European common frog (*Rana temporaria*) recolonised Switzerland from multiple glacial refugia in northern Italy via trans- and circum-Alpine routes

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Abstract

The high mountain ranges of western Europe have had a profound effect on the recolonisation of Europe from glacial refugia. The Alps present a particularly interesting case, because they present an absolute barrier to dispersal to most lineages, obstructing recolonisation from multiple refugia in the Italian Alps. Here we investigate the effect of the European Alps on the the phylogeographic history of *Rana temporaria* across its range in Switzerland. Based on partial *cytochrome b* and *COX1* sequences we find two mitochondrial lineages that occur roughly north and south of the alpine ridge bisecting Switzerland, with contact zones between them in the east and west. The northern haplogroup falls within the previously identified common western European haplogroup, while the southern haplogroup is unique to Switzerland. We find that the lineages diverged ~110 kya, approximately the onset of the last glacial maximum, indicative of origins in separate refugia. Phylogenetic analyses suggest that the lineages originate from two refugia in northern Italy, and colonised Switzerland via trans- and curcum-alpine routes. Our results show that the European Alps is a semi-permeable barrier to dispersal for *R. temporaria*, and have contributed to the complex recolonisation history of Switzerland.

35 **Introduction**

36

37 Contemporary geographic distributions of genetic variation result from
 38 processes occurring on diverse time scales. Climate oscillations and
 39 associated ice-ages that occurred during the last ~700 ky had a major
 40 influence on contemporary European ecosystems (Taberlet *et al.* 1998; Hewitt
 41 1999, 2000). The extent of the ice-sheets and inhospitable habitat restricted
 42 many species to three major refugia: the Balkans, Italy, and the Iberian
 43 Peninsula (Taberlet *et al.* 1998). After the retreat of the ice-sheets, Europe
 44 was recolonised by the surviving populations. Differing dispersal abilities,
 45 residual habitat discontinuities, and geographic complexities have determined
 46 these recolonisation routes. This has had a fundamental impact on the extant
 47 geographic distribution of species and their genetic diversity (Roy *et al.* 1996;
 48 Taberlet *et al.* 1998; Hewitt 2000; Willis & Whittaker 2008; Avise & Riddle
 49 2009).

50 High mountain ranges such as the European Alps and Pyrenees were
 51 the last to deglaciate. For taxa adapted to warm climates, the persistence and
 52 extent of alpine ice-sheets and the east-west orientation of the mountain
 53 ranges hindered biotic recolonisation of Europe from their refugia (Taberlet *et*
 54 *al.* 1998). Cold-tolerant organisms exhibit a more complex history. Many
 55 expanded their range during glacial periods, some persisted in nunataks
 56 within the Alps, and trans-alpine recolonisation has occurred in several
 57 species (Mátyás & Sperisen 2001; Lugon-Moulin & Hausser 2002; Parisod
 58 2008; Yannic *et al.* 2008; Braaker & Heckel 2009). However, the effect of the
 59 Alps on the biogeography of cold-tolerant species remains understudied,
 60 particularly for vertebrates (but see Yannic *et al.* 2008, Braaker & Heckel
 61 2009).

62 Here we describe the phylogeographic history of a cold-tolerant
 63 amphibian, the European common frog (*Rana temporaria*) by densely
 64 sampling populations across the Alps in Switzerland. This species is the most
 65 widespread anuran in Europe, occurring from northern Italy and Spain to the
 66 sub-arctic tundra in Fennoscandia in the north and the Ural mountains in the
 67 east. *R. temporaria* is ubiquitous in the European Alps and occurs up to an

elevation of 2600 m (Sillero *et al.* 2014). Two deeply diverged (~0.7 Mya) mitochondrial lineages occur in western and eastern/northern Europe, respectively (Palo *et al.* 2004). A contact zone has been identified that extends from northern Germany (Schmeller *et al.* 2008) to the northern lowlands of Switzerland and north western Italy (Teacher *et al.* 2009). The precise location and structure of the contact zone in the Alps has not been resolved by existing studies (Stefani *et al.* 2012; Rodrigues *et al.* 2013). The western haplogroup has higher genetic diversity than the eastern haplogroup which suggests that *R. temporaria* recolonised western Europe from multiple glacial refugia. Both Iberia (Teacher *et al.* 2009) and Italy (Stefani *et al.* 2012) have been considered as the main refugia for the western haplogroup. However, sampling has not been available to reconstruct the main routes of post-glacial recolonisation of the western haplogroups, and it remains unclear whether the Alps was a barrier to re-colonisation into western Europe.

Our main aims are to determine the colonisation history and extant population structure of *Rana temporaria* in Switzerland. Specifically we ask whether 1) a contact zone exists between Eastern and Western haplotypes in Switzerland, 2) Switzerland was initially colonised from one or multiple glacial refugia, and 3) determine whether the Alps was a barrier to colonisation.

The geography of the Alps between northern Italy and Switzerland are characterised by two main high mountain ridges running roughly east to west. We refer to the first as the southern Alpine ridge. This comprises the highest peaks and roughly constitutes the border between Italy and Switzerland. The second ridge is called the northern Alpine ridge, and roughly bisects Switzerland into a northern and southern part.

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Materials and Methods

Sampling

To investigate the geographic distribution of genomic variation in Switzerland, we sampled 82 populations across a ~2300 m gradient of elevation in the Swiss Alps during the 2013 breeding season (Fig. 1, Table 1). Approximately 10 eggs from each of 20-30 freshly laid clutches were collected from each site. The eggs were transported to the University of Zürich, where they were hatched in separate water-filled containers in a water bath. The tadpoles were euthanised and stored in ethanol when they reached stage 36 (Gosner, 1960). Each clutch is assumed to represent a single family; thus one tadpole per clutch was used for all work presented here.

To establish phylogeographic patterns, we selected a subset of the samples collected; 2-9 eggs were collected from freshly-laid clutches in 72 populations of *R. temporaria* in the Swiss Alps during the 2013 breeding season. Locations, elevations, and sample sizes of the populations are given in Table S1. The eggs were transported to the University of Zürich, where they were reared at 23-25 °C until they hatched and reached stage 36 (Gosner, 1960). One individual from each clutch ($N = 368$) was euthanised and stored in ethanol.

Molecular lab work

Total DNA was extracted from tadpole tails by overnight digestion in 10% proteinase K solution at 56 °C and extracted using the Qiagen Biosprint 96 DNA blood kit (Qiagen, CA, USA). DNA was eluted in 100 - 200 µL buffer AE (QIAGEN).

mitochondrial DNA

To establish phylogeographic patterns at a European scale, we sequenced a 448bp fragment of *cytb* to correspond with previous studies (Teacher *et al.* 2009; Vences *et al.* 2013). The gene was amplified by PCR using *Rana-cytb-F2* (5' TTAGTAATAGCCACAGCTTTTGTAGGC), and *Rana-Cytb-R2* (5' AGGGAACGAAGTTTGGAGGTGTGG) primers (Vences *et al.*

2013) with an annealing temperature of 53 °C.

The aforementioned studies do not include fine-scale sampling of the Italian Alps. To determine the phylogenetic relationship between Swiss and Italian samples generated sequence data to compare with Italian samples from Stefani *et al.* (2012). For a subset of 44 populations (151 families), we PCR-amplified a 628bp fragment of COXI. Primers L4437 (5' AAGCTTTCGGGCCCCATACC) and H6564 (5' GGGTCTCCTCCTCCAGCTGGGTC) with an annealing temperature of 49 °C.

Amplified COXI and *cytb* fragments were subjected to Sanger sequencing after we purified them using a standard ExoSap protocol: 0.25 µl Exonuclease I (20 U/µl; New England Biosystems), 0.5 µl rAPID Alkaline Phosphatase (SAP) (1 U/µl), 7.25 µl nuclease free water, 8 µl PCR product. Cycling conditions were as follows: 37 °C for 45 min, 80 °C for 15 min, and held at 10 °C. From the cleaned product, 2 µl per sample was used in 10 µl sequencing reactions (BigDye Sequencing Kit, Applied Biosystems). Samples were sequenced in one direction for each gene using an automated 3130xl DNA Analyzer (Applied Biosystems, CA, USA) and the sequences were aligned using CLC Main Workbench 5.0.2 (CLC Bio) and BioEdit (Hall 1999). Sequences were verified as *cytb* and COXI using the BLASTN 2.2.24 algorithm (Altschup *et al.* 1990) implemented on the National Center for Biotechnology Information (NCBI) online platform.

Palo *et al.* (2004) identified a restriction enzyme cut site at position 277 in the *cytb* sequence that distinguishes between the eastern and the western mitochondrial clades. We used this assay to assign our samples to clades. Restriction digests were performed using 8 µl of the PCR product, 0.1 µl 10000 U/ml *StyI* restriction enzyme (New England Biolabs), 0.9 µl water, and 1 µl 10X Buffer (Qiagen). PCR products were digested for 30 minutes at 37 °C, where after they were resolved on a 1% agarose gel and assessed by eye under UV-light.

Analysis

Geographic distribution of mitochondrial variation. – To visualise the geographic distribution of genetic variation, haplotype networks were

constructed with *cytb* and COX1 sequences using statistical parsimony (Templeton *et al.* 1992) as implemented in TCS version 1.21 (Clement *et al.* 2000). Within Switzerland, data generated in this study were used: 368 individuals sequenced at 448bp *cytb*, and 151 individuals sequenced at 1076bp (628bp COX1 and 448bp *cytb*). At a broader geographic scale, we visualised genetic variation by combining our data with those from elsewhere in Europe obtained from NCBI (accession numbers for *cytb*: KC799522.1 - KC800122.2; Vences *et al.* 2013). This dataset comprised 969 individuals sequenced at 331bp *cytb*. Note that these data do not include Italian samples described in Stefani *et al.* (2012), as a different *cytb* fragment was amplified in their study. A separate analysis was conducted based on COXI to determine the phylogenetic relationship between Swiss and Italian samples (see below).

Phylogenetic analyses. – Evolutionary relationships among geographic groups in the haplotype network were determined with phylogenetic analyses on three datasets: 1) *cytb*1, 2) COXI, 3) combined *cytb*1 and COXI. All datasets comprised of data from this study and all available data from Vences *et al.* (2013). Outgroup species were chosen based on the *Rana* phylogeny in Veith *et al.* (2003) and data available from NCBI. For *cytb*, the outgroups were *R. pyrenaica* (KC799521.1), *R. arvalis* (KC800123), *R. iberica* (KC799485), and *R. italica* (KC799493). For COXI, the outgroups were *R. pyrenaica* (KC977251.1) and *R. arvalis* (JN971596.1). As a first step we identified the evolutionary model that best fit each dataset, and secondly we determine the most likely phylogenetic tree under the chosen model. Phylogenetic trees were estimated using both Bayesian and likelihood methods.

The most likely evolutionary mutation model was determined for each dataset by comparing 88 alternative models in jModeltest2 (Darriba *et al.* 2012) and using the Akaike Information Criterion (Akaike 1973) to select the model best supported by the data.

Phylogenetic analyses were conducted using maximum likelihood and Bayesian methods. Maximum likelihood analyses were implemented in RAxML v.8.2.2 (Stamatakis 2006). For each dataset, 1000 rapid bootstrap replicates were conducted followed by a maximum likelihood search for the

193 most likely phylogenetic tree. Bayesian phylogenetic analyses were
194 conducted using MrBayes v.3.2.1 (Huelsenbeck & Ronquist 2001). Analyses
195 were run for 1×10^6 generations and sampled every 1000 generations. Four
196 Metropolis-coupled Markov chain Monte Carlo (MCMCMC) chains were used
197 for two replicate runs of each dataset, with chain heating kept at default
198 temperatures. Convergence was assessed using the diagnostic output from
199 the *sump* command: Adequate effective samples sizes (ESS) for each
200 parameter (>200), appropriate mixing of chains, and an average standard
201 deviation of split frequencies between independent runs <0.05 . A burnin of
202 25% was used to obtain the consensus phylogram and posterior probabilities
203 for each bipartition.

204 Nomenclature for the major lineages determined either side of the Alpine
205 ridge will be CH-North (northern Swiss lineage) and CH-South (southern
206 Swiss lineage). A third lineage referred to as CH-brown is phylogenetically
207 closer to the CH-North lineage, but occurs exclusively south of the bisecting
208 alpine ridge. To estimate the geographic distribution of genetic diversity,
209 haplotype diversity (H) and nucleotide diversity (π) were calculated in DnaSP
210 5.0 (Librado & Rozas 2009) for all Swiss populations and for CH-North and
211 CH-South separately.

212 We estimated the coalescence date for all *R. temporaria* haplotypes and
213 the Swiss subset of haplotypes using a Bayesian Markov chain Monte Carlo
214 analysis with *R. arvalis* and *R. pyrenaica* as outgroups (BEAST v. 1.8.2;
215 Drummond *et al.* 2012). COX1 and *cytb1* were analysed as a single dataset,
216 totaling 1076bp, because jModeltest inferred a similar nucleotide substitution
217 model for both gene fragments. We used the GTR + I substitution model, with
218 estimated base frequencies, and 6 rate categories. Priors were set to their
219 defaults except for the date of divergence of the three *Rana* species, which
220 was specified as a lognormal distribution with a mean of 3.18 Mya and
221 standard deviation of 0.43 Mya (Veith *et al.* 2003). Tests were run with all
222 demographic and molecular clock combinations as priors, and the most likely
223 model was selected based on a marginal likelihood test. The final analysis
224 was run using a relaxed molecular clock, and a demographic model of
225 constant population size.

226 To test the hypothesis that Switzerland was colonised from the Italian

refugium, we estimated the phylogenetic relationship between 569 bp COX1 data from this study, and data available on NCBI. This analysis was based on COX1 data only, because the publicly available *cytb* fragment sequenced for samples from northern Italy (Stefani *et al.* 2012) does not overlap with that of samples from Europe (Vences *et al.* 2013). We included *R. temporaria* haplotypes from the Italian Alps (Stefani *et al.* 2012; FN813783-FN813812), Europe (Vences *et al.* 2013; KC977228.1 - KC977251.1) and outgroups *R. pyrenaica* (KC977251.1) and *R. arvalis* (JN971596.1). Phylogenetic analyses were conducted as described above.

Demographic history. – To test for signals recent changes in population size we used Tajima's D, Fu's F, and the DNA sequence mismatch distribution (Rogers & Harpending 1992). A mismatch distribution tests for signatures of population expansion based on the number of nucleotide differences (mismatches) between DNA sequences. When population size remains stable the distribution of mismatches is expected to be multimodal, reflecting the stochastic nature of gene trees. When populations expand, we expect a unimodal distribution of mismatches. Signals of population expansion were tested in the haplogroups using Harpending's raggedness index (RI). The expansion time was estimated under a model of pure demographic expansion with parameters set to default in Arlequin (Excoffier & Lischer 2010). The parameter of demographic expansion, Tau, was estimated according to Schneider & Excoffier (1999). The validity of the expansion model was tested using the sum of square deviations (SSDs) between the observed and expected mismatches as implemented in Arlequin.

Results

The final dataset comprised 368 families from 72 populations sequenced at 448bp of the *cytb* gene, and 151 families from 44 populations sequenced at 628bp of COXI. Based on the absence of the diagnostic *cytb*

restriction site, all Swiss samples were determined to be from the western clade.

Geographic distribution of genetic variation

Haplotype network - Overall the haplotype network shows an eastern (blue) and western (other colours) clade with much higher genetic diversity found in the western clade (Fig. 1). The Swiss samples assigned to three western haplogroups; a green haplogroup which is widespread across western Europe and Great Britain, a brown haplogroup found almost exclusively in the Pyrenees (Vences *et al.* 2013), and a Swiss specific haplogroup shown in purple described here for the first time. Within Switzerland the green haplogroup is found north of the Alpine ridge bisecting Switzerland, while the purple and brown haplogroups occur south of the ridge (Fig. 2). This division was confirmed when Swiss samples were analysed using *cytb* alone and in combination with *COXI* (Fig. S2.1). The haplogroup found north of the Alps (CH-North) corresponded with the widespread western European haplogroup, which is thought to have colonised western Europe from the Pyrenees (green haplotypes in Fig. 1; Vences *et al.* 2013). The brown haplogroup (CH-brown) was closely related to the common western European haplogroup, and occurs almost exclusively in the Pyrenees (brown haplotypes in Fig. 1). The southern haplogroup was unique to Switzerland, and fell between the haplogroups sampled in the Pyrenees and those sampled in Croatia and Greece in the haplotype network. CH-North and CH-South co-occurred in some populations but across all elevations in eastern Switzerland and at low elevation in western Switzerland. A second western European lineage (CH-brown: brown haplotypes in Fig. 1), found in the Pyrenees and restricted roughly to the southern part of Europe, co-occurred with CH-South haplotypes in southern Switzerland (Fig. 2).

292 Phylogenetic analyses - The same phylogenetic relationships were
 293 recovered using ML and Bayesian approaches, as well as between the *cytb1*,
 294 COXI, and concatenated datasets, and were concordant with previously
 295 published phylogenies (Fig. 3) (Veith *et al.* 2002; Vences *et al.* 2013). A basal
 296 split in the *R. temporaria* clade separates three Spanish clades that occur
 297 exclusively in Iberia (Vences *et al.* 2013) from the rest of the samples. *R.*
 298 *temporaria* is further split into an eastern and a western clade with high
 299 posterior probability (0.99). Within the western clade, a further split is found
 300 between populations from southern Switzerland and all other western
 301 populations (including northern Switzerland/western Europe; posterior
 302 probability = 0.97).

303 The estimated mean coalescent times in Fig. 3 support a divergence
 304 between the eastern and western clades at ~210 kya, and a more recent
 305 divergence between CH-South and the rest of the western clade at ~110 kya.
 306 The most likely coalescent model based on marginal likelihood tests was a
 307 relaxed molecular clock and constant population size. The effective sample
 308 size (ESS) for all parameters was above 200, and all independent runs
 309 showed convergence and stationarity.

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313 Distribution of genetic variation - Genetic diversity within Switzerland
 314 was higher in populations found south of the alpine ridge bisecting
 315 Switzerland. Haplotype and nucleotide diversity based on 448bp *cytb* gene
 316 fragment was roughly double in CH-South when compared to CH-North
 317 populations, and roughly three times higher based on 628bp COX1 sequence
 318 (Table 1; diversity measures for individual populations are reported in Tables
 319 S1 and S2).

320

321 *Demographic history*

322 The mismatch distribution analyses did not converge for CH-North for
 323 any of the datasets, but Tajima's D and Fu's F were negative and significant
 324 for all CH-North datasets indicative of a population expansion. Tajima's D and

Fu's F were non-significant for CH-South, and there was no evidence for population expansion for any of the CH-South analyses.

Origin of haplotypes in Switzerland

Phylogenetic analysis based on 569 bp of COX1 revealed that haplotypes from the Italian Alpine Lineage (AP) I described in Stefani *et al.* (2012) grouped with CH-South haplotypes with high posterior probability (0.94; Fig. 4). Three haplotypes identified by Stefani *et al.* (2012) – FN813810.1, FN813785.1, FN813786 – were identical to CH-South haplotypes. API occurs in the central Italian Alps and contains the most abundant Italian Alpine haplotype (CA2), which was identical to CHS03. In our sample, this haplotype was found only in a population on a high pass in southern Switzerland, on the border with Italy. The most widespread CH-South haplotype was identical to the widespread API haplotype DE10. A third haplotype (API: VF6) was identical to CHS06, found only at high elevation in southwestern Switzerland (Figs. 2.4 & 2.5).

The northern Swiss lineage (CH-North) clustered with APIII with high posterior probability (1.0). One previously identified haplotype from APIII (FN813809.1 = VF3) was identical to the most common CH-North haplotype (CHN01). Geographically, APIII and VF3 are found in northwestern Italy, but in Switzerland CHN01 occurs mostly north of the bisecting alpine ridge (Fig. 5). APIII clusters with a haplotype from southern France with high posterior probability (0.93; Fig. 4), which suggests that this haplogroup is analogous to the green haplogroup described by Vences *et al.* (2013) and is widespread across France, Germany, and Great Britain.

Discussion

We interpret our results to indicate that *Rana temporaria* recolonised western Europe almost exclusively from multiple refugia in the Italian Alps. Populations originating from hypothesised refugia in the Iberian Peninsula occur only in Spain and southwestern France. Fine-scale sampling of populations across Switzerland provides evidence of a trans-alpine

colonisation from the central Italian Alps into Switzerland, and recolonisation from the western Italian Alps around the alpine arc into much of western Europe. We found two deeply diverged mitochondrial lineages occurring roughly north and south of the northern Alps, with contact zones in eastern and western Switzerland. These results strongly suggest that the Alps did not present a significant barrier to dispersal for this species during the recolonisation of Europe. This has resulted in complex regional variation in the genetic structure of *R. temporaria* across Switzerland, which could have important implications for population persistence and adaptive potential.

Eastern and Western European lineages

Consistent with previous research, we found a deep phylogenetic split between eastern and western European lineages of *R. temporaria* (Palo *et al.* 2004). The age of the east-west divergence, calibrated based on the *R. dalmatina* divergence time (3.18 Mya; Veith *et al.* 2003), was placed in the late Pleistocene (~0.215 Mya). The divergence date was previously estimated as ~0.71 Mya (CI 0.53-0.95 Mya) based on *cytb* sequences (Palo *et al.* 2004), corresponding to the onset of strong Pleistocene climatic cycles. The discrepancy may be attributed to the large confidence intervals associated with the statistical priors, as well as the slightly different calibration points included in both studies. However, our estimated date and that of Palo *et al.* (2004) both suggest that the divergence between the eastern and western clades is attributable to the dramatic climatic cycles that occurred during the Pleistocene, which began ~1.8Mya.

Two previous studies disagreed about the existence of a contact zone between the eastern and western lineages in northern Switzerland and along the French Mediterranean into Spain (Teacher *et al.* 2009, Rodrigues *et al.* 2013). Our thorough sampling of northern Switzerland, including several populations in the lowlands where the contact zone was said to lie (Teacher *et al.* 2009), uncovered no eastern haplotypes whatsoever and hence no evidence of contact between clades. Presumably, a secondary contact zone between the eastern and western haplogroups occurs somewhere farther to the east.

390

391 ***Post-glacial recolonisation of western Europe***

392 Several studies have identified multiple glacial refugia in the Italian
393 peninsula, that consequently lead to multiple deeply diverged lineages
394 (Canestrelli *et al.* 2006, 2008; Crottini *et al.* 2007; Canestrelli & Nascetti
395 2008). Our results support the assertion that *R. temporaria* re-colonised
396 western Europe from multiple sub-refugia within the Italian refugium (Stefani
397 *et al.* 2012). We identified two western mitochondrial lineages distributed
398 roughly north and south of the northern Alps within Switzerland. The two
399 lineages are estimated to have diverged ~110 kya, at the onset of the most
400 recent glacial cold period. Four Alpine lineages have been described in
401 northern Italy, two of which (API and APIII) are distributed south of
402 Switzerland roughly east and west of the Ticino valley (Fig. 5). Our
403 phylogenetic analyses suggest that the southern (CH-South) haplogroup was
404 most likely colonised by API from the central Italian Alps, while the northern
405 (CH-North) haplogroup was colonised by APIII.

406 A thorough geographic analysis of the CH-North and CH-South
407 lineages suggests that they recolonised western Europe via two different
408 routes (Fig. 6). The geographic distribution of the southern lineage across the
409 central Alpine ridge is evidence of multiple trans-alpine colonisations into
410 Switzerland (Fig. 5). Three CH-South haplotypes were identical to AP1
411 haplotypes, and their geographic distribution can be used to narrow down
412 colonisation routes. Assuming a conservative maximum habitable elevation of
413 2000 m (as in Braaker & Heckel 2009), there are seven possible passes
414 across the central Alpine ridge (Fig. 5). The most widespread AP1 haplotype
415 (CA2) was identical to a CH-South haplotype found in south-eastern
416 Switzerland, which suggests colonisation across the Maloja Pass (5, Fig. 5) or
417 Bernina Pass (6, Fig. 5).

418 Southwestern Switzerland contained several sites with secondary
419 contact between API and APIII, which could suggest a southern trans-alpine
420 colonisation route of APIII into Switzerland. Possible routes include the Col de
421 Ferdaz (1, Fig. 5) in the west or Simplonpass (2, Fig. 5), but the most likely
422 point of colonization is difficult to identify because the most common CH-

South haplotype (API haplotype DE10) was geographically widespread in northern Italy.

In other European taxa, studies of trans-alpine colonisation have identified the same trans-Alpine colonisation routes for both plants and animals (Lugon-Moulin & Hausser 2002; Yannic *et al.* 2008, 2012; Braaker & Heckel 2009; Parisod 2008). The example of European white oaks (Mátyás & Sperisen 2001), which presently occur only rarely above 1200 m in the Alps (Rigling *et al.* 2013), illustrates that trans-Alpine recolonisation was not limited only to taxa adapted to cold climates. Presumably, dispersal over Alpine passes was more extensive when the treeline was ~200 m higher than it is today, which occurred regularly during the period of 5'000-10'000 years ago (e.g. Ammann *et al.* 2000; Tinner & Theurillat 2003; Heiri *et al.* 2006). Exact colonisation routes could be investigated with the inclusion of nuclear markers and fine-scale geographic sampling of populations across the putative passes used for colonisation.

CH-North haplotypes appear to have colonized Switzerland from the west probably up the Rhone River or from the north west over the Jura Mountains (Fig. 6). The geographic distribution of the derived CH-North haplotypes towards southern Switzerland (light green haplotypes; Fig. 2) and overall low genetic diversity (Table 1) suggest that this lineage expanded southwards in Switzerland rather than colonising via trans-alpine routes as with the CH-South lineage. Three possible origins of this haplogroup have been proposed: Iberia, a cyptic refugium in southern Ireland, and northern Italy (Teacher *et al.* 2009; Stefani *et al.* 2012; Vences *et al.* 2013). Although we cannot definitively rule out Iberia or Ireland, our results seem to suggest an Italian origin. Specifically, the previously identified common western European haplogroup (T4; Vences *et al.* 2013) occupies northern Switzerland, and we found that this haplogroup is identical to Italian APIII found in the north western Italian Alps. In addition, the rest of the CH-North haplotypes form a monophyletic group with APIII (Fig. 4). Finally, genetic diversity is expected to decrease with distance from glacial refugia as populations experience bottleneck events with colonisation (Hewitt 1996). The genetic variation in the green/APII/T4 haplogroup is substantially higher in northern Italy than in Ireland or Iberia, suggesting that this was the main refugium of the

haplogroup.

The identification of a second CH-brown lineage exclusively south of the central Alpine ridge in southern Switzerland suggests that there may be eastward expansion from Iberia south of the Alps (Fig. 2 & 2.5). This lineage is ubiquitous in the Pyrenees, and is found in southern France, northern Italy, and Croatia (Vences *et al.* 2013). Deglaciation of the Tinee Valley on the border between France and Italy occurred during the Oldest Dryas (~15kya), earlier than the rest of the Alps where glaciers retreated during the Younger Dryas (~11kya) and the late ice retreat (~9kya) (Darnault *et al.* 2011). This potential early colonisation corridor could have provided the way for an eastward expansion from the Pyrenees, across northern Italy, to Croatia. Although westward expansion from the Carpathian refugium through northern Italy has been described (Demesure *et al.* 1996; Taberlet *et al.* 1998), we found no examples of an eastward expansion from Iberia into northern Italy. Longer mtDNA fragments and more extensive sampling would be needed to investigate this unusual colonisation route further.

Conclusions

The *R. temporaria* western mitochondrial clade is exceptional in that it harbours more genetic diversity than any other European brown frogs (Palo *et al.* 2004; Vences *et al.* 2013). We show that much of this variation can be attributed to the European Alps. The topographic complexity of the Alps has resulted in multiple glacial refugia and subsequent divergence between *R. temporaria* lineages. After the retreat of the glaciers, the Alps presented a semi-permeable barrier to dispersal for these lineages, resulting in multiple colonisation events in the south. However, this also limited colonisation from other refugia in northern Italy, which subsequently followed a circum-Alpine route to colonise western Europe and Switzerland (Fig. 6). This has resulted in two deeply diverged lineages that occur in the north and the south of Switzerland with contact zones in the east and west. Our work highlights how fine-scale phylogeographic studies across the Alps can elucidate of the phylogeographic history of cold-adapted species in Europe.

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 949 **Author contributions**

950 AJvR and JVB conceived of the project. AJvR, MR, and SB generated and
 951 analysed the data. All authors contributed to the writing of the manuscript.
 952 This manuscript forms part of AJvR's PhD thesis.

953
 954 **Data accessibility**

955 All sequences are available on NCBI. Accession numbers for *cytb*:
 956 MF624310-MF624355; COX1:
 957

Figures

Figure 1 Haplotype network of 331bp of the *cytb* gene sequenced for this study and combined with data from Vences *et al.* (2013). Geographic distribution of the haplotypes within the eastern (blue) and western (other colours) clades is shown on a map of Europe. Swiss haplotypes (shown with bold outlines) fall within the green (CH-North) and brown (CH-brown) haplogroups, and a Swiss-specific purple haplogroup (CH-South). Numbers in the green and brown haplotypes show the proportion of samples from this study assigning to that haplotype.

Figure 2 Swiss *cytb* (448bp) haplotypes are distributed roughly north (CH-North)

and south (CH-South and CH-brown) of the northern Alps. Pie charts show the proportion of individuals that had northern or southern haplotypes in each population, with pie chart size reflecting the number of samples per population. The inset depicts the *cytb* haplotype network calculated for all Swiss samples (368 individuals from 72 populations sequenced at 448 bp). The most common haplotype within each haplogroup is shaded in a darker colour compared to the derived haplotypes. Thus the distribution of the common and derived haplotypes are shown with pie charts on the map.

Figure 3 Bayesian phylogenetic tree of concatenated *cytb1* and COXI genes. The eastern and western lineage, the CH-South, CH-North, and CH-brown haplogroups are defined as in Fig. 1. The eastern and CH-South haplogroups are coloured as in Fig. 1. CH-North, CH-brown and western European haplotypes are indicated by green, brown, and pink circles (colours as in Fig. 1). Posterior probability is shown above the line, and the inferred coalescent time (Mya) as determined by BEAST is shown below the line. The calibration used (3.18Mya; Vences *et al.* 2003) is shown at the root of the tree.

Figure 4 Bayesian phylogenetic tree of COX1 haplotypes (569 bp) sampled across the *R. temporaria* range. Italian lineages described by Stefani *et al.* (2012) (Alpine lineage I-IV and Apennine lineage; NCBI code FNxx) are shown with vertical black bars. Four haplotypes from this study match Stefani *et al.* (2012) haplotypes and are indicated with an equal (=) sign. Coloured regions match the haplogroups identified in Fig.

998 1. Haplotypes from this study are indicated with CHN (CH-North) and CHS (CH-South). CH-brown haplotypes are not visible in this figure as
999 these populations were not sequenced at COX1. Haplotypes from Vences *et al.* (2013) are shown with their NCBI codes (KCxx) and their
1000 sample locations. Posterior probability of >0.5 is shown above each node.

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1003
1004 **Figure 5** Possible trans-alpine colonisation routes from the Italian Alps into
1005 Switzerland. The map shows elevation >2000 m in dark grey; thus the light grey
1006 highlights the potential regions available for colonising populations. The thick solid
1007 grey line roughly denotes the central Alpine ridge. Haplotype colours correspond with
1008 the haplogroups identified in Fig. 2 and 4. We show the geographic distribution of the
1009 COX1 haplotypes identified by Stefani *et al.* (2012) in Northern Italy bordering
1010 Switzerland. The dashed line indicates the Ticino valley that roughly separates API
1011 (light and dark purple) from APIII (light and dark green). For illustration purposes we
1012 show the *cytb* haplotypes from Switzerland (i.e. Fig. 3) as mitochondrial haplotypes
1013 are linked and this gene was sequenced more geographically extensively than COX1
1014 for our study. The Italian populations are defined as in Stefani *et al.* (2012); Loc8:
1015 Pramollo (46.36 N 11.86 E), Loc9: M.Pora (45.88 N 10.1 E), Loc10: Lemna (45.85 N
1016 9.18 E), Loc11: S.Caterina (46.41 N 10.49 E), Loc12: V.Martello (46.49 N 10.69 E),
1017 Loc13: V.Ferret (45.85 N 7.02 E), Loc14: Traversella (45.53 N 7.72 E), Loc15:
1018 Issiglio (45.46 N 7.32 E). Four haplotypes matched sequences from this study and
1019 their geographic locations are shown as follows: VF3: Dark green; DE10: Dark
1020 purple; CA2:#; VF6:***. The numbered blocks are mountain passes less than 2000 m
1021 elevation presenting possible cross-alpine colonisation points: 1=Col de Forclaz,
1022 2=Simplonpass, 3=Lukmanierpass, 4=San Bernardino Pass, 5=Malojapass,
1023 6=Bernina Pass, 7=Reschenpass.

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Figure 6 Proposed *R. temporaria* post-glacial recolonisation routes. Coloured arrows indicate the proposed origin and colonisation routes of five haplogroups. Our results suggest that Italy is the major glacial refugia for *R. temporaria*, with the common green haplogroup colonising western Europe from the north western Italian Alps, and a transalpine colonisation of the purple haplogroup into Switzerland. Dashed lines show probable regions of secondary contact between diverged lineages. 1=common western European lineage meets *R. temporaria* from Irish refugium (Teacher *et al.* 2009); 2=Likely original contact zone between eastern and western haplogroup, but only the western haplogroup is currently found here (Palo *et al.* 2004); 3=Contact zone between eastern and western haplogroup in northern Germany (Schmeller *et al.* 2008), but contact zone south of this site still needs to be described; 4=contact zone between CH-South and CH-North described in this paper; ?=Possible contact zone between the Iberian (CH-brown) and Italian lineages described in this paper. The asterisk (*) denotes an alternative origin and colonisation route of the green haplotype from the Irish refugium into western Europe.

Table 1 Diversity measures and neutrality tests for the *cytb*, COX1, and concatenated datasets generated in this study. CH-North includes all samples with haplotypes from the Northern haplogroup, and CH-South from the Swiss-specific haplogroup. N = Number of samples; length = sequence length in base pairs; nr Hap = the number of haplotypes detected, Hd = haplotype diversity (standard deviation); Nd = nucleotide diversity (standard deviation). Three tests for selection are included. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Pop Group	N	length (bp)	nr Hap	Hd (SD)	π (SD)	Tajima's D	Fu & Li's D	Fu & Li's F
<i>cytb</i>								
All	368	448	29	0.712 (0.020)	0.00857 (0.00019)	-0.377	-2.896*	-2.202
CH-North	217	448	17	0.347 (0.042)	0.00126 (0.00021)	-2.207**	-3.945**	-3.918**
CH-South	151	448	12	0.633 (0.034)	0.00249 (0.00016)	-1.068	-1.429	-1.550
<i>COX1</i>								
All	151	628	15	0.614 (0.033)	0.00517 (0.00023)	-0.1242	-1.797	-1.381
CH-North	92	628	7	0.167 (0.053)	0.00031 (0.00011)	-2.039*	-3.117*	-3.264**
CH-South	59	628	8	0.485 (0.078)	0.00103 (0.00021)	-1.468	-1.179	-1.497
<i>concatenated cytb + COX1</i>								
All	141	1076	31	0.792 (0.029)	0.00658 (0.00026)	-0.067	-2.848	-2.034
CH-North	86	1076	17	0.557 (0.065)	0.00080 (0.00014)	-2.209***	-3.415**	-3.545**
CH-South	55	1076	14	0.706 (0.064)	0.00157 (0.00018)	-1.194	-2.147	-2.158

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.0$