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Biogeography of the smooth snake (*Coronella austriaca*): origin and conservation of the northernmost population

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Understanding historical range expansions and population demography can be crucial for the conservation and management of endangered species. In doing so, valuable information can be obtained regarding, for example, the identification of isolated populations, associations to particular habitats and distribution range shifts. As poikilotherms, snakes are vulnerable to environmental changes that can greatly shape their distribution ranges. Here we used mitochondrial data to elucidate the origin of the smooth snake population in Åland island, which is the northernmost location where the species is found. In Åland, we used mitochondrial and microsatellite data to fine-map its spatial genetic structure, infer its demographic dynamics and determine its effective population size. We found three independent lineages, which expanded north from Iberian, the Balkans and Caucasus regions. The central lineage originating in the Balkans was the only one that reached Scandinavia. The Åland population belongs to this lineage and potentially colonized the island from the west via Sweden. This population appeared to be critically small and fragmented into two genetically isolated subpopulations. We discuss our results in light of previous findings regarding colonization routes in Europe and Scandinavia. Moreover, we discuss the origin and current genetic status of the Åland population relative to other co-occurring snakes and suggest conservation measures based on our findings. *Biological Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: Åland – edge populations – endangered species – genetic diversity – population structure.

INTRODUCTION

Understanding the origin of populations throughout the species' distribution range is of primary importance when devising conservation–management strategies. In doing so, valuable information can be obtained on, for example, the identification of isolated populations, associations to particular habitats and distribution range shifts (Long, Trussell & Elliman, 2009). Of particular importance are populations at the edge of the distribution. Such populations demark the limits at which the species can successfully persist and are expected to be less genetically diverse and less dense, occur in less favourable habitats and have more variable densities relative to more central populations (Lawton, 1993; Vucetich & Waite, 2003).

Due to their strong dependence of environmental conditions and high sensitivity to habitat loss, terrestrial snakes can provide illustrative examples about how environmental factors such as latitude and habitat fragmentation can shape the distribution range of a species and affect its genetic diversity (Goldingay & Newell, 2000; DiLeo, Row & Lougheed, 2010). The smooth snake (*Coronella austriaca*, Laurenti 1768) is a Colubrid species distributed across Europe, from the Iberian Peninsula to the Ural Mountains (Arnold, Burton & Ovenden, 1978). Its northernmost population occurs in Åland, an island between Sweden and Finland (60°N, 20°E), which is naturally fragmented by numerous inlets and islets, as well as by anthropogenic factors, mainly

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urbanization and crop fields. No information is currently available about the origin and genetic status of this edge-population. Only a partial biogeography has been described focused mainly in Iberia (Santos *et al.*, 2008).

Here we describe the full biogeography of the smooth snake. We aim to reveal the extant lineages, as well as the geographical distribution of genetic diversity within them. Furthermore, we elucidate the potential colonization route to Åland and assess its current population size, genetic structure and demographic stage. Based on our results, we re-evaluate the current conservation status of this edge-population.

MATERIAL AND METHODS

Study species

The smooth snake is a medium-sized (up to 80 cm) Colubrid species widely distributed across Europe. Presently, it is classified as specially protected in the European Union (Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora, annex IV). In Åland it is currently listed as vulnerable (Rassi *et al.*, 2010). This diurnal highly secretive species feeds mainly on lizards and invertebrates (Goddard, 1984). Smooth snakes can be found in various habitats, but in the northern parts of its range (Sweden and Åland) the typical habitats are relative dry, open rocky hillsides.

Sample collection

Samples were collected in 2010 and consisted of 41 buccal swabs and 21 tissue samples (shed skins and road-kills) from nine different localities throughout Åland. Likewise, 81 tissue samples were collected from different European museums, as well as from shed skins provided by collaborators. Additionally, we included 12 sequences of the 16S rRNA gene, and 24 sequences of the Cytochrome b (Cyt-b) gene reported by Santos *et al.* (2008). In total, we analysed 145 samples originating from 17 different countries throughout the species' distribution (Fig. 1, Supporting information, Table S1).

Total DNA was extracted from buccal swabs using a DNeasy Blood + Tissue extraction kit (Qiagen). For museum samples stored in formalin, we performed a pre-extraction based on ethanol washes and rehydration with Tris/EDTA prior to digestion with proteinase K as previously described (Coura *et al.*, 2005). In total, we analysed 1449 bp of mitochondrial DNA from partial sequences of the 16S (495 bp), Cyt-b (303 bp) and cytochrome oxidase subunit 1 (COI) (651 bp) genes. The COI gene was analysed in the museum and Åland samples only. Primer pairs were designed for both the 16S and the Cyt-b gene from an alignment composed of the sequences reported by Santos *et al.* (2008) using Primer3 (Untergasser *et al.*, 2012) (Table S2). For the COI gene, we used the primers LCO and HCO (Folmer *et al.*, 1994). However, for most museum samples it was not possible to recover the full COI sequence in a single amplification reaction. Therefore, we designed nested primers to amplify the gene in four shorter segments that were subsequently assembled. All primers included a universal AM13 sequence adaptor. The amplification conditions for all primer pairs and adaptor sequences are given in Table S2. The sequences are deposited in GenBank (accession KC997577–KC997598). Furthermore, to evaluate the genetic structure of the Åland population, we genotyped all Åland samples at 11 microsatellite loci (Ca19, Ca26, Ca27, Ca43, Ca47, Ca79, Ca612, Ca20, Ca40, Ca16, Ca30; Bond *et al.*, 2005). The DNA extractions were performed as above and amplification conditions are given in Table S4.

Data analyses

European biogeography

The level of genetic diversity in the three genes indicated by the number of haplotypes (h) was calculated in dnaSP v. 5.10 (Librado & Rozas, 2009). We implemented Bayesian and maximum-likelihood (ML) approaches to elucidate the species' biogeography and establish the most likely colonization route to Åland. First, we used jMODELTEST v. 0.1.1 (Posada, 2008) to determine the best evolutionary model of each gene according to Akaike's information criterion. For the COI and 16S genes, GTR+I was identified as the best model, whereas the HKY model was suggested for the Cyt-b gene. We then performed a partition homogeneity test (Farris *et al.*, 1995) on a concatenated alignment of the 16S and Cyt-b sequences (803 bp) implemented in PAUP v. 4 (Swofford, 2003). The result showed that the two-gene partitions were congruent ($P = 0.67$). Therefore, this concatenated dataset was also used as a two-partition alignment, each partition having its own substitution model. In total, five datasets were analysed (16S, Cyt-b, 16S+Cyt-b, COI and microsatellites).

For the Bayesian approach we used MrBayes, v. 3.1.2 (Huelsenbeck & Ronquist, 2001) implementing the substitution models suggested by jMODELTEST. The analyses were performed by running a total of three million generations in two chains sampling every 100th tree, and ensuring that the standard deviation of the split frequencies remained below 0.01. The posterior probabilities for lineages were then established by constructing a majority-rule consensus tree after completion of the burnin phase,

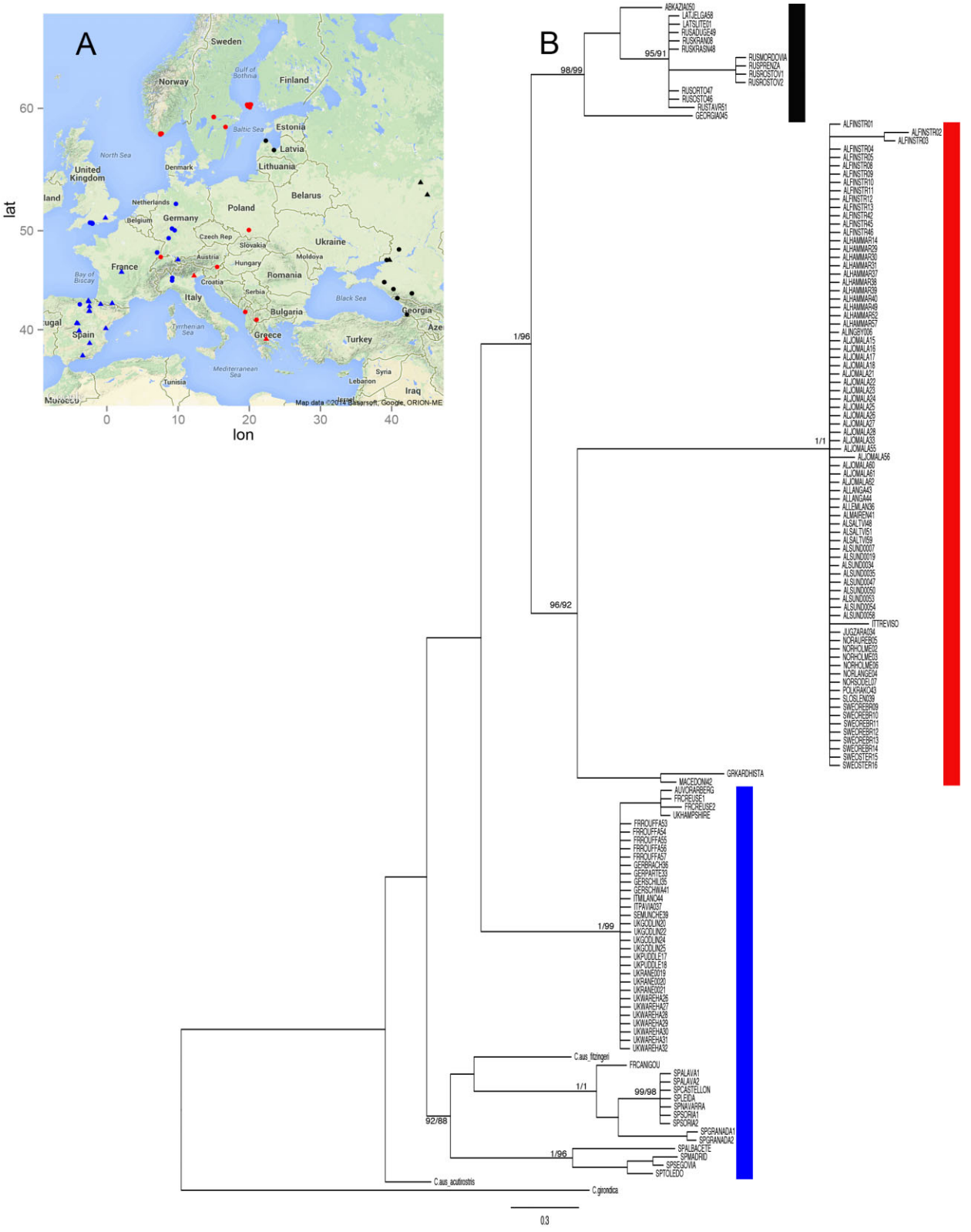

Figure 1. Smooth snake (*Coronella austriaca*) biogeography. A, sampling sites across Europe and their respective lineage indicated by different colours. Triangles represent samples from Santos *et al*. (2008). B, Cyt-b combined Bayesian and ML majority-rule phylogeographical tree showing posterior probabilities and bootstrap support; only values > 85 are displayed.

which represented one-quarter of the total sampled trees. For the ML approach we used RAxML v. 8.0.X (Stamatakis, 2014). We performed rapid bootstrap analyses using the extended majority-rule bootstrap convergence criterion, followed by a full ML search of the best-scoring tree. The HKY model is not implemented in RAxML and hence the simpler GTR model was used with a gamma distribution of rate heterogeneity. In both approaches, we included two subspecies (*C. austriaca fitzingeri* and *C. austriaca acutirostris*) reported by Santos *et al.* (2008) and one congener (*C. girondica*) (Utiger *et al.*, 2002) as outgroup. For the COI gene, no sequences are available for the two subspecies, and thus only the congener was included.

To evaluate the geographical distribution of genetic diversity within lineages, we grouped samples in bins of increasing geographical latitude, and compared the number of haplotypes within the bins. The number of bins (k) for each lineage was determined according to the 2k rule, $2^k \geq n$, where n is the total number of samples. The number of samples contained in each bin was determined as n/k (Table S3). The theoretical expectation is that genetic diversity (i.e. number of haplotypes) would be lower in edge-populations relative to more central ones (Lawton, 1993).

Demography and population genetic structure in Åland

The demographic history of the Åland samples was examined through a mismatch distribution test in Arlequin v. 3.5.1.3 (Excoffier & Lischer, 2010) according to Harpending (1994). As a complementary analysis, we estimated effective population size changes through time according to the Bayesian skyline plot (BSP) method implemented in BEAST v. 1.7.0 (Drummond *et al.*, 2012). We used a strict molecular clock, a GTR+I heterogeneity model for COI and HKY for Cyt-b. Chains were run for ten million iterations, of which the first 25% was discarded as burnin, sampling genealogies and model parameters every 1000 iterations. The results were analysed and summarized as BSPs in TRACER v. 1.5 (available from http://beast.bio.ed.ac.uk/Tracer). We tested for departures from neutrality by computing Tajima's D using dnaSP v. 5.10 (Librado & Rozas, 2009). Finally, we obtained an overall estimate of the female effective population size (N_{ef}) by calculating θ according to Watterson (1975) setting a generation time of 3.5 years and a nucleotide substitution rate of 5.2×10^{-9} substitutions per site year^{-1}, recently estimated for the family Coloubridae (Eo & DeWoody, 2010). The above analyses could not be performed in the 16S dataset because only one haplotype was found in Åland.

For the microsatellite dataset, the total number of alleles per locus and sample was obtained using GENETIX v. 4.01 (Belkhir *et al.*, 1997). We used FSTAT v. 2.9.3 (Goudet, 1995) to quantify the effective number of alleles independently of sample size (i.e. allelic richness). The level of observed and expected heterozygosity was calculated in Arlequin v. 3.5.1.3 (Excoffier & Lischer, 2010). Deviations from Hardy–Weinberg equilibrium (HWE) were estimated according to the level of significance determined by means of 10 000 Markov chain Monte Carlo (MCMC) iterations executed in GENEPOP v. 4.0 (Rousset, 2008). To correct for possible type I errors, we employed a false discovery rate (FDR) approach (Benjamini & Hochberg, 1995; Verhoeven, Simonsen & McIntyre, 2005). Gene flow levels (F_{ST}) among populations and their estimated probabilities were calculated based on 10 000 permutations using GENETIX v. 4.01 (Belkhir *et al.*, 1997).

We implemented a spatial genetics approach to detect possible genetic discontinuities in Åland using GENELAND (Guillot *et al.*, 2005). Geographical coordinates for each sampling location were obtained by GPS, and from digital maps based on recorded information of museum samples. Individual coordinates were then assigned to each sample by randomly choosing n points within a 5-km radius from each sampling location using the Mapping toolbox in MATLAB v. 7. (Mathworks), where n equals the number of samples per location. Hence, each sample was assigned a unique geographical coordinate with an uncertainty of 10 km to account for imprecision of museum records and/or dispersal. The inference algorithm was launched for 20 000 MCMCs using spatial information and setting a Dirichlet distribution as prior for allele frequencies. Then, the algorithm was re-run with an additional 20 000 MCMCs, fixing the value of K (i.e. number of populations) to that determined by the mode of the posterior distribution of the MCMC chain, and setting the Poisson processes equal to the number of samples. In both runs, sampling was conducted every 100th iteration and a correlated allele frequency model was specified.

RESULTS

European biogeography

For the COI and Cyt-b gene sequences we found a total of 21 and 11 haplotypes, respectively. Only ten haplotypes were observed for the 16S gene. Both, the Bayesian and the ML approaches identified three main lineages in all datasets. The subdivision among the samples appeared longitudinally structured into Eastern, Western and Central lineages. The Bayesian posterior probabilities and ML bootstrap support

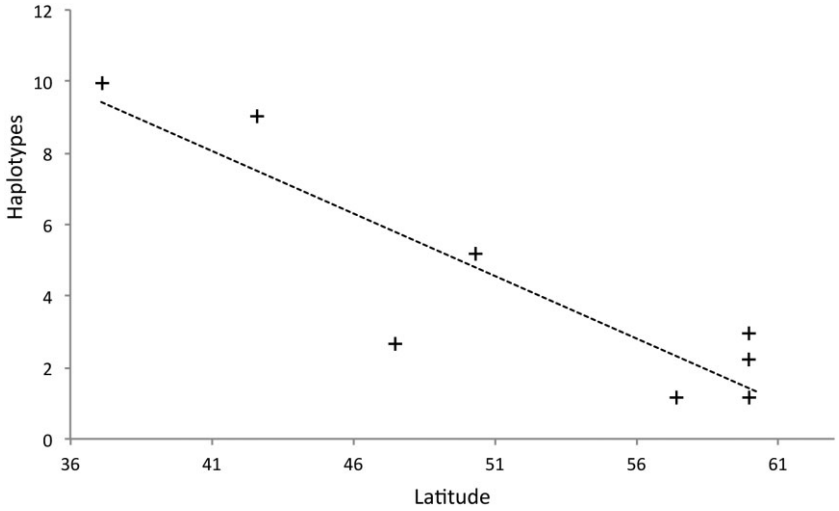

Figure 2. Smooth snake (*Coronella austriaca*) Cyt-b haplotypes observed across all samples grouped in latitudinal bins (°N).

values were high (> 87) in all datasets (Figs S2–S9). Within clades, the number of haplotypes decreased as geographical latitude increased when examining the three lineages together (Fig. 2), and each lineage separately (Fig. S1).

Demography and population genetic structure in Åland

Only four haplotypes were observed for the COI and Cyt-b genes in Åland, whereas only one haplotype was observed for the 16S gene. Both the mismatch distribution test and the BSP analyses suggested that the Åland population has experienced a recent population expansion rather than being a relict population (Fig. 3). This was also indicted by the Tajima's *D* test, which showed significant negative values (COI: −0.68, $P < 0.05$; Cyt-b: −1.76, $P < 0.05$). The female effective population size (N_{ef}) resulted in only 41.35 (COI) and 31.81 (Cyt-b) estimated breeding females.

In the microsatellite dataset, the total number of alleles per locus and sampling location ranged from one to nine (Table S4). We found no evidence of linkage disequilibrium between locus pairs. Significant deviations from HWE were observed after FDR correction for the loci Ca19, Ca40 and Ca47. However, departures occurred only at some sampling locations, indicating biological processes rather than technical issues, and thus the loci were kept in the dataset. Two loci (Ca20 and Ca30) were monomorphic across most or all populations and were removed from subsequent analyses.

Population pairwise F_{ST} values indicated significant differentiation ($P < 0.005$) between JOMALA vs. FINSTRÖM, JOMALA vs. SUND, and HAMMARLAND vs. SUND (Table S5). In line with this result, GENELAND suggested the existence of two genetically differentiated subpopulations, namely JOMALA+HAMMARLAND and FINSTRÖM+SALTVIK+SUND+LEMLAND areas (Fig. 4).

DISCUSSION

We found three independent lineages which expanded north from Iberia, the Balkans, and Caucasus regions. A trend of latitudinal reduction in genetic diversity was observed in the three lineages. The central lineage originating in the Balkans was the only lineage that reached Scandinavia. The Åland population belongs to this lineage and potentially colonized the island from the west via Sweden. The population in Åland appeared to be critically small and fragmented into two subpopulations. We discuss our results in light of previous findings regarding colonization routes in Europe and Scandinavia. Moreover, we discuss the origin and current genetic status of the Åland population relative to other co-occurring snakes and suggest conservation measures based on our findings.

European biogeography

Snake molecular biogeographies in Europe have focused mainly on southern and central regions. Such studies have found similar patterns to ours with lineages divided longitudinally into Eastern (Caucasus and Near East), Central (Balkans and Carpathians) and Western (Iberia, Alps and Italian peninsula) (Goddard, 1984; Carranza, Arnold & Pleguezuelos, 2006; Guicking, Joger & Wink, 2008;

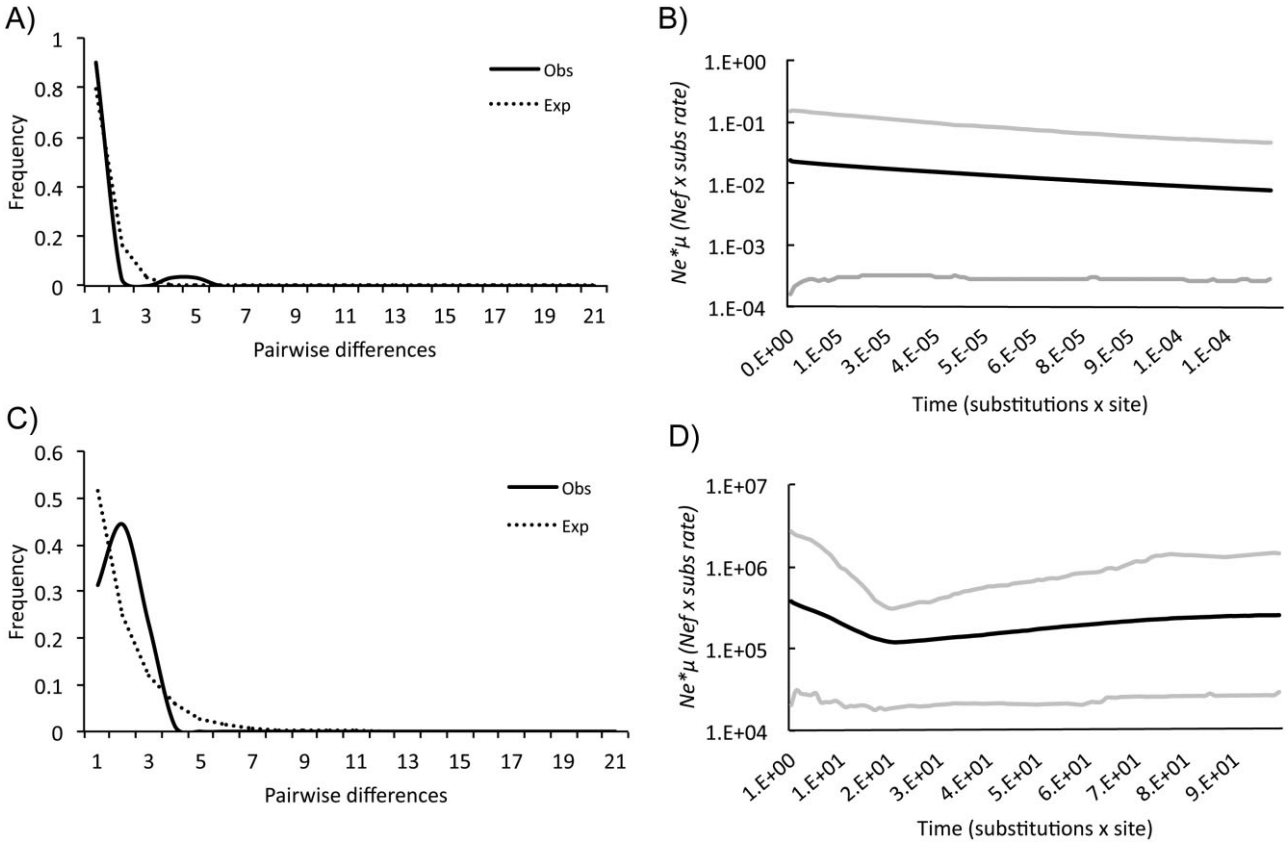

Figure 3. Demographic history of the smooth snake (*Coronella austriaca*) in Åland based on Cyt-b (A, B) and COI (C, D) haplotypes. A, C, mismatch distribution analyses indicating the number of pairwise base differences against their relative frequency. The observed distributions are compared for their goodness-of-fit to a distribution under a model of sudden expansion. B, D, Bayesian skyline plots showing the effective population size fluctuation throughout time. The centre line shows the median estimate; upper and lower confidence limits (95% highest posterior density) are indicated by grey lines.

Ferchaud *et al*., 2012; Santos *et al*., 2012). It is commonly accepted that these regions acted as glacial refugia for several animal and plant species (Fedorov & Stenseth, 2001; Crnobrnja-Isailovic, 2007). The origin of the Western and Central lineages has been relatively well studied in a number of snake species (Goddard, 1984; Blum *et al*., 2004; Carranza *et al*., 2006; Joger *et al*., 2007; Schmitt, 2007). The common consensus is that geological and climate events such as the Messinian Salinity Crisis, the Pliocene aridification and Pleistocenic glaciations have all contributed to multiple range expansions and vicariance events, which resulted in the isolation of these two lineages. In the smooth snake, it has been shown that dynamic shifts in distribution ranges during the Pleistocene, with multiple expansions and retreats, contributed to structure part of the Western clade (Iberia) into at least three sub-clades (Santos *et al*., 2008). This was later confirmed using meristics and morphometric traits (Llorente *et al*., 2012), and has been interpreted as refugia-within-refugia (Gomez & Lund, 2006). In the present study, no fine-scale sampling was available at the European level. However, within the Western clade, we did find different haplotypes in some locations from which we had multiple samples such as Bollenberg in France and Dorset in the UK. This sub-structuring could reflect historical isolation processes influenced by the Pyrenees and the Alps, as well as by the insularity of the UK, in which genetic fragmentation has previously been reported for smooth snake populations (Pernetta *et al*., 2011).

Much less is known about the origin of Eastern lineages. For snake species with Eurasian distributions such as the adder, *Vipera berus* (Linnaeus 1758), and the grass snake, *Natrix natrix* (Linnaeus 1758), it has been argued that their distribution results from post-Pleistocene eastward expansions of northern European lineages (Ursenbacher *et al*., 2006; Guicking, Joger & Wink, 2009). For other species such as the dice snake, *Natrix tessellata* (Laurenti 1768), populations from the Near East

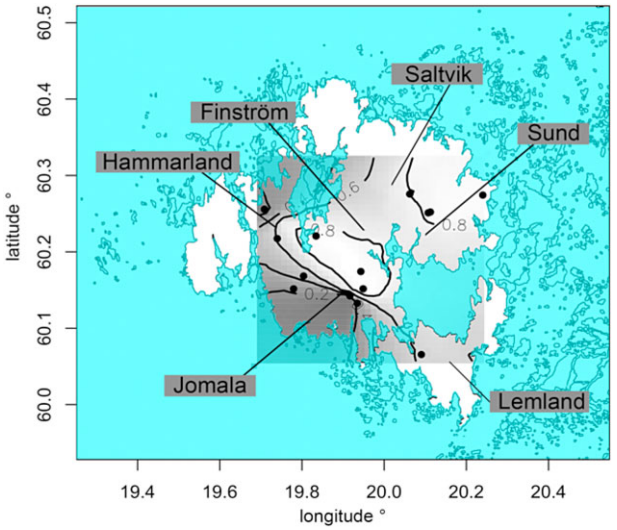

Figure 4. Smooth snake (*Coronella austriaca*) sampling sites in Åland (dots), and map of posterior probabilities of population membership for samples within each site. Contour lines indicate genetic discontinuities. Grey gradient indicates probability of population membership.

(Iran) have been reported as ancestral (Guicking *et al*., 2009). In our case, the two samples from Georgia occupied the basal positions of the Eastern clade. However, the lack of intermediate samples from Eastern Europe such as Ukraine and Belarus impede a full assessment of the geographical evolution of this clade.

Our data clearly support the existence of three independent lineages with no apparent overlap in their distribution ranges (Fig. 1). The relatively high genetic diversity of southern populations compared with northern ones supports the idea of south to north expansions (Fig. 2, Supporting information, Fig. S1). However, more samples and intermediate locations are needed to confirm this. Of the three lineages, the Central one expanded the furthest north. This could indicate a slightly better aptitude for dispersal or differences in life-history traits, such as brumation and cold tolerance. Further work is needed to corroborate this, and more detailed sampling along contact zones would be valuable. Overall, this study together with those of Santos *et al*. (2008) and Pernetta *et al*. (2011) provide a good representation of the overall biogeography of the smooth snake and its fine-scale structuring patterns in edge-populations.

Demography and population genetic structure in Åland

In Scandinavia, two main colonization routes – from south-west and north-east – have been proposed based for several plants and animals (Hewitt, 2000; Blum *et al*., 2004). This is also the case for the two other snake species that inhabit Åland, the grass snake and the adder (Carlsson, Söderberg, & Tegelström, 2004). A previous study showed that Åland adders are mostly of Swedish origin, with a contact zone between Eastern and Western lineages occurring at the Finnish archipelago (Marek & Bond, 2009). No grass snakes from Åland or Finland have been studied, although samples from Sweden and Russia show a distinct origin, suggesting two separate lineages (Thorpe, 1984). Interestingly, both the grass snake and the adder have spread further north than the smooth snake, reaching up to 67 and 69°N, respectively (Arnold *et al*., 1978). Both the grass snake and the adder also occur in Finland, whereas the smooth snake does not. Furthermore, no smooth snake expansion into Finland has occurred from the Eastern lineage through Russia, suggesting that the Eastern lineage hardly occurs at such high latitudes or has a reduced dispersal. Moreover, the smooth snake is restricted to the southern part of Sweden. We cannot be sure, however, if the smooth snake reached Åland naturally by swimming across the Baltic Sea from Sweden, or by anthropogenic introductions. However, natural range expansion is plausible given the short distances (< 30 km) separating the islands between Sweden and Åland.

The population subdivision in Åland may be a consequence of various factors. First, the habitat is naturally fragmented by an island landscape mosaic and anthropogenic influences (e.g. urbanization and agriculture). Previous empirical and theoretical studies have shown that the smooth snake is highly susceptible to habitat fragmentation. Habitat-suitability models and field observations have shown that smooth snakes occur mainly in small and fragmented areas in the Iberian peninsula (Santos *et al*., 2009). Secondly, intrinsic life-history traits can also be a major factor influencing population substructure. Snakes in temperate climates often use the same hibernacula for overwintering year after year, thereby limiting their home range (Viitanen, 1967; Larsen, 1987; Weatherhead & Housak, 1998) and thus gene flow between different areas. It has also been proposed that the low vagility of the smooth snake can have a strong effect in determining population subdivision (Pernetta *et al*., 2011). Thirdly, Åland is an island, and thus gene flow through immigration can be outweighed by drift and selection, giving rise to the evolution of local adaptations and genetic isolation between populations. This has already been shown to be possible for the smooth snake, where differences in scale counts and head shape were found between different genetic lineages (Llorente *et al*., 2012).

Fourthly, smooth snakes mimic the venomous viper (*Vipera* spp.) by flattening their head and making its shape triangular, which can enhance their survival as some predators avoid vipers (Valkonen, Nokelainen & Mappes, 2011; Valkonen *et al*., 2012). Vipers are often abominated and killed by humans, who may accidently kill smooth snakes (Valkonen & Mappes, 2014). Thus, habitat fragmentation, phylopatric behaviour, isolation and human-induced mortality can contribute to erode the genetic diversity of this edge-population, making it vulnerable to local extinctions.

In conclusion, the smooth snake displays three distinct lineages of which the Central one expanded into Åland, representing the northernmost population. Here, the population is fragmented into two genetically isolated subpopulations with low mitochondrial variability, and is critically small. According the IUCN red list categories and criteria (IUCN, 2012b), if population size is estimated below 250 but not as low as 50 (threshold for critically endangered) adult individuals it should be considered as endangered. Additionally, if a population is isolated, recommendations for national classifications should follow international ones (IUCN, 2012a). Therefore, we recommend reclassification of the current threatened status to endangered.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Number of Cyt-b haplotypes observed across samples grouped in latitudinal bins. A, Western clade; B, Central clade; C, Eastern clade.

Figure S2. Smooth snake (*Coronella austriaca*) biogeography. 16S gene Bayesian majority-rule phylogeographical tree showing posterior probabilities; only values > 90 are displayed.

Figure S3. Smooth snake (*Coronella austriaca*) biogeography. 16S gene ML majority-rule phylogeographical tree showing bootstrap support; only values > 90 are displayed.

Figure S4. Smooth snake (*Coronella austriaca*) biogeography. Cyt-b gene Bayesian majority-rule phylogeographical tree showing posterior probabilities; only values > 90 are displayed.

Figure S5. Smooth snake (*Coronella austriaca*) biogeography. Cyt-b gene ML majority-rule phylogeographical tree showing bootstrap support; only values > 85 are displayed.

Figure S6. Smooth snake (*Coronella austriaca*) biogeography. Concatenated 16S + Cyt-b gene Bayesian majority-rule phylogeographical tree showing posterior probabilities; only values > 85 are displayed.

Figure S7. Smooth snake (*Coronella austriaca*) biogeography. Concatenated 16S + Cyt-b gene ML majority-rule phylogeographical tree showing bootstrap support; only values > 85 are displayed.

Figure S8. Smooth snake (*Coronella austriaca*) biogeography. COI gene Bayesian majority-rule phylogeographical tree showing posterior probabilities; only values > 85 are displayed.

Figure S9. Smooth snake (*Coronella austriaca*) biogeography. COI gene ML majority-rule phylogeographic tree showing bootstrap support; only values > 90 are displayed.

Table S1. Source details of smooth snake (*Coronella austriaca*) samples included in this study.

Table S2. Primers and adaptor combinations used to amplify 16S, Cyt-b and COI genes in the smooth snake (*Coronella austriaca*).

Table S3. Observed clades of smooth snake (*Coronella austriaca*). Shown are the number of bins used, their latitudinal range, the number of sequences contained and the number of haplotypes found within each bin.

Table S4. Microsatellite diversity in the smooth snake (*Coronella austriaca*) from Åland. N, number of individuals genotyped; A, number of alleles; Rs, allelic richness; He, expected heterozygosity; Ho, observed heterozygosity; FIS, inbreeding.

Table S5. F_{ST} pairwise comparisons between smooth snake (*Coronella austriaca*) populations in Åland based on microsatellite markers. $^*P < 0.005$.