Phylogeny and phylogeography of the caddisfly *Rhyacophila pubescens*, PICTET 1834, (Trichoptera), with special consideration of its habitat specificity

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

des Fachbereichs

Biologie und Geografie

an der Universität Duisburg-Essen

vorgelegt von

Christine Engelhardt

aus München

Februar 2009

Tag der mündlichen Prüfung

22. Juni 2009

Prüfer:

Vorsitzender des Prüfungsausschusses: Prof. Dr. B. Sures
Prof. Dr. D. Hering
PD Dr. P. Haase
Prof. Dr. H. Burda

Die der vorliegende Arbeit zugrunde liegenden Experimente wurden in der Abteilung für Limnologie und Naturschutzforschung des Forschungsinstituts Senckenberg und in der Abteilung für Hydrobiologie der Universität Duisburg-Essen durchgeführt.

1. Gutachter:
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Vorsitzender des Prüfungsausschusses:

Tag der mündlichen Prüfung:

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Abbreviations

Asl above sea level

bp base pairs

DNA deoxyribonucleic acid

mt mitochondrial

mts mountains

nu nuclear

PCR Polymerase chain reaction

Introduction

General introduction

In biological research the field of phylogeography is relatively young. Phylogeography comprises many disciplines, like molecular genetics, population genetics, phylogenetic biology, geology and historical geography (Avise 2000). In the 1980ies mtDNA studies of natural populations showed that branches in intraspecific gene trees were linked to geographical distribution. The term phylogeography was created to describe relationships between gene genealogies and geography. Phylogeography is a subdiscipline of biogeography and extends the knowledge on the effects of contemporary natural forces shaping species distribution (Avise 2000), by acknowledging that population history also influences spatial distribution and plays an important role in the development of genetic differentiation. For example, processes like migration of a small number of individuals into new habitats can cause founder effects (Neal 2004) that result in a loss of genetic variation when compared to the original population. This allows spatial changes to be inferred from molecular data. Another situation occurs if populations become gradually isolated, e.g. when they are surrounded by an unsuitable habitat. This can affect gene flow with other populations and can lead to genetic signatures such as the accumulation of specific point mutations.

Recent advances in molecular techniques have contributed to a more precise understanding of how organisms´ spatial distributions changed, for example, due to major climatic changes such as the European ice ages. During glaciations -the last glacial maximum (LGM) was 18 000 to 22 000 years BP (Beebee & Rowe 2008)- temperate species survived in southern refugia located on the Iberian peninsula, Italy and the Balkans. Populations in the northern cool regions of Europe went extinct (Hewitt 1996). Many studies using molecular data have shown different recolonization routes to the periglacial area (see reviews Taberlet et al. 1998, Hewitt 1999, 2004, Schmitt 2007) with postglacial climate warming. One example is the scenario found in species such as the grasshopper *Chorthippus parallelus* or the newt *Triturus cristatus*, where the main route was from the Balkans northwards, while populations coming from the area of the Apennines were stopped by the Alps and populations from Iberia stopped by the Pyrenees (Hewitt 1999). Another scenario was found in other species such as the hedgehog *Erinaceus* sp. or the silver fir *Abies alba* that recolonized Central Europe from all three refugial areas (Hewitt 1999). It has since become evident that despite the existence of some common patterns, there are many differences in the effects of climatic changes

depending on each species´ life cycle, habitat specificity and dispersal capacity. Bottlenecks reducing genetic variation (Neal 2004) occurred during glaciation (Grivet & Petit 2003, Dubey et al. 2006) and may have acted differently on the population genetic structure of each species depending, for example, on the original population size or generation time. Geographical barriers such as unsuitable habitats or mountains with high altitudes and other barriers to gene flow also influenced species differently depending on their specific habitat requirements and ecological plasticity. Phylogeographic studies have highlighted patterns in plants and different animal groups, like birds, mammals and invertebrates.

Phylogeographic patterns in aquatic insects

Aquatic insects are particularly useful for phylogeographic studies since they are supposed to show pronounced genetic structure compared to terrestrial organisms (Avise 2000). This is due to the fact that their distribution range is not continuous but restricted to water bodies, that can be many kilometers apart. Often the dispersal between these habitats is restricted to or dominated by the adult life stage of aquatic insects. Adults are able to fly while the larval stages are not. Besides the general utility of aquatic species in phylogeographical studies, caddisflies (order Trichoptera) exhibit a variety of feeding types, ecological niche specificity and geographic distribution (e.g. lowland or highland species) (Mackay & Wiggins 1979). Caddisflies are globally distributed moth-like insects, that, together with their sister group Lepidoptera, make up the superorder Amphiesmenoptera (Kjer et al. 2002). Currently more than 12 000 extant species are described worldwide (Morse 2009). Trichoptera are divided in three suborders (Kjer et al. 2002), Annulipalpia, Integripalpia and Spicipalpia. The first three life stages - egg, larva, and pupa - are aquatic (except for two genera); only the mostly short-lived adult stage is terrestrial. Trichoptera larvae use silk to build shelters or capture nets. Annulipalpian larvae make fixed shelters and integripalpian larvae make portable tube cases from mineral or organic materials (Mackay & Wiggins 1979, Kjer et al. 2002). Spicipalpia show differing behavior: there are free-living Rhyacophilidae, “purse-case-makers” (Hydroptilidae) and “saddle-case makers” (Glossosomatidae). Larval caddisflies have adapted to a variety of habitats. They occur in lotic habitats, such as springs, streams and rivers and in lentic water bodies such as ponds, lakes and temporary pools (Mackay and Wiggins 1979). The order also exhibits different feeding types, including herbivorous, detritivorous and carnivorous species. Like other merolimnic insects caddisflies are only able to disperse among water bodies as adults when they are able to fly. Some species are known to be strong fliers such as *Stenophylax* or *Mesophylax* (Malicky 1987) which are known to fly distances up to 5

km. Other studies have shown that species stay close to the stream where they hatched (Sode & Wiberg-Larsen 1993, Petersen et al. 2004, Winterbourn et al. 2007).

The ecological diversity of Trichoptera offers a broad spectrum to investigate questions about distribution patterns and population genetics. Population genetics and phylogeography of caddisflies was for example studied by Smith et al. (2006) in New Zealand and Baker et al. (2003) and Múrria & Hughes (2008) in Australia. In Europe Wilcock et al. (2001, 2005, 2007) studied *Plectrocnemia conspersa* and *Plectrocnemia flavomaculatus* in parts of their range. Kelly et al. (2001) provided a study of *Mesophylax aspersus* on the Canary islands. Recent studies are available that consider the whole biogeographic range of three European montane Trichoptera species, using molecular data of mitochondrial DNA (Pauls et al. 2006, Lehrian et al. 2009, Bálint 2008). These studies yielded interesting and differing results concerning population genetic structure and location of glacial refugia, illustrating species specific phylogeographic patterns in the group of Trichoptera. Pauls et al. (2006), for instance, found a genetic pattern with divergent haplotypes in the former periglacial area for *Drusus discolor*. They inferred refugia in Central European highlands supporting the Dinodal-theory of Malicky (1983), which states that cold-tolerant caddisflies were able to remain north of the Alps during cold periods. Lehrian et al. (2009) found a different pattern for *Hydropsyche tenuis*, that shows little haplotype diversity across its range and probably recolonized Central Europe from one or more refugia in Southern Europe. These findings represent different genetic patterns in montane species that occur in fragmented populations due to their restriction to mountain ranges with peaks above 800 m asl (Haase 1999). To study fragmentation not only by certain altitudes, we chose a caddisfly with a Central European range that is restricted to certain geological conditions, namely limestone bedrock. *Rhyacophila pubescens* shows high niche specificity and is not distributed evenly in its distribution area in Central Europe, but bound to mountainous areas with limestone geology. *R. pubescens* populations are thus bound to “habitat islands”. In these areas, the species only occurs in calcareous streams with lime precipitation (Haase 1998, 1999), which we will from here onwards refer to as tufa streams. This is very remarkable since such a strict obligation to a geological factor has not been reported to our knowledge of other caddisflies in Central Europe. This habitat specificity could lead to increased genetic structure compared to aquatic species that have a wider tolerance concerning stream types. This makes *R. pubescens* particularly suitable as a study species. It occurs in the Central European highlands from France in the west to Hungary in the east (Fauna Europaea Web Service 2004), in altitudes above approximately 180 m asl (personal observation). *R. pubescens* also occurs in lower

altitudes of the Alps (< 1500 m asl), ranging from the Eastern Alps to Liguria, in the Apennines and on the island of Corsica (Fig.1, GTOPO30, ESRI). It is thus recently covering a range that was in the northern part affected by glaciation and offered conditions for survival in the southern part.

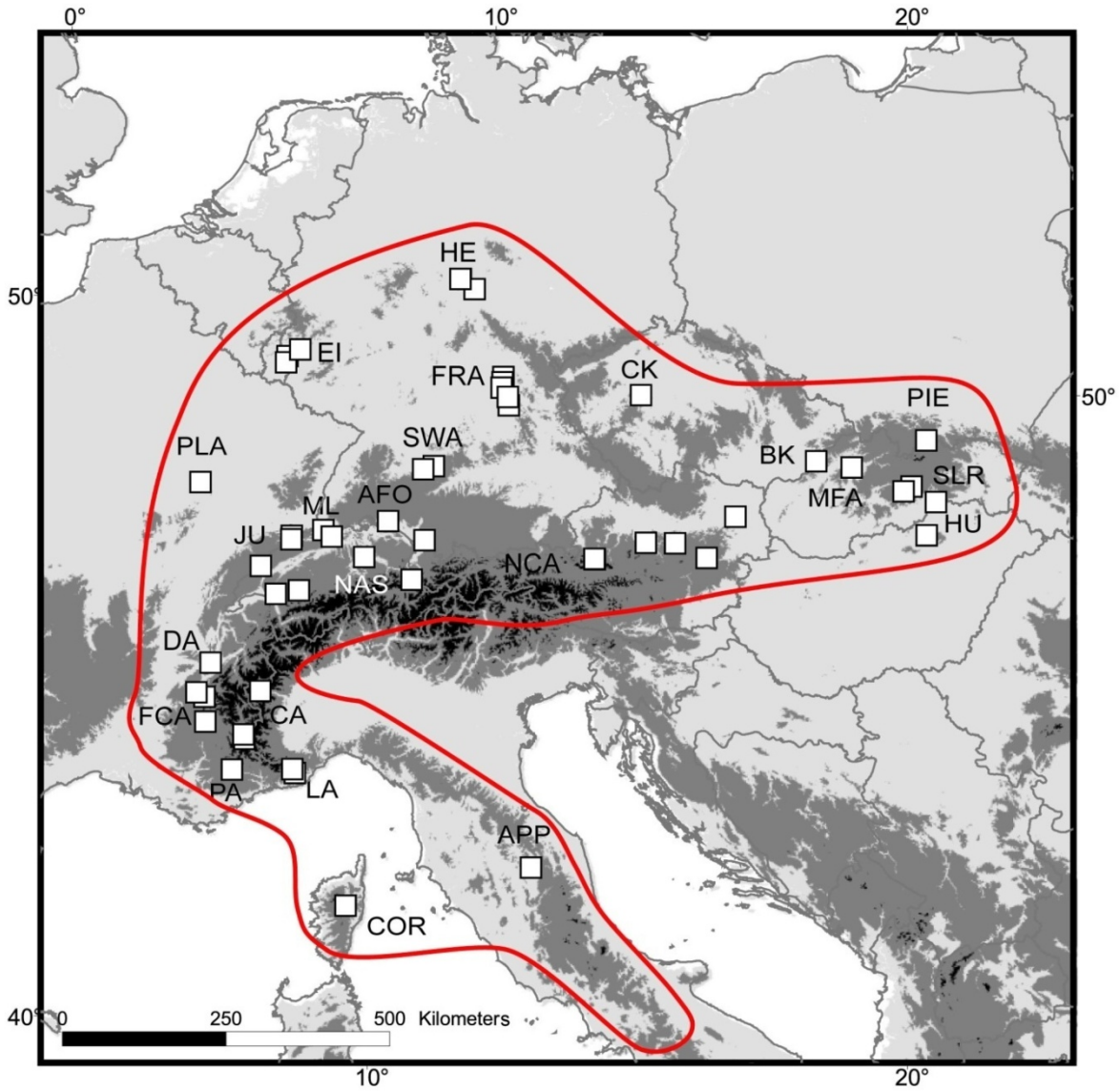

Fig.1. Map of the entire distribution range of *R. pubescens*. Letters indicate mountain regions sampled in this study. HE: Northern Hessian mountains. FRA: Franconian Alb. SWA: Swabian Alb. EI: Eifel. NCA: Northern Calcareous Alps. AFO: Alpine foothills. ML: Mittelland. JU: Swiss Jura. NAS: Northern Alpine slope. PIE: Pieniny mountains. BK Bilé Karpaty mountains. CK: Český Kras. MFA: Malá Fatra. SLR: Slovenské Rudohorie. HU: Northern Hungarian mountains. PLA: Plateau de Langrès. DA: Dauphiné Alps. FCA: French Calcareous Alps. CA: Cottic Alps. PA: Provence Alps. LA: Ligurian Alps. APP: Apennines. COR: Corsica. Red line indicates distribution range of the species.

Rhyacophilidae are living in lotic water bodies, and most occur in fast flowing creeks or streams because they require water with high oxygen content (Bálint 2008), as larvae and pupae. *R. pubescens* larvae are strictly bound to the crenal, hypocrenal and epirithral (Graf et al. 2002) and are thus probably not able to use lower stream zones to disperse in a watershed, therefore dispersal is entirely restricted to the adult stage. Larvae live in microlithal (pebbles with diameter of 2-6 cm) and mesolithal (pebbles with diameter of 6-20 cm) substrate in shallow water, and sometimes in hygropetric habitats (personal observation). Flight period is from June to October (Tobias & Tobias 1981). Population sizes of *R. pubescens* are usually not high (Haase 1999, Engelhardt pers. observation). One reason for this could be that tufa streams present extreme environmental habitats; another reason would be that since almost all *Rhyacophila* are predators, they are not as numerous as phytophagous benthic organisms. When studying population genetics it can be advantageous to investigate species with comparatively smaller populations, because in divided populations allelic frequencies fluctuate independently thus causing genetic diversity between populations (Neal 2004), and this process of genetic drift happens more rapidly in smaller than in larger populations (Neal 2004). All outlined factors – adult only dispersal, high habitat specificity, small effective population sizes – could lead to quicker manifestation of genetic differentiation among isolated populations. The main aim of this thesis is to demonstrate how the insular distribution pattern, caused by a geological factor, affects the genetic pattern of *R. pubescens*. This is to our knowledge the first range wide phylogeographical study about fragmentation of an aquatic insect by geology.

In order to conduct a phylogeographical intraspecific study it is essential to know whether the species can be considered a true species. Since *R. pubescens* was collected from the entire Central European range it is important to reveal possible cryptic species. Therefore in the first chapter phylogenetic relationships of *R. pubescens* from different mountain ranges with several other species of the *Rhyacophila tristis*-group will be examined. This species group was defined by Schmid (1970) based on morphological similarities of the adult genital appendices. The main question of interest is whether *R. pubescens* specimens sampled in different regions of its distributions range are a monophyletic group and thus a good species in the sense of the monophyletic species concept (De Queiroz 2007). Another objective of this chapter is to highlight the relatedness of *R. pubescens* and other *Rhyacophila* species, e.g. which species are sister taxa. One nuclear and two mitochondrial markers are used, and a distance method and two character-based methods are applied to the data set to estimate phylogenetic trees.

The second chapter deals with the population genetic structure in populations situated north of the Alps. This area was in the permafrost and tundra zone during the Pleistocene and therefore not populated by temperate species (Hewitt 2004, Schmitt 2007). By using a sequence fragment of mitochondrial DNA the genetic differentiation is examined in mountain regions north of the Alps, where many isolated populations exist. MtDNA is the most popular phylogeographic marker apart from microsatellites and cpDNA (Beebee & Rowe 2008). It has the advantage of evolving neutrally and faster than nuclear DNA and is not subject to recombination. If rapid recolonization of *R. pubescens* occurred after the last glacial maximum, signatures of this process should be detectable in a haplotype network and in statistical analyses identifying past demographic changes. Another aim of this part of the study is to draw conclusions from the results on recent dispersal and gene flow between mountain ranges. The results of the DNA sequence analysis of the northern part of the caddisfly´s range will be discussed in a phylogeographic context. This part of the thesis has been published in a slightly modified version (Engelhardt et al. 2008).

In the third chapter specimens from the whole biogeographic range of *R. pubescens* are examined using a mitochondrial marker and Amplified Fragment Lengths Polymorphisms (AFLP´s). A second marker system was chosen in order to use multilocus DNA profiles that may behave differently than a single marker (Bensch & Åkesson 2005), and to gain results not only reflecting maternal inheritance. In particular three questions are addressed in this chapter. The first question of interest is whether the genetic population structure in the southern areas is different from the structure present in the area north of the Alps. We would expect higher genetic diversity in the south if this region was continuously inhabited compared to the periglacial area. The second question is whether the results of the two marker systems agree or disagree concerning e.g. genetic differentiation of populations or population structure inferred by assignment methods. The third question of interest is what can be deduced from the results of both markers with regard to potential refugial areas, postglacial range expansion and recolonization of *R. pubescens*. Based on the results it is possible to reveal whether *R. pubescens* survived the ice ages in the periglacial area or recolonized this area coming from a refugium in the south.

In the last chapter the results obtained by the different analyses will be summarized and general conclusions on the phylogeny and phylogeography of the study species *R. pubescens* will be given.

Chapter 1

Phylogeny of the *Rhyacophila tristis*-group with special consideration of *R. pubescens* (Insecta: Trichoptera)

Introduction

Taxonomy of the genus *Rhyacophila*

The Trichoptera are a diverse group of holometabolous insects with aquatic larval stages (Kjer et al. 2002) that live in lotic and lentic water bodies. The genus *Rhyacophila*, PICTET 1834, belongs to the Spicipalpia as used by Wiggins (2004), which include the families *Rhyacophilidae* and *Hydrobiosidae*, that are both "free-living" carnivores (with very few exceptions), and the *Hydroptilidae* and *Glossosomatidae* (Holzenthal et al. 2007). The genus is the largest in Trichoptera with currently over 700 species described (Holzenthal et al. 2007). *Rhyacophila* species live in almost all regions of the holarctic region (Schmid 1970). In Europe and the Mediterranean 125 species are described (Malicky 2005, Graf et al. accepted). Döhler (1950) grouped the European species of the genus in six different categories according to larval morphology: *Rhyacophila s.str., Hyperrhyacophila, Pararhyacophila, Prosrhyacophila, Metarhyacophila* and *Hyporhyacophila*. Morphological features that were mainly considered were presence or absence of sword process, gills and number of bristles on the pronotal ridge. These categories were also used in the study of Buholzer (1978) who described the larval morphology and distribution of Swiss *Rhyacophila*. Pitsch (1993) also followed Döhlers larvae types when he wrote his comprehensive work about Central European caddisfly morphology, faunistics and ecology. Adult characteristics of the genus were described by Schmid in his monographic work "Le genre *Rhyacophila* et la famille des Rhyacophilidae (Trichoptera)" (1970) where he constructed phyletic trees in which he grouped the species mainly according to similarities in male genitalia morphology.

The *R. tristis*-group and questions addressed in this chapter

Schmid (1970) created the *R. tristis*-group in the branch "invaria". The *R. tristis*-group, as described by Schmid (1970), comprises twelve European *Rhyacophila* species characterized by a reduced aedeagus compared to the other species in the branch "invaria". This group is an ideal species complex for studying diversification of freshwater aquatic invertebrates in Europe since it is very diverged with many regional endemites (Malicky 2004, Bálint 2008). For this study a subset of several species of the *R. tristis*-group was collected. Three widely

distributed and three endemic species (Carinthia, Balkans) were chosen. Other species of the group, e.g. *R. trescaviscensis,* occur only in areas on the Balkan where land mines inhibit field work. The main focus of this study is *R. pubescens*, PICTET 1834, which is distributed in Central Europe, Italy and Corsica. Mountain regions north of the Alps, Western Alps, Liguria, Apennines and the island of Corsica (Tab. 1.1) were sampled to compare how specimens from these regions differ genetically. The main objective was to test monophyly of the species using partial sequence data of two mitochondrial genes (mtCOI, mt large subunit ribosomal DNA) and one nuclear gene (wingless). The results shall serve as the basis for an intraspecific phylogeographical study of this species using a fragment of mtCOI and AFLP´s (see chapter 2 and 3). Furthermore, I aimed to investigate the phylogenetic relationships between several species of the *R. tristis*-group. Conclusions drawn from the phylogeny can provide valuable insight in understanding how these aquatic insect species evolved. Until now molecular phylogeny studies of caddisflies have looked at the whole order Trichoptera (Kjer et al. 2001, 2002, Holzenthal et al. 2007) and at the subfamily level (Pauls et al. 2008). Some deeper level studies have focused on the genus level, e.g. Hayashi et al. (2008) worked on *Nothopsyche* and Malm and Johanson (2008) on *Gracilipsodes*. However to date there is no phylogeny available for *Rhyacophila* species that is based on molecular data, I therefore aim at taking a first step towards filling this gap.

This study includes, besides *R. pubescens*, *R. tristis*, PICTET 1834, *R. aquitanica*, MCLACHLAN 1879, *R. obtusa*, KLAPÁLEK 1894, and *R. producta*, MCLACHLAN 1879. *R. pubescens*, *R. tristis* and *R. aquitanica* have a relatively wide European distribution range compared to *R. obtusa* who is a Balkan endemic. *R. producta* occurs exclusively in the Eastern Alps, in Carinthia and Upper Austria. I also included two specimens of *R. margaritae*, a Balkan endemic species restricted to Bulgaria which was described by Kumanski (1998), and which he proposed to be closely related to the other species of the *R. tristis*-group. Malicky (2004) also places *R. margaritae* close to other species of the *R. tristis*-group. Remaining species of the *R. tristis*-group, that were not included in this study are: *R. trescavicensis*, *R. bosnica*, *R. cibinensis*, *R. vranitzensis*, *R. orghidani*, *R. aberrans*, *R. spinulata* and *R. borcka. R. pendayica, R. braasch,i R. pirinica,* and *R. pseudotristis.* Some of these species were described after Schmid´s work (1970).

The main questions I try to answer with this phylogenetic study are:

-Are specimens of *R. pubescens*, collected in different regions of its range, derived from the same ancestor?
-Is the *R. tristis*-group monophyletic and is *R. margaritae* included in this group?

Materials and methods

Specimens examined

For this study I used 17 specimens of *R. pubescens*, PICTET 1834 (Tab. 1.1). These originated from different regions of the distribution range, from the Central European highlands, the Western Alps, the Apennines and Corsica. The dataset also includes two specimens of each of the following species: *R. tristis*, PICTET 1834, *R. aquitanica*, MCLACHLAN 1879, *R. obtusa*, KLAPÁLEK 1894, *R. margaritae,* KUMANSKI *1998* and *R. producta*, MCLACHLAN 1879 (Tab.1.1). As outgroup species I used one specimen of *R. italica*, MORETTI 1981 and one *R. ferox*, GRAF 2006, both belonging to the Pararhyacophila-group. All larval *R. pubescens* specimens were determined using Waringer & Graf (1997), adults of *R. pubescens* and the other species were determined using Malicky (2004), *R. ferox* was determined by Wolfram Graf.

DNA extraction and amplification

Specimens were kept in 70-96% ethanol until DNA extraction. DNA extraction of larval tissue followed the protocol outlined in Pauls et al. (2006). DNA of adults was extracted from two legs using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer´s instructions. PCR primers for mtCOI were LCOI490 (5´GGTCAACAAATCATAAAGATA TTGG3´) and HCO2198 (5´TAAACTTCAGGGTGACCAAAAAATCA3´) (Folmer et al. 1994). For the mitochondrial large subunit (mtLSU) primers were LR-J-12887 (5´CCGGTCTGAACTCAGATCACGT3´) and LR-N-13398 (5´CGCCTGTTTAACAAAAA CAT3´) (Simon et al. 1994). For the nuclear gene wingless (nuWG) I used Wingnut1a (5´ GAAATGCGNCARGARTGYAA 3´) and Wingnut3 (5´ ACYTCRCARCACCARTGRAA 3´) (Pauls et al. 2008). 25 µl PCR reactions contained 1 puReTaq Ready-To-Go Bead (GE Healthcare) and 10 pmol of each primer. The PCR for mtCOI included 5 cycles of 95°C for 60 s, 45°C for 90 s and 72°C for 90 s and 35 cycles of 94°C for 60 s, 50°C for 90 s, 72°C for 60 s and a final extension of 72°C for 300 s. Amplification of mtLSU included 36 cycles of

95°C for 45 s, 46°C for 45 s and 72°C for 80 s and a final extension of 72°C for 600 s. I amplified nuWG following Pauls et al. (2008), but using an annealing temperature of 54°C.

Sequencing and sequence editing

Sequences were generated by LGC AGOWA, Berlin. Sequences for *R. ferox* were provided by Steffen Pauls.

The software Sequencher 4.8. (Genecodes) was used to check and manually edit ABI traces. I used Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) to compare the nucleotide sequences with data of NCBI database to make sure that I amplified the correct fragments. Sequences of mtCOI and of nuWG were aligned with CLUSTAL W as implemented in BioEdit (Hall 1999). For aligning sequences of the mtLSU region I used the software G-Blocks 0.91b (Castresana 2000) with a stringent method, since there were length variations between different species in the dataset and a repetitive sequence in *R. margaritae* specimens. The final lengths of the used fragments were 633 bp of mtCOI, 388 bp of nuWG and 375 bp of mtLSU. The combined dataset of all three genes consisted of 1396 bp.

Phylogenetic inference

I carried out calculations for the single genes and for the combined dataset with the Neighbor-joining (NJ) method, Maximum Parsimony (MP) and the Bayesian Markov Chain Monte Carlo (B/MCMC) method. Gaps were treated as missing data. Software programmes used were Paup* 4.0b10 (Swofford 2001) and MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). For MP analysis I carried out a heuristic search with 100 replicates of random taxon addition, the branch swapping algorithm was tree-bisection-reconnection (TBR). The MulTrees option was in effect. The MaxTrees option was set to auto-increase. Bootstrap support values (Felsenstein 1985) were estimated with 2000 bootstrap replicates. I used the consistency index (CI), retention index (RI) and rescaled consistency (RC) index (Farris 1989) to assess levels of homoplasy for each marker and the combined data set. For the Bayesian analysis a substitution model was selected for each gene partition with the software Modeltest 3.7 (Posada & Crandall 1998). B/MCMC analysis for the combined dataset was carried out in two parallel runs with four chains each. Number of generations was 5×10^6, sumt burn-in was set to 3000. Tree sample frequency was 1000. I calculated a majority rule consensus tree with posterior probabilities for each clade. To compare the results in tree topology of each gene partition I used the Bayesian method according to Buckley et al. (2002). The tree topology of each single gene was compared with each other in order to examine the 0.95 posterior

probability for the clades. In case no conflict occurred it was concluded that the three datasets could be combined.

Results

The datasets

New sequences for two mitochondrial and one nuclear gene were generated for 28 *Rhyacophila* specimens belonging to eight taxa as outlined in the material and method section. Sequence information and tree characteristics for the single gene partitions and the combined dataset are summarized in Tab. 1.2. Each partition had a different substitution model (Tab. 1.2) and the combined dataset was calculated partitioned according to these models.

Tab. 1.2 Results of single genes and combined dataset using Maximum Parsimony and Bayesian approach.

	mtCOI	**mtLSU**	**nuWG**	**combined**
Number of characters	633	375	388	1396
Number of variable characters (%)	205 (32.39)	93 (24.8)	119 (30.67)	417 (29.87)
Maximum Parsimony				
Uninformative characters	24	41	18	83
Informative characters (%)	181 (28.59)	52 (13.86)	101 (26.03)	334 (23.92)
Consistency index (CI)	0.5969	0.7817	0.7130	0.6545
Retention index (RI)	0.8306	0.8735	0.8772	0.8454
Rescaled consistency index (RC)	0.4958	0.6828	0.6254	0.5532
Tree length	454	142	216	819
Bayesian/MCMC				
Selected model under HLRT	GTR+I+G	TVM+G	TrNef+G	According to models for each gene partition
Average standard deviation of split frequencies	0.003787	0.009997	0.003736	0.002960
Log-likelihood	-2922.54	-1186.64	-1607.35	-5845.08

Numbers of parsimony informative characters ranging between 13.86% (52 sites) and 28.59% (181 sites) were detected. They were highest in mtCOI, followed by nuWG and mtLSU (Tab. 1.2). When comparing the 95% majority-rule consensus trees of single gene fragments there were no significantly supported conflicts. Thus it can be assumed that all three gene regions follow the same way of evolution and lead to the same phylogenetic topology. Therefore I used the three sequence fragments in one combined dataset.

Levels of homoplasy deduced from the consistency index according to Farris (1989), 1-CI, were highest in mtCOI, followed by nuWG and mtLSU. MtLSU performed slightly better than nuWG and nuWG performed much better than mtCOI. In the combined dataset the fraction of change attributed to homoplasy is 0.35.

Likelihood parameters are given in Tab. 1.2 for each of the gene partitions and the combined dataset. G/C content was highest in the nuclear gene fragment (50%) and lower in the mitochondrial fragments (0.30% in mtCOI, 0.25% in mtLSU). An A/T bias is commonly found in insect mtDNA genes (Lunt et al. 1996, Langor & Sperling 1997, Jamnongluk et al. 2003).

Monophyly of *R. pubescens*

Support for *R. pubescens´* monophyly in single gene analyses and in the combined dataset is summarized in Tab. 1.3. All bootstrap values of neighbor-joining and Maximum Parsimony method show that *R. pubescens* specimens are monophyletic. Support is a bit weaker in the mtLSU gene fragment. Posterior probabilities of the Bayesian/MCMC analysis strongly support monophyly of *R. pubescens*, again the value of the mtLSU gene is a bit weaker. Combining the three partitions leads to a larger dataset and significant support for the same ancestor of *R. pubescens* specimens (Tab.1.3).

Tab. 1.3 Maximum Parsimony bootstrap support values and posterior probabilities for the clade of *R. pubescens* specimens.

Monophyletic clade ***R. pubescens***	**mtCOI**	**mtLSU**	**nuWG**	**combined**
NJ	100.00	100.00	100.00	100.00
Maximum Parsimony	96.00	57.20	100.00	100.00
Posterior probability Bayesian/MCMC	1.00	0.62	1.00	1.00

Relationships in the *R. tristis*-group inferred by different phylogenetic methods

The clade of the *R. tristis*-group is not supported with the Neighbor-joining (NJ) method. The NJ tree of the combined dataset shows a well supported clade for the *R. pubescens* specimens (bootstrap value 100.00) (Fig. 1.1).

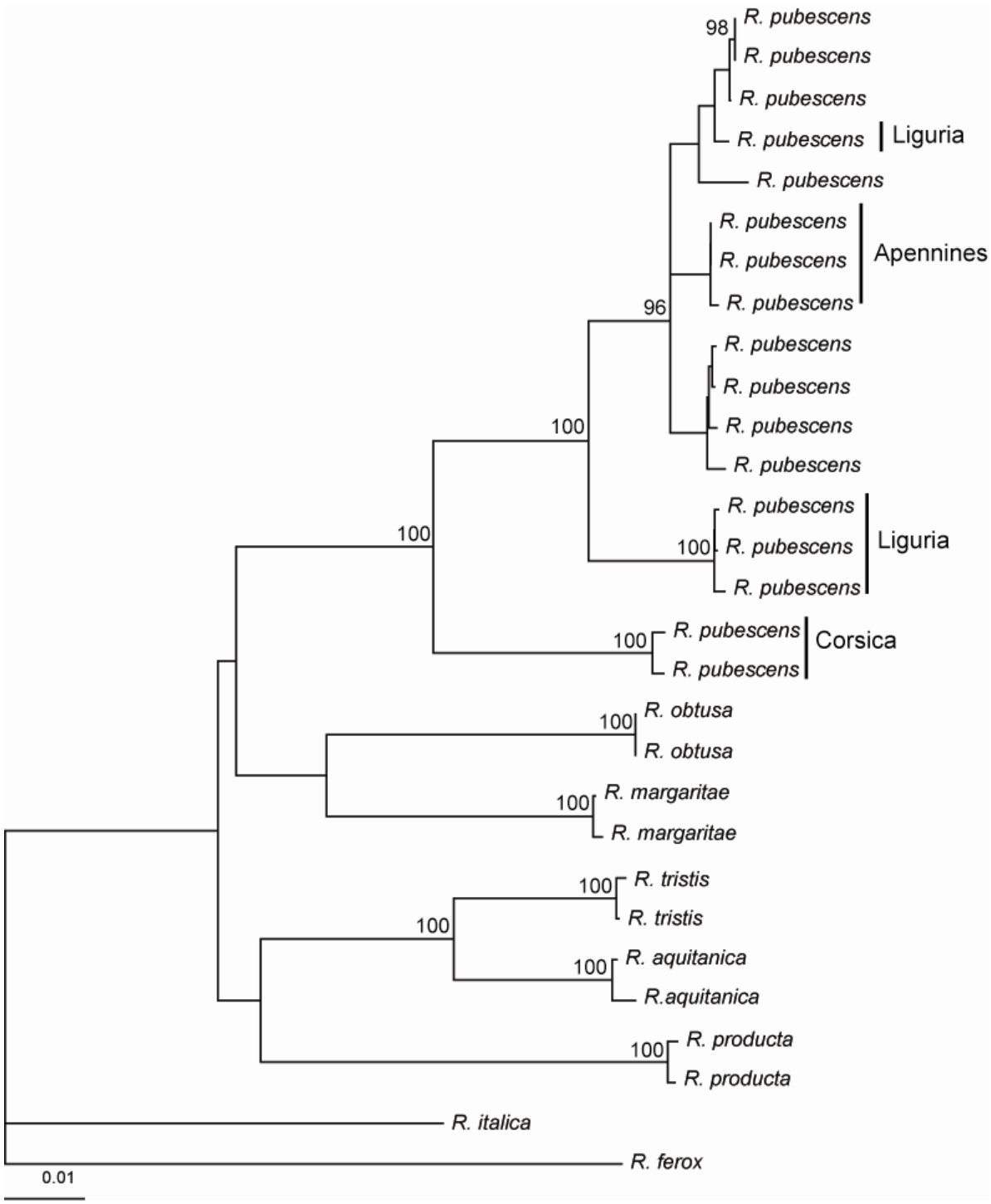

Fig. 1.1 Neighbor-joining phylogenetic tree of *Rhyacophila* specimens for combined dataset. Scale indicates percentage of sequence difference. Numbers indicate bootstrap values above 95.

The Corsican specimens are on their own branch compared to the other regions and also the three Ligurian specimens from Valle di Pietra stream are a bit distant from all other regions. *R. obtusa* and *R. margaritae* are in the same clade and form a sister clade to *R. pubescens*. *R. tristis* and *R. aquitanica* are sister taxa and form a clade that is the next clade to *R. producta* which is on its own branch.

In the eight most parsimonious trees obtained using the Maximum Parsimony method the *R. pubescens* specimens also form a well supported clade (bootstrap value 100%) (Fig. 1.2). The trees show only slight differences in the relatedness of *R. pubescens* individuals, but otherwise topologies are the same.

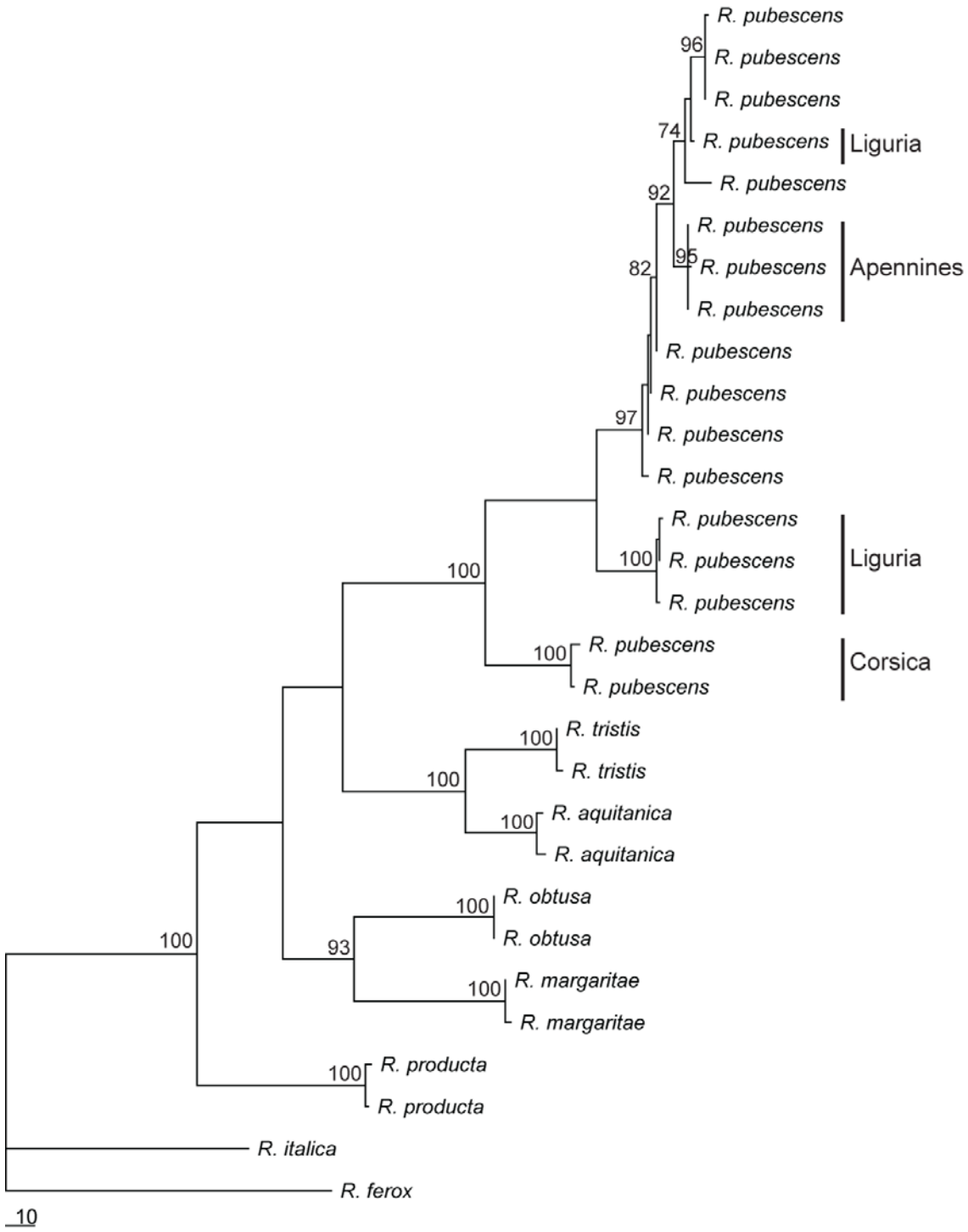

Fig. 1.2 One of the eight most parsimonious trees obtained by Maximum Parsimony analysis for combined dataset. Above nodes bootstrap support values above 70% are shown. Scale indicates number of steps.

Again Corsican and Ligurian specimens from Valle di Pietra seem to be a bit separated from the other regions. In this tree *R. tristis* and *R. aquitanica* build a sister clade to *R. pubescens*. *R. obtusa* and *R. margaritae* build a clade. *R. producta* is on its own branch apart from all other studied species of the *R tristis*-group. All species of the *R. tristis*-group build a monophyletic clade that is supported with a bootstrap support value of 100. The topology of

the Bayesian 50% majority rule consensus tree (Fig. 1.3) is the same as found with the Maximum Parsimony tree.

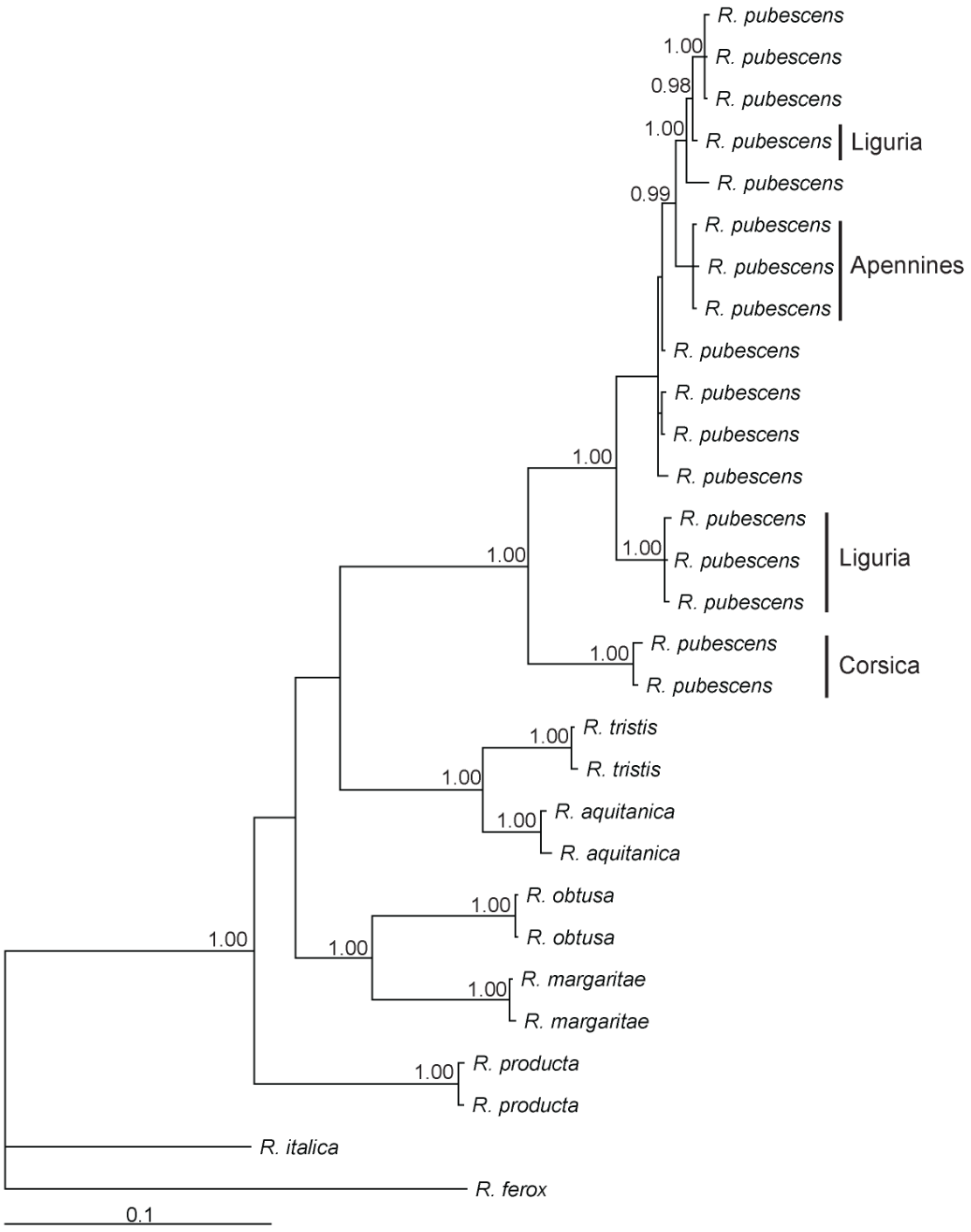

Fig. 1.3 50% majority rule consensus tree for combined dataset, obtained with B/MCMC method from 4006 trees calculated in two parallel runs with three heated chains and one cold chain. Numbers above branches indicate posterior probabilities. Scale indicates model based distance.

R. pubescens form a clade with a posterior probability of 1.00. Again *R. tristis* and *R. aquitanica* build a sister clade to *R. pubescens* and another clade consists of *R. obtusa* and *R.*

margaritae. *R. producta* is a sister taxon to all other studied species. With the Bayesian approach the *R. tristis*-group is supported with a probability of 1.00.

Discussion

In this study I used single gene fragments and a combined dataset of two mitochondrial (mtCOI, mtLSU) and one nuclear (nuWG) marker to gain insight in the phylogenetic relationships between several species of the *R. tristis*-group as circumscribed by Schmid (1970).

Phylogenetic relationships of *Rhyacophila pubescens*

The question concerning the phylogenetic status of *R. pubescens* specimens originating from different mountain regions (north of the Alps, Western Alps, the Apennines, the island of Corsica) could be resolved. Specimens cluster in the same highly supported clade with all three methods (Tab. 1.3). The individuals of Corsica and the ones found in the Valle di Pietra region in Liguria seem to have separated earlier since they show some genetic distance compared to the other investigated individuals of *R. pubescens*. It seems reasonable that the Corsica specimens occupy a separate branch since the population on the island must have reached it through dispersal when the Mediterranean sea level was lower before flooding 3 or 5 Myrs ago (Steininger & Rögl 1984) or have been present there since the land mass split from the mainland in the early Oligocene 32 Myrs ago (Meulenkamp & Sissingh 2003). Presumably they developed separately from the mainland populations for several million years. The population in Liguria seems to be isolated from the population in the Southwestern Alps, possibly this is due to the geographic location in this mountainous area with many deep valleys and different watersheds. Altogether the data demonstrate that *R. pubescens* specimens across Europe are more closely related to each other than to any other species included in our study. The fact that they are all descendants from the same ancestor means that they can be considered a good species according to the monophyletic species concept (De Queiroz 2007) and implies that they share the same evolutionary history. This allows us to study the population structure and genetics of this species. A phylogeographic study based on sequence analysis of a highly variable region of mtCOI and on AFLP´s (chapter 2 and 3) was conducted to reveal migration and colonization processes of this caddisfly species.

Monophyly of the *R. tristis*-group and relatedness between species

Another aim of this study was to examine whether the categorization of species into the *R. tristis*-group based one male genitalia morphology can be supported with molecular data. All three methods that were used, NJ, Maximum Parsimony and Bayesian/MCMC support monophyly of the *R. tristis*-group with respect to two outgroup taxa (Pararhyacophila-group), although this is not significantly supported with the NJ method. Maximum Parsimony and Bayesian 50% majority rule consensus tree, however, show highly supported clades for the *R. tristis*-group. When looking at the relationships of the species it is interesting that *R. tristis* and *R. aquitanica* are clearly differentiated from each other in all obtained tree topologies and are sister taxa. This supports the opinion of Pitsch (1993) who states that *R. aquitanica* is a true species and not a variation of *R. tristis*, as Döhler assumed due to larval and imaginal features (1950). Recent evidence from a study of Bálint et al. (2008) shows that adult male *R. aquitanica* and *R. tristis* could clearly be distinguished from each other by measurement of several parameters like spur length, characters of the 10th abdominal segment and the phallic apparatus. Our study supports this distinction of these two species. *R. tristis* and *R. aquitanica* are sister taxa to *R. producta* in the NJ tree. In both other methods they are sister to *R. pubescens*, but this was not supported. In the larval stage *R. tristis* and *R. aquitanica* are morphologically very similar to *R. pubescens* (Waringer & Graf 1997), except that their heads are darker. The larval morphology would thus suggest a closer relationship of *R. tristis* and *R. aquitanica* with *R. pubescens* than with *R. producta*. The male characteristics of the adult stage (Malicky 2004) of *R. pubescens* are also more similar to *R. tristis* and *R. aquitanica* than to *R. producta*. At the time Schmid described the *R. tristis*-group, the larva of *R. producta* was not known. The species only occurs in a mountain range located in Carinthia in the Eastern Alps. According to the description of Graf (1993), *R. producta* is the only one of the investigated species of my study that possesses larval abdominal gills. This could explain why it is separated from the other species in the Maximum Parsimony tree and in the Bayesian consensus tree. Pitsch (1993) also stated that larvae of this species cannot be assigned to any of the *Rhyacophila* larvae types. Schmid (1970) stated that *R. producta* is a very specialized species that is derived from *R. pubescens*. The results of my analyses do not support this origin. Although *R. producta* adults can be assigned to the *R. tristis*-group due to similarities, the precise position of the species remains unclear due to different larval morphology. *R. margaritae* (Central Balkan), whose adult stage was described by Kumanski (1998) and whose larval form is not yet described, and *R. obtusa* (Rhodopi mts.) are both Bulgarian endemics. All phylogenetic methods that I used in the present study indicate that they are

closely related to each other. Thus it can be suggested that they have been derived from a common ancestor in the Balkan area. The topologies obtained with the single genes and the combined dataset with all three methods show that *R. margaritae* clusters in the *R. tristis*-group. Therefore my study supports the opinion of Kumanski (1998) who stated that this caddisfly species is closely related to other Bulgarian and Bosnian species of the *R. tristis*-group.

Performance of the genetic markers

Combining different gene fragments for phylogenetic analyses is advisable since the results provide tree topologies based on a number of independent loci and allow more significant conclusions. Recent studies of neotropical caddisfly phylogenies are based on sequence data from three to four nuclear and mitochondrial loci (Hayashi et al. 2008, Johanson & Keijsner 2008, Malm & Johanson 2008). The three single gene regions that were chosen for the present study proved to be useful and variable enough for calculation of phylogenetic trees, and could be combined since no significant conflicts occured. The mtLSU fragment seems to be a bit weaker at resolving the relationships of the study organisms, which may be due to the smallest percentage of variable characters compared to mtCOI which had the highest percentage and nuWG. Nevertheless levels of homoplasy were lowest in mtLSU. In a recent study of Pauls et al. (2008) dealing with feeding ecology evolution of caddisflies of the Drusinae family also two mitochondrial (mtCOI, mtLSU) and one nuclear (nuWG) gene were used. I can only compare performance of mtLSU and nuWG to their findings, because they used a different part of the COI gene. Comparing the two markers in the two caddisfly families there are higher percentages of informative characters in the *Rhyacophila* of this study than in the Drusinae. The consistency index is slightly higher in *Rhyacophila,* as well.

Conclusions and outlook

In the present study I could infer that *R. pubescens* individuals sampled from different mountain regions all across their distribution range form a monophyletic clade based on three markers and three different methods of inferring phylogenies (NJ, Maximum Parsimony, Bayesian). According to my results *R. tristis* and *R. aquitanica* are sister taxa to *R. pubescens*, less related are *R. obtusa*, *R. margaritae* and *R. producta*. The *R. tristis*-group is monophyletic, although *R. producta* occupies its own branch within this group, due to genetic differences found with our markers. *R. margaritae* is included in the *R. tristis*-group, supporting Kumanski´s (1998) assignment. Since my main objective was testing monophyly

of specimens determined as *R. pubescens*, I use this investigation to gain a preliminary insight in the phylogenetic relationships of the *R. tristis*-group. To fully understand relatedness between these species it is necessary to expand the dataset with the remaining species of the group, as described by Schmid (1970) and species that were described later like *R. pendayica* MALICKY 1975, *R. braaschi* MALICKY&KUMANSKI 1976, *R. pirinica* KUMANSKI 1982 and *R. pseudotristis* KUMANSKI 1987. It would also be interesting to include *R. tsurakiana* MALICKY 1984, a species occurring in Greece whose adult stage is placed next to *R. pubescens* in the determination key of Malicky (2004), to determine whether they are sister taxa. Three other species belonging to the *R. tristis*-group should also be included: *R. aberrans* MARTINOV 1913, *R. spinulata* MARTINOV 1913 both from the Caucasus and *R. borcka* SIPAHILER 1996 from Anatolia. With a larger dataset it would be possible to gain more knowledge on molecular phylogenetics and diversification of the genus *Rhyacophila* and European caddisflies in general on the species level.

Tab. 1.1 Sampling sites of *Rhyacophila* specimens. Country codes are according to ISO 3166. Abbreviations: L: Larva, A: Adult, m: male, f: female.

Species	**Country**	**Mountain region**	**Number of individuals/ Life stage**	**Locality**	**Latitude (°N)**	**Longi-tude (°E)**	**Collector**	**Vouchers deposited at**
R. pubescens	DE	Franconian Alb	1 L	Hundshaupten	49.72	11.23	Engelhardt	Senckenberg
	CH	Swiss Jura	2 L	Ocourt	47.35	7.06	Engelhardt & Lehrian	Senckenberg
	FR	Cottic Alps	3 A	La Condamine-Châtelard	44.45	6.74	Bálint	Senckenberg
	FR	Provence Alps	2 L	L´Iscle de Vergons	51.3	9.88	Engelhardt & Kind	Senckenberg
	FR	Corsica	2 L	Tributary Tavignano	42.26	9.21	Engelhardt & Kind	Senckenberg
	IT	Ligurian Alps	1 L	Rezzo	44.03	7.87	Engelhardt & Kind	Senckenberg
	IT	Ligurian Alps	3 A m	Valle di Pietra	44.08	7.81	Delmastro	Senckenberg
	IT	Apennines	3 L	Tributary of Fiume Tescio	43.10	12.68	Engelhardt & Lehrian	Senckenberg
R. aquitanica	RO	Retezat	1 A m, 1 A f	Galeş Lake	45.38	22.90	Bálint	Coll. Bálint
R. tristis	RO	Lotru	1 A m	Obarsia Lotrului	45.46	23.62	Nagy & Bálint	Coll. Bálint
	RO	Retezat	1 A m	Cimpu lui Neag	45.30	22.97	Bálint & Theissinger	Coll. Bálint

Tab. 1.1 (continued) Sampling sites of *Rhyacophila* specimens. Country codes are according to ISO 3166. Abbreviations: L: Larva, A: Adult, m: male, f: female.

Species	**Country**	**Mountain region**	**Number of individuals/ Life stage**	**Locality**	**Latitude (°N)**	**Longi-tude (°E)**	**Collector**	**Vouchers deposited at**
R. obtusa	BG	Stara Planina	2 A m	Teteven	42.81	24,37	Bálint	Coll. Bálint
R. producta	AT	Eastern Alps	2 A m	Nockberge	46.85	13.76	Graf	Senckenberg
R. margaritae	BG	Stara Planina	2 A m	Ribaritsa	42.76	24.37	Pauls & Kumanski	Senckenberg
R. italica	IT	Apennines	1 A m	Purello	43.32	12.77	Engelhardt & Lehrian	Senckenberg
R. ferox	AT	Eastern Alps	1 A m	Saualpe	46.85	14.67	Graf	Coll. Graf

Chapter 2

Population genetic structure of the caddisfly *Rhyacophila pubescens*, PICTET 1834, north of the Alps

Introduction

Effects of population fragmentation

Understanding the processes leading to fragmented species distribution is a major issue in biogeography. Species that live in fragmented populations due to habitat specificity and/or narrow ecological niches are interesting models to study fragmentation processes. Fragmentation of large habitats into smaller "habitat islands" can develop through anthropogenic influence (e.g. changes in land use or construction of dispersal barriers) or naturally through vicariant events or disjunct distribution of suitable altitudes, landscape units or underlying geology. Past and present gene flow are often examined with the help of molecular approaches to indirectly reconstruct fragmentation processes (e.g. Piertney et al. 2005, Martinez-Solano et al. 2005, Qi et al. 2007). Fragmentation studies in Central Europe have dealt with a varied biota including different groups of animals, e.g. the European polecat (Pertoldi et al. 2006), the capercaillie (Segelbacher et al. 2003) and several species of butterflies (Vandewoestijne & Baguette 2004, Louy et al. 2007). Among freshwater species, Geist & Kuehn (2005) recently studied the freshwater pearl mussel *Margaritifera margaritifera,* Vainio & Väinölä (2003) the amphipod *Gammarus lacustris* and several studies have focussed on fishes (e.g. Gum et al. 2005, Barluenga et al. 2006). There is still a lack of studies concerning aquatic insects living in fragmented populations in Central Europe, considering the diversity of this group. Some of the existing studies deal with Central European caddisflies and mayflies (Wilcock et al. 2001, 2003, 2007, Monaghan et al. 2002, Pauls et al. 2006). These species are either widespread with somewhat isolated larval populations or limited in their distribution due to a preference for high altitudes. Wilcock et al. (2001, 2003, 2007) observe contrasting patterns of local population structure in two regions in England for the widespread caddisfly *Plectrocnemia conspersa* and much more local population structure in *Polycentropus flavomaculatus* using allozymes and microsatellites. They conclude that adult dispersal may counterbalance fragmentation of larval habitats at the local scale in *P. conspersa* (Wilcock et al. 2001) and that the observed population structure is maintained by landscape formations and anthropogenic dispersal

barriers (Wilcock et al. 2007). Monaghan et al. (2002) find different patterns of differentiation in three alpine aquatic insect species inferred from allozymes. In the mayfly *Baetis alpinus* they detect differentiation within and among streams, but not among major drainages of the Alps. They interpret this lack of differentiation as a result of historical rather than present gene flow. In their study of the montane caddisfly *Drusus discolor*, which also exhibits an insular distribution resulting from its limitation to higher altitudes, Pauls et al. (2006) reveal high levels of genetic differentiation between mountain ranges in Europe and significant divergence between numerous isolated refugial populations.

Habitat specificity of *R. pubescens*

The outlined examples show that there is a wealth of different patterns of population structure to be expected in caddisflies and other aquatic insects depending on life histories and ecological niches. Nevertheless, to date there are no studies that have explicitly examined whether population fragmentation due to geological factors affects population structure. This question is particularly interesting as species with very narrow geological niches are rare and the current geological situation is generally considered old and stable in an evolutionary time frame. Therefore one would expect that species have not "moved around" much with the geology but actually disperse among disjunct geologic formations and suitable habitats, or remain in place and represent highly diverged, independent evolutionary units. In our study we analyze the genetic population structure of a caddisfly with high niche specificity related to calcareous geology. The species was chosen as a model for examining whether geology is a determining factor for population fragmentation and subsequent molecular diversification. *Rhyacophila pubescens* is a free-living predatory caddisfly that occurs in Central European mountain ranges, in lower elevations of the Alps, in the Apennines and on the island of Corsica, mostly in submontane and montane altitudes (Graf et al. 2006). The larvae of the species live in springs and headwaters of small streams (Graf et al. 2002). The species is restricted to mountain ranges with calcareous geology due to its stenotopic binding to tufa streams (Haase 1998, 1999, Malicky 1990) and thus exhibits an insular distribution pattern (Fig.2.1.). The term tufa is used differently across subject areas. We refer to tufa as calcium carbonate deposits which form by precipitation in calcareous streams. Due to the stenotopic binding of the studied caddisfly to calcareous mountain ranges we expect limited gene flow between populations of different mountain ranges. As it was shown in the phylogenetic study in chapter 1, *R. pubescens* can be considered as a true species according to the monophyletic

species concept (De Queiroz 2007). This is a necessary assumption for studying intraspecific phylogeographic patterns.

Questions addressed in this chapter

The present chapter focuses on the investigation of the genetic population structure of *R. pubescens* in the northern part of its range by sequence analysis of a fragment of the mitochondrial cytochrome c oxidase I gene (mtCOI). The region north of the Alps was chosen to analyze the genetic population structure of several calcareous mountain ranges in detail. This region is an interesting study area because of its history that has been influenced by glaciations and recolonization processes (e.g. Hewitt 1999, 2004). The aim of this chapter is to detect whether the species binding to tufa streams and thus to calcareous geology leads to isolation of populations by surrounding areas of unsuitable habitat, or if *R. pubescens* is capable of dispersing between suitable habitat islands. We also use the observed patterns of haplotype divergence to formulate phylogeographic hypotheses that will be studied in detail in chapter 3.

Materials and methods

Specimens examined

We collected and analyzed larval specimens of *R. pubescens* from 33 sites across the northern distribution range (Fig. 2.1, Tab. 2.1).

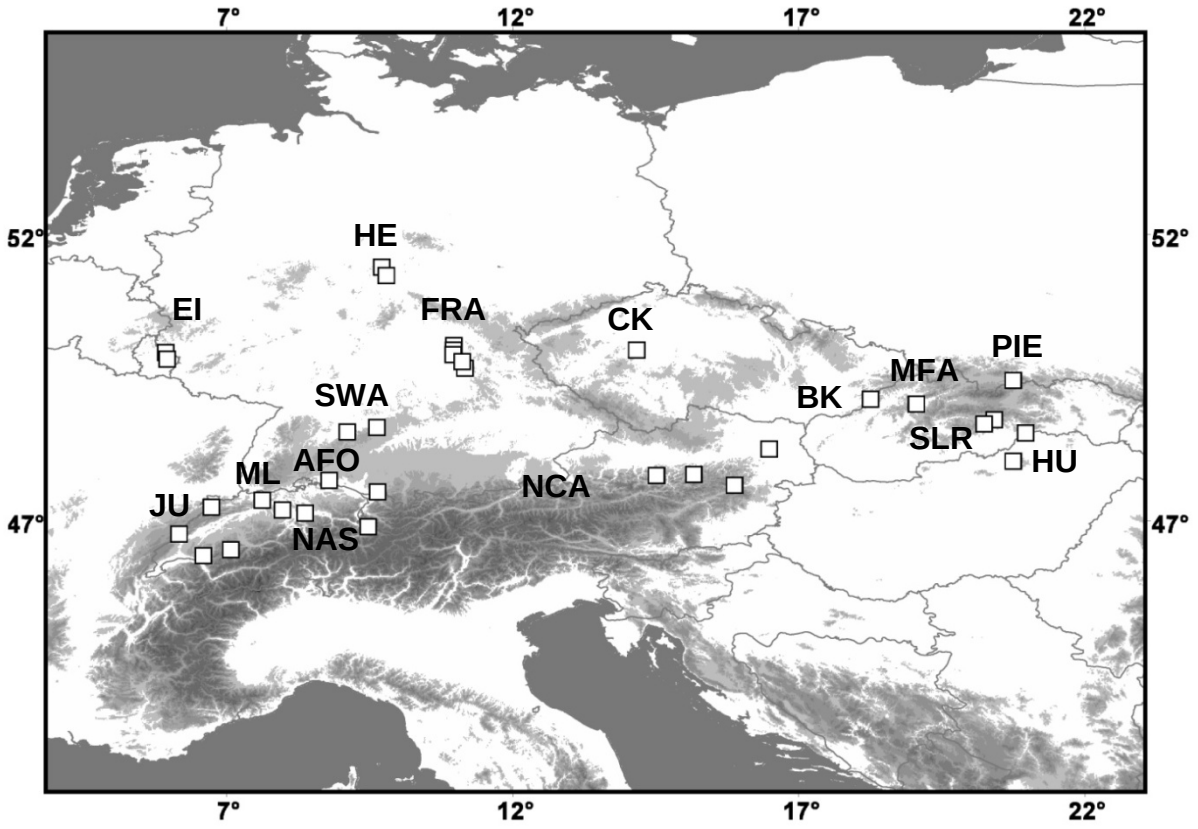

Fig. 2.1 Sampling locations across the northern part of the distribution range of *R. pubescens*. Shading of landmasses reflects altitude. Numbers around edges refer to geographic coordinates. Letters indicate mountain ranges according to Tab. 2.1. (Map Source: GTOPO30, ESRI).

Tab. 2.1 Sampling locations and haplotypes of *R. pubescens* populations. Country codes according to ISO 3166. Populations are sorted by mountains. Haplotypes are numbered, numbers in brackets refer to the number of individuals carrying each haplotype.

Mountain region	**Country**	**Population Number**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Altitude (m)**	**Collector**	**Haplotypes**
Northern Hessian mountains (HE)	DE	1	Flachsbach above Wendershausen	51 18 06	09 53 16	263	Engelhardt & Hövelborn	H1(5)
Northern Hessian mountains (HE)	DE	2	Gatterbach above Wanfried	51 10 59	10 13 35	350	Engelhardt & Hövelborn	H2(7)
Franconian Alb (FRA)	DE	3	Burglesauer Bächlein above Burglesau	49 59 46	11 05 14	395	Engelhardt	H1(6)
Franconian Alb (FRA)	DE	4	tributary Ellerbach above Tiefenellern	49 55 00	11 04 47	425	Engelhardt	H1(5), H3(3)
Franconian Alb (FRA)	DE	5	brook below Tiefenhöchstadt	49 50 28	11 04 34	443	Engelhardt	H1(3), H4(3), H5(1)
Franconian Alb (FRA)	DE	6	Rüsselbach at Kirchrüsselbach	49 36 05	11 16 18	481	Engelhardt	H1(4), H6(2), H7(1)
Franconian Alb (FRA)	DE	7	Hundshauptener Bach below Hundshaupten	49 43 17	11 13 49	490	Engelhardt	H1(5), H2(3)
Swabian Alb (SWA)	DE	8	Attenriedbach , Geislingen	49 58 56	06 34 52	-	Mayer	H1(2), H2(1)
Swabian Alb (SWA)	DE	9	Fils above Wiesensteig	48 33 34	09 35 56	623	Engelhardt & Schlünder	H1(8)
Eifel (EI)	DE	10	hygropetric, Tränenlay	49 51 18	06 19 25	180	Engelhardt, Pauls & Neu	H1(8)
Eifel (EI)	LU	11	spring near Haalerbach	49 46 00	06 19 00	-	Graf	H2(5)
Northern Calcareous Alps (NCA)	AT	12	brook near Möggers	47 33 42	09 49 01	-	Graf	H1(8)
Northern Calcareous Alps (NCA)	AT	13	Bertaquelle, Hollensteingraben	47 40 04	15 45 41	-	Graf	H1(1)
Northern Calcareous Alps (NCA)	AT	14	Schreiberbach, Wiener Wald	48 16 27	16 20 04	400	Graf & Pauls	H1(2)
Northern CalcareousAlps (NCA)	AT	15	Mayrgraben, Lunz	47 51 00	15 05 00	-	Malicky	H1(9)

Tab. 2.1 (continued) Sampling locations and haplotypes of *R. pubescens* populations. Country codes according to ISO 3166. Populations are sorted by mountains. Haplotypes are numbered, numbers in brackets refer to the number of individuals carrying each haplotype.

Mountain region	**Country**	**Population Number**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Altitude (m)**	**Collector**	**Haplotypes**
Northern Calcareous Alps (NCA)	AT	16	Weißenbach, Reichraming	47 49 52	14 27 41	-	Graf	H1(1)
Alpine foothills (AFO)	DE	17	Mühltalbach above Möggingen	47 45 45	09 00 29	424	Sundermann	H8(4), H9(2)
Mittelland (ML)	CH	18	Talbach above Pratteln	47 30 19	07 41 10	420	Engelhardt & Lehrian	H1(2), H10(2), H11(1), H12(1)
Swiss Jura (JU)	CH	19	La Motte above Ocourt	47 21 00	07 03 24	438	Engelhardt & Lehrian	H1(3), H13(2), H14(1), H24(1)
Swiss Jura (JU)	CH	20	Dénériax, Noirvaux	46 51 26	06 31 02	1020	Engelhardt & Lehrian	H1(3), H10(4), H18(1)
Swiss Jura (JU)	CH	21	brook above Soubey	47 18 09	07 03 31	525	Engelhardt & Lehrian	H1(6), H21(1), H25(1)
Northern Alpine slope (NAS)	CH	22	nameless brook, Bächenmoos	47 12 31	08 36 47	625	Vicentini	H1(5)
Northern Alpine slope (NAS)	CH	23	nameless brook, Prantin	46 29 49	06 55 27	1212	Engelhardt & Lehrian	H1(2) H19(4)
Northern Alpine slope (NAS)	CH	24	Warmbach above Weissenbach	46 36 02	07 22 42	869	Engelhardt & Lehrian	H1(3), H20(1)
Northern Alpine slope (NAS)	CH	25	brook near Fanas	46 58 53	09 39 40	700	Lubini	H1(7), H22(1)
Pieniny mountains (PIE)	PL	26	Pieninski Potok	49 24 58	20 23 56	-	Sczesny	H1(2)
Bílé Karpaty mountains (BK)	CZ	27	Tributary of Kloboucký Potok	49 06 09	18 01 06	420	Chvojka	H27(3)
Český Kras (CK)	CZ	28	Císařská rokle SW of Srbsko	49 55 05	14 08 00		Engelhardt & Schlünder	H26(6)
Malá Fatra (MFA)	SK	29	Valcansky Potok, Martin	49 01 22	18 47 02	576	Engelhardt & Bieber	H1(8)
Slovenské Rudohorie (SLR)	SK	30	Biele Vody, Murán	48 45 36	20 04 37	428	Engelhardt, Blanár & Trebulová	H1(6), H4(1), H28(1)
Slovenské Rudohorie (SLR)	SK	31	Potok Kamenárka, Tisovec	48 41 25	19 54 40	480	Engelhardt, Blanár & Trebulová	H15(6), H23(1)

Tab. 2.1 (continued) Sampling locations and haplotypes of *R. pubescens* populations. Country codes according to ISO 3166. Populations are sorted by mountains. Haplotypes are numbered, numbers in brackets refer to the number of individuals carrying each haplotype.

Mountain region	**Country**	**Population Number**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Altitude (m)**	**Collector**	**Haplotypes**
Northern Hungarian mountains (HU)	HU	32	Tributary, Menes Völgy, Aggtelek	48 32 27	20 35 53	-	Engelhardt & Bieber	H2(4), H16(2)
Northern Hungarian mountains (HU)	HU	33	Ban, Bükk mountains	48 04 03	20 23 40	-	Kiss	H1(5), H17(1)

The sampling covered all known regions of occurrence of the species north of the Alps. Larval and adult specimens were collected using a hand net and were stored in 70-96% ethanol until DNA was extracted. Larvae and adults were determined using Waringer & Graf (1997) and Malicky (1983), respectively. All specimen vouchers are deposited at Senckenberg Research Institute and Natural History Museum, Germany.

DNA extraction and amplification

DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's supplementary protocol for insects. A fragment of mtCOI was amplified via PCR. 25 µl PCR reactions contained 1 puReTaq Ready-To-Go Bead (GE Healthcare) and 10 pmol of the primers Jerry (5´-CAACATTTATTTTGATTTTTTGG-3´: Simon et al. 1994) and S20 (5´-GGGAAAAAGGTTAAATTTACTCC-3´: Pauls et al. 2003) following the protocol outlined in Pauls et al. (2006). Annealing temperature was 40°C. Sequences were generated by Nano+Bio Center Kaiserslautern, Germany using the PCR primers.

Sequence editing and alignment

ABI traces were aligned, checked, and manually edited using the software Seqman (DNASTAR Inc.). We used Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) to verify the identity of sequences. Sequences were aligned using CLUSTAL W as implemented in BioEdit (Hall 1999).

Calculation of networks and statistical analyses

The sequence alignment was imported into DnaSP (Rozas et al. 2003) to generate a haplotype file as input for calculating a median-joining haplotype network (Bandelt et al. 1999) in Network (Fluxus Technology). We calculated exact tests of population differentiation (Raymond & Rousset 1995) and population pairwise F_{ST} as implemented in Arlequin 3.1 (Excoffier et al. 2005) to test whether mountain range populations are significantly differentiated. The Markov chain for exact tests was run for 100 000 steps, with a burn-in phase of 10 000 steps. Settings were default. We performed Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) by grouping the 15 sampled mountains into six major geological units, the Central European Highlands, northern edges of Western Alps and Eastern Alps, northern Alpine Foothills, the Český Kras (due to its isolated position) and the

Western Carpathians. 16 000 permutations were run to estimate genetic structure indices using information of haplotypes as well as their frequency. To measure the loss of genetic variation in populations fixation indices (Wright 1943, 1951, 1965) were calculated. Each of these approaches differs in its algorithm, allowing us to avoid overlooking potential methodological biases. A Mantel (1967) test was applied to the matrices of pairwise F_{ST} and geographical distance between populations to assess isolation-by-distance. 10 000 permutations were run.

Pairwise mismatch distributions (Rogers & Harpending 1992) were calculated for every mountain range and for the whole data set. For mountain ranges and the whole data set we calculated Tajima´s *D* (Tajima 1989) and Fu´s *F* (Fu 1997). Neutrality tests were calculated under default settings. All calculations were performed in Arlequin 3.1 (Excoffier et al. 2005).

Results

Sequence data and haplotype networks

We generated and analysed 197 new mtCOI sequences from *R. pubescens* individuals from the northern range of the species. The 475 bp alignment contained no gaps or ambiguous positions and had twenty-seven variable positions. Twenty-eight different haplotypes were identified (GenBank Accession Numbers EU885387-EU885414).

The median-joining network (Fig. 2.2) showed one common central haplotype, H1, which occurred in every region of the area that was studied, except for the Český Kras and the Bílé Karpaty.

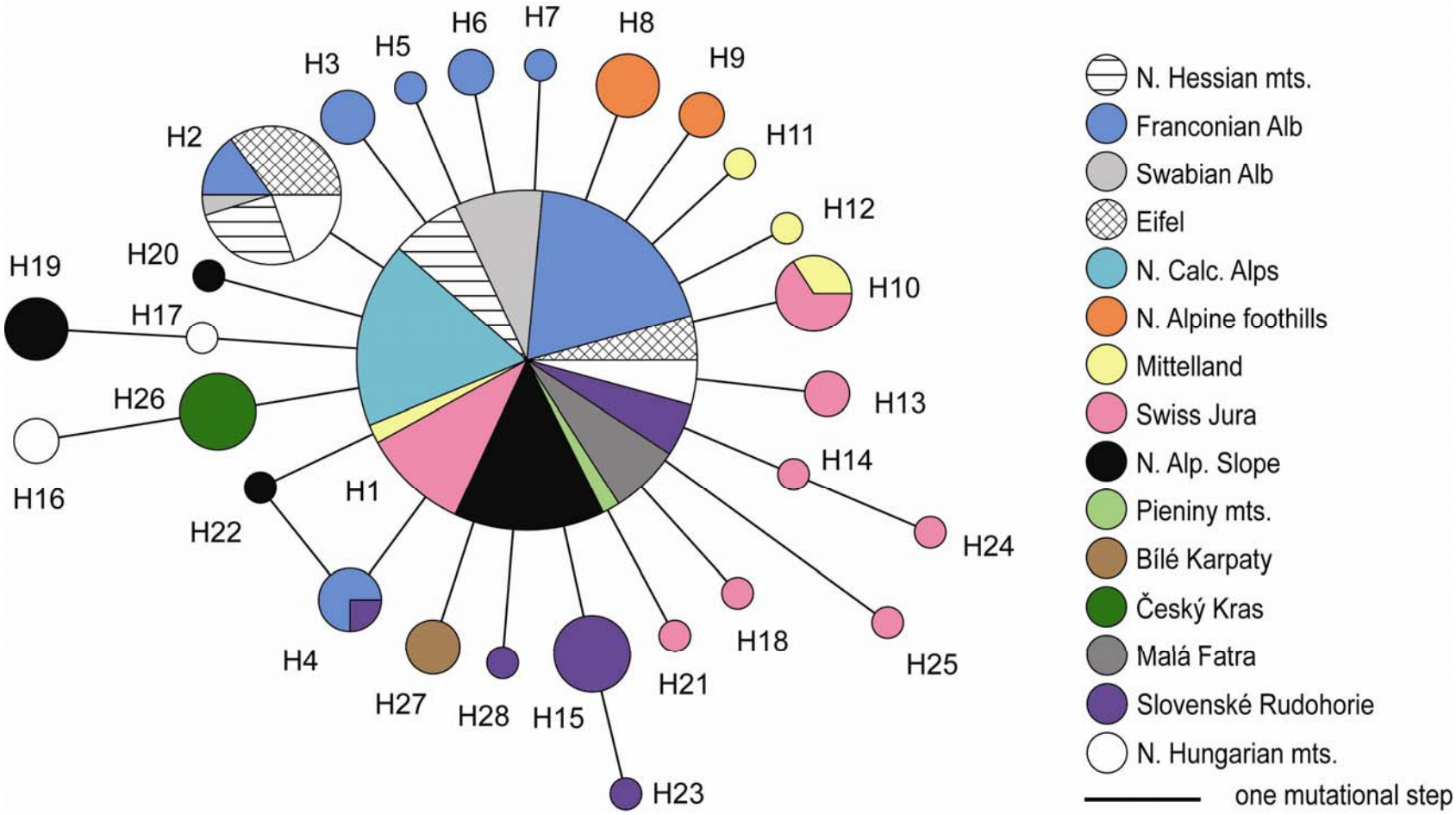

Fig. 2.2 Median-joining network of *R. pubescens* haplotypes in mountain ranges north of the Alps. Coloured circles represent haplotypes and their diameter is relative to the number of individuals carrying any given haplotype. Colors indicate origin of specimens carrying individual haplotypes. Lines between haplotypes indicate genetic distance between haplotypes. Two different mutations occurred at position 284 (H4: T-G, H22: T-C). N. Northern, mts.: mountains, Calc.: Calcareous, Alp.: Alpine.

All other haplotypes differed from H1 by one or two (H16, H19, H23, H24, H25) base pair changes. Most haplotypes occurred in only one region or a single stream. For example H3, H5, H6 and H7 were only found in the Franconian Alb. H3 was endemic to a single stream, as was H5 to another stream, and H6 and H7 to a third stream site (Tab. 2.1). Besides H1, only haplotype H2, H4 and H10 occurred in more than one mountain region.

Population differentiation

Exact tests of population differentiation (Raymond & Rousset 1995) showed that 76 of 105 (72.4%) of mountain range pairs were significantly differentiated from other mountain ranges ($p \leq 0.05$) (Tab. 2.2). The population from Pieniny mountains that only showed differentiation from the Northern Alpine slope and the Český Kras, comprised two individuals with the common haplotype H1. The Český Kras population was significantly differentiated from all other mountain ranges. Pairwise F_{ST} values were relatively high (average of significant values 0.512) and significant ($p \leq 0.05$, with Bonferroni correction for 105 tests, adjusted $\alpha < 0.00047$) for 55 of 105 mountain comparisons (Tab. 2.2).

Tab. 2.2 Population differentiation by exact tests of population differentiation and pairwise F_{ST}. Above diagonal are results of exact tests of population differentiation. Significantly differentiated populations are indicated by + ($p < 0,05$). Below diagonal are F_{ST} values. Significant values (with Bonferroni correction, adjusted $\alpha < 0.00047$) are printed in bold letters. Abbreviations of populations correspond to mountain regions in Tab. 2.1.

	HE	FRA	SWA	EI	NCA	AFO	ML	JU	NAS	PIE	BK	CK	MFA	SLR	HU
HE		+	+	-	+	+	+	+	+	-	+	+	+	+	-
FRA	**0.263**		-	-	-	+	+	+	+	-	+	+	-	+	+
SWA	0.366	-0.028		-	-	+	+	-	+	-	+	+	-	+	-
EI	-0.004	0.105	0.134		+	+	+	+	+	-	+	+	-	+	-
NCA	**0.635**	0.020	0.063	**0.414**		+	+	+	+	-	+	+	-	+	+
AFO	**0.537**	**0.408**	**0.513**	**0.479**	**0.714**		+	+	+	-	+	+	+	+	+
ML	**0.359**	**0.136**	0.171	0.241	**0.370**	0.320		-	+	-	-	+	+	+	+
JU	**0.276**	**0.057**	0.012	0.147	0.050	**0.318**	-0.008		+	-	+	+	-	+	+
NAS	0.028	**0.352**	**0.330**	0.124	**0.486**	**0.474**	0.356	**0.355**		+	+	+	+	+	+
PIE	0.290	-0.292	-0.325	0.023	0.000	0.226	-0.200	-0,279	0.213		-	+	-	-	-
BK	**0.735**	**0.582**	**0.866**	**0.706**	**1.000**	**0.642**	0.526	**0.471**	0.566	1.000		+	+	+	+
CK	**0.780**	**0.615**	**0.891**	**0.753**	**1.000**	**0.733**	**0.640**	**0.526**	**0.611**	1.000	**1.000**		+	+	+
MFA	0.486	-0.033	-0.032	0.262	0.000	**0.525**	0.158	-0.017	**0.375**	0.000	**1.000**	**1.000**		+	+
SLR	**0.405**	**0.216**	0.235	0.306	**0.352**	**0.410**	**0.219**	**0.185**	**0.418**	-0.018	**0.567**	**0.626**	0.216		+
HU	0.053	0.106	0.070	-0.005	**0.244**	**0.326**	0.130	**0.122**	0.134	-0.164	**0.472**	**0.459**	0.109	**0.231**	

AMOVA results (Tab. 2.3) showed that molecular variance was lowest among major mountain ranges (0.98 % of variation, p = 0.31). 30.48 % of variation (p = <0.0001) accounted for variation among mountains within major mountain ranges. Highest percentage of variation (68.54%) was detected within mountains (p = <0.0001). AMOVA results showed that there was very low genetic variation at the highest hierarchical level but that differentiation and diversification existed within each mountain (ϕ = 0.30783, p = <0.0001).

Tab. 2.3 Analysis of molecular variance (AMOVA) for grouping of the 15 sampled mountains into six major mountain ranges.

Nr. of Groups	**Source of variation**	**Sum of squares**	**Variance components**	**% of Variation**	**ϕ**	**P-value**
6	Among major mountain ranges	13.502	0.00493	0.98	0.00979	0.31
	Among mountains within major mountain ranges	19.344	0.15345	30.48	0.31460	<0.0001
	Within mountains	62.798	0.34505	68.54	0.30783	<0.0001

Mantel test did not demonstrate a significant isolation by distance effect in the data set (r = -0.19, P = 0.98), suggesting that isolation by distance is not the main process structuring populations of *R. pubescens*.

The majority of pairwise mismatch distributions within populations showed unimodal distribution of haplotypes in mountain ranges across the study area. This result is indicative of recent demographic population expansion processes (Rogers & Harpending 1992) (see examples in Fig. 2.3).

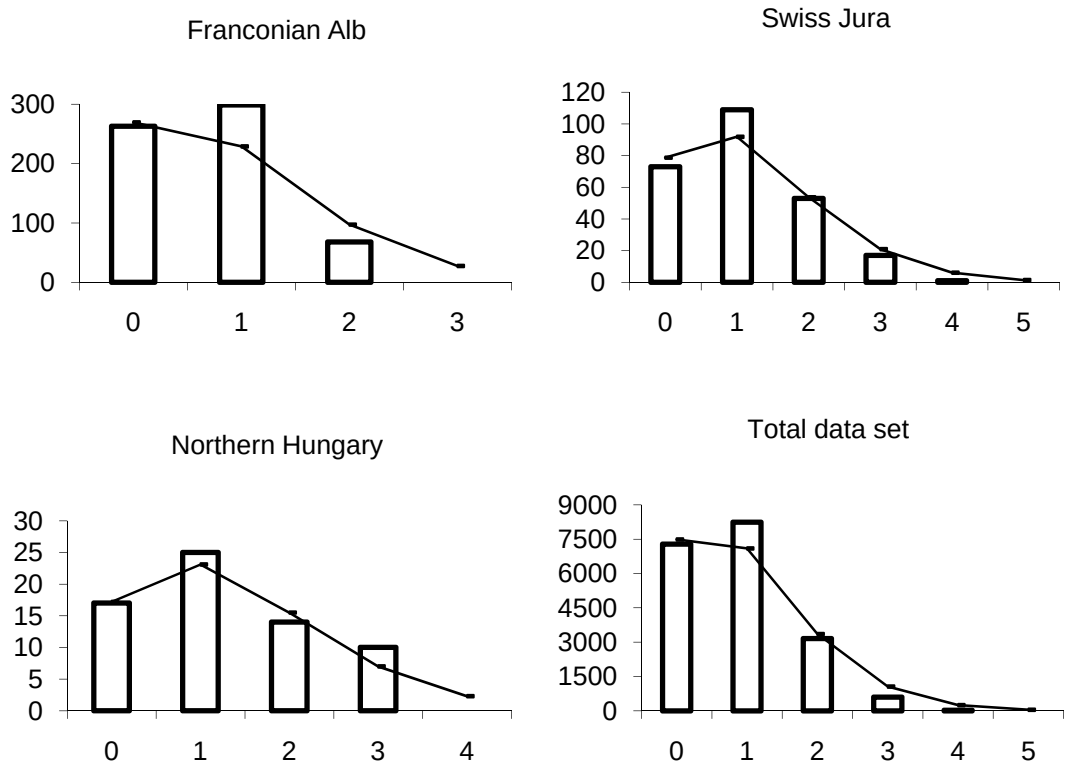

Fig. 2.3 Pairwise mismatch distributions of selected mountain ranges (Franconian Alb, Swiss Jura, Northern Hungary) and for the complete data set. Abscissa: distance between pairs of haplotypes. Ordinate: frequency of pairwise distance. White bars: observed frequency, line: model frequency.

Mismatch distributions were multimodal only in the northern Alpine foothills and in the northern Alpine slope. Calculation of mismatch distributions for the whole dataset showed unimodal distribution of haplotypes (Fig. 2.3).

Neutrality tests supported the results of mismatch distributions. Significant negative values were observed in both tests, Tajima´s D and Fu´s F_S, for the total data set and for the Swiss Jura (Tab. 2.4). In other regions, the Swabian Alb, Mittelland, Slovenské Rudohorie and in the northern Hungarian mountains values of both tests were negative, albeit not always significant in both tests (Tab. 2.4).

Tab. 2.4 Neutrality test results for selected mountain regions. Significant (at $P < 0.05$ (Tajima's D) or at $P < 0.02$ (Fu´s F_S) values are highlighted in bold print. N.: Northern, mts.: mountains, Calc.: Calcareous, Alp.: Alpine, Slov.: Slovenské.

Region	**Tajima´s *D***	**p**	**Fu´s F_S**	**p**
N. Hessian mts.	1.381	0.96	1.152	0.63
Franconian Alb	-1.429	0.07	**-3.853**	0.002
Swabian Alb	-1.129	0.19	**-0.410**	0.02
Eifel	1.301	0.93	1.151	0.62
N. Calc. Alps	0.000	1.000	-	-
N. Alp. foothills	1.032	0.86	1.723	0.76
Mittelland	-0.447	0.36	-1.454	0.04
Swiss Jura	**-1.647**	0.04	**-4.328**	< 0.001
N. Alpine slope	-0.383	0.39	0.535	0.63
Pieniny mts.	0.000	1.000	-	-
Bile Karpaty	0.000	1.000	-	-
Český Kras	0.000	1.000	-	-
Malá Fatra	0.000	1.000	-	-
Slov. Rudohorie	-0.774	0.25	-1.701	0.06
N. Hungarian mts.	-0.178	0.47	-0.127	0.42
Total data set	**-2.280**	< 0.001	**-29.272**	< 0.001

Discussion

Genetic differentiation of *Rhyacophila pubescens* and possible microendemism

The results of our molecular study show that one common haplotype, H1, is present all over the distribution range of *R. pubescens* north of the Alps. A network with one common central haplotype is unexpected since this is typical for common and widespread species which exchange genes over a wide range, e.g. the mayfly *Baetis rhodani* (Williams et al. 2006). The star shaped pattern that we observe for *R. pubescens* indicates recent demographic expansion (Slatkin & Hudson 1991, Jesus et al. 2005, Matthews et al. 2007). The results from neutrality tests and mismatch distributions for the whole data set also indicate recent demographic expansion. The endemic haplotypes we found in almost every region north of the Alps, and the lacking isolation-by-distance-pattern, which suggests strong dispersal barriers between populations (Slatkin 1993), both indicate that genetic differentiation in the species seems to

take place on a much smaller scale. For example in the relatively small region of sampling localities in the Franconian Alb – with distances between 8 to 17 km between streams – we found endemic haplotypes in 3 of the 5 investigated streams. In one stream we found individuals carrying one endemic haplotype (H3), in two streams, we found individuals carrying endemic haplotypes, H5, and H6 and H7, respectively. These haplotypes are only found in 1-3 specimens in our samples. The private haplotypes could thus be the result of small sample size within populations. For example in a recently expanded huge panmictic population, young, locally arisen haplotypes may still be so rare in populations outside their region of origin, that they were not observed in our sampling. However, the consistency of the pattern across several regions we studied, suggests the pattern is real, in particular for those private haplotypes that are carried by two or more specimens. Also, the species is rarely found in large numbers (Haase 1999) suggesting that the entire population is not exceptionally large. It seems more likely that the pattern results from differentiation within mountain ranges where geographically close populations of *R. pubescens* are genetically different and possibly isolated from one another. Unfortunately the haplotype divergence in our data set is too shallow to allow explicit testing of these two scenarios using coalescent simulations-based analyses. A larger sample size and data from an independent nuclear marker could provide sufficient variability in terms of depth to clarify if recent diversification and local differentiation is indeed taking place.

Population differentiation within streams coinciding with no population differentiation among catchments has been observed in several studies in the Australian tropics and led to the patchy recruitment hypothesis (Bunn & Hughes 1997, Hughes et al. 1998, Schultheis & Hughes 2005). According to the patchy recruitment hypothesis, low genetic differentiation among catchments results from widespread adult dispersal, while limited larval movement and most offspring being produced by a limited number of females lead to population differentiation within streams (Bunn & Hughes 1997, Schultheis & Hughes 2005). Under this hypothesis one would expect a limited number of haplotypes to arise from only a few females producing the majority of offspring (Schultheis & Hughes 2005). The observed population differentiation at the local scale in *R. pubescens* is based on many private and potentially endemic haplotypes, which in turn comprise most of the variability observed in the data set. There is no evidence that only few females are producing offspring to each sampled population, but our sample size for some populations is too small to exclude a recruitment effect. The star-shaped topology of the haplotype network and our tests of demographic history, however, suggest that the pattern is more likely related to recent expansion and diversification.

In general, habitat specificity and thus insular or fragmented distribution combined with limited gene flow can facilitate accumulation of genetic differences and, in the long run, lead to new species (Muths et al. 2006, Schluter 2001). This is for example shown for freshwater and other taxa on the island of Madagascar (Benstead et al. 2003, Wilmé et al. 2006) where species richness is high due to a long period of isolation from the mainland and manifold niche occupation. Considering its restriction to single regions or streams indicated by the presence of numerous private haplotypes, *R. pubescens* may currently be diversifying into a complex of microendemic lineages that could under long-term stable conditions evolve into independent genetic lineages and eventually species. In *R. pubescens* we consider the observed microendemism to result from the species' niche specificity and fragmented distribution of suitable calcareous mountainous habitats, which are separated by lowlands and/or regions with a different geology.

Insular distribution pattern and demographic history

Haplotype H1 seems to be ancestral to all other haplotypes, since many of the individuals (60.4%) carry this haplotype and due to its central position in the network (Posada & Crandall 2001). The presence of this haplotype all across the study area could indicate that the species was historically present all over the study area and did not exhibit its currently insular distribution pattern. Most haplotypes in the study area differ by one or two mutational step from haplotype H1. Therefore maximum difference between all haplotypes is four steps (0.84%). Compared to another European caddisfly species with insular distribution, *Drusus discolor,* this is relatively low. Maximum difference between haplotypes of *D. discolor* at the same geographical range is 21 mutational steps (4.21%) in a largely homologous stretch of mtCOI (Pauls et al. 2006). In a study of a New Zealand caddisfly, *Orthopsyche fimbriata* (Smith at al. 2006), a maximum difference of 16 mutational steps (5.2%) between haplotypes was detected. The comparison shows that the situation of *R. pubescens* is different from what might have been expected based on previous studies of caddisflies with insular distributions.
The close relatedness of all haplotypes north of the Alps suggests that this differentiation pattern is quite young. This is in stark contrast to *D. discolor*, where the differentiation between isolated populations in different mountains north of the Alps appears to be in place since well before the last glacial maximum ~18 000 years ago (Pauls et al. 2006). Although any attempt at calculating divergence times between lineages based on a single base pair difference for *R. pubescens* is not sensible, it would appear that the observed population differentiation is a much younger phenomenon. We would expect regional haplotypes of

greater divergence to accumulate within any given region if they were isolated for longer periods of time (Schönswetter et al. 2004, Johnson 2005). The picture we observed rather indicates that differentiation between lineages has not been in place long enough to allow accumulation of base pair changes and lineage divergence. On the other hand, the recent within region diversification appears to be quite strong, and isolation and differentiation have allowed potentially endemic haplotypes to form, even between geographically proximate localities. This is in contrast to results observed in *D. discolor*, where haplotype endemism is more prevalent at the regional and not the stream scale (Pauls et al. 2006). The results in *R. pubescens* also differ from those in an allozyme study of *Plectrocnemia conspersa*, which exhibits little genetic substructuring between populations less than 50 km apart (Wilcock et al. 2001). A more recent microsatellite study (Wilcock et al. 2007) shows that *Plectrocnemia conspersa* is able to disperse overland efficiently but it was also found that geological barriers, in that case circular bands of limestone bedrock, play an important role in shaping genetic population structure. Compared to another montane caddisfly *Hydropsyche tenuis* (Lehrian et al. in press) that exhibits only 9 different haplotypes in its Central European distribution range including the Alps and Appenines, the number of haplotypes we found in our study area seems to be rather high. Population structure in *Hydropsyche tenuis* is quite low, indicating that this species is able to disperse across long distances and seems to have a higher level of gene flow than *R. pubescens*.

Based on the star-shaped network topology and the results from our tests of demographic history it would appear that recent differentiation in *R. pubescens* follows a period of population and/or range expansion of the species. For the total data set, we can infer that the whole population of *R. pubescens* experienced a recent demographic expansion north of the Alps, based on the unimodal distribution of pairwise differences (Slatkin & Hudson 1991) and significantly negative values for Tajima's D and Fu's F. This is also the case in the majority of individual mountains. This suggests that populations of *R. pubescens* either experienced an increase in effective population size under demographic expansion that could have coincided with range expansion.

Postglacial history

While the aim of this study was to examine the population structure of *R. pubescens* in a part of its distribution range, we think the results warrant some phylogeographic interpretation. We limit this interpretation to formulating hypotheses on Pleistocene survival and postglacial

migration. These hypotheses shall serve as the basis for future range wide, multi-locus studies of *R. pubescens* and other species.

Our data indicate that there was a shift in the gene flow between *R. pubescens* populations from past to present. This shift could be the result of a variety of processes and events that shape the population genetic structure in the species. Based on our data rapid postglacial (re-)colonization of Central Europe from a limited refugial source e.g. in the calcareous mountain ranges of Italy or Southern France, where *R. pubescens* still occurs today, seems a reasonable explanation. Within such a refugium, *R. pubescens* could have also experienced a major bottleneck in the period of the Pleistocene, as did many other species (Grivet & Petit 2003, Dubey et al. 2006). This would have reduced genetic variability allowing for one dominant haplotype to thrive with renewed demographic expansion (Hewitt 1999).

As pointed out in the introduction, all the data taken from the literature, provided by colleagues, or based on our own sampling clearly show a strong and unusual restriction of *R. pubescens* to tufa stream habitats (Haase 1998, 1999, Engelhardt pers. observation, Graf pers. communication, Malicky pers. communication). Today these habitats are fragmented since they occur only on a few geological formations (e.g. limestone). However, in the early postglacial period the situation was different as loess accumulations were widespread across the Central European highlands (Pye 1995, Haase et al. 2007). These loess accumulations could have provided a basis for the necessary tufa substrate to form and allow *R. pubescens* to (re-)colonize wide expanses of the Central European highlands. Although it is not fully understood to which extent calcium carbonate washout from loess deposits into watercourses affected water chemistry (Anderson 2007), tufa deposits dating from the holocene have been recorded from loess layers (e.g. in Belgium: Rommens et al. 2006). After an initial (re-)colonization the loess cover declined and calcium carbonate was gradually dissolved and carried away by precipitation and runoff. This could have forced *R. pubescens* into tufa streams in mountain ranges with underlying calcareous geology.

Competition could also have played a role during postglacial (re-)colonization and in shaping the current distribution pattern. *R. pubescens* is restricted to tufa streams that are characterized by stretches of heavy lime precipitation and represent one of the most extreme environments in freshwater systems. Lime precipitation affects both the microhabitat composition and the organisms themselves, because precipitation takes place on the surface of organisms and can reduce their respiration abilities (e.g. Dürrenfeldt 1978). For these reasons the number of species and individuals in these streams is comparably low (Haase 1999). Under such extreme environmental conditions interspecific competition is reduced, favoring species which are capable of tolerating those conditions but are otherwise poor competitors. Thus *R. pubescens*

could have had a competitive advantage over other species during (re-)colonization that were not able to survive in such carbonate-rich habitats. Also, *R. pubescens'* cold tolerance may have served as a competitive advantage allowing the species to rapidly (re)colonies Central Europe postglacially as our demographic expansion analyses would suggest. After the (re-)colonization of other aquatic species and increasing competition, *R. pubescens* might have retreated to tufa habitats that are not suitable for most species.

Conclusions and outlook

Our study shows that *R. pubescens* exhibits a very unusual genetic structure across its northern distribution range related to its occurrence in calcareous mountains. The occurrence of a ubiquitous mtCOI haplotype together with very closely related, but locally private haplotypes that indicate high in-stream differentiation differs from patterns observed in all other aquatic insects investigated in Europe to date. It seems reasonable that postglacial history of the species was shaped by various environmental and internal factors. *R. pubescens* may have rapidly (re-)colonized Central Europe by means of loess deposits, which possibly provided suitable habitat and/or a competitive advantage over other species in the early postglacial. However a full range phylogeographic study also using a nuclear marker, like AFLPs, is needed to test the hypotheses outlined in our discussion. The range wide phylogeography of *R. pubescens* will be described in chapter 3. Another possible explanation for the observed pattern lies in ecological plasticity, but rearing and competition experiments are required to evaluate this idea. Independent of its genesis, the population structure of *R. pubescens* is uncommon and confirms that species reactions to past climate change varies dramatically among aquatic insects and is highly dependent on the ecology and habitat requirements of each species (Pauls et al. 2006, Lehrian, pers. communication). Our findings highlight the need for more studies of taxa with insular distributions, especially of aquatic insects, as the variety of life histories appears to have brought forth a wealth of unique population genetic and phylogeographic patterns. In particular more multi-locus studies including nuclear and mitochondrial markers may help explain patterns of recent diversification, as they will allow incorporating more rapidly evolving loci and more comprehensive use of novel coalescent-based phylogeographic analyses.

Chapter 3

Range wide phylogeography of *Rhyacophila pubescens* inferred from mtCOI and AFLP´s

Introduction

Phylogeographic patterns in Europe

In recent years our knowledge on phylogeographic patterns of European animal and plant species (Taberlet 1998, Hewitt 2000, Petit et al. 2003, Koch & Kiefer 2006, Beheregaray 2008) has tremendously grown. Many studies involving the use of mitochondrial DNA have highlighted intraspecific genetic structure and population history during and after glaciations, in such diverse animal groups as mammals (Rajabi-Hamam et al. 2008, Scandura et al. 2008, Yannic et al. 2008), birds (Haring et al. 2007, Wennerberg et al. 2008) and fish (Mäkinen & Merila 2008, Sediva et al. 2008). Species bound to freshwater habitats are particularly interesting study objects for phylogeographical patterns, due to the linear structure of these habitats which allow dispersal of fully aquatic species only along streams. Aquatic insects like caddisflies (Trichoptera) or stoneflies (Plecoptera) possess larval stages that are only capable of short distance dispersal within streams. The short-lived adult stages are able to fly and can thus fly distances ranging from several meters to several kilometers (e.g. Malicky 1987, Griffith et al. 1998) which can allow for long distance dispersal. To date aquatic insects are underrepresented in studies using mitochondrial DNA that consider the whole species range. To our knowledge only three range wide studies about caddisflies using mtCOI sequences (Pauls et al. 2006, Bálint 2008, Lehrian et al. 2009) have been published. Several other studies have so far discovered very diverse European patterns of genetic structure in European macroinvertebrates considering large parts of the distribution range (Monaghan et al. 2002, Wilcock et al. 2001, 2007, Williams et al. 2006). Besides natural barriers like e.g. regions with high altitudes or other unsuitable habitats, niche specificity acts as a factor shaping distribution patterns and thus genetic structure of species. By examining the whole range of an organism it is possible to gain a comprehensive picture of the large-scale spatial patterns of its genetic diversity (Vucetich & Waite 2003). This is important because not only historical processes like e.g. past range expansion affect genetic diversity and structure (e.g. Anducho-Reyes et al. 2008, Debes et al. 2008), but also contemporary processes like for example higher

genetic drift in peripheral populations than in core populations (Vucetich & Waite 2003). Recent studies have shown that living in suboptimal conditions in the periphery of the species´ range can lead to smaller population sizes and loss of genetic diversity (Beatty et al. 2008) and/or to higher differentiation in the form of endemic haplotypes (Fritz et al. 2006). Our study aims to contribute to the knowledge about large-scale phylogeographic patterns in aquatic European insects by studying the caddisfly *Rhyacophila pubescens* across its entire range.

In chapter two, sequences from a fragment of mtCOI DNA were analyzed, from specimens originating from populations north of the Alps. That chapter served to study the former periglacial area, which was presumably not inhabited by the study species during the Würm ice age. We could reveal recent range expansion in this area and recent differentiation in mountain ranges related to niche specificity, namely habitat restriction to calcareous geology.

Questions addressed in this chapter

In this chapter we investigate recolonization of *R. pubescens* from a southern refugium. We expand our mtDNA dataset with samples from its remaining distribution (the Western Alps, Italy and Corsica) and additionally use Amplified Fragment Length polymorphisms (AFLP´s) to examine range wide genetic population structure and phylogeography. In particular we address the following questions:

Is the genetic population structure inferred from mtDNA sequences different in the Western Alps and south of the Alps compared to the area north of the Alps?

Do the AFLP results about genetic population structure agree with the mtCOI results?

What inferences can be drawn from the results of both markers about potential refugia, recolonization and postglacial history of *R. pubescens*?

Material and methods

Mitochondrial DNA: Specimens examined

We collected and analysed specimens of *R. pubescens* from 51 sites across the distribution range (Fig. 1 in Introduction, Tab. 3.1). The sampling covered all known regions of occurrence of the species. Larval and adult specimens were collected using a hand net and

were stored in 7096% ethanol until DNA was extracted. Larvae and adults were determined using Waringer & Graf (1997) and Malicky (1983), respectively. All specimen vouchers are deposited at the Senckenberg Research Institute and Natural History Museum, Germany.

DNA extraction and amplification

DNA extraction and PCR protocol for amplification of mtCOI are described in chapter two. Sequences were generated by Nano+Bio Center Kaiserslautern, Germany and AGOWA GmbH Berlin, Germany using the PCR primers.

ABI traces were aligned, checked, and manually edited using the software Sequencher Vers. 4.8 (Gene Codes Corporation, Michigan, USA). We used Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) to verify the identity of sequences. Sequences were aligned using CLUSTAL W as implemented in BioEdit 7.0.9.0. (Hall 1999).

Statistical methods

The sequence alignment was imported into DnaSP 4.50.2 (Rozas et al. 2003) to generate a haplotype file as input for calculating an unrooted median-joining haplotype network (Bandelt et al. 1999) in Network 4.5.0.1. (Fluxus Technology). When mtCOI lineages are very divergent, the connection among lineages becomes more ambiguous. This means some connections are less trustworthy than others. We thus also calculated another haplotype network in TCS1.21 (Clement et al. 2000) using the statistical parsimony method. This method defines a cut-off limit for divergent lineages whereafter the lineages cannot be linked with 95% confidence. A Bayesian analysis for mtCOI haplotypes was conducted with MrBayes (Ronquist & Huelsenbeck 2003) using the Markov chain Monte Carlo (MCMC) method and the model selected by Modeltest 3.7. (Posada & Crandall 1998). The best model selected by likelihood ratio tests in Modeltest 3.7. was the HKY+G. Four chains were run for 5×10^6 generations, tree sample frequency was 1 000 and the first 2.5×10^6 generations were set as burn-in. We used only one individual per haplotype because a high number of individuals would not add information to haplotype relations discovered with this method.

For our population structure analyses we pooled the 51 sampled sites by mountain region and uniform geological units. This grouping is non-random but reflects the natural geographic isolation of the samples across the distribution range. We thus analyzed the data set grouped in 23 different geological units, i.e. mountain regions (Tab. 3.1). Exact tests of population differentiation (Raymond & Rousset 1995) and pairwise F_{ST}-values were calculated using the

default settings as implemented in Arlequin 3.1 (Excoffier et al. 2005) to detect differentiation among mountain range populations. An Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) for all 23 mountains regions was calculated in Arlequin 3.1 (Excoffier et al. 2005) with 16 000 permutations. We also grouped the mountain regions in nine and ten major geological units and used these datasets for AMOVA calculations. The units were the Central European Highlands, the Northern Calcareous Alps , the northern Alpine Foothills, Plateau Langrès, the Western Alps (divided into Northwestern and Southwestern Alps in the 10 unit scenario), the Český Kras, the Western Carpathians, the Apennines and Corsica.

We used the software Barrier 2.2 (Manni et al. 2004) to investigate barriers to gene flow. This method is based on Monmonier´s (1973) maximum difference algorithm. Barriers are computed associating a genetic (in this case pairwise F_{ST}) distance matrix with a geographic data matrix. We conducted the analysis for the mtCOI dataset and also for the AFLP dataset. A Mantel (1967) test was applied to the matrices of pairwise F_{ST}-values and geographical distance between all analyzed populations to assess isolation-by-distance in Arlequin 3.1 (Excoffier et al. 2005). 10 000 permutations were run. To analyze the genetic structure of the 23 different mountain regions where *R. pubescens* occurs, mismatch distributions were calculated in Arlequin 3.1 (Excoffier et al. 2005) with 1 000 bootstraps. We also calculated mismatch distributions for the whole dataset under the same settings. To test for neutrality in each of the 23 mountain regions and in the whole dataset, we calculated neutrality tests under the infinite sites model, Tajima´s *D* and Fu´s F_S, in Arlequin 3.1 with default settings. Significant negative *D* and *Fs* values can arise under selective effects but can also indicate population expansion or bottlenecks (Tajima 1993, 1996). Fu´s *F* has been shown to be especially sensitive to population expansions (Fu 1997).

Migrate 3.0 (Beerli 2008) was used to estimate genetic parameters like effective population sizes and migration rates. We calculated a stepping stone model for the populations Provence Alps, French Calcareous Alps, Dauphiné Alps, Swiss Jura, Mittelland and Northern Alpine slope to estimate numbers of effective migrants. We wanted to investigate past gene flow in the Western Alps region to examine from where the northward recolonization originated. The model was set with asymmetric migration and unrestricted theta estimates. Migration was set to be possible into one (for edge populations) or two neighboring populations. Starting values were estimated from F_{ST}-values. We conducted two independent runs with 10 short chains each with 25 000 recorded genealogies and a sampling increment of 20 and two long chains with 200 000 recorded genealogies and a sampling increment of 50. Thus 500 000 and 10 000

000 genealogies were visited by the short and long chains. Burn-in was set to 15 000 genealogies. We used an adaptive heating scheme with four chains (1.00, 1.5, 2.5, 5.0) and a swapping interval of one to ensure sufficient mixing.

Amplified Fragment Length Polymorphism: DNA amplification

DNA of specimens of the whole distribution range of *R. pubescens* was used for Amplified Fragment Length Polymorphism (AFLP) – analysis (Tab. 3.1). The AFLP protocol followed Vos et al. (1995) with minor modifications: EcoRI-primers used for selective PCR´s were fluorescence-labeled (6-FAM, NED, HEX, Applied Biosystems (ABI), Foster City, California, USA). For each sample, genomic DNA concentration was determined (ND-1000, Peqlab GmbH, Erlangen, Germany) and standardized to 50 ng DNA/μL. For digestion 250 ng genomic DNA were used. The initial restriction-ligation step was performed for 14 h at 20°C with EcoRI and MseI (New England Biolabs GmbH, Frankfurt, Germany) and EcoRI and MseI adapters (Metabion International AG, Martinsried, Germany). The digested-ligated products were diluted 1:100. Preselective amplification was carried out in 10 µl-reactions with primers with one selective base (EcoRI primer E+A: 5´-GACTGCGTACCAATTCA-3´, MseI primer M+C: 5´-GATGAGTCCTGAGTAAC-3´) using 0.5 U Taq DNA-Polymerase (New England Biolabs). Selective primers with three additional selective bases (EcoRI primer E+3, MseI primer M+3) were used. Primer combinations were: E37/M54: E + ACG/M+ CCT; E39/M61: E + AGA/M + CTG; E45/M57: E + ATG/M + CGG.

Fragment analysis of multiplex products was run on an ABI Prism 3100 DNA capillary sequencer (University of Mainz, Germany) together with an internal size standard (GeneScan ROX 500, ABI). Fragments were scored with Genemarker Vers. 1.7 (SoftGenetics, Pennsylvania, USA) and automatically scored as present when the peak height exceeded the standard parameter-setting threshold (300). Trace files were also reexamined visually. Fragments in the size range of 100-250 bp were used for analysis. We did 18 replicate samples to assess scoring error according to Bonin et al. (2007). 12 fragments were not used because of scoring error. 19 fragments were only present in one or two individuals and were not used for further analysis. One fragment was monomorphic and was thus excluded from analysis.

Statistical methods

We used AFLP-SURV 1.0 to calculate Nei´s D (after Lynch & Milligan 1994) for mountain regions with 1 000 bootstrap replicates. These datafiles were used as input for NEIGHBOR in the PHYLIP 3.67-package (Felsenstein 1993) to calculate neighbor-joining phenograms (Saitou & Nei 1987) with 1 000 bootstrap replicates. With a distance matrix of squared Euclidean distances a principal coordinate analysis (PCA) was calculated to visualize individual or group differences using GenAlEx 6.1 (Peakall & Smouse 2006). To analyze molecular variance in the studied mountain regions Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) was calculated for the AFLP data in Arlequin 3.1 (Excoffier et al. 2005) with 16 000 permutations.

To evaluate population structure, three different assignment methods were used. Using two or more assignment methods is considered good practice (Beebee & Rowe 2008) in order to get reliable results. A Bayesian Analysis of Population Structure was calculated in BAPS 3.2 (Corander et al. 2004, Corander & Marttinen 2006) using stochastic optimization. We calculated admixture of individuals in the given populations (populations where only one specimen was collected were excluded from analysis) based on mixture clustering using the recommended settings. Setting for estimation of admixture coefficients for individuals was 100, number of reference individuals from each population for simulation was 200, and 20 iterations were calculated for estimation of admixture coefficients for reference individuals. An analysis with an MCMC approach was conducted in Structurama (Huelsenbeck et al. submitted) assuming the model described by Pella and Masuda (2006) with 500 000 generations. This model allows the number of populations to be a random variable following the Dirichlet process prior. Another assignment test was carried out in Structure 2.2 (Pritchard et al. 2000, Falush et al. 2003, 2007) using model based clustering. A model with K populations is assumed, and individuals are assigned probabilistically to populations. We ran ten replicate analyses each for K=1 to K=10 using 200 000 steps as burn-in and 1 000 000 steps for the analysis. We assumed a model with admixture (alpha inferred) and correlated allele frequencies among populations (lambda=1).

As mentioned above, Barrier 2.2 (Manni et al. 2004) was used to calculate barriers to gene flow with a pairwise F_{ST} distance matrix and a geographic data matrix. To estimate ongoing geneflow between populations, a Mantel (1967) test was conducted using pairwise F_{ST}-values and geographical distance between all analysed populations. 999 permutations were run in GenAlEx 6.1 (Peakall & Smouse 2006).

To assess gene diversity, we calculated the proportion of polymorphic markers (95% confidence) and Nei´s gene diversity H (Nei 1987) in each mountain region with AFLPdat (Ehrich 2006). The same software was used to calculate frequency down weighed marker values DW (Schönswetter & Tribsch 2005). The Shannon index of phenotypic diversity S, derived from the Shannon-Weaver index (Shannon 1948) was calculated in Popgene 3.2. (Yeh & Boyle 1997). We assessed private fragments, defined as fragments that only occur in one region, to gain insight into the degree of divergence of a certain population or group of populations (Ronikier et al. 2008).

Results

Mitochondrial DNA

Haplotype networks and haplotype distribution

We generated mtCOI sequences, 475 bp in length, for 333 individuals. The alignment contained no gaps or length invariants. 94 positions are variable, 83 sites are parsimony informative. 59 haplotypes were detected. Maximum difference between all haplotypes is 70 steps (14.74%). The unrooted median-joining haplotype network (Fig. 3.1) shows that the northern populations are dominated by one common haplotype, H1, which is carried by almost half of the specimens examined (N=149).
This haplotype has a central position and is surrounded by several haplotypes that differ from it by one or two mutational steps. H1 is present in all mountain regions north of the Alps except for the Český Kras and the Bilé Karpaty. In these regions we found individuals carrying a haplotype endemic to each population, which was one mutational step apart from H1 (Fig. 3.1 and chapter two). In all mountain regions, whether north or south of the Alps, endemic haplotypes are present that only occur in one region or in single streams, like for example H3, H5, H6 and H7 in the Franconian Alb or H37, H38 and H39 in the Provence Alps (see also Tab. 3.1). In the Western Alps, Apennines and on the island of Corsica, however, haplotypes are more diverged and connected with each other by up to 44 mutational steps. H1 is not present in the Cottic and Ligurian Alps, the Apennines and on the island of Corsica. Maximum difference between haplotypes in the total dataset is 15.73% which corresponds to 59 mutational steps.

The TCS network connects the haplotypes in four separate networks using a 95% confidence interval. One network represents all the northern populations and the Western Alps, with 53 haplotypes and 311 individuals. The other three networks are smaller. The first one comprises

only haplotype H55, which was found in all specimens from one stream in Liguria. The second network groups H42, H43 and H44 from the Apennines, and the third network connects H40 and H41 from the island of Corsica. The segregation into four networks according to TCS is shown by dotted lines in the median-joining network (Fig. 3.1).

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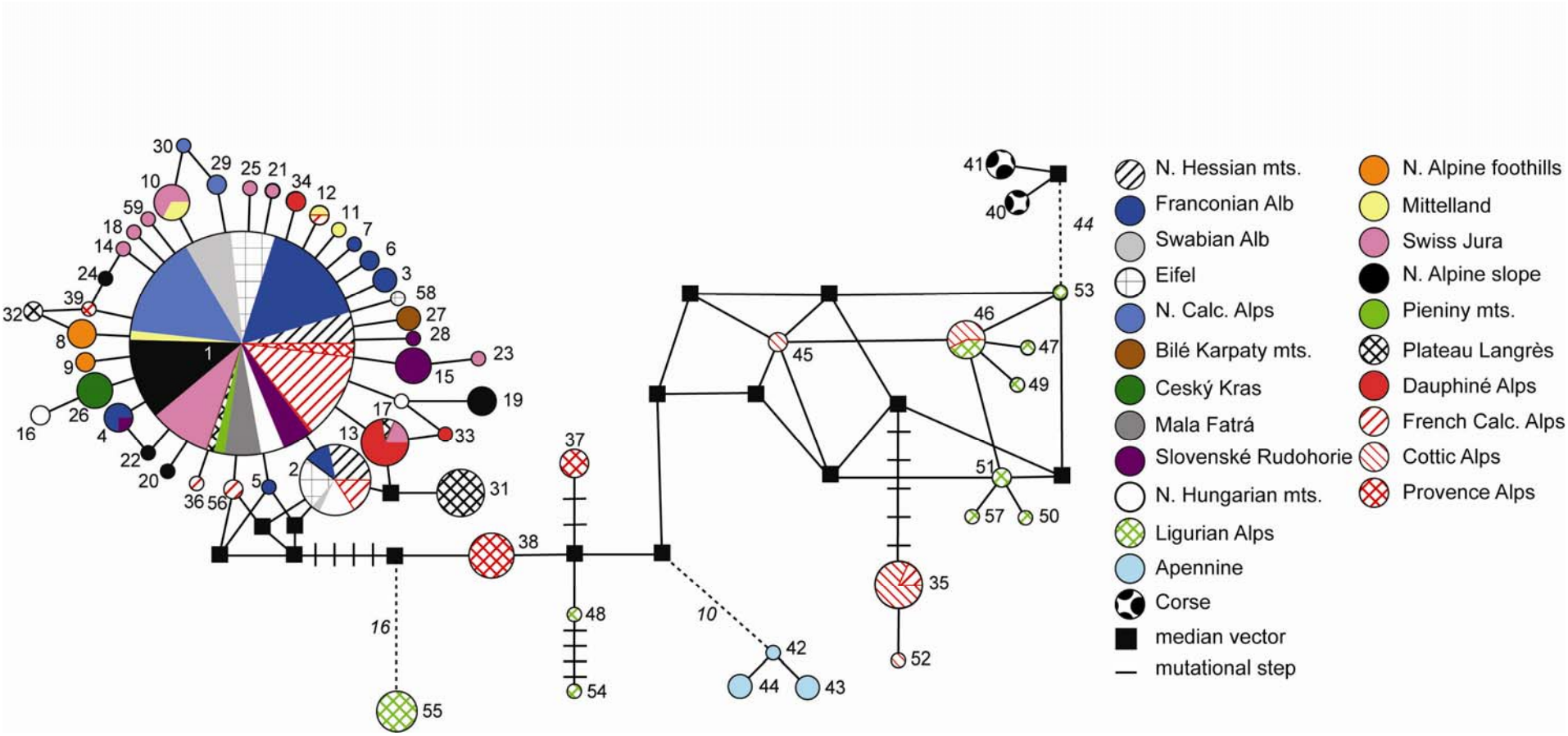

Fig. 3.1 Median-joining haplotype network of *R. pubescens*. Colors code for mountain regions. Size of haplotypes is relative to the number of individuals carrying this haplotype. Cutoffs of networks according to TCS are shown by dotted lines. N. Northern, mts.: mountains, Calc.: Calcareous.

The 50% majority rule consensus tree (Fig. 3.2) shows one node that is supported with 0.99 posterior probability including all haplotypes found north of the Alps and in the Plateau Langrès.

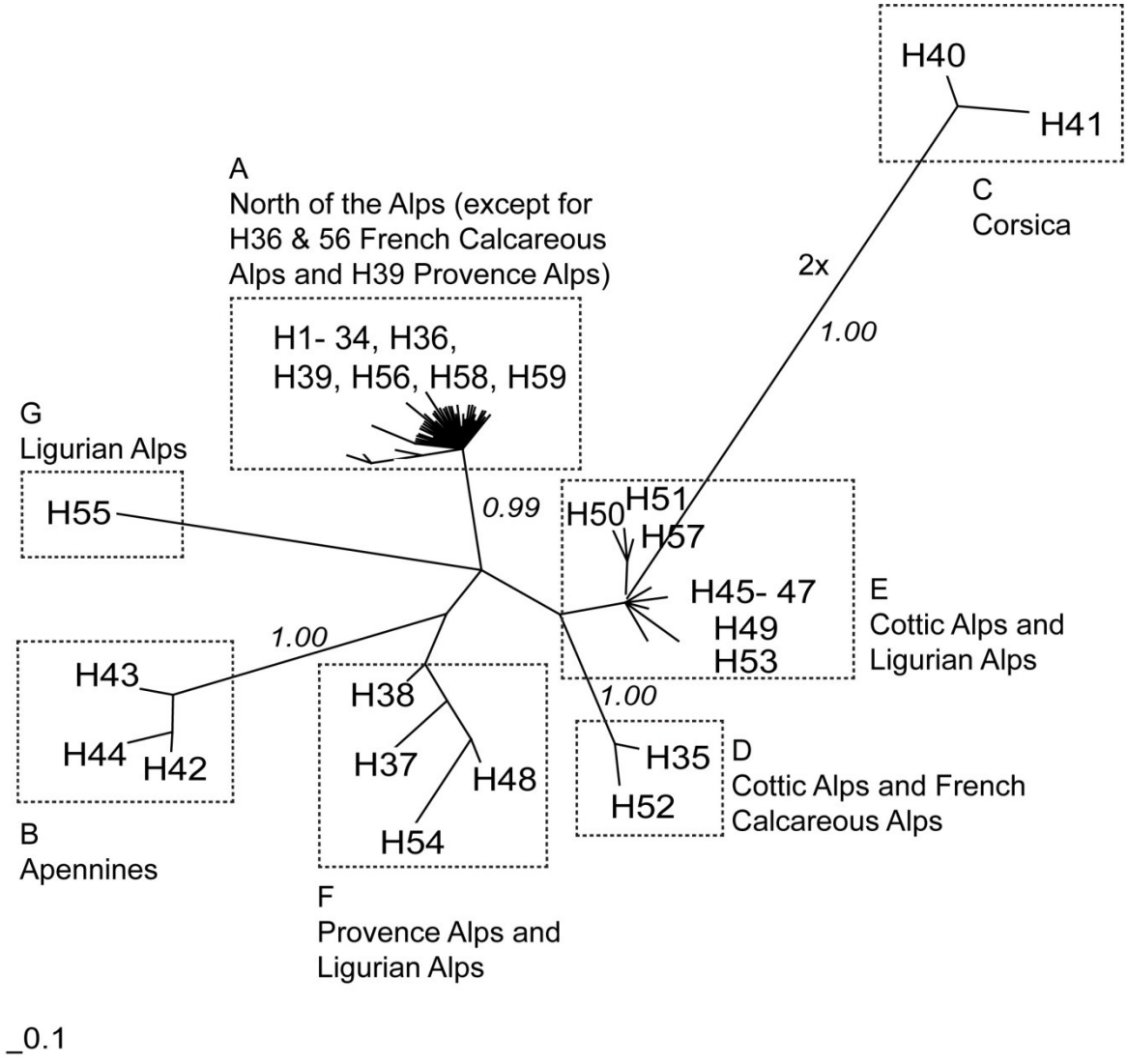

Fig. 3.2 Unrooted 50% majority rule consensus tree of *R. pubescens* haplotypes calculated using a Bayesian approach. Clades are boxed with dotted lines and capital letters. Haplotype numbers are according to table 3.1. Text indicates geographical regions. Numbers on branches show posterior probabilities (pp) ≥ 0.95. Branch lengths refer to genetic distances; the branch connecting H40 and H41 with the others is twice as long as shown in this figure.

In this clade A there are also haplotypes H36 and H56 that both occur only in the French Calcareous Alps and H39 that occurs only in the Provence Alps. This clade shows little internal structure segregating two haplotypes found in the Northern Alpine slope (pp=0.97). Another clade, B, groups the haplotypes found in the Apennines (pp=1.00). Clade C groups the two haplotypes from Corsica together (pp=1.0) and clade D comprises haplotypes H35 and H52, both found in the Cottic and French Calcareous Alps. Clades E, F and G are not

supported (pp<0.95). The Bayesian inference shows a more branched topology for the southern part of the distribution range of *R. pubescens* when compared with the northern region.

Population differentiation

Exact tests of population differentiation show that 214 of 253 of mountain region comparisons were significant (Tab. 3.2.). Results of pairwise F_{ST}-values are significant for 182 of 253 comparisons ($p \leq 0.05$, Bonferroni adjusted α-value = 0.00020, Tab. 3.2).

Results of AMOVA showed that populations in different mountain regions are genetically different from each other (73.06%, $p < 0.001$) and that variance among populations within mountain regions was much lower (26.94%, $p < 0.001$). Grouping of the 23 studied regions into nine or ten (see material and methods section) major mountain ranges led to the same significant results (data not shown), showing that most differentiation is found among mountains and not within mountains or populations. When only taking the mountain ranges north of the Alps into account, the AMOVA result shows that more variation is present within mountain regions (63.78%, $p < 0.001$) than among regions (36.22%, $p < 0.001$). Analyzing only the southwestern and southern mountain regions the result is the opposite, less variation is found within regions (24.95%, $p < 0.001$) and a high proportion of variation is found among regions (75.05%, $p < 0.001$). This illustrates the genetic structure in the dataset, with low levels of genetic variance in the area north of the Alps and higher levels of variation in the south.

The Mantel test revealed a significant isolation-by-distance effect in the data set ($r = 0.151759$, $p = 0.02$). A Mantel test for only the populations north of the Alps does not show a correlation ($r = 0.038254$, $p = 0.30$), see also chapter two. In the southern populations a significant isolation-by-distance effect could be detected with $r = 0.434078$ ($p < 0.01$). Thus we can infer that the southern populations are comparatively closer to equilibrium between genetic drift and geneflow than the northern ones.

Barriers to gene flow

When analyzing the datasets to locate potential barriers to gene flow, three barriers were detected with both marker sets and we will only describe these here (Fig. 3.3). One population that is separated by a barrier is the one in the Český Kras, which is the only known location of *R. pubescens* in Bohemia. Another barrier separates the Apennine and the Corsican

population from all other populations. A third barrier that both marker sets have in common is one in the Southwestern Alps. It separates the Ligurian, the Cottic Alps, the Provence populations and one population from the French Calcareous Alps (torrent de la Sapie) from the remaining populations in the Alps and north of the Alps.

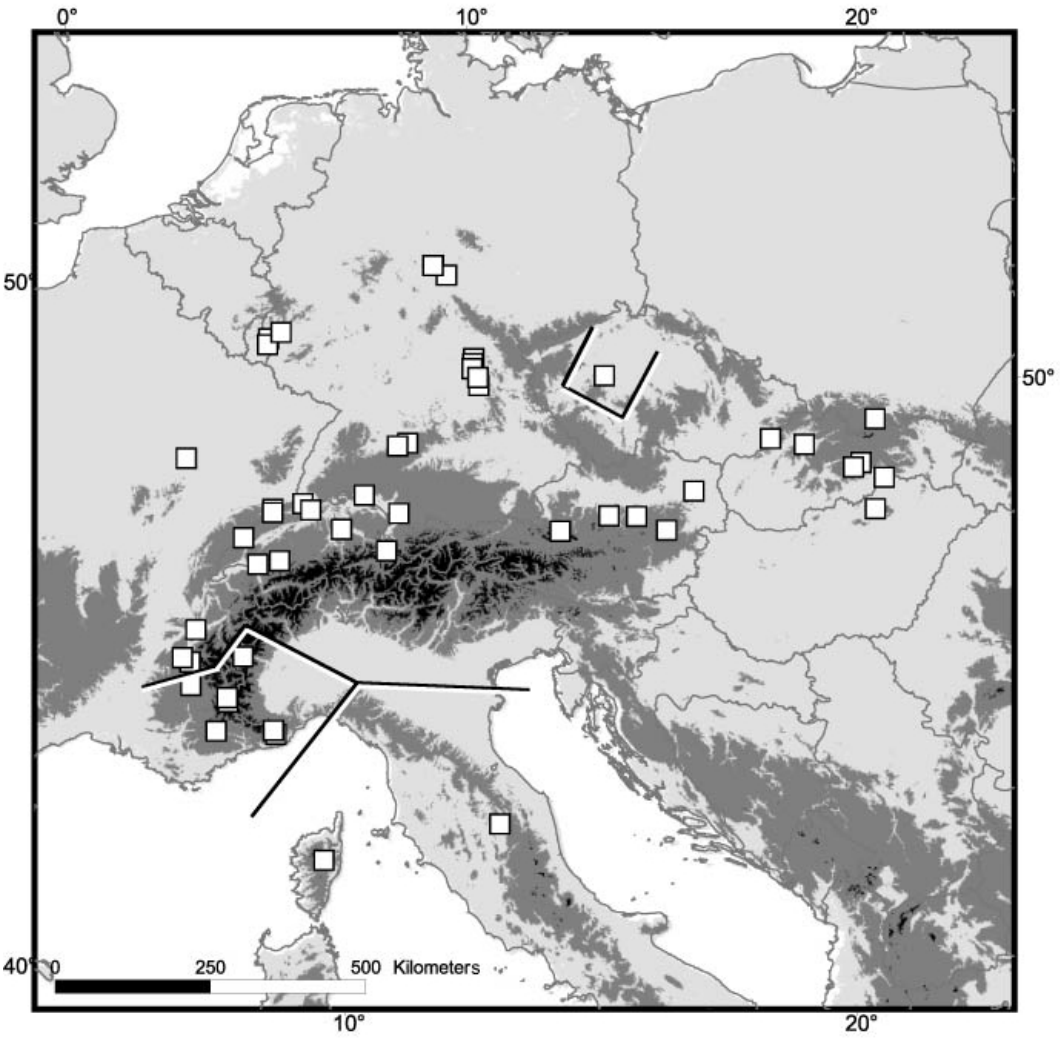

Fig. 3.3 Map of *R. pubescens´* range with sampled sites marked with white squares. Lines indicate barriers detected with both markers, mtCOi and AFLP data, by Barrier 2.2.

Demographic expansion

In the studied regions north of the Alps, all mismatch distributions are unimodal (see also chapter two), only the data from the Northern Alpine foothills, the Northern Alpine slope and from the Plateau Langrès show multimodal distribution. Unimodal distribution of pairwise differences indicates recent population growth and occurrence of nucleotide mutations (Rogers & Harpending 1992), therefore we can infer population size expansion or

demographic expansion in the northern mountain regions. In the southern mountain regions most of the mismatch distributions are bi- or multimodal. Only the Dauphiné Alps and the Apennines exhibit a unimodal distribution. This indicates that in the south most population sizes are stable and that these populations do not carry signals of recent expansion processes. Mismatch distributions for only the northern populations are unimodal, while the distribution is multimodal for the southern populations (Fig. 3.4).

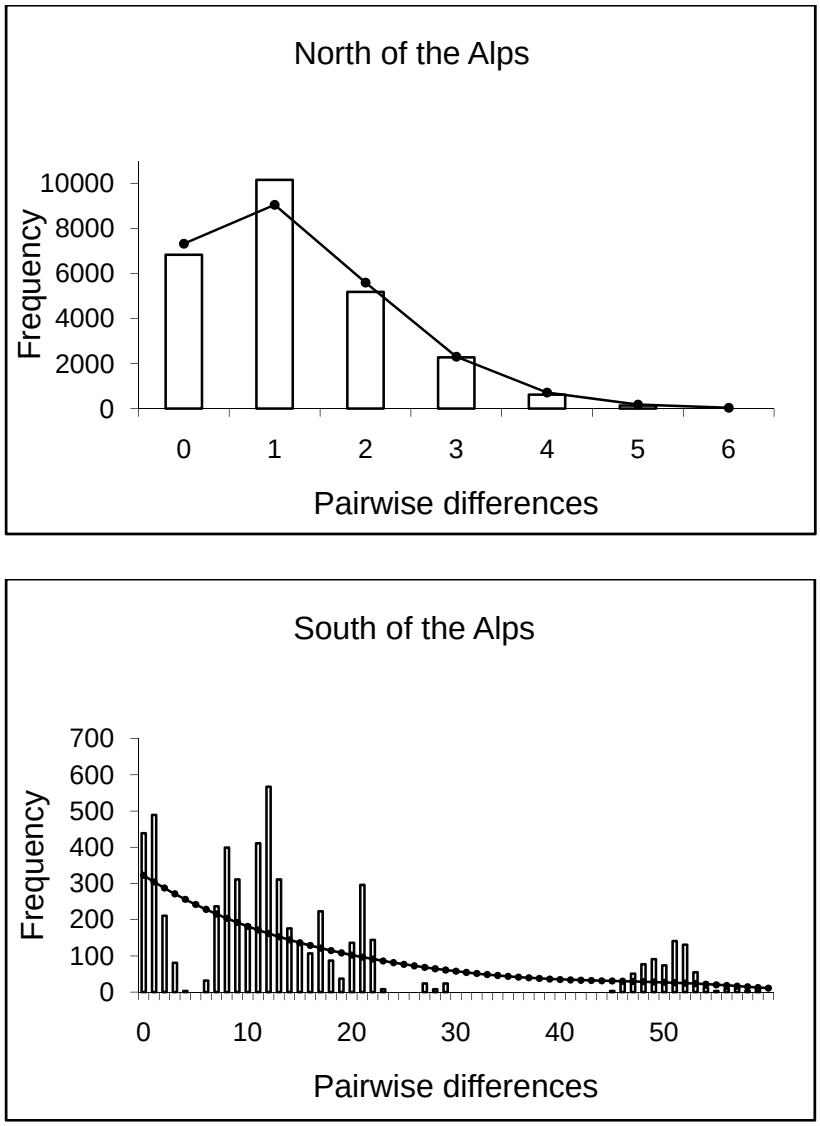

Fig. 3.4 Mismatch distributions for populations north and south of the Alps. Bars indicate observed frequencies, the dotted line indicates model frequencies.

Negative significant values for Tajima´s D were found in the Swiss Jura, the French Calcareous Alps and the whole dataset, indicating a high number of low frequency polymorphisms and potential population size expansion (Tajima 1993, 1996). Values of Fu´s F_S test were significant for the Franconian Alb and the whole dataset and highly significant

for the Swiss Jura. Thus we can infer demographic expansion especially in these mountain regions and also for the total population. Negative, but not significant values for both tests were found in the Swabian Alb, Northern Calcareous Alps, Mittelland, Slovenské Rudohorie, Northern Hungarian mountains and the Dauphiné Alps.

Migration

We used Migrate 3.0 to test the hypothesis that the refugial source for the northern populations is located in the SW Alps. We calculated a stepping stone model for the populations Provence Alps, French Calcareous Alps, Dauphiné Alps, Swiss Jura, Mittelland and Northern Alpine slope to estimate numbers of effective migrants and the direction of migration from the populations in the SW Alps to the northern populations. Both runs of Migrate 3.0 yielded similar results. Estimation of past migration between populations in the Western Alps shows that the Dauphiné Alps seem to play an important role (Fig. 3.5). Gene flow in form of effective migrants is detected from the Dauphiné Alps southwards to the French Calcareous Alps, and from these to the Provence Alps. Northwards there is gene flow from the Dauphiné Alps to the Swiss Jura, to a higher degree from the Swiss Jura to the Mittelland and from there to the Northern Alpine slope. No gene flow was detected by Migrate 3.0 from the Provence or Calcareous Alps northwards or from the Swiss Jura southwards.

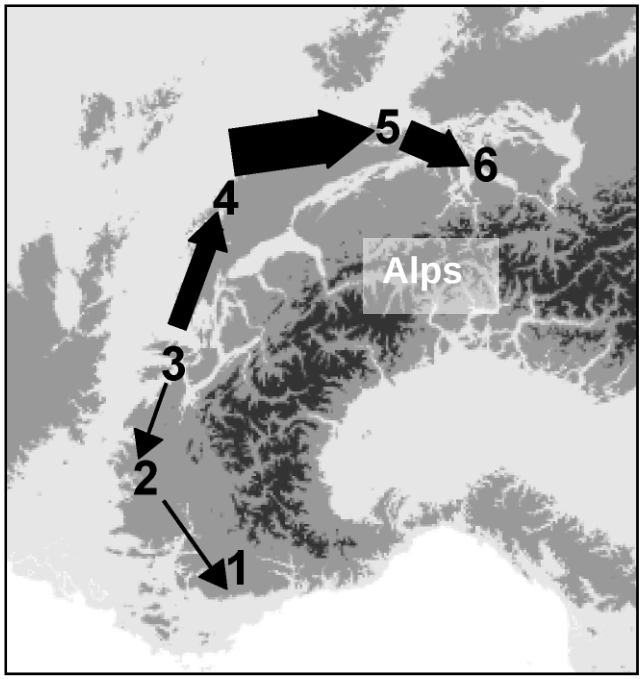

Fig. 3.5 Relative migration rate values (N_m) between each population pair for the stepping stone model for the Western Alps region. Line width is relative to number of migrants. Regions: 1: Provence Alps 2: French Calcareous Alps 3: Dauphiné Alps 4: Swiss Jura 5: Mittelland 6: Northern Alpine slope.

Results

Amplified Fragment Polymorphism: Structure of the AFLP-dataset

The final dataset comprised 132 fragments. Maximum scoring error at individual loci was 0.11, mean mismatch value per fragment over all samples was 0.05. The neighbor-joining phenogram calculated with Nei´s D shows four different clades that are supported with bootstrap values above 70 % (Fig. 3.6). There is a separate clade comprising all mountain regions in the Western Alps and north of the Alps that are obviously genetically quite similar (bootstrap value 93%). Another clade consists of Apennines and Ligurian Alps (bootstrap value 79%). The third clade comprises the regions Česky Kras, Bílé Karpaty and Corsica (bootstrap support value 100%). The similarity of these three regions conflicts with mtDNA data. A fourth subclade consists of Bílé Karpaty and Corsica (bootstrap value 100%).

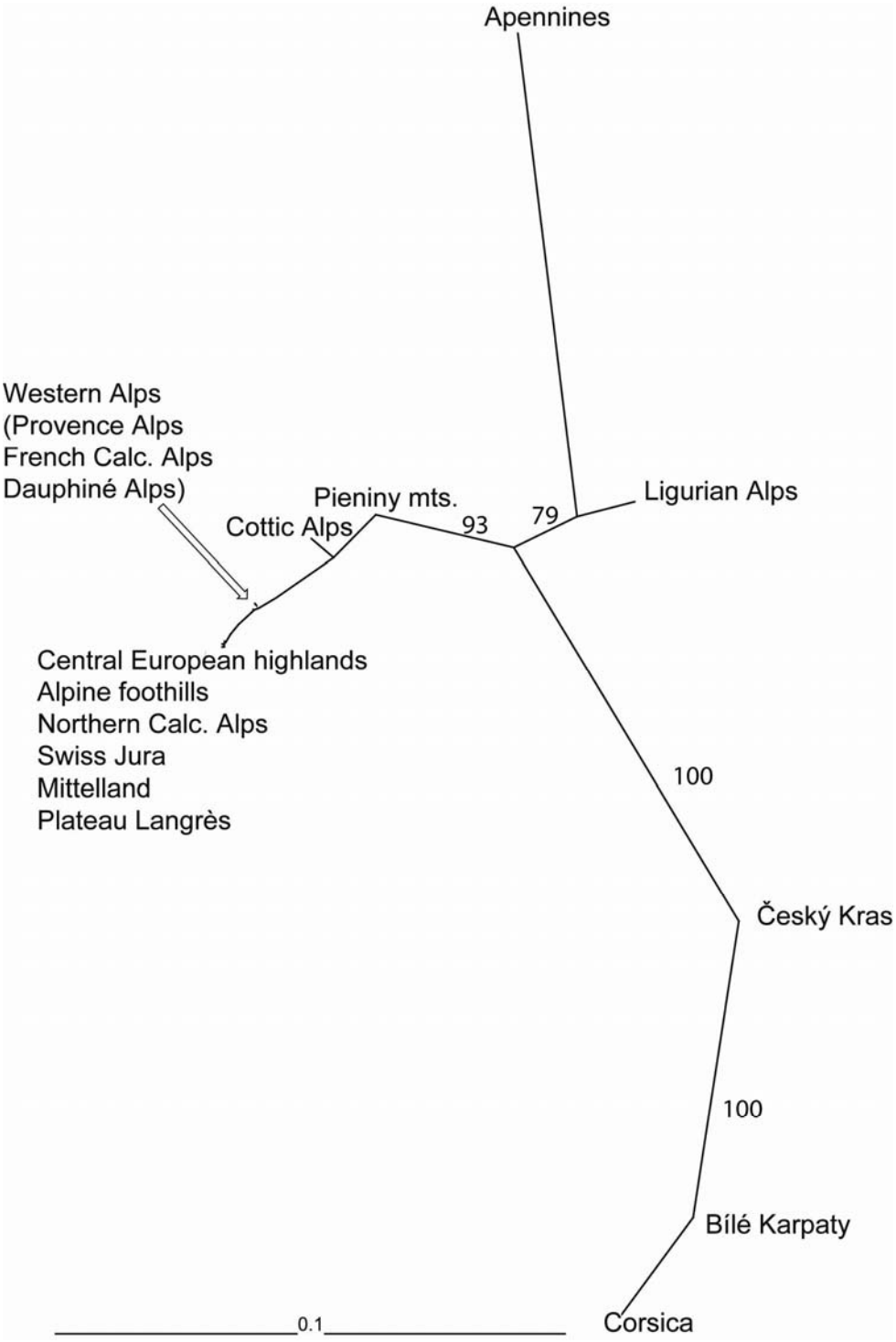

Fig. 3.6 Neighbor-joining phenogram of Nei´s *D* values for mountain regions. Numbers show bootstrap support values above 70% of 1000 bootstrap replicates.

The first axis of the PCA analysis (Fig. 3.7) explains 67.09% of the variation in the dataset, the second one explains 16.25% of the variation. There are five clearly distinct clusters.

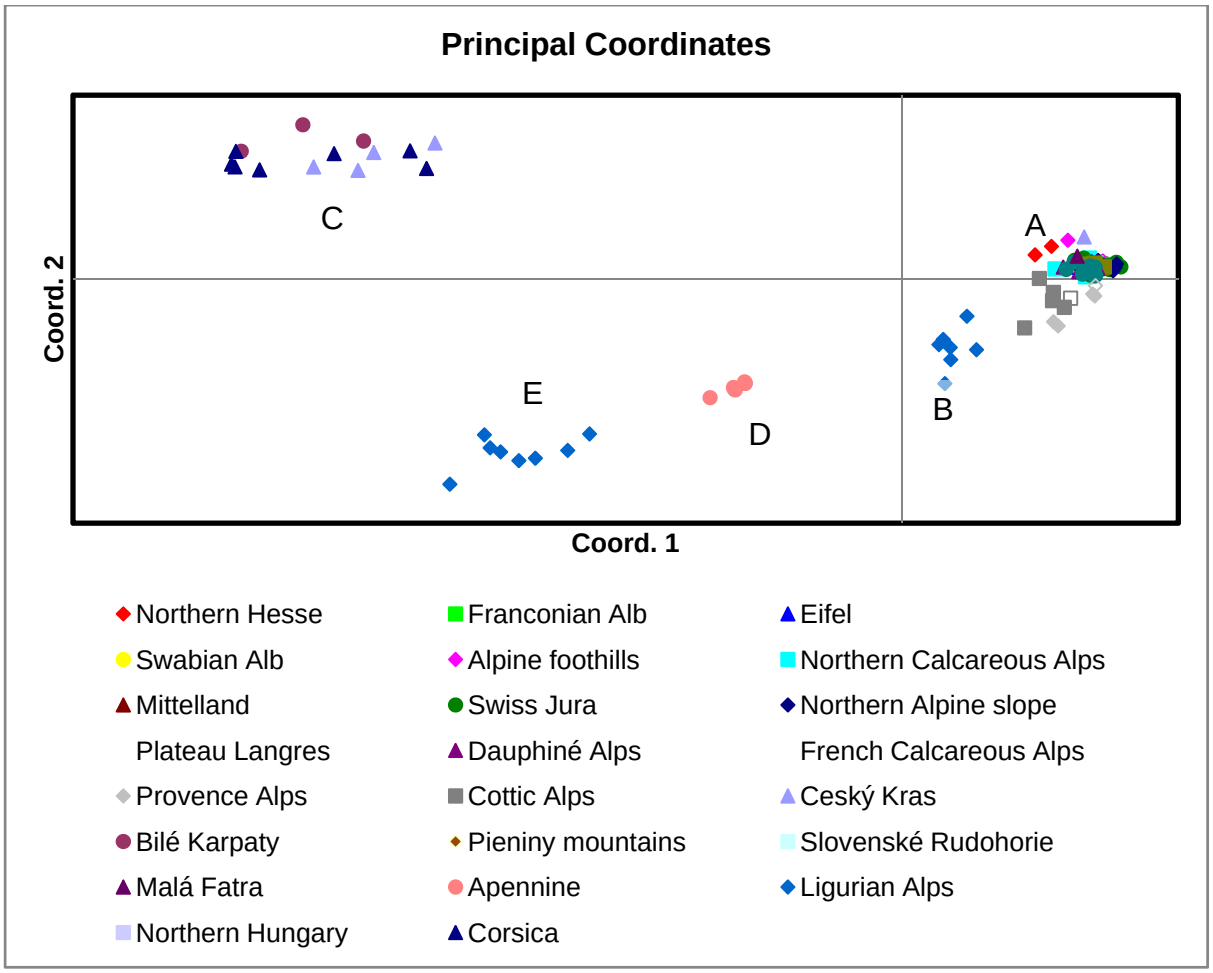

Fig. 3.7 Principal Coordinate Analysis based on squared Euclidean distances. Clusters are marked with letters.

One cluster (A) comprises the populations in the Central European highlands north of the Alps and those in the Western Alps. One population of the Ligurian Alps (B), clusters within this group but is separated from most of the samples, occurring closer to the middle. The *R. pubescens* individuals from the two Czech Republic populations cluster together with the ones from Corsica (C). As already mentioned above, this is most probable due to homoplasy of fragments in these populations. The Apennine population (D) and the second population from Liguria (E) are well separated. In terms of spread there is a clear indication that some populations, e.g. the second population of Liguria and the Corsica populations have greater variability than others, e.g. the Apennines.

Results of the AMOVA were highly significant ($p < 0.001$) showing that 42.47% accounted for variance within mountain regions and that the major part (57.53%) of molecular variance was detected among mountain regions. These results obtained from the nuclear markers support the results observed in mtDNA, namely that populations of *R. pubescens* differ more

strongly among regions than within. AMOVA of the northern populations showed that 45.72% variation is within mountain ranges and that 54.28% variation is among mountain ranges ($p < 0.001$ for both values). This is in contrast to the results of the mitochondrial marker, where a higher percentage of variance was present within mountain ranges than among. AMOVA of the southern populations showed that 46.46% variation is within mountain ranges and 53.54% variation is among mountain ranges ($p < 0.001$ for both values). The differentiation among mountain ranges is less pronounced than for mtDNA analysis of the southern populations.

Assignment tests

BAPS 3.2 analysis (Corander et al. 2004, Corander & Marttinen 2006) of the dataset resulted in five clusters (prob = 1, log (ml) value -3898) (Fig. 3.8), each colored differently. The horizontal bars are split proportionally into different colors if there is evidence for admixture. Cluster 1 (red) comprises 186 individuals, originating from all populations north of the Alps except for Bilé Karpaty and also contains individuals from Plateau Langrès, the Dauphiné Alps, the French Calcareous Alps and the Provence Alps. Cluster 2 (green) comprises 14 individuals from the Český Kras, the Bilé Karpaty and from Corsica. Cluster 3 (blue) includes all eight individuals from the Valle di Pietra stream in Liguria. In cluster 4 (yellow) there are 34 individuals from populations in the Western Alps, from the regions French Calcareous Alps, Provence Alps, Cottic Alps and Ligurian Alps. Cluster 5 (pink) comprises all individuals originating from the Apennines. In cluster 1 some individuals show that part of their genome is from ancestral sources that are represented in the four other clusters though mostly not in high proportions. Population Saint Philibert (French Calcareous Alps), Ravin de Chambières (Provence Alps), Condamine and tributary of Dora Riparia (Cottic Alps) show shared ancestry of clusters 1 (red) and 4 (yellow). This admixture is significant for one individual in the Provence Alps (Bayesian p-value 0.035), and for 3 individuals in the Dora Riparia tributary (Bayesian p-value two times 0.02, one time 0.04). One population in Liguria belonging to cluster 4 shows partial ancestry from cluster 5, although this is not significant.

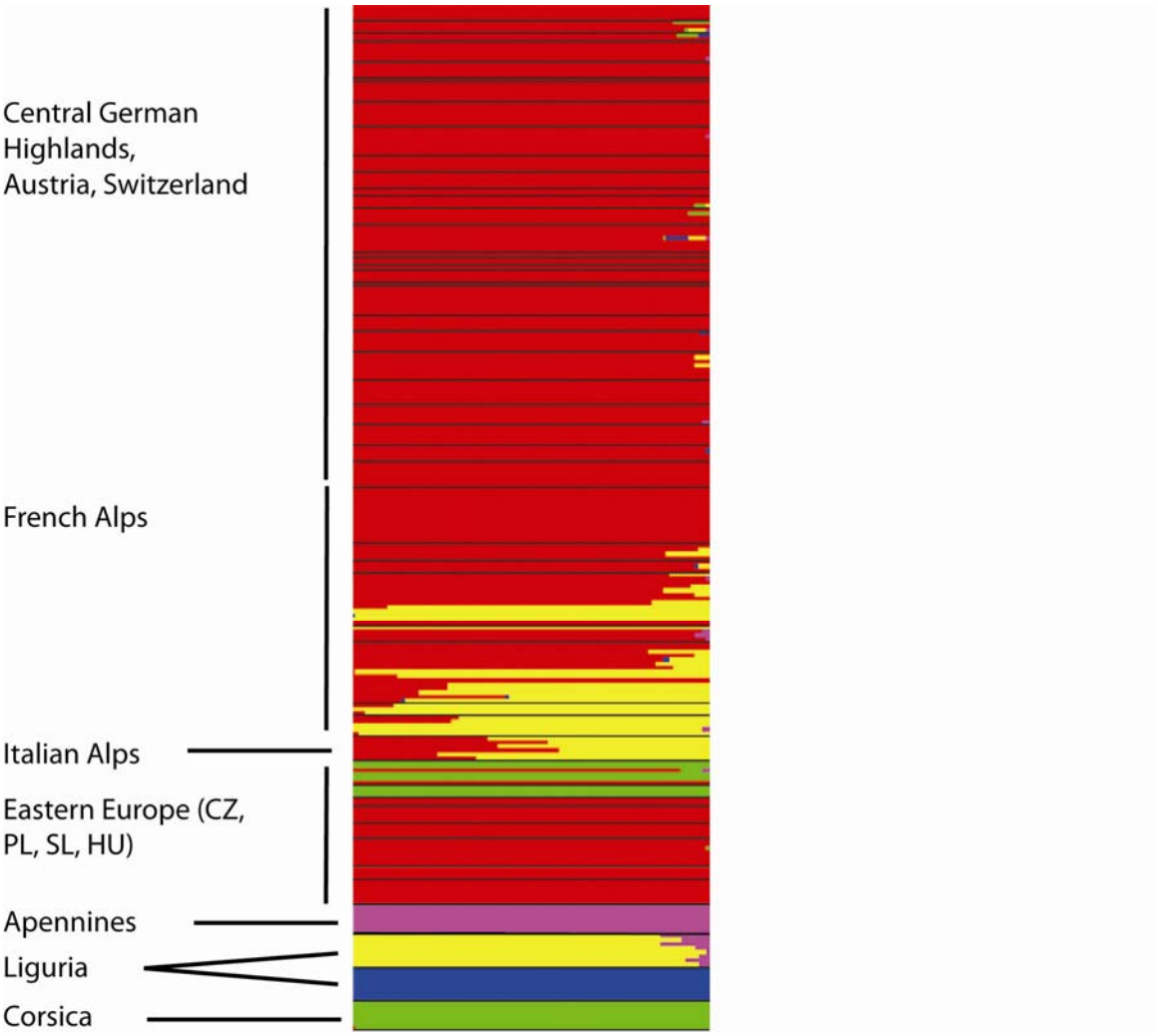

Fig. 3.8 Results of BAPS analysis with admixture based on mixture clustering. Major geographical regions corresponding to clusters are noted on the left side. Colors of clusters 1: red, 2: green, 3: blue, 4: yellow, 5: pink. Populations are separated by horizontal black lines.

Assignment analysis with Structurama yielded almost the same results as the BAPS 3.2 analysis concerning the clusters. Fig. 3.9 shows the results of Structurama analysis representing the clusters on the distribution map of *R. pubescens*.

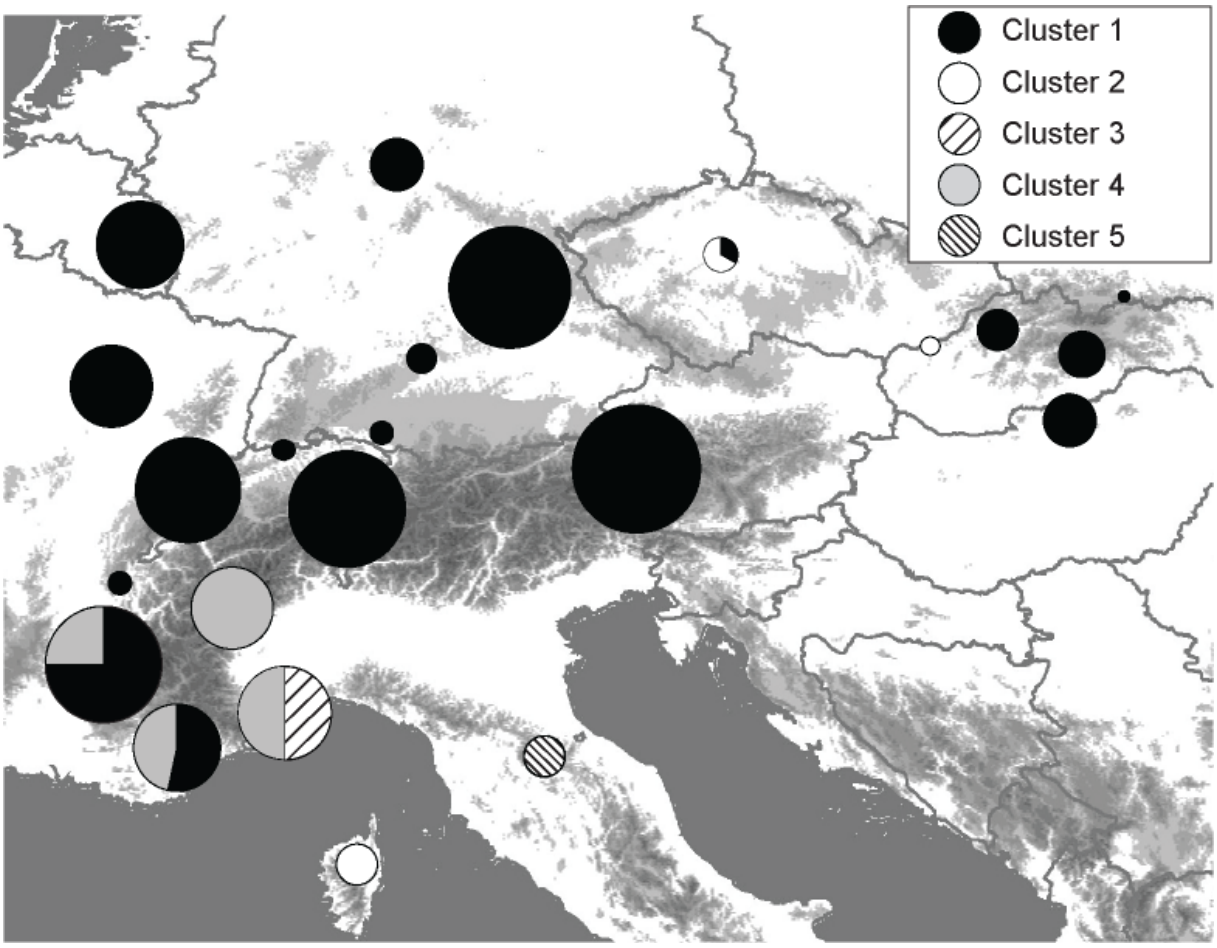

Fig. 3.9 Clusters found in the AFLP-dataset with Structurama assignment test.

5 clusters were detected that are almost identical to the clusters found by BAPS. In cluster 1 there are 187 individuals from the region north of the Alps except for Bilé Karpaty, and also individuals from the Plateau Langrès, the Dauphiné Alps, the French Calcareous Alps and the Provence Alps. Cluster 2 comprises individuals from the Bilé Karpaty, the Česky Kras and Corsica. In cluster 3 there are all individuals from the Valle di Pietra stream in Liguria. In cluster 4 there are 34 individuals from populations in the Western Alps, from the regions French Calcareous Alps, Provence Alps, Cottic Alps and Ligurian Alps, in cluster 5 there are all Apennine individuals.

Another assignment test was conducted in Structure 2.2. The summary of estimated log likelihoods for K=1 to K=10 are given in Fig. 3.10 (dots). One can see that the likelihood value begins to converge on a likelihood value around -4400, starting at K=5. At K=5 the estimated value alpha also converged to around 0.025 and remains stable for successively higher values of K (Fig. 3.10, crosses). Thus the best estimate of population subdivision is

made based on 5 groups. This value was also observed in the other assignment tests. The individuals clustered in the same way as described for BAPS.

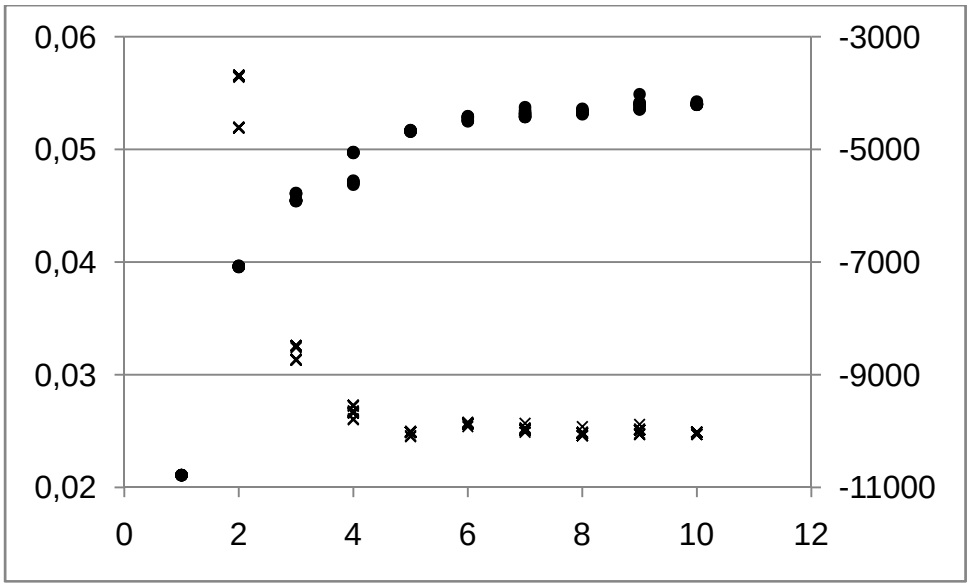

Fig. 3.10 Results of assignment test calculated in Structure 2.2. X axis: number of K. Y axis on the left: alpha values, Y axis on the right: log likelihood. Dots: estimated likelihood, crosses: estimated alpha.

As with the mtDNA, Mantel test of AFLP data demonstrates an isolation-by-distance effect in the total data set ($r = 0.226$, $p < 0.01$). Unlike the mtDNA, a Mantel test of AFLP data calculated only for the populations situated north of the Alps also showed an isolation-by-distance correlation, albeit a weaker one ($r = 0.244$, $p < 0.01$). In the southern populations the correlation was stronger ($r = 0.666$, $p < 0.01$), in concordance with the results from the mitochondrial marker used in this study.

Results of calculations for assessing gene diversity are summarized in Tab. 3.3. The percentage of polymorphic loci (95% confidence) and Nei´s gene diversities (Nei 1987) were highest in the Ligurian Alps, followed by the Česky Kras, Cottic Alps, French Calcareous Alps and the Provence Alps. Values of the Shannon Index of phenotypic diversity were high in the Western Alps, on Corsica and in the two populations of the Czech Republic. We expect high values of these estimators in populations that are older relative to others or in hybrid zones. As a measure of divergence the frequency down-weighed marker value (DW) was calculated. We found the highest value in Liguria, and high values also in the Apennine and on Corsica, and in the Czech populations. High DW-values are expected in older populations and low values in recently established populations (Schönswetter & Tribsch 2005). Shannon Index and DW values are shown by region in Fig. 3.11.

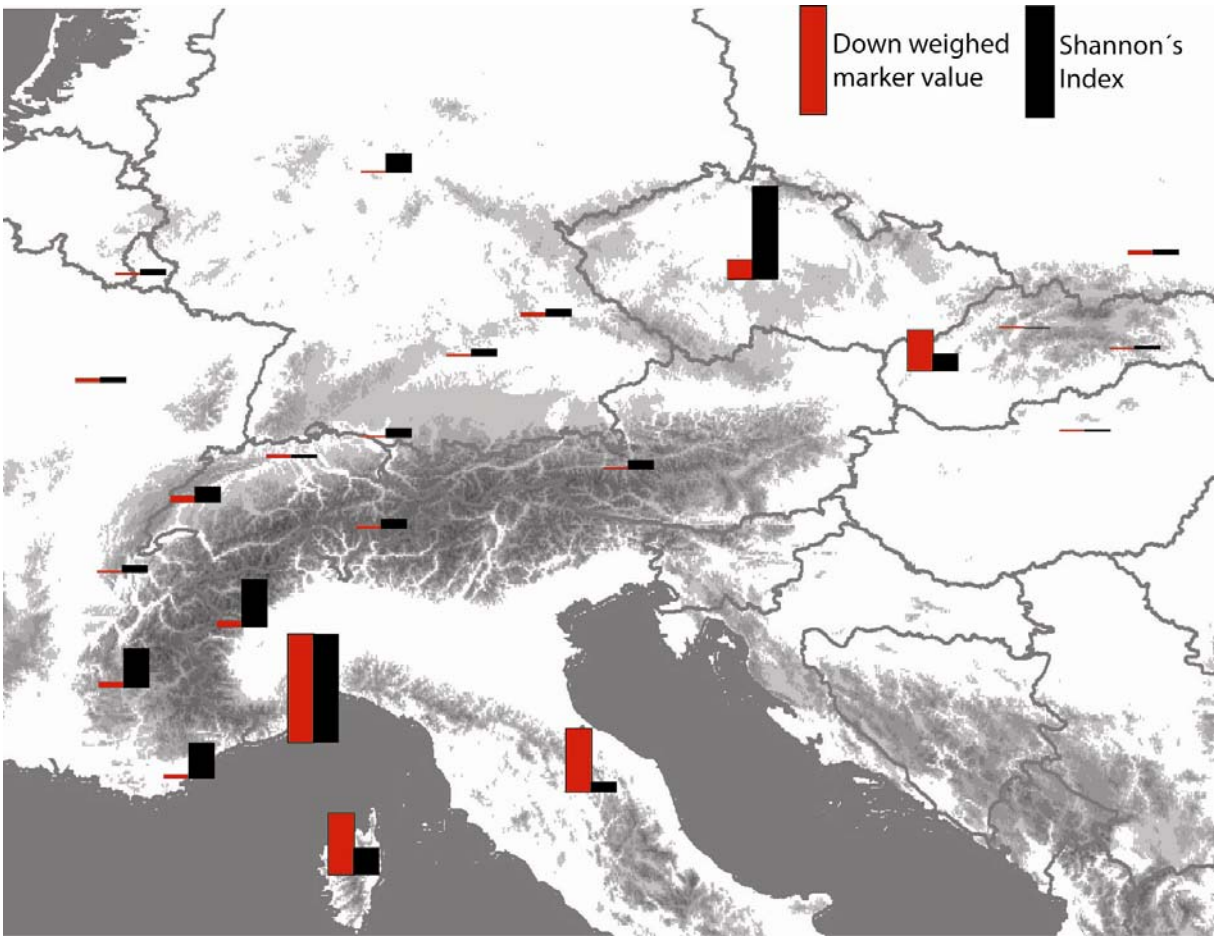

Fig. 3.11 Shannon´s index and down-weighed marker value for AFLP samples for each mountain region. Heights of bars indicate relative values compared to the highest value found for each index.

Private fragments were present in the mountain regions of the Apennines, Corsica, Liguria and the Cottic Alps and one private fragment was found in the Franconian Alb. Fixed private fragments (private fragments that occur in all individuals in the population) were found in the Apennines and on Corsica. We expect private fragments in regions that are inhabited for a long time or that are very isolated allowing single nucleotide mutations to occur that are unique to these regions.

Discussion

Population genetic structure north and south of the Alps

In chapter two of this thesis we demonstrated that mitochondrial sequence data from the cytochome oxidase 1 gene show a specific pattern of genetic population structure in *R. pubescens* specimens from north of the Alps. This pattern is characterized by a common

ancestral haplotype H1 and many endemic haplotypes that are only one mutational step apart from H1. In the present chapter we included mtCOI-data of *R. pubescens* from the Southwestern Alps, from the Apennines and from the island of Corsica. This way we investigate the population structure across the species entire distribution range to better understand its population history and thus its Pleistocene persistence and postglacial migration.

Our extended data set of mtCOI-data shows a commonality between northern and southern regions. Unique haplotypes are present in almost all mountain ranges and in single streams across the whole range of the caddisfly (Tab. 3.1).This indicates that higher genetic drift in marginal populations is not the main reason for this occurrence. We find endemic haplotypes in streams in the core regions for example in the Northern alpine slope, Northern Calcareous Alps and in the Dauphiné Alps. The streams that we sampled south of the Alps were also without exception tufa water bodies, which underlines the obligation of *R. pubescens* to this stream type. As mentioned in chapter two the species is rarely found in large numbers (Haase 1999, Engelhardt pers. observation), so it can be deduced that population size per stream is rather low. Low effective population sizes combined with low or zero gene flow between habitats can lead to genetic drift and thus change the makeup of each gene pool. Additionally separate evolution in distinct populations due to habitat specificity could cause accumulation of unique point mutations all over the species range and could be a possible explanation for the high numbers of endemic haplotypes in *R. pubescens*.

Overall haplotype divergence is much larger in the southern part of the distribution range than in the north as seen in the haplotype network. Additionally, a distance of seven mutational steps is dividing the northern circular haplogroup from the southern haplogroup. Molecular variance shown with AMOVA results of mitochondrial data is also much higher among mountains regions in the south (75.05%, $p < 0.001$) than in the north (36.22%, $p < 0.001$).

The results from the second marker system that we used, the AFLP analysis, show concordant results for the South, namely a more diverged pattern in the southern regions. However the AMOVA results of the AFLP data show slightly higher percentage attributed to variation among (54.28%, $p < 0.001$) northern mountain ranges than within (45.72%, $p < 0.001$). This could be due to the fact that variability between samples from the area north of Alps is quite low, as seen in the principal coordinates analysis.

Genetic diversity detected by AFLP analysis seems to be considerably greater in the southern regions compared to the north. This is supported by the pattern of the PCA and comparatively higher values of gene diversity indices in the Southwestern Alps, the Apennines and Corsica. Unique fragments occur in the Apennines, Corsica, Liguria and in the Cottic Alps and characterize these regions as long term stable habitats for *R. pubescens*. Both markers illustrate that genetic population structure in the older occupied regions in France and Italy is different compared to the recently colonized northern areas. The impoverishment from former refugial regions to recently colonized areas is well known from a simulation study (Ibrahim et al. 1996) and studies of temperate species (e.g. Hewitt 1999, Pinceel et al. 2005, Rowe et al. 2006). This genetic pattern is also not uncommon in European aquatic species like e.g. the bryozoan *Cristatella mucedo* (Freeland et al. 2004) that exhibits a decrease of genetic diversity in mtDNA towards Northern Europe attributed to postglacial expansion. Another aquatic species with a similar pattern is the gastropod *Theodoxus fluviatilis* (Bunje 2005), that shows low genetic diversity in mtDNA in populations in Northern Europe, which are all derived from a single ancestral haplotype, similar to the situation in *R. pubescens*. Central European aquatic insects in general, and caddisflies in particular, show very different population genetic patterns. Pauls et al. (2006), who investigated *Drusus discolor* across its range, found that the genetic structure supported the presence of several refugial zones during the Pleistocene, some of them in former periglacial area in Germany. This is clearly a different pattern than in *R. pubescens,* that can be associated with the cold tolerance of *Drusus discolor*, which allowed persistence in streams of the permafrost zone. The caddisfly *Hydropsyche tenuis* shows little differentiation across its distribution range with only nine haplotypes (Lehrian et al. 2009). A south-north haplotype gradient along a possible recolonization route starting from Italy was visible although low haplotype numbers do not allow further conclusions. In *H. tenuis* the genetic pattern seems to be more gradual or is possibly masked by a high level of gene flow between the Alps and surrounding regions.

Overall it seems convenient to use mtDNA and AFLP´s for molecular ecological studies. The combination of a neutrally evolving and maternally inherited marker with a nuclear marker allows a comprehensive data basis. Mitochondrial and nuclear markers are indicators of demographic population structure in the intermediate (mtDNA) and distant (nuDNA) past. (Zink & Barrowclough 2008). NuDNA is lagging more behind because the effective population size of mtDNA is smaller. AFLP´s have the advantage that no prior knowledge is required about the genomic sequence of the study species (Vos et al. 1995) and that a high number of fragments can be achieved in a single assay. However the disadvantage of the

AFLP method is that heterozygous fragments cannot be distinguished from homozygous ones. Our results show almost concordant evidence concerning genetic population structure and diversity. We can therefore exclude sex specific dispersal behavior for *R. pubescens* based on the nuclear DNA data. Other studies have also used mtDNA and AFLP´s , like e.g. Mock et al. 2007 in their study of genetic variation in pine beetles. They found concordant results in their datasets and also detected gender-specific AFLP markers in the study species. Another study on the Colorado potato beetle (Grapputo et al. 2005) showed that while mtDNA data were not informative for understanding population history in Europe, nuclear data supported single invasion of this continent.

Population history of *R. pubescens*

Diversity indices and down-weighed marker values derived from AFLP data indicate that the Southwestern Alps, the Apennines and Corsica have been inhabited by the study species continuously for a long time. MtDNA and AFLP results suggest isolation on the island of Corsica. In the haplotype network (Fig. 3.2) H53 from Liguria is the closest haplotype to the Corsican ones, and is 45 mutational steps apart (9.47 % of 475 bp), so long term separation of the Corsican population from the remaining mainland populations seems reasonable. If we assume a divergence rate of 2.3% per million years for mitochondrial insect DNA (Brower 1994) we can infer the separation having occurred approximately 4 million years ago. This is in line with the work of Meulenkamp & Sissingh (2003) who state that a land bridge was present between the Corsican-Sardinian microplate and the area that is now Liguria and Piedmont region before the Pliocene. Between 5 and 3 Myrs ago a flood occurred in the Mediterranean Basin (Steininger & Rögl 1984), which ended the land connection. Subsequently the Mediterranean sea acted as a barrier to migration and gene flow between these areas. This was shown by Ketmaier et al. (2006) for the land snail genus *Solatopupa*. The scenario seems reasonable for *R. pubescens* but since there are no fossils to calibrate the molecular clock we are not able to verify this hypothesis. It would be promising to test this observation with a larger dataset also comprising molecular data of other *Rhyacophila* species occurring on Corsica and the mainland.

Based on both of our data sets, we can also dismiss the Apennine as a likely potential refugial source for the northern populations. The genetic makeup of these populations differs dramatically from those of Central European populations. Instead, our data show that the location of the refugium was most probably in the western part of the Alps, not on the Italian Peninsula. This is supported by the fact that haplotype H1, which is the ancestral haplotype of

the northern populations, is not present in the Italian Peninsula or Liguria but in the French part of the Alps. Thus it seems likely that the northern edge of the distribution of *R. pubescens* during the last glacial maximum was in the region of the French Calcareous or Dauphiné Alps below the permafrost line and that northward expansion started from there. The Migrate-N results indicate that the Dauphiné Alps are the only region from which there was migration northwards and southwards in the Western Alps. The northward migration presumably coincided with gradual climate warming after the last glacial maximum. It would seem plausible, that the French and Swiss Jura were recolonized first, as the glacial retreat was slower in the higher regions of the main alpine ridge. Gene flow and migration rates indicate a recolonization route along the Western Alps to Switzerland and then to Central European highlands and to Northern Hungary that represents the most eastern recent population. Recolonization from a refugium in the southwestern Alps seems plausible since potential peripheral refugia with calcareous bedrock have also been inferred for mountain plants (Schönswetter et al. 2005). A southwestern Alps refugium and subsequent recolonization from there was shown for the plant *Eryngium alpinum* (Naciri & Gaudeul 2007) that also exhibits a strong binding to calcareous substrate like our study species. A recolonization route from the southwestern Alps northwards along the Western Alps was also inferred for the butterfly *Polyommatus coridon* in an allozyme study (Schmitt & Krauss 2004).
Interestingly a glacial tongue was present in the area of Gap (Schönswetter et al. 2005) during the last glacial maximum which could have caused a period of separation between Liguria/Provence populations and the French Calcareous Alps/Dauphiné Alps populations. This is in concordance with the barrier to geneflow that we detected with Barrier 2.2 with both markers in this area. When this glacial tongue retreated gene flow would have been possible again between the French Calcareous Alps/Dauphiné Alps and the Provence Alps. This scenario is concordant with the results of our Migrate-N analysis and would explain why we find both the "northern" haplotype H1 and southern haplotypes H37, H38 in the Provence Alps. Based on the haplotype distribution and Migrate results we can thus infer a secondary contact zone for *R. pubescens* in the Provence Alps.

Results from both mitochondrial and nuclear DNA of *R. pubescens* throughout its range support a scenario of rapid postglacial expansion from the refugial zone to the former periglacial area north of the Alps. Concerning the mtDNA data, a starlike network structure like in Fig. 3.1 is expected in species that expanded recently in size from a small number of founders (Avise 2000). This is supported by demographic and/or population size expansion processes detected by the distribution of mismatches in the northern *R. pubescens*

populations. The fact that haplotype divergence in the north is very low, only up to two mutational steps, is also an indicator of recent and rapid recolonization. These findings also imply that recolonization took place once and was not characterized by several waves of expansion. Assignment tests show that the largest cluster in the data set consists of individuals north of the Alps and also of some from the French Calcareous Alps, the Provence and Cottic Alps. This also illustrates relatedness of these specimens and supports the hypothesis of glacial recolonization from southwest to northeast.

Today low gene flow between population and differentiation at the stream level are the main processes shaping genetic population structure of *R. pubescens*. Almost all populations across the range possess unique haplotypes. An especially separate entity is the population of the Česky Kras, as shown by Barrier results, that seems to be reproductively quite isolated. This is plausible because it is the only known location in Bohemia where *R. pubescens* lives today. Populations that served as stepping stones during colonization of this area have probably died out. The Česky Kras is the only bigger karst area in the Czech Republic apart from the Moravský Kras and several small isolated karst areas. While mtDNA data show recently low gene flow between populations of *R. pubescens*, AFLP data show highly similar profiles for the individuals sampled north of the Alps. Most probably the period since recolonization of this area was too short for development of detectable differences in the nuclear DNA. This way our scenario of postglacial expansion after the last ice age was supported. As mentioned above nuclear markers can help to understand processes in the distant past, and served well in studying diversity in the southern regions. To overcome the fact that AFLP profiles in the north are very similar, it would be advisable to use additional primer pairs to obtain a higher number of fragments for analysis.

Conclusions

Our mitochondrial (COI sequences) and nuclear data (AFLP´s) show concordant results concerning intraspecific genetic structure in the caddisfly *R. pubescens* across Central Europe, Italy and Corsica. We provide the first example of a Central European aquatic insect that started postglacial recolonization from a southwestern alpine refugium along the western edge of the Alps to the former periglacial area north of the Alps. Comparable rangewide European studies using mitochondrial data of caddisflies (Pauls et al. 2006, Lehrian et al. 2009) show different genetic patterns than *R. pubescens* and highlight the need of more studies of benthic invertebrates to possibly detect general phylogeographic patterns in these species.

Tab. 3.1 Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Northern Hessian mountains (HE)	DE	5/3	Flachsbach above Wendershausen	51.30167	9.88778	Engelhardt & Hövelborn	H1(5)	
		7/4	Gatterbach above Wanfried	51.18306	10.22639	Engelhardt & Hövelborn	H2(7)	
		2/2	Griesbach	51.30278	9.87583	Engelhardt & Hövelborn	H1(2)	0
Franconian Alb (FRA)	DE	6/5	Burglesauer Bächlein above Burglesau	49.99611	11.08722	Engelhardt	H1(6)	
		8/4	Tributary Ellerbach above Tiefenellern	49.91667	11.07972	Engelhardt	H1(5), H3(3)	
		7/6	Brook below Tiefenhöchstädt	49.84111	11.07611	Engelhardt	H1(3), H4(3), H5(1)	
		7/1	Rüsselbach at Kirchrüsselbach	49.60139	11.27167	Engelhardt	H1(4), H6(2), H7(1)	
		8/5	Hundshauptener Bach below Hundshaupten	49.72139	11.23028	Engelhardt	H1(5), H2(3)	4

Tab. 3.1 Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Swabian Alb (SWA)	DE	3/3	Attenriedbach , Geislingen	48.62139	9.81639	Mayer	H1(2), H2(1)	
		8/2	Fils above Wiesensteig	48.55944	9.59889	Engelhardt & Schlünder	H1(8)	0
Eifel (EI)	DE	8/7	Hygropetric, Tränenlay	49.85500	6.32361	Engelhardt, Pauls & Neu	H1(8)	
	LU	5/4	Spring near Haalerbach	49.76667	6.31667	Graf	H2(5)	
	LU	4/4	Walpengraben near Metterich	49.98222	6.58111	Balint & Neu	H1(2), H2(1), H58(1)	1
Northern Calcareous Alps (NCA)	AT	8/7	Brook near Möggers	47.56167	9.81694	Graf	H1(8)	
		1/1	Bertaquelle, Hollensteingraben	47.66778	15.76139	Graf	H1(1)	
		2/2	Schreiberbach, Wiener Wald	48.27417	16.33444	Graf & Pauls	H1(2)	
		9/7	Mayrgraben, Lunz	47.85000	15.08333	Malicky	H1(9)	

Tab. 3.1 (continued) Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Northern Calcareous Alps (NCA)		1/1	Weißenbach, Reichraming	47.83111	14.46139	Graf	H1(1)	
		3/3	Teufelsgraben	47.54528	13.41944	Pauls & Theissinger	H29(2), H30(1)	
		1/1	Bach above Dygrub	47.55139	13.41389	Engelhardt	H1(1)	2
Alpine foothills (AFO)	DE	6/4	Mühltalbach above Möggingen	47.76250	9.00806	Sundermann	H8(4), H9(2)	2
Mittelland (ML)	CH	6/4	Talbach above Pratteln	47.50528	7.68611	Engelhardt & Lehrian	H1(2), H10(2), H11(1), H12(1)	1
Swiss Jura (JU)	CH	8/7	La Motte above Ocourt	47.35000	7.05667	Engelhardt & Lehrian	H1(3), H13(2), H14(1), H24(1), H59(1)	
		8/5	Dénériax, Noirvaux	46.85722	6.51722	Engelhardt & Lehrian	H1(3), H10(4), H18(1)	
		8/6	Brook above Soubey	47.30250	7.05861	Engelhardt & Lehrian	H1(6), H21(1), H25(1)	

Tab. 3.1 (continued) Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Swiss Jura (JU)		1/0	Chrintelbachquellen	47.43083	7.88361	Pauls	H1(1)	6
Northern Alpine slope (NAS)	CH	5/5	Nameless brook, Bächenmoos	47.20861	8.61306	Vicentini	H1(5)	
		6/5	Nameless brook, Prantin	46.49694	6.92417	Engelhardt & Lehrian	H1(2) H19(4)	
		4/4	Warmbach above Weissenbach	46.60056	7.37833	Engelhardt & Lehrian	H1(3), H20(1)	
		8/6	brook near Fanas	46.98139	9.66111	Lubini	H1(7), H22(1)	3
Pieniny mountains (PIE)	PL	2/2	Pieninski Potok	49.41611	20.39889	Szczesny	H1(2)	0
Bílé Karpaty mountains (BK)	CZ	3/3	Tributary of Kloboucký Potok	49.10250	18.01833	Chvojka	H27(3)	1

Tab. 3.1 (continued) Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Český Kras (CK)	CZ	6/6	Císařská rokle SW of Srbsko	49.91806	14.13333	Engelhardt & Schlünder	H26(6)	1
Malá Fatra (MFA)	SK	8/7	Valcansky Potok, Martin	49.02278	18.78389	Engelhardt & Bieber	H1(8)	0
Slovenské Rudohorie (SLR)	SK	8/4	Biele Vody, Murán	48.76000	20.07694	Engelhardt, Blanár & Trebulová	H1(6), H4(1), H28(1)	
		7/4	Potok Kamenárka, Tisovec	48.69028	19.91111	Engelhardt, Blanár & Trebulová	H15(6), H23(1)	3
Northern Hungarian mountains (HU)	HU	6/6	Tributary, Menes Völgy, Aggtelek	48.54083	20.59806	Engelhardt & Bieber	H2(4), H16(2)	
		6/3	Ban, Bükk mountains	48.06750	20.39444	Kiss	H1(5), H17(1)	2
Plateau de Langrès (PLA)	FR	16/14	Cascade d´Etuf	47.87500	4.96528	Engelhardt & Kind	H1(2),H13(1), H31(11), H32(2)	2

Tab. 3.1 (continued) Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Dauphiné-Alps (DA)	FR	12/4	Nameless brook near Les Miards	44.88722	5.85167	Engelhardt & Kind	H1(1), H13(8), H33(1), H34(2)	2
French Calcareous Alps (FCA)	FR	7/4	Lalley	44.92361	5.67472	Engelhardt & Kind	H1(4), H2(2), H12(1)	
		4/3	Torrent de la Sapie	44.53833	5.95083	Engelhardt & Kind	H1(1), H35(2), H36(1)	
		17/13	Saint-Philibert, Grande Chartreuse	45.37972	5.84917	Balint	H1(13), H2(2), H56(2)	2
Cottic Alps (CA)	FR	5/5	Jausiers	44.39000	6.77600	Balint	H35(4), H52(1)	
	FR	5/3	La Condamine-Châtelard	44.45100	6.74100	Balint	H35(5)	
	IT	6/6	Tributary of Dora Riparia	45.10000	6.93333	Engelhardt & Kind	H45(2), H46(4)	2
Provence Alps (PA)	FR	18/15	Ravin de Chambiéres	43.93278	6.63694	Engelhardt & Kind	H1(3), H37(4), H38(10), H39(1)	3

Tab. 3.1 (continued) Sampling sites of *R. pubescens*, listed by mountain ranges, separated by horizontal lines. Country codes according to ISO 3166.

Mountain region	**Country**	**Number of individuals for mtCOI/AFLP**	**Stream name, locality**	**Latitude (°N)**	**Longitude (°E)**	**Collector**	**Haplotypes**	**Nr. of endemic haplotypes/ mountain region**
Ligurian Alps (LA)	IT	12/8	Nameless brook near Rezzo	44.02583	7.86667	Engelhardt & Kind	H46(3), H47(1), H48(1), H49(1), H50(1), H51(2), H53(1), H54(1),H57(1)	
		8/8	Valle di Pietra	44.07722	7.80639	Delmastro	H55(8)	9
Apennines (APP)	IT	7/7	Tributary of Fiume Tescio	43.09722	12.67556	Engelhardt & Lehrian	H42(1), H43(3), H44(3)	3
Corsica (COR)	FR	7/7	Tributary of Tavignano	42.25639	9.20583	Engelhardt & Kind	H40(3), H41(4)	2

Tab. 3.2 Results of exact tests of population differentiation (Raymond & Rousset 1995) are shown above diagonal, significant values indicated by +. Below diagonal are results of pairwise $F_{ST,}$ bold print marks significant (Bonferroni adjusted α-value = 0.00020) values.

	1 HE	2 FRA	3 SWA	4 EI	5 NCA	6 AFO	7 ML	8 JU	9 NAS	10 PIE	11 BK	12 CK	13 MFA
1 HE		+	+	-	+	+	+	+	+	-	+	+	+
2 FRA	**0.198**		-	-	+	+	+	+	+	-	+	+	-
3 SWA	0.262	-0.028		-	-	+	+	-	+	-	+	+	-
4 EI	-0.022	0.090	0.081		+	+	+	+	+	-	+	+	-
5 NCA	**0.389**	**0.042**	0.025	**0.226**		+	+	+	+	-	+	+	-
6 AFO	**0.514**	**0.408**	**0.513**	**0.458**	**0.549**		+	+	+	-	+	+	+
7 ML	**0.314**	0.136	0.171	0.220	0.193	**0.320**		-	+	-	-	+	+
8 JU	**0.220**	**0.051**	0.004	**0.131**	0.034	**0.316**	-0.004		+	-	+	+	-
9 NAS	0.067	**0.352**	**0.330**	**0.153**	**0.453**	**0.474**	**0.356**	**0.353**		+	+	+	+
10 PIE	0.176	-0.292	-0.325	-0.057	-0.273	0.226	-0.200	-0.287	0.213		-	+	-
11 BK	**0.712**	**0.582**	**0.866**	**0.656**	0.777	0.642	0.526	**0.467**	**0.566**	1.000		+	+
12 CK	**0.756**	**0.615**	**0.891**	**0.701**	**0.800**	**0.733**	**0.640**	**0.520**	**0.611**	1.000	1.000		+
13 MFA	0.381	-0.033	-0.032	0.176	-0.014	**0.525**	0.158	-0.025	**0.375**	0.000	1.000	**1.000**	
14 SLR	**0.366**	**0.216**	0.235	**0.292**	**0.291**	**0.410**	0.219	**0.181**	**0.418**	-0.018	**0.567**	**0.626**	0.216
15 HU	0.026	**0.106**	0.070	0.005	**0.202**	**0.326**	0.130	**0.119**	0.134	-0.164	**0.472**	**0.459**	0.109
16 PLA	**0.459**	**0.580**	**0.539**	**0.486**	**0.634**	**0.521**	**0.481**	**0.508**	**0.426**	0.390	**0.586**	**0.641**	**0.540**
17 DA	**0.533**	**0.442**	**0.505**	**0.485**	**0.548**	**0.525**	**0.396**	**0.315**	**0.507**	0.310	**0.660**	**0.716**	**0.502**
18 FCA	0.041	0.037	-0.008	0.015	**0.065**	0.196	0.036	**0.061**	**0.169**	-0.265	0.277	0.344	-0.009
19 CA	**0.785**	**0.845**	**0.789**	**0.799**	**0.846**	**0.750**	**0.740**	**0.813**	**0.788**	0.695	**0.737**	**0.774**	**0.774**
20 PA	**0.625**	**0.722**	**0.636**	**0.646**	**0.722**	**0.600**	**0.579**	**0.678**	**0.637**	0.500	**0.587**	**0.638**	**0.617**
21 LA	**0.550**	**0.675**	**0.545**	**0.577**	**0.642**	**0.500**	**0.481**	**0.625**	**0.592**	0.383	**0.457**	**0.518**	**0.519**
22 APP	**0.956**	**0.955**	**0.968**	**0.955**	**0.972**	**0.938**	**0.934**	**0.938**	**0.930**	0.943	0.953	**0.965**	**0.969**
23 COR	**0.982**	**0.984**	**0.985**	**0.982**	**0.989**	**0.973**	**0.971**	**0.977**	**0.975**	0.972	**0.976**	**0.982**	**0.985**

Tab. 3.2 (continued) Results of exact tests of population differentiation (Raymond & Rousset 1995) are shown above diagonal, significant values indicated by +. Below diagonal are results of pairwise $F_{ST,}$ bold print marks significant (Bonferroni adjusted α-value = 0.00020) values.

	14 SLR	15 HU	16 PLA	17 DA	18 FCA	19 CA	20 PA	21 LA	22 APP	23 COR
1 HE	+	-	+	+	-	+	+	+	+	+
2 FRA	+	+	+	+	-	+	+	+	+	+
3 SWA	+	-	+	+	-	+	+	+	+	+
4 EI	+	-	+	+	-	+	+	+	+	+
5 NCA	+	+	+	+	+	+	+	+	+	+
6 AFO	+	+	+	+	+	+	+	+	+	+
7 ML	+	+	+	+	+	+	+	+	+	+
8 JU	+	+	+	+	+	+	+	+	+	+
9 NAS	+	+	+	+	+	+	+	+	+	+
10 PIE	-	-	-	-	-	+	-	-	+	+
11 BK	+	+	+	+	+	+	+	+	+	+
12 CK	+	+	+	+	+	+	+	+	+	+
13 MFA	+	+	+	+	-	+	+	+	+	+
14 SLR		+	+	+	+	+	+	+	+	+
15 HU	**0.231**		+	+	-	+	+	+	+	+
16 PLA	**0.551**	**0.422**		+	+	+	+	+	+	+
17 DA	**0.455**	**0.385**	**0.394**		+	+	+	+	+	+
18 FCA	0.133	0.021	**0.388**	**0.258**		+	+	+	+	+
19 CA	**0.794**	**0.759**	**0.764**	**0.789**	**0.735**		+	+	+	+
20 PA	**0.653**	**0.596**	**0.609**	**0.654**	**0.582**	**0.570**		+	+	+
21 LA	**0.578**	**0.532**	**0.552**	**0.565**	**0.582**	**0.328**	**0.355**		+	+
22 APP	**0.944**	**0.929**	**0.907**	**0.944**	**0.886**	**0.800**	**0.770**	**0.592**		+
23 COR	**0.978**	**0.972**	**0.966**	**0.978**	**0.960**	**0.932**	**0.930**	**0.814**	**0.975**	

Tab. 3.3 Gene diversity estimators of *R. pubescens* in mountain ranges across the range detected by AFLP´s.

	1 HE	2 FRA	3 SWA	4 EI	5 NCA	6 AFO	7 ML	8 JU	9 NAS	10 PIE	11 BK	12 CK	13 MFA
Prop. of polymorphic loci	0,14	0,05	0,05	0,04	0,11	0,05	0,02	0,11	0,09	0,02	0,08	0,48	0,01
Nei´s gene diversity H	0,043	0,015	0,020	0,011	0,017	0,028	0,008	0,034	0,017	0,023	0,056	0,233	0,002
DW-value from means	57.98	98.40	46.91	45.31	53.41	59.22	77.59	178.34	77.20	103.11	943.30	457.82	39.12

Tab. 3.3 (continued) Gene diversity estimators of *R. pubescens* in mountain ranges across the range detected by AFLP´s.

	14 SLR	15 HU	16 PLA	17 DA	18 FCA	19 CA	20 PA	21 LA	22 APP	23 COR
Prop. of polymorphic loci	0,02	0,02	0,05	0,04	0,30	0,30	0,21	0,58	0,05	0,15
Nei´s gene diversity H	0,007	0,005	0,009	0,020	0,078	0,104	0,076	0,241	0,026	0,063
DW-value from means	39.74	40.19	110.81	39.65	144.69	151.61	93.13	2504.97	1484.31	1423.44

Summary and conclusions

Caddisflies (Trichoptera) are an ecologically diverse group of aquatic insects that exhibit a variety of feeding types, habitat requirements and life cycles (Mackay & Wiggins 1979). Some species are, for example, adapted to streams in mountain ranges with peaks above 800 m asl (Haase 1999). These montane caddisflies are not distributed evenly in their distribution ranges but live on “habitat islands”. This insular distribution pattern was investigated in three range wide molecular studies (Pauls et al. 2006, Bálint 2008, Lehrian et al. 2009) in Central Europe, showing different genetic patterns in the study organisms. Fragmentation due to restriction to certain altitudes seems to affect each species differently. Proceeding from the results that habitat specificity influences the genetic population structure of species, the caddisfly *Rhyacophila pubescens* was chosen to study fragmentation by a geological factor. This species is strictly bound to tufa streams in calcareous mountains (Haase 1999, personal observation). Populations are therefore separated by regions of unsuitable habitat, such as lowlands or regions with different geology. This thesis represents the first investigation of an aquatic insect bound to limestone across its entire distribution range using molecular methods. The main question of interest was, how the geographical distribution pattern affects the genetic pattern, and what could be inferred with respect to genetic differentiation and gene flow. Furthermore the results were used to deduce the population history of the species. The phylogeographic pattern is supposed to indicate whether the species survived the ice ages in a Southern European refugium or stayed in the periglacial area north of the Alps.

First of all it is essential to reveal whether there are cryptic species among the specimens collected from the entire distribution range. To solve this question, sequences from three gene regions, two mitochondrial (mtCOI, mtLSU) and one nuclear (nuWG), were used to estimate phylogenies for *R. pubescens* and a subset of species from the *R. tristis*-group (Schmid 1970). The phylogenetic study revealed that *Rhyacophila* specimens collected from the former periglacial area north of the Alps, from the Western Alps and the Apennines and from Corsica are all derived from the same ancestor. This was significant for all three used methods: a neighbor-joining algorithm and two character-based methods (Maximum Parsimony, Bayesian/MCMC). According to the monophyletic species concept (De Queiroz 2007) this allows us to regard *R. pubescens* as a true species based on molecular data. Additionally there were no conspicuous morphological features in individuals originating from different regions of the range when determining the specimens for this study. The monophyly of the *R. tristis*-

group in relation to the outgroup species, two *Rhyacophila* of the *Pararhyacophila* larva type (Döhler 1950), is significantly supported with the Maximum Parsimony and the Bayesian/MCMC method. *R. tristis* and *R. aquitanica* were shown to be the sister taxa of *R. pubescens*. The taxonomic position of *R. producta*, a Carinthian endemic, remains uncertain and needs to be evaluated using species from other *Rhyacophila* larval types. This would possibly allow classification of this species and reveal its evolutionary history. Both Bulgarian Carpathian endemics, *R. margaritae* and *R. obtusa* cluster together, which indicates descent from a common ancestor. By investigating the phylogenetics of a subset of the *R. tristis*-group, it became evident that the chosen markers are suitable to study relatedness on the genus level. It would be profitable to complete the dataset with the remaining species of the group in the future, as a basis for a comprehensive molecular phylogeny of Rhyacophilidae, a much needed if daunting effort in Trichoptera research. To date, the only published phylogenetic literature on Rhyacophilidae is a spermatological analysis of Friedlander (1993) and the work of Schmid (1970) which is not based molecular data. Rhyacophilidae are an especially interesting group, as they are basal within the Trichoptera (Ross 1967, Kjer 2001, 2002). Mackay and Wiggins (1979) state that Rhyacophilidae show similarities concerning use of silk and feeding behavior with ancestral Trichoptera species. It would thus be beneficial to examine this family more closely, taking behavioral, morphological and genetic characteristics into account, to better understand evolution of Trichoptera in general, and how they split from Lepidoptera.

Based on the findings that *R. pubescens* can be considered a true species, it is possible to investigate the intraspecific population structure. In chapter 2 the genetic population structure was examined in detail for the populations north of the Alps using sequences of mtCOI. All of these populations are now in areas that were permafrost regions during the last glacial maximum (Hewitt 1999, 2004). Molecular data from mtCOI reveal that one common haplotype and many endemic haplotypes are present throughout this area. The shallow structure of the haplotype network with one or two mutational steps connecting all haplotypes to the widespread, common, and putative ancestral haplotype, suggests that *R. pubescens* did persist the last glacial maximum in Central Europe. We would expect a more diverged haplotype network if *R. pubescens* had sustained in Central European highland refugia, as demonstrated for the caddisfly *Drusus discolor* (Pauls et al. 2006).

Differentiation in *R. pubescens* populations as indicated by unique haplotypes is remarkable in the Central European highlands. Even streams that are only few kilometers apart show different haplotype composition for example in the Franconian Alb or in the Swiss Jura. The

lacking isolation-by-distance pattern in the study area could be another indicator that dispersal between streams is very limited and therefore gene flow between populations is low. We lack precise empirical data about dispersal distances of *R. pubescens* but it was shown for other aquatic insects that they tend to stay close to the stream where they hatched and most probably lay their eggs in the same habitat. Petersen et al. (2004) found that caddisflies, including *Rhyacophila dorsalis*, travelled distances well below 100 m inland and mostly stayed close to the water course. Sode and Wiberg-Larsen (1993) found that females of *Rhyacophila fasciata* dispersed only a few meters away from the brook under investigation. Another study of Winterbourn et al. (2007) also shows that caddisflies exhibit different distribution patterns, but mostly are found close to streams up to 300 meters distance. These observations suggest that dispersal behavior of *R.pubescens* might also be characterized by site fidelity and that long distance dispersal only occurs rarely. Also only the number of effective migrants, that are the ones that successfully reproduce (Bohonak & Jenkins 2003, Neal 2004) is the crucial factor for bringing new alleles into another population. So if *R.pubescens* disperses to another creek, it also needs to reproduce to make migration successful.

Our findings suggest that niche specificity is another factor besides dispersal capacity that is shaping the genetic population structure of *R. pubescens*. The distribution of the species is linked both at broad scales and in terms of microhabitat distribution to highland calcareous bedrock “islands” separated by landscapes with unsuitable geology or altitudes. In its biotope *R. pubescens* is restricted to tufa stretches of the eucrenal, hypocrenal and epirithral zone (Graf 2002). Limited gene flow between recently disjunct habitat islands seems to have brought fourth high levels of local genetic differentiation that we found in *R. pubescens*. Numerous minimally diverged microendemic lineages are evidence that species could be in a state of incipient speciation, making it an interesting model for studying speciation in general terms. Speciation is an ecological process that is difficult to detect, because the time-scale is usually too great for direct observation or experiments (Barraclough et al. 1998). Recent advances in molecular methods offer an opportunity to test hypotheses about evolution and speciation in groups at the species level and within species. Caddisflies would therefore be an ideal group to study speciation processes either with morphological or genetic methods. A first step in this direction was made by Pauls et al. (2008) who studied the evolution of feeding types in the family Limnephilidae.

Summary

Since results of the investigation of genetic differentiation north of the Alps showed that *R.pubescens* must have colonized this area after the last ice age, it was promising to study the entire distribution range to locate a southern glacial refugium. MtDNA and a second nuclear marker system, Amplified Fragment Lengths Polymorphisms (AFLP´s), were used to study the phylogeography of *R. pubescens* across its range. AFLP´s were chosen to detect processes that do not follow maternal inheritance, like it is indicated by mitochondrial DNA data. Endemic haplotypes restricted to single streams or mountain regions were detected also in the southern regions. We could thus exclude that this phenomenon is related to margin effects and that it is more likely due to low gene flow between populations and low population sizes. Both markers showed genetic impoverishment of the northern part of the range consistent with rapid colonization of this area after the last ice age (Hewitt 1999). This is supported by higher genetic diversity indices of AFLP´s and presence of unique fragments in the south. Populations of the Apennines and Liguria are genetically very different from the French Alps populations. Presumably the Alps have acted as a barrier to gene flow. Based on haplotype distribution and genetic diversity indices we can conclude that in the Provence region at the most southern tip of the Western Alps there is a secondary contact zone between the Italian peninsula and the French populations. Based on our estimates of gene flow it seems most plausible that northwards recolonization started from the region of the French Calcareous Alps and Dauphiné Alps. These populations may present the sources from which the refugial populations recolonized the French and Swiss Jura, and eventually the Central European highlands. This scenario is consistent with the fact that the region south of Grenoble remained largely unglaciated and potential refugia on calcareous bedrock were present in this area (Schönswetter et al. 2005). Alternative refugia regions are the zones in the French and Swiss Jura which were free of ice during the last glacial maximum (Jäckli 1962). However, our data provide no evidence for this scenario. It seems very unlikely that the species remained close to alpine ice sheets, because the species is not as cold-tolerant as other caddisfly species like for example *Drusus discolor* and *Hydropsyche silfvenii*. However, with the present data set we cannot entirely rule out this scenario. To our knowledge *R. pubescens* is one of the few examples of a species recolonizing Central European regions from a southwestern Alps refugium. Expansion along the western margins of the Alps from the Maritime Alps has been proposed for the butterfly species complex *Melanargia galathea/lachesis* in an allozyme study (Habel et al. 2005) and for the butterfly *Polyommatus coridon* (Schmitt & Krauss 2004). Another study with the AFLP method (Lihová et al. 2009) showed that the alpine plant *Cardamine alpina* survived the last ice age in a single refugium in the Maritime Alps and

colonized the entire Alps starting from there. This example may not be completely comparable to *R. pubescens* due to different ecological requirements of the species, but clearly shows that the Southwestern Alps appear to have served several species as a refugial region. This region was also proposed as a refugial zone by several other authors (Malicky 1983, Schmidt & Seitz 2001, Schönswetter et. al. 2002).

The phylogeographic pattern we found in *R. pubescens* generally corresponds well with results from other European phylogeographical studies (e.g. Taberlet 1998, Hewitt 1999, Schmitt 2007), namely high genetic diversity in Southern Europe and low genetic diversity in the northern areas most probably due to a bottleneck. This genetic pattern is typical for a temperate species recolonizing Northern Europe with the onset of climate warming after the Würm ice age. As opposed to other aquatic organisms, *R. pubescens* does not show different reactions to climatic oscillations in the Pleistocene than terrestrial species.

In caddisflies in particular, vastly different patterns have been observed. Molecular studies of *Drusus discolor* (Pauls et al 2006) and the close relative of *R. pubescens*, *Rhyacophila aquitanica* (Bálint 2008) propose multiple extra-mediterranean refugia. The caddisfly *Hydropsyche tenuis*, on the contrary, exhibits low overall genetic diversity and location of the refugium was most probably south of the Alps (Lehrian et al. 2008), but the recolonization route cannot be fully resolved. Based on these observations many phylogeographical patterns are present in caddisflies.

The present thesis could reveal that *R. pubescens* is a true species according to the monophyletic species concept (De Queiroz 2007). The investigation represents one of very few studies on macroinvertebrates that cover the entire biogeographical range of the study species. For the first time it was demonstrated that geology is causing habitat fragmentation in an aquatic insect and that this is detectable on a molecular scale. It could be shown that habitat specificity of *R. pubescens* leads to genetic differentiation in mountain ranges and even single streams. The data clearly indicate that the study species survived cool periods in warmer regions in the Southwestern Alps and started recolonization from these regions along the margins of the Western Alps. It is, to our knowlegde, the first time that such a recolonization pattern was detected in an aquatic insect species.

The results obtained by the molecular study of the former periglacial area were published in Fundamental and Applied Limnology (Engelhardt et al. 2008). The range wide phylogeographical study is planned for submission in an international scientific journal.

Summary

Many questions still remain about the way species survived the glaciations and how they colonized their present range. New approaches like ecological niche modeling or applying model selection to several recolonization scenarios might help us in the future to give us a more realistic idea how these processes worked. An interesting example where these methods were used, is the study of a land snail (Dépraz et al. 2008), where the authors were able to demonstrate that the species most probably survived in two climatically suitable and ice free regions in Switzerland. With further development of molecular techniques and statistical methods for data analysis, there will be new opportunities for interpretation of phylogeographic patterns and inference of population histories.

Phylogeny and phylogeography of the caddisfly *Rhyacophila pubescens* (Trichoptera) with special consideration of its habitat specificity - Deutsche Zusammenfassung

Das Forschungsfeld der Phylogeographie ist ein relativ junges Feld der biologischen Forschung und vereint verschiedene Disziplinen wie Molekulargenetik, Populationsgenetik, phylogenetische Biologie, Geologie und historische Geographie (Avise 2000). Das Hauptziel ist es, Zusammenhänge zwischen phylogenetischen und geographischen Mustern zu untersuchen, um daraus Rückschlüsse auf die Populationsgeschichte einer oder mehrerer Arten ziehen zu können. Europa ist in den letzten Jahren in vielen Studien (vgl. Taberlet et al. 1998, Hewitt 1999, 2004, Schmitt 2007) untersucht worden, da dieses geographische Gebiet durch den Einfluss der Eiszeiten während des Pleistozäns (vor 1,8 Mio. Jahren bis vor 11 500 Jahren) besonders interessant ist. Durch abwechselnde Kalt- und Warmzeiten und daraus resultierende Klimaschwankungen veränderten sich die Verbreitungsgebiete temperater Tier- und Pflanzenarten stark. Durch phylogeographische Studien konnte gezeigt werden, dass es zum Aussterben von Populationen im Periglazialgebiet, einer kaltklimatischen Zone mit Permafrostboden, kam. Dieses Gebiet dehnte sich während der Eiszeiten nördlich der Alpen über ganz Mitteleuropa aus. Viele Tier- und Pflanzenarten überdauerten in wärmeren, südlichen Refugialzonen und breiteten sich in den Warmzeiten und nach dem Ende der letzten Eiszeit (der Würmeiszeit, vor 10 000 Jahren) wieder nach Norden aus. In den letzten Jahren haben Untersuchungen gezeigt, dass es gemeinsame Wiederbesiedelungsmuster gibt (Taberlet 1998, Hewitt 1999, 2004). Obwohl einige generelle Trends feststellbar sind, hat sich jedoch gezeigt, dass die phylogeographischen Muster sehr unterschiedlich sind und stark abhängen von Lebenszyklus, Habitatbindung und Verbreitungsfähigkeit einer Art. Auch innerhalb einer Ordnung, wie der der Köcherfliegen (Trichoptera), wurden verschiedene phylogeographische Muster entdeckt, die stark mit der jeweiligen Ökologie der Art zusammenhängen (Pauls et al. 2006, Lehrian et al. 2009). In der vorliegenden Arbeit wurde die Köcherfliege *Rhyacophila pubescens,* PICTET 1834, hinsichtlich ihrer Phylogenie und Phylogeographie mithilfe molekularer Marker untersucht. Die Art eignet sich für eine phylogeographische Studie, da sie ein Verbreitungsgebiet besiedelt, das nördlich der Alpen durch die Eiszeit beeinflusst war und in welchem sie südlich der Alpen vermutlich dauerhaft leben konnte. Die Art kommt von Frankreich im Westen bis nach Ungarn im Osten vor (Fauna Europaea Web Service 2004), in den niedrigen Lagen der Alpen (bis 1500 m ü. N.N.) von Österreich bis in die französischen und italienischen Südwestalpen, sie fehlt jedoch am Südrand der Alpen. Weiterhin kommt sie

im Apennin und auf Korsika vor. Der Hauptgrund für die Auswahl von *R. pubescens* war die bemerkenswerte Habitatbindung dieser Art. Sie ist ausschließlich auf Kalksinterbäche beschränkt (Haase 1998, 1999) und besiedelt dort den Bereich von der Quelle bis 5 km unterhalb davon (Pitsch 1993). Kalksinterbäche sind durch Ausfällung von Kalk auf organische und mineralische Substrate im Bach gekennzeichnet, unter Umständen kann auch die Respirationsfähigkeit durch Kalkablagerungen auf den Wasserorganismen selbst herabgesetzt sein (Dürrenfeldt 1978). Die Nachweise von *R. pubescens* stammen ausschließlich aus kalkreichen Gebirgsformationen, die Verbreitung kann somit als inselartig bezeichnet werden. Eine so ausgeprägte Habitatbindung an einen bestimmten geologischen Untergrund ist, nach derzeitiger Kenntnis, bei keiner anderen europäischen Köcherfliege bekannt. Durch ihr inselartiges Verbreitungsmuster stellt *R. pubescens* daher ein interessantes Untersuchungsobjekt für eine phylogeographische Studie dar. Sowohl Regionen der Ebene oder des Hochgebirges als auch Regionen mit ungeeigneter Geologie können als Verbreitungsbarrieren wirken, was sich auf genetischer Ebene zeigen kann, wenn es in isolierten Populationen zu genetischer Differenzierung durch geringen oder nicht vorhandenen Genaustausch mit benachbarten Populationen kommt. Das Hauptziel dieser Arbeit bestand darin, zu untersuchen, ob sich das geographische Verbreitungsmuster, geprägt von Fragmentierung durch einen geologischen Faktor, auch auf genetischer Ebene auswirkt. Weiterhin sollte aufgedeckt werden, ob *R. pubescens* während der Eiszeiten nördlich der Alpen überdauern konnte, oder das Gebiet erst nach dem Ende der letzten Eiszeit, aus einem südlichen Refugium kommend, wiederbesiedelte.

Da Individuen aus dem gesamten Verbreitungsgebiet untersucht wurden, ist es essentiell zuerst zu klären, ob eventuell kryptische Arten vorhanden sind. Dazu wurden in einem phylogenetischen Teil (Kapitel 1) *R. pubescens*-Individuen aus verschiedenen Regionen sowie nah verwandte *Rhyacophila*-Arten mithilfe von Sequenzdaten dreier Marker untersucht. Im zweiten Kapitel wurden mitochondriale Sequenzen von *R. pubescens*-Individuen aus dem Raum nördlich der Alpen analysiert, um erste Hinweise auf die Populationsstruktur und eine mögliche glaziale Überdauerung zu erhalten. Ausgehend von den Ergebnissen wurde im dritten Kapitel die Phylogeographie des gesamten Verbreitungsgebietes mit einem mitochondrialen Marker und Amplified Fragment Length Polymorphismen (AFLP) untersucht, um Refugialzonen und mögliche Rekolonisierungsrouten zu erkennen.

Für die phylogenetische Untersuchung wurden drei genetische Marker verwendet: die mitochondriale Cytochrom c Oxidase I–Region (mtCOI), die Region, die für die

mitochondriale große Untereinheit (mtLSU 16S) kodiert und die Region der Kern-DNA, die für das wingless-Gen (nuWG) kodiert. Sequenzen wurden von *R. pubescens, R. tristis, R. aquitanica, R. obtusa, R. margaritae* und *R. producta* generiert. Die ersten drei gehören dem Hyporhyacophila-Larventyp nach Döhler (1950) an, alle genannten Taxa außer *R. margaritae* wurden von Schmid (1970) in die *R. tristis*-Gruppe gestellt. *R. margaritae* wurde erst 1998 von Kumanski beschrieben und ebenfalls in die *R. tristis*-Gruppe gestellt. Als Außengruppentaxa wurden *R. italica* und *R. ferox* verwendet, die als Pararhyacophila nach Döhler (1950) eingestuft werden. DNA der Larven wurde nach dem Protokoll von Pauls et al. (2006) extrahiert, die DNA der Adulten wurde aus 2 Beinen mit dem QIAamp DNA Micro Kit (Qiagen) nach Anleitung des Herstellers extrahiert. PCR (Polymerase-Ketten-Reaktion) - Primer für mtCOI waren LCOI490 (5´GGTCAACAAATCATAAAGATATTGG3´) und HCO2198 (5´TAAACTTCAGGGTGACCAAAAAATCA3´) (Folmer et al. 1994). Für mtLSU wurden die Primer LR-J-12887 (5´CCGGTCTGAACTCAGATCACGT3´) und LR-N-13398 (5´CGCCTGTTTAACAAAAACAT3´) (Simon 1994) verwendet. Für nuWG wurden die Primer Wingnut1a (5´ GAAATGCGNCARGARTGYAA 3´) und Wingnut3 (5´ ACYTCRCARCACCARTGRAA 3´) (Pauls et al. 2008) benutzt. 25 µl PCR Reaktionen enthielten ein puReTaq Ready-To-Go Bead (GE Healthcare) und 10 pmol jedes Primers. Die Sequenzierung wurde von LGC AGOWA, Berlin, durchgeführt. Die Sequenzen wurden anschließend in Bioedit (1999) aliniert. Phylogenetische Bäume wurden für die Einzeldatensätze und für den aus allen drei Markern kombinierten Datensatz (1396bp) mit der distanzbasierten Neighbor-joining (NJ)-Methode und der charakterbasierten Maximum Parsimony (MP)-Methode in Paup* 4.0b10 (Swofford 2001) berechnet. MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) wurde verwendet, um Bäume mit der Bayesian Markov Chain Monte Carlo (B/MCMC) Methode zu berechnen.

Für die Untersuchung der genetischen Populationsstruktur und für die phylogeographischen Untersuchungen wurden Sequenzen der mtCOI-Region benutzt. DNA-Extraktion und PCR-Protokoll folgten Pauls et al. (2006). 333 Sequenzen des mtCOI-Gens von Tieren des gesamten Verbreitungsgebiets wurden generiert und die 475 bp langen Sequenzabschnitte in Bioedit (Hall 1999) aliniert. Zunächst wurde ein ungewurzeltes median-joining-Netzwerk mit Network 4.5.0.1. (Fluxus Technology) und ein Netzwerk mit dem statistischen Parsimonieverfahren in TCS1.21 (Clement et al. 2000) berechnet. Weiterhin wurde eine Bayesische Analyse der Haplotypen im Programm MrBayes 3.1.2 durchgeführt. Zur Analyse der genetischen Populationsstruktur wurden verschiedene statistische Methoden eingesetzt, wie exact tests of population differentiation (Raymond & Rousset 1995) und pairwise FST im

Programm Arlequin 3.1 (Excoffier et al. 2005). Mit dem gleichen Programm wurden Fixationsindices nach Wright (1943, 1951, 1965), AMOVA (Analysis of Molecular Variance) (Excoffier et al. 1992), Mantel (1967) –Test, pairwise mismatch distributions (Rogers & Harpending 1992) und Neutralitätstests (Fu 1997, Tajima 1989) berechnet. Migrate-N 3.0 (Beerli 2008) wurde benutzt, um Migrationsvorgänge, ausgehend von den Westalpen als mögliches Refugium, aufzuklären.

Als zweiter Marker wurden Amplified Fragment Length Polymorphismen (AFLP) verwendet, die Multi-Locus-Profile aus der Kern-DNA darstellen. Das Protokoll für die AFLP-Analyse folgte Vos et al. (1995) mit geringen Modifikationen, Fragmente wurden mit Genemarker Vers. 1.7 ausgewertet und visuell überprüft. Für 250 Tiere konnten AFLP-Profile mit jeweils 132 Fragmenten erzeugt werden. Analysen für die AFLP-Daten beinhalteten die Berechnung von Nei´s D in AFLP-SURV 1.0 (Vekemans 2002), zur Erstellung eines neighbor-joining Phänogramms (Saitou & Nei 1987) in PHYLIP 3.67 (Felsenstein 1993). Eine Hauptkoordinaten-Analyse wurde mit GenAlEx 6.1 (Peakall and Smouse 2006) durchgeführt. Verschiedene Diversitätsmaße, wie Anteil polymorpher Marker, Nei´s Gen-Diversität H (Nei 1987) und DW-Wert (Schönswetter & Tribsch 2005) in jeder Gebirgsregion wurden mit AFLPdat (Ehrich 2006) berechnet. Der Shannon Index S (Shannon 1948) der phänotypischen Diversität wurde in Popgene 3.2. (Yeh & Boyle 1999) berechnet. Mit den AFLP-Daten wurden assignment-Tests mit drei verschiedenen Methoden durchgeführt, mit BAPS 3.2 (Corander et al. 2004, Corander & Marttinen 2006), Structure 2.2 (Pritchard et al. 2000, Falush 2003, Falush 2007) und dem neu entwickelten Structurama (Huelsenbeck et al. submitted). Für beide Marker (mtDNA, AFLP) wurden eine AMOVA (Analysis of Molecular Variance) in Arlequin 3.1., sowie Mantel-Tests (Mantel 1967) berechnet. Weiterhin wurde mit beiden Datensätzen eine Barrier-Analyse zur Lokalisierung genetischer Barrieren in Barrier 2.2 (Manni et al 2004) durchgeführt.

Im phylogenetischen Teil dieser Studie wurden die Verwandtschaftsbeziehungen zwischen *R. pubescens* und mit ihr verwandter Arten der *R. tristis*-Gruppe untersucht. Hauptziel war es, zu untersuchen, ob alle Individuen von *R. pubescens*, die aus verschiedenen Regionen des Verbreitungsgebiets stammen, eine monophyletische Gruppe bilden und als gute Art nach dem monophyletischen Artkonzept (De Queiroz 2007) betrachtet werden können.

Wie die Ergebnisse, bei allen drei Methoden hoch unterstützt, zeigen, stammen die untersuchten Individuen von *R. pubescens* von einem gemeinsamen Vorfahren ab. Die Individuen von Korsika sind genetisch distanziert von den übrigen untersuchten, vermutlich

weil sie sich seit dem Anstieg des Meeresspiegels vor 3 bis 5 Mio. Jahren (Steininger & Rögl 1984) dort isoliert entwickeln. Die Monophylie der *R. pubescens* Individuen ist eine wichtige Voraussetzung für die Untersuchung der Phylogeographie dieser Art.

Die Monophylie der *R. tristis*-Gruppe in Bezug auf die gewählten Außengruppentaxa konnte mit signifikanten Ergebnissen bei der Maximum Parsimony und der B/MCMC - Methode bestätigt werden. *R. tristis* und *R. aquitanica* sind in den phylogenetischen Bäumen klar getrennt, womit die Trennung beider Arten anhand morphologischer Merkmale (Bálint et al. 2008) unterstützt wird. *R. margaritae* clustert in die *R. tristis*-Gruppe, womit die morphologische Einordnung von Kumanski (1998) mit molekularen Daten bestätigt werden konnte. Die Stellung von *R. producta* ist unklar und müsste im Rahmen eines umfangreicheren Datensatzes, welcher die restlichen Taxa der *R. tristis*-Gruppe und andere mitteleuropäische *Rhyacophila*-Arten umfasst, untersucht werden. Zum jetzigen Zeitpunkt existiert neben der Phylogenie Schmid´s (1970) und einer spermatologischen Studie von Friedlander (1993) keine Phylogenie der Rhyacophilidae, somit ist die vorliegende Studie die erste molekulare Phylogenie der Rhyacophilidae auf Artebene. Es zeigte sich, dass die verwendeten Marker für die Untersuchung von Verwandtschaftsbeziehungen zwischen Arten geeignet sind, sodass auf den Datensatz aufbauend eine umfassender Analyse der Gattung *Rhyacophila* angestrebt werden sollte.

Im zweiten Teil der Arbeit wurde die Populationsstruktur und genetische Differenzierung von *R. pubescens* aus 15 Gebirgsregionen nördlich der Alpen mit mtDNA-Sequenzen untersucht. Die Ergebnisse der Untersuchung sollten erste Hinweise auf die Populationsgeschichte während der Eiszeiten und eine mögliche Wiederbesiedelung dieses Gebietes liefern. Mitochondriale DNA ist besonders geeignet für phylogeographische Studien (Beebee & Rowe 2008), da sie nicht der Rekombination unterliegt und neutral evolviert. Aufgrund der Tatsache, dass die effektive Populationsgröße der mtDNA geringer ist als bei diploiden Kerngenen, sind Unterschiede der Haplotypenfrequenzen zwischen Populationen schneller erkennbar.

Der Datensatz enthält 28 Haplotypen (einzigartige Basenabfolgen). Ein intraspezifisches phylogenetisches Netzwerk nach der median-joining-Methode zeigt einen häufigen Haplotyp, H1, der von 119 Individuen getragen wird und im gesamten Untersuchungsgebiet vorhanden ist. Aufgrund seiner zentralen Position kann er als ursprünglicher Haplotyp aufgefasst werden. Alle anderen Haploytpen unterscheiden sich durch einen oder zwei Mutationsschritte von H1. In fast allen untersuchten Gebirgsregionen (außer Eifel, Nordhessen, der schwäbischen Alb, den Nördlichen Kalkalpen, dem Pieniny Gebirge und der Malá Fatra)

wurden endemische Haplotypen gefunden, die zum Teil nur auf einen Bach beschränkt sind. Dies lässt sowohl auf Differenzierung in einzelnen Gebirgen und in einzelnen Bächen schließen, als auch darauf dass sich durch geringen Genfluss bestimmte Haplotypen in einem kleinen Areal gebildet haben und sich nicht weiter ausbreiten. Die Werte der exact tests of population differentiation und die F_{ST}-Werte zeigen genetische Differenzierung in den Gebirgsregionen und unterstützen damit diese Hypothese. Eine AMOVA ergab hochsignifikant, dass der höchste Prozentsatz der Variation innerhalb von Gebirgen gefunden wurde. Die hohe Anzahl endemischer Haplotypen und kleinräumige genetische Differenzierung lassen darauf schließen, dass sich die inselartige Verbreitung auch auf genetischer Ebene zeigt. Die Fragmentierung der Populationen durch ungleichmäßiges Vorkommen von Kalkstein führt nach diesen Ergebnissen zu geringem Genfluss zwischen Populationen. Weiterhin weisen die Verteilung der Haplotypen (mismatch distributions) und die Neutralitätstests auf demographische Expansionsprozesse hin, die kürzlich erfolgt sind. Dies lässt den Schluss zu, dass der Raum nördlich der Alpen nach dem Ende der Würmeiszeit wiederbesiedelt wurde. Die geringe maximale Differenz zwischen Haplotypen (0,84%) stützt diese Hypothese, da beispielsweise bei der Köcherfliege *Drusus discolor* im selben geographischen Gebiet eine Differenz von 4,21% in einem nahezu übereinstimmenden mtCOI-Fragment festgestellt wurde (Pauls et al. 2006). Diese Art hat in periglazialen Refugien überdauert und ist somit stärker divergiert. Wenn man davon ausgeht, dass der Periglazialraum von *R. pubescens* Individuen mit dem heute noch häufigen Haplotyp H1 wiederbesiedelt wurde, kann es anschließend zu Differenzierung in den Gebirgen gekommen sein. Da die Wiederbesiedelung erst vor ca. 10 000 Jahren einsetzte, ist die Zahl der Mutationsschritte gering. Ein mögliches Szenario könnte eine rasche Wiederbesiedelung aus südlichen Populationen sein, die durch das weiträumige Lössvorkommen (Pye 1995, Haase et al. 2007) erleichtert wurde. Im Löss, der im Periglazialraum vorhanden war, könnten kalkreiche Habitate vorhanden gewesen sein, sodass sich die Art schnell ausbreiten konnte. Durch den Rückgang des Lösssubstrats wurde *R. pubescens* möglichweise in Kalkgebirge zurückgedrängt und weist heute ein inselartiges Verbreitungsmuster auf. Ebenso könnte die Art bei der Wiederbesiedelung im Vorteil gewesen sein, da sie im Gegensatz zu anderen Makroinvertebraten hohen Kalkgehalt des Wassers toleriert. Später könnte sie durch den erhöhten Konkurrenzdruck in Kalksinterbäche verdrängt worden sein. Um diese Theorien zu überprüfen, wären jedoch experimentelle Untersuchungen, z.B. zur Konkurrenzfähigkeit der Art notwendig.

Ausgehend von den Ergebnissen der Untersuchungen im Gebiet nördlich der Alpen, wurde im dritten Kapitel das gesamte Verbreitungsgebiet von *R. pubescens* mit mtCOI-Sequenzen und zusätzlich mit Fragmenten der Kern-DNA (AFLP´s) untersucht. AFLP´s wurden gewählt, um Prozesse zu erkennen, die nicht der maternalen Vererbungslinie folgen, wie sie von der mitochondrialen DNA angezeigt werden. In diesem Teil der Studie sollte zum einen aufgedeckt werden, ob sich die genetische Populationsstruktur der nördlichen und südlichen Gebiete voneinander unterscheidet. Weiterhin war von Interesse, ob beide Marker übereinstimmende Ergebnisse bezüglich der genetischen Differenzierung von Populationen oder der Populationsstruktur zeigen. Aus den Ergebnissen wurden Rückschlüsse auf potentielle Refugien, postglaziale Ausbreitung und Wiederbesiedelung gezogen.

Analysen zur Populationsstruktur zeigen bei beiden Markern, dass sich die südlichen Populationen deutlich von den nördlichen unterscheiden. Haplotypennetzwerke (median-joining, TCS) zeigen, dass die südlichen Haplotypen wesentlich divergenter sind und viele Mutationsschritte zwischen ihnen liegen. Beispielweise liegen zwischen einem Haplotypen aus Ligurien und den Haplotypen aus Korsika 44 Mutationsschritte. Der im Norden häufige Haplotyp H1 ist jedoch auch in den Südwestalpen bis in die Provence vorhanden. Im gesamten Verbreitungsgebiet wurden endemische Haplotypen, die z.T. nur auf einen Bach beschränkt sind, gefunden. Somit kann davon ausgegangen werden, dass es auch im Süden rezent geringen Genfluss zwischen Populationen gibt, der mit der Habitatbindung und dem inselartigen Vorkommen der Art zusammenhängt. Das Vorkommen von privaten AFLP-Fragmenten in italienischen und korsischen Populationen weist darauf hin, dass diese Gebiete seit langem besiedelt werden. Hohe Diversitätswerte (Shannon´s Index, DW-Wert) in den Populationen in Ligurien, dem Appenin und auf Korsika bestätigen dies. Die Barrier-Analyse (Manni et al. 2004) für beide Marker, die genetische Barrieren lokalisiert, zeigt Abgrenzung von Korsika, dem Apennin, den Südwestalpen und des Ceský Kras. Die assignment tests des Fragmentdatensatzes zeigen, dass sich die Individuen nördlich der Alpen genetisch ähnlich sind. Die drei Programme, Structure 2.2., Barrier 3.2 und das neuentwickelte Structurama ergaben übereinstimmend, dass eine Einteilung der Gesamtpopulation in fünf Gruppen die wahrscheinlichste ist. Cluster 1 umfasst alle Populationen nördlich der Alpen, außer den Tieren aus den tschechischen Bilé Karpaty, und auch Individuen aus den französischen Westalpen. Cluster 2 umfasst Individuen aus Bilé Karpaty, Česky Kras und Korsika. Diese Ähnlichkeit ist vermutlich auf Homoplasie von AFLP-Fragmenten zurückzuführen, da die mtCOI-Daten eine hohe genetische Distanz zwischen diesen Populationen zeigen. Cluster 3 enthält ligurische Individuen, Cluster 4 enthält übrige Tiere der Westalpen und Cluster 5

enthält die des Apennins. Da Cluster 1 Individuen aus den nördlichen Gebieten und Tiere aus den Westalpen umfasst, liegt es nahe, dass die Wiederbesiedelung durch Migration ausgehend von einer Refugialzone in den Westalpen erfolgte. Da die Tiere aus dem Apennin und aus Ligurien genetisch zu unterschiedlich von den Individuen im Norden waren, scheint einer Wiederbesiedelung aus Italien nicht wahrscheinlich.

Diese Hypothese zur Wiederbesiedelung aus den Westalpen wurde in Migrate-N mithilfe eines stepping-stone-Modells getestet. Die Südwestalpen wurden auch in anderen Arbeiten als Refugialzone postuliert (Malicky 1983, Schmidt & Seitz 2001, Schönswetter et. al. 2002). Es zeigte sich, dass von den Dauphiné-Alpen Migration Richtung Norden und in geringerem Maß nach Süden erfolgte. Somit ist es wahrscheinlich, dass das Gebiet der Dauphiné-Alpen das „leading edge" für die Wiederbesiedelung darstellte, die entlang der Westalpen über das Schweizer Jura zu den Zentralen Europäischen Mittelgebirgen erfolgte. Eine postglaziale Route ausgehend von den Südwestalpen entlang der Westalpen wurde auch für den Schmetterlingsartenkomplex *Melanargia galathea/lachesis* in einer Allozym-Studie (Habel et al. 2005) nachgewiesen. Weiterhin wurde eine solche Rekolonisationsroute für den Schmetterling *Polyommatus coridon* (Schmitt & Krauss 2004) beschrieben. Durch die vorliegende Studie wurde bestätigt, dass die Hypothese einer postglazialen Wiederbesiedelung des ehemaligen Periglazialgebiets für *R. pubescens* zutrifft. Die verwendeten Marker zeigen größtenteils übereinstimmende Ergebnisse, wobei die Tiere der nördlichen Regionen im AFLP-Datensatz zum großen Teil nicht individuell unterscheidbar waren, sodass weitere Aussagen zur Differenzierung nicht möglich waren. Hier wäre eine höhere Anzahl von Fragmenten nötig gewesen, die durch Amplifikation mit zusätzlichen Primerpaaren erreicht werden könnte. Andererseits bildet die Kern-DNA Prozesse ab, die weiter in der Zeit zurückliegen, im Gegensatz zur mitochondrialen DNA, welche eine geringere effektive Populationsgröße besitzt. Eventuell war die Zeit seit der Wiederbesiedelung zu kurz, sodass Mutationen in der Kern-DNA in den nördlichen Populationen noch nicht sichtbar sind. Die übereinstimmenden Ergebnisse der Kernmarker und des mitochondrialen Markers zur Populationsstruktur zeigen, dass es keine geschlechtsspezifischen Unterschiede beim Ausbreitungsverhalten gibt.

Zusammenfassend wurde bei *R. pubescens* ein deutlich anderes phylogeographisches Muster gefunden, als bei anderen arealweit untersuchten Köcherfliegen, wie z.B. *Drusus discolor*, die in periglazialen Refugien überdauerte. Auch bei der nah verwandten *R. aquitanica* wurden mehrere extramediterrane Refugien vorgeschlagen (Balint 2008). Das genetische Muster von *R. pubescens* wird von der Habitatbindung an kalkhaltige Geologie stark beeinflusst. Die

vorliegende Arbeit ist, nach derzeitigem Kenntnisstand die erste, in der untersucht wurde, wie sich Fragmentierung durch einen geologischen Faktor bei Wasserinsekten auf molekularer Ebene auswirkt. Die Ergebnisse der populationsgenetischen Studie wurden bei Fundamental and Applied Limnology veröffentlicht (Engelhardt et al. 2008). Es ist geplant, die arealweite Phylogeographie zur Veröffentlichung bei einer internationalen Fachzeitschrift einzureichen.

Nach wie vor sind viele Aspekte der Migration während der Eiszeit und den postglazialen Wiederbesiedelungsmechanismen ungeklärt. Neue Ansätze wie „ecological niche modeling“ oder das Testen verschiedener Wiederbesiedelungsszenarien mithilfe von Modellen könnten zukünftig einen realistischeren Einblick in diese Prozesse ermöglichen. Ein interessantes Beispiel, bei welchem diese Methoden angewendet wurden, ist die Untersuchung einer Landschnecke (Dépraz et al. 2008), in der die Autoren zeigen, dass die Art höchstwahrscheinlich in zwei klimatisch geeigneten und eisfreien Regionen der Schweiz überdauerte. Mit der Weiterentwicklung von molekularen und statistischen Methoden wird die Deutung phylogeographischer Muster und die Rekonstruktion von Populationsgeschichten in Zukunft sehr viel genauer und wirklichkeitsgetreuer werden.

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Acknowledgments

I wish to thank my supervisors PD Dr. Daniel Hering and PD Dr. Peter Haase for their support throughout this study and valuable comments on the manuscript. Peter Haase had the initial idea for this thesis and supported collection trips across Europe and laboratory work.

I particularly thank Dr. Steffen Pauls for introducing me to the world of molecular ecology, for sharing his knowledge on phylogeography and for his patience to discuss research ideas or problems. Steffen Pauls and his wife Imke is thanked for giving me a warm welcome in Minnesota and for help with statistical analyses during my stay.

I would like to acknowledge the help of many colleagues who contributed to my study. Dr. Miklos Bálint sent material for this study and provided literature that was very difficult to obtain. Special thanks to Dr. Pavel Chvojka, Dr. Wolfram Graf, Prof. Dr. Otto Kiss, Dr. Verena Lubini, Prof. Dr. Hans Malicky, Johannes Mayer, Dr. Andrea Sundermann, Prof. Dr. Bronislaw Szczesny and Dr. Heinrich Vicentini, who provided specimens.

I also like to thank my colleagues at the Research Institute Senckenberg: Stephanie Lehrian accompanied me to many exciting collection trips, I also would like to thank her for many valuable discussions on phylogeography and caddisflies. I wish to thank Dr. Carsten Nowak for his help with tricky statistical analyses. Dr. Stefan Huck is thanked for providing the idea on Loess faciliated recolonization, Dr. Jürgen Jung for providing the maps, Miriam Gerhardt, Elisabeth Heigl, Stephanie Lehrian, Anna Liebrich and Thomas Michl for proof-reading.

Special thanks to Julia Weindl for proof-reading of the german part of the text.

I greatly acknowledge the financial support of the Konrad-Adenauer-Stiftung during the work on this thesis. They also supported a collection trip to Switzerland and a trip to a phylogeographic conference in Luxembourg. Furthermore I was able to attend many interesting seminars organized by the Konrad-Adenauer-Stiftung.