.8388	0.8975	0.8511	0.8492	0.8297	0.7062	0.8019	0.8328	0.5580
M	-	0.8360	0.8388	0.7691	0.8243	0.8052	0.8096	0.7370	0.7180	0.7487	0.8400	0.5069
N	0.1791	-	0.7330	0.8215	0.7558	0.8554	0.7961	0.8085	0.7180	0.7116	0.7229	0.4527
O	0.1758	0.3106	-	0.8136	0.8968	0.8369	0.8700	0.7844	0.7730	0.8663	0.9161	0.5357
P	0.2625	0.1967	0.2063	-	0.8246	0.8978	0.8814	0.8960	0.7920	0.8275	0.7831	0.4868
Q	0.1932	0.2800	0.1089	0.1928	-	0.8776	0.8141	0.7873	0.6769	0.8373	0.9274	0.5801
R	0.2166	0.1562	0.1781	0.1078	0.1306	-	0.8912	0.8924	0.7622	0.8179	0.8298	0.5018
S	0.2113	0.2280	0.1393	0.1262	0.2057	0.1152	-	0.9077	0.8379	0.7695	0.7796	0.5293
T	0.3052	0.2126	0.2428	0.1098	0.2392	0.1138	0.0968	-	0.8227	0.7852	0.7493	0.4870
U	0.3313	0.3293	0.2575	0.2332	0.3902	0.2715	0.1769	0.1952	-	0.7033	0.6853	0.3988
V	0.2894	0.3402	0.1435	0.1893	0.1776	0.2010	0.2620	0.2418	0.3519	-	0.8791	0.3675
W	0.1743	0.3245	0.0876	0.2445	0.0754	0.1866	0.2490	0.2886	0.3779	0.1289	-	0.5121
X	0.6794	0.7926	0.6242	0.7198	0.5445	0.6896	0.6363	0.7195	0.9192	1.0010	0.6692	-
POP	M	N	O	P	Q	R	S	T	U	V	W	X
Y	0.2054	0.2093	0.3244	0.3114	0.2949	0.1726	0.3495	0.2236	0.3195	0.2439	0.2452	1.0633
Z	0.2128	0.3024	0.1077	0.2630	0.1103	0.1716	0.2244	0.2119	0.4317	0.2303	0.1053	0.5731
AB	0.3107	0.2469	0.2744	0.1726	0.1924	0.1437	0.2123	0.1026	0.3993	0.2218	0.2533	0.5139
AC	1.6151	1.3124	2.3086	1.6510	1.8247	1.4558	2.0393	1.6862	2.3184	1.8343	1.9075	0.6388
AD	1.9044	1.5510	2.4338	1.8509	1.9650	1.6168	2.2208	1.9626	1.8941	1.9560	2.0952	0.6425
AE	1.4730	1.4980	1.6511	1.6894	1.6809	1.1880	1.6096	1.6340	2.4868	2.0447	1.8370	0.6465
AF	0.1580	0.4187	0.1710	0.4148	0.2212	0.3167	0.3345	0.4399	0.4184	0.1334	0.1135	0.9021
AG	1.3047	0.9999	1.3281	1.1231	1.1130	1.0733	1.2783	1.4071	1.6187	1.5335	1.2489	0.5035
AH	0.0696	0.2448	0.2184	0.2762	0.2627	0.2356	0.2005	0.2643	0.3467	0.4093	0.2786	0.6760
AI	0.1275	0.3213	0.1169	0.2663	0.0551	0.1821	0.2645	0.2710	0.3494	0.1841	0.0777	0.6048
AJ	1.1163	1.1964	1.2787	1.7725	1.3291	1.6911	1.8860	1.9549	1.7291	1.5855	1.2733	0.9498

Appendix 7.6: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

POP	Y	Z	AB	AC	AD	AE	AF	AG	AH	AI	AJ
A	0.8149	0.7685	0.7680	0.2456	0.1971	0.1835	0.7746	0.3578	0.7374	0.8404	0.1694
B	0.6258	0.6468	0.7544	0.1818	0.1512	0.1513	0.6058	0.2345	0.7428	0.6597	0.1390
C	0.6969	0.8472	0.6840	0.1199	0.0908	0.1751	0.8628	0.3169	0.7997	0.9031	0.2786
D	0.7219	0.7779	0.8420	0.2419	0.1976	0.1942	0.7338	0.3842	0.7484	0.7863	0.1881
E	0.7637	0.6514	0.7787	0.2753	0.2623	0.2473	0.6849	0.2699	0.6770	0.6542	0.1895
F	0.8434	0.7879	0.7754	0.1654	0.1322	0.1842	0.7821	0.2371	0.8905	0.8516	0.2190
G	0.8284	0.7430	0.7764	0.2594	0.2142	0.1873	0.8019	0.2881	0.8426	0.7858	0.2475
H	0.7784	0.7273	0.7968	0.1937	0.1768	0.1959	0.7104	0.2418	0.8775	0.8023	0.2091
I	0.7131	0.5343	0.5560	0.1763	0.1543	0.2270	0.4429	0.2948	0.8315	0.5684	0.1541
J	0.7480	0.7627	0.8190	0.1476	0.1073	0.1341	0.6546	0.2712	0.6883	0.7734	0.1006
K	0.6918	0.8865	0.8055	0.1415	0.0628	0.1684	0.7903	0.2341	0.8040	0.8469	0.3294
L	0.7005	0.8324	0.8264	0.1906	0.1380	0.2250	0.7951	0.2811	0.7928	0.8852	0.2737
M	0.8143	0.8083	0.7329	0.1989	0.1489	0.2292	0.8539	0.2713	0.9327	0.8803	0.3275
N	0.8111	0.7390	0.7812	0.2692	0.2120	0.2236	0.6579	0.3679	0.7829	0.7252	0.3023
O	0.7230	0.8979	0.7601	0.0994	0.0877	0.1918	0.8428	0.2650	0.8038	0.8897	0.2784
P	0.7324	0.7688	0.8415	0.1919	0.1571	0.1846	0.6604	0.3253	0.7586	0.7662	0.1699
Q	0.7446	0.8956	0.8250	0.1613	0.1401	0.1862	0.8016	0.3286	0.7689	0.9464	0.2647
R	0.8415	0.8423	0.8661	0.2332	0.1985	0.3048	0.7285	0.3419	0.7901	0.8335	0.1843
S	0.7051	0.7990	0.8087	0.1301	0.1085	0.2000	0.7157	0.2785	0.8183	0.7676	0.1517
T	0.7996	0.8091	0.9025	0.1852	0.1405	0.1951	0.6441	0.2448	0.7677	0.7626	0.1416
U	0.7265	0.6494	0.6708	0.0984	0.1504	0.0832	0.6581	0.1982	0.7070	0.7051	0.1774
V	0.7836	0.7943	0.8011	0.1597	0.1414	0.1294	0.8752	0.2158	0.6641	0.8318	0.2048
W	0.7825	0.9000	0.7763	0.1485	0.1230	0.1593	0.8927	0.2868	0.7568	0.9252	0.2799
X	0.3453	0.5638	0.5981	0.5279	0.5260	0.5239	0.4057	0.6044	0.5087	0.5462	0.3868
Y	-	0.7620	0.7376	0.2895	0.2575	0.2658	0.7801	0.2476	0.7641	0.8368	0.2473
Z	0.2718	-	0.8822	0.1708	0.1344	0.2906	0.7365	0.3315	0.7788	0.8576	0.2850
AB	0.3043	0.1253	-	0.2677	0.2715	0.3220	0.6332	0.3537	0.7265	0.7514	0.2564
AC	1.2397	1.7671	1.3179	-	0.8455	0.8668	0.1728	0.6205	0.1175	0.2015	0.4320
AD	1.3567	2.0071	1.3039	0.1678	-	0.8264	0.1420	0.7203	0.0657	0.1200	0.4379
AE	1.3250	1.2358	1.1333	0.1430	0.1906	-	0.1453	0.6047	0.2078	0.2044	0.4041
AF	0.2484	0.3058	0.4570	1.7556	1.9517	1.9287	-	0.2339	0.7052	0.8894	0.2372
AG	1.3961	1.1040	1.0393	0.4773	0.3281	0.5030	1.4529	-	0.2280	0.2684	0.3097
AH	0.2691	0.2500	0.3195	2.1412	2.7228	1.5711	0.3492	1.4782	-	0.8134	0.3171
AI	0.1782	0.1536	0.2858	1.6021	2.1204	1.5877	0.1172	1.3152	0.2065	-	0.2999
AJ	1.3973	1.2553	1.3610	0.8393	0.8257	0.9062	1.4386	1.1721	1.1484	1.2044	-

8. General Discussion

The study of the population structure of *Reissita simonyi* (REBEL, 1899; Lepidoptera, Zygaenidae) and *Hyla savignyi* (AUDOUIN, 1827; Amphibia, Hylidae) in Southern Arabia demonstrated to be quite demanding. Despite the scarcity of literature available dealing with the two species and/ or with the geographical region (NAUMANN & EDELMANN, 1984; SCHÄTTI & GASPERETTI, 1994), long term climatic data as well as population genetic reference studies for this particular geographical region were lacking. For this reason, this work should also be understood as a basis for further studies in this still hardly accessible area of the world. Therefore, a special focus was put on a faunistic, methodological and finally a population genetic approach for each species in order to provide a detailed picture for following studies.

The study area proved to be particularly exciting, because it provided a variety of features making it especially interesting for biologists (chapter 1/ General introduction). In spite of the fact that this geographical area has a high degree of endemism and represents a melting-pot of Afrotropical and Palaearctic species, the *status quo* of research was mainly based on faunistic studies. In fact, regarding the fast human population growth and increasing agricultural land use, further population genetic and conservation genetic studies in addition to the presented ones here would be highly desirable in order to examine the influence of habitat fragmentation and loss on a variety of systems. Based on the conducted investigations, it became apparent that diverse systems are differently affected by habitat fragmentation, habitat loss and additional forces like isolation by distance and isolation by altitude (chapter 6 and 7). Population genetic and conservation genetic approaches may also build the basis for extended nature conservation projects with the intention to protect the unique natural heritage of Southern Arabia. In connection to this aspect, it would be highly desirable to extent teaching in biology at the universities and in schools in Yemen, *e. g.* teaching ecology and population genetics has proven to contribute much to the acceptance of conservation acts.

In the present study, I inferred the population genetic structure of two faunal elements, *Reissita simonyi* and *Hyla savignyi,* in Southern Arabia. I selected microsatellites as a

primary source of population genetic information, because these turned out to be ideal markers for the specific questions tackled in population genetic studies (OLIVEIRA *et al.*, 1998; MOSSMAN & WASER, 2001; SCHREY & HEIST, 2003; MARQUADT & EPPERSON, 2004; GOMEZ-UCHIDA & BANKS, 2005; PATT, 2005). However, in the present work, microsatellites were not only applied to investigate the population structure, possible migration routes and the impact of a variety of forces like isolation by distance and isolation by altitude, but the marker system itself was critically examined and discussed.

In spite of their obvious advantages, putative functions and the evolution of microsatellites are rarely touched in the literature (LI *et al.*, 2002). The careful verification of potential cross-utility and size homoplasy of cross-amplified microsatellites is often still lacking (but see ESTOUP *et al.*, 1995; AMOS & RUBINSZTEIN, 1996; AMOS, 1999; MAKOVA *et al.*, 2000). Therefore, a special focus was put on the intensive treatment of this marker system, beginning with the isolation and optimization (chapter 4 and 5), followed by the discussion of cross-utility, size homoplasy and evolution of microsatellites within the genus *Hyla* (chapter 4) as well as the statistical analyses in chapter 6 and 7 including a discussion of potential pitfalls of the isolation and applicability of microsatellites in Lepidoptera. It became obvious that further analyses about the complex evolution of microsatellites are highly warranted. The potential influence of hidden polymorphism caused by undetected mutations in the core and flanking regions of microsatellites should be treated with caution and deserve more attention at a larger scale in order to examine the contribution of hidden polymorphism and refinement of today´s mutation models.

In addition, the widespread utility of microsatellites in species, for which the microsatellite set was not originally developed, should be applied with care, because size homoplasy can lead to erroneous results. Although some authors (ESTOUP *et al.*, 2002) suggested that the impact of size homoplasy may be marginal, it would be of extraordinary interest to conduct a study, in which the influence of size homoplasy on a system is studied at a large scale with the aim to get profound empirical data on this issue. A further aspect concerns the difficult isolation of microsatellites in Lepidoptera. Potential explanations are rare (MEGLÉCZ *et al.*, 2004; MEGLÉCZ *et al.*, submitted). It would be of great importance to explore potential reasons, why various isolation

protocols fail to yield a sufficient number of microsatellites in Lepidoptera. A second phenomenon refers to the apparent accumulation of null alleles in Lepidoptera (KEYGHOBADI, 1999; MEGLÉCZ *et al.*, 2004; chapter 7). It would be interesting to explore, why especially Lepidoptera exhibit null alleles so often and how to deal with them in population genetic analyses in general.

An extraordinary focus was put on the incorporation of new approaches in statistical analyses for population genetic analysis. In the population genetic analysis of *H. savignyi* (chapter 6), I applied a Bayesian assignment test (GENECLASS2, PIRY *et al.*, 2004) to examine regional population structure. For *R. simonyi*, I applied a very recently developed approach to investigate population structure and genetic variability. DYER & NASON (2004) developed a network analysis for population genetic analyses, which compares all data simultaneously instead in the traditional pairwise fashion (F_{ST}). The analysis has the advantage to project populations in multidimensional space, where all connections to other populations are illustrated. I think that both approaches present a step forward in the analysis of population genetic and conservation genetic studies, because both propose to compare all populations in a way that all probable connections and origins of populations are taken into account. Therefore, attention is drawn to the connectivity and differentiation of populations at the same time. In addition, isolated populations and highly variable populations are easily identified. This approach may represent an advance in conservation genetics, because it may enable scientists to identify most important populations to save populations and species. Conversely, populations which are endangered can be discovered and to put it in a very simple way, translocation of individuals from highly variable populations could rescue endangered populations. More generally, areas with low genetic connectivity are easily identified and conservation actions may concentrate on these areas.

In contrast, assignment tests present a breakthrough to study potential migration routes at the individual level. Thus, migration can be studied in more detail and in a direct manner. For example, the identification of real-time migrants or migration in past generations can be distinguished and therefore, changes in number of migrants can be discovered. In addition, the total amount of migration can be examined and again, isolated populations may be differentiated from populations which are essential to maintain migration. Finally, preferred directions of gene flow can be examined and may

give insight in the underlying factors of population structure of species (chapter 6) when contrasted with, for example, landscape topology features. In conclusion, both analyses permitted to draw a detailed picture of the population structure of *R. simonyi* and *H. savignyi* and potential underlying factors (chapter 6 and 7).

I intended to offer population genetic data for two presumable vulnerable species regarding habitat fragmentation. The analyses dealt with a vertebrate and an invertebrate, which differed in dispersion ability and habitat preferences. Therefore, I covered a broad spectrum of different characteristics of examined species. The molecular data revealed that *R. simonyi* is less affected by habitat fragmentation than *H. savignyi*. Two obvious reasons for the strong connectivity of populations of *R. simonyi* are the higher mobility of a flying insect in comparison to a tree frog species with high pond fidelity and low migration power. The second reason is the well known phenomenon of top-hopping in lepidopteran species. As a consequence, *R. simonyi* is less vulnerable to habitat fragmentation (at least at a small geographic scale) than *H. savignyi*. The lower vulnerability is due to higher dispersion ability and top-hopping behavior which enables this species to successfully disperse over longer geographic distances. The low dispersion ability of *H. savignyi* makes this species particular exposed to habitat fragmentation or loss, because the persistence of populations highly depends on low between-pond geographic distances. Further studies could concentrate on invertebrate species with presumable low migration abilities, like wingless insects in order to contrast the results found in *R. simonyi*. PATT (2005) contrasted the population genetic results of a winged and a wingless beetle species in order to study the population structure and the differential impact of habitat fragmentation on these two coleopteran species in Kenya. An extension at a larger geographical scale could be possible in Southern Arabia. This approach would be of particular interest in order to extent already collected data.

In both population genetic studies (chapter 6 and 7), an impact of isolation by altitude on population genetic differentiation was found. However, there was no significant correlation between altitude and neither expected or observed heterozygosity nor other parameters like allelic richness or inbreeding coefficient (chapter 6 and 7) found. Thus, altitudinal differences seemed to have no impact on the variability within populations and there were no hints for an ecological differentiation of populations. It would be of

particular interest to examine the influence of isolation by altitude on population differentiation as well as other landscape topology features like mountain ridges or rivers in more detail. This approach would be essential to complement our understanding of population differentiation and to build a bridge between population genetic markers and landscape characteristics (MANEL *et al.*, 2004). Again, wingless insects may be suitable model organisms. Specific transect analysis (STORZ & DUBACH, 2004; FUNK *et al*., 2005b) of wingless insects in order to study the influence of isolation by altitude in more detail would be of special interest in this context. Transect analysis could be applied to systematically investigate the effect of altitudinal difference on population genetic structure. In combination with the collection of climatic data like temperature, solar radiation, and humidity, along the same transects, a potential ecological gradient along an altitudinal gradient could be examined at the same time. A further potential step forward in the analysis of the already obtained data and potentially new one could be the inclusion of a GIS data approach (*e. g.* MICHELS *et al.*, 2001).

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