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ORIGINAL ARTICLE

Insect Conservation and Diversity

Strong genetic differentiation between fragmented alpine bush-cricket populations demands preservation of evolutionary significant units

Isabel Martinez-Sañudo[1] | Andrea Basso[2] | Giacomo Ortis[1] | Federico Marangoni[3] | Gionata Stancher[3] | Luca Mazzon[1]

[1]Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padua, Legnaro, Padova, Italy

[2]Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Legnaro, Padova, Italy

[3]Fondazione Museo Civico (FMC), Rovereto, Trentino, Italy

Correspondence
Luca Mazzon, Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padua, Viale dell'Università 16, 35020 Legnaro, PD, Italy.
Email: lmazzon@unipd.it

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Abstract

1. The eastern alpine bush cricket, *Anonconotus italoaustriacus* Nadig, 1987, is a grassland species historically present in a few disjunct ranges in north-eastern Italy and High Tauern in Austria. The species has recently been included in the Red List of the International Union for Conservation of Nature (IUCN) as an endangered species with a decreasing population trend.
2. Information regarding the genetic structure of endangered insect populations and the delineation of evolutionary significant units (ESUs) are nowadays a useful and integral component of many conservation plans. The genetic differentiation of *A. italoaustriacus* populations was studied through the analysis of four fragments of the mitochondrial DNA in five disjunct populations, covering the entire Italian geographical distribution known for this taxon and partially the Austrian distribution.
3. Results revealed a strong geographical structure among populations and complete absence of gene flow suggesting the need to protect these evolutionary distinct lineages. Divergence time estimation analyses suggested that an ancient separation of bush-cricket populations occurred approximately 1–1.5 Mya.
4. Since the *A. italoaustriacus* populations represented genetically differentiated entities, conservation efforts should consider each population as a management unit. Conservation actions, preceded by detailed ecological studies and focused on monitoring, preserving and enlarging the existing habitat patches are thus proposed.

KEYWORDS
dispersal ability, flightless species, gene flow, haplotype, katydid

INTRODUCTION

Many species, especially from mountainous regions, occur in subdivided populations due to spatial heterogeneity in the landscape (Segelbacher & Storch, 2002; Watson, 2002; Weston & Robertson, 2015). In high-altitude species with narrow distribution, populations typically exist as small, isolated subunits that are at elevated risk of extinction due to the combined influences of

demographic, climate or habitat changes (Caughley, 1994; Elsen & Tingley, 2015). In particular, for taxa with micro-endemic patterns, geographic range size is widely correlated with extinction risk (Gaston & Fuller, 2009).

The absence of gene flow among subdivided populations makes them evolve in isolation from each other, leading to the formation of evolutionary significant units (ESUs) (Fraser & Bernatchez, 2001). Identifying ESUs is still a preliminary step in conservation. Efforts to preserve a threatened species without considering its genetic differentiation may fail to conserve the full spectrum of diversity the species encompasses (Pearse & Crandall, 2004). Consequently, knowledge on the distribution of genetic differentiation within species combined with a demographic history of the studied species are necessary for defining ESUs, which provides a framework for identification of separate, intra-specific management units (MUs) for conservation prioritisation (Crandall et al., 2000; Fraser & Bernatchez, 2001; Moritz, 1994; Moritz, 2002; Palsbøll et al., 2007). Mitochondrial DNA data are known to provide meaningful estimates of genetic differentiation. However, it could sometimes present some drawbacks such as presence of pseudogenes or hybridisation. Despite this general criticism, it has been shown to be worthwhile, as it possibly reflects several evolutionary processes (Hawlitschek et al., 2017).

Many semi-natural grassland species, with the recent decline and deterioration of their habitats, are now facing decline or extinction (Bubová et al., 2015; Isselstein et al., 2005; Uchida & Ushimaru, 2014). *Anonconotus* is a genus of endemic Ensifera present in grasslands of the Alps and Apennines. In Italy, there are nine *Anonconotus* species mostly located in the western Alps, and only one present in the eastern Alps (Galvagni, 2005; Galvagni & Fontana, 2004; Massa & Fontana, 2020). Here, we focus on the eastern alpine bush-cricket, *Anonconotus italoaustriacus* Nadig, 1987, which is a grassland species present exclusively in north-eastern Italy and in the High Tauern in Austria (Galvagni, 2005; Galvagni & Fontana, 2004; Illich et al., 2010; Zuna-Kratky et al., 2016). This flightless species inhabits fairly small habitat patches usually located above 2000 m in alpine and pre-alpine grasslands and dwarf shrub heaths, characterised by the presence of *Juniperus* sp., *Rhododendron* sp., and *Erica* sp. (Fontana, 2002; Massa et al., 2012). It prefers steep sunny slopes with a southeast to southwest exposure. Only four populations of the bush cricket have been reported to date in Italy, more precisely: the population of the Belluno Dolomites (Vette Feltrine, Busa delle Vette, BL), Scilliar Group (Alpe di Siusi, BZ), the population of San Candido (Monte Elmo, BZ), and the Baldo Group (Monte Altissimo, TN) (Massa et al., 2012). Because of its range restricted to a few locations scattered between Italy and Austria, the severely fragmented populations and a continuing decline in its area of occupancy as well as in the extent of occurrence, *A. italoaustriacus* has been included in the Red List of the International Union for Conservation of Nature (IUCN) as an endangered species with a decreasing population trend (www.iucnredlist.org). Furthermore, the extent and quality of suitable habitats, the number of populations as well as number of mature individuals seem to be declining, mainly due to abandonment of extensive grazing and mowing (Zuna-Kratky et al., 2016).

We predicted that *A. italoaustriacus* may be spatially split into distinct genetic groups, isolated because of the geographic barriers in its range. To verify this and delineate ESUs for conservation, the population structure, phylogenetic relationships, and evolutionary history of the four known Italian populations as well as one population from Austria were investigated by analysing four fragments of mitochondrial DNA.

MATERIALS AND METHODS

Sample collection

Samples of *A. italoaustriacus* were collected from the four disjunct populations covering its entire known Italian geographical distribution. In particular, the study areas were located in north-eastern Italy and included sites where the species has been historically reported in the literature: Monte Elmo (ME), Alpi di Siusi (AS), Monte Altissimo (MA) and Vette Feltrine (FV) (Galvagni & Fontana, 2004). In addition, a site within the Austrian distribution range of High Tauern (Heinkaralm) (AiO) was also considered for sample collection. The mean distance between all sample sites was 88 km (14 $\pm$ SE), ranging from 50.5 km to 167.1 km. In all sites, the sampling was conducted at altitudes between 1886 and 2362 m a.s.l. (Table 1).

Adults of *A. italoaustriacus* were collected by a combination of manual catching and sweep netting, throughout the late summer of two successive years (2018 and 2019). Due to its endangered status no more than 15 samples were collected from each site. To avoid sampling relatives, bush-cricket specimens were collected by sweeping randomly at different points in each sampling site. After capturing and identification, a leg was removed from each individual for DNA analysis, and in most cases specimens were subsequently released in the same point of collection, as described in other studies involving endangered Orthoptera (e.g. Contreras-Díaz et al., 2006). Samples were immediately kept in 95% ethanol and stored in the laboratory in individual vials at -20°C until DNA extraction.

Furthermore, four related tettigoniid species: *A. alpinus*, *A. ghilianii*, *A. occidentalis* and *Barbitistes vicetinus*, collected in previous orthopteran surveys, were used as outgroups to encompass a representative sample for the following molecular analysis (Table 1).

Molecular analysis

Total DNA was extracted separately from a femur of each specimen using DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD, USA) according to the indications provided by the manufacturer. Before starting with the lysis step, each sample was manually homogenised in lysis buffer and then incubated at 56°C overnight for the digestion process.

Five fragments of the mitochondrial genome were chosen for amplification: the COX1fragment, the NAD4 fragment, the 5' of 12S rRNA and the adjacent part of control region (CR) and a second part

TABLE 1 Sampled sites (approximate midpoint) of *Anonconotus italoaustriacus* across all its distribution range and the four tettigoniid species used as outgroups in the molecular analyses

Species	Site (country)	ID	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)
Anonconotus italoaustriacus	Monte Elmo (I)	ME	46°42′49″	12°23′12.7″	2362
	Alpi di Siusi (I)	AS	46°30′41″	11°35′22″	2139
	Monte Altissimo (I)	MA	45°48′36″	10°53′17″	2075
	Vette Feltrine (I)	VF	46°5′34″	11°50′38″	1886
	Heinkaralm (A)	AiO	46°52′12″	12°25′9″	2138
Anonconotus alpinus	La Thuille (I)		45°42′29″	6°55′5″	1831
Anonconotus ghilianii	Lago Moncenisio (I)		45°13′40″	6°57′44″	2006
Anonconotus occidentalis	Colle degli Agnelli (I)		44°41′41″	6°58′44″	2440
Barbitistes vicetinus	Monselice (I)		45°14′53″	11°44′39″	70

of CR with the primers listed in table (Table S1). The amplification thermal protocol for the fragment including COX1 and NAD4 involved the following conditions: denaturation at 95°C for 5 min; followed by 35 cycles at 95°C for 30 s, 45–56°C × 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. For 12S rRNA-CR amplification, the thermal profile followed the conditions described by Eweleit et al. (2015) consisting of 2 min at 92°C followed by 35 cycles with a denaturation step of 92°C for 2 min, an annealing step of 52°C for 30 s, and extension step of 60°C for 3 min, with a final extension of 72°C for 7 min. Each reaction was performed at the following final concentrations: 1× PCR Go Taq Flexi buffer – Promega, 2.5 mM MgCl2, 0.1 mM dNTPs, 0.5 μM for each primer, 0.5 U of Taq polymerase – Promega, 2 μl DNA template for a final volume of 20 μl. Then amplicons were visualised on 1% Agarose gel and all suitable PCR products were purified with ExoSap enzymes (ExoSAP-IT; USB Corp.) before sequencing at the BMR Genomics Company (Padua, Italy).

DNA sequence chromatograms were quality checked and manually corrected when necessary using MEGA X (Kumar et al., 2018). Low-quality regions found at the beginning and end of each sequence were trimmed and low-quality sequences were not included in the analysis.

The protein coding sequences were translated with Transeq (EMBOSS: http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) to exclude the presence of any nuclear mitochondrial pseudogenes. The bioinformatics tool translates nucleic acid sequences to their corresponding peptide sequences and identifies stop codons. Since these pseudogenes (NUMTs) are characterised by the accumulation of in-frame stop codons and indels (Bensasson et al., 2001) the absence of stop codons in the protein sequence can allow the presence of NUMTs to be excluded.

Protein coding genes were aligned using TranslatorX software with MAFFT (http://translatorx.co.uk/) (Abascal et al., 2010). Not coding protein genes were aligned by the ClustalW approach available in MEGA X. To study the phylogenetic relationship among datasets, only unique sequences were retrieved using DNAsp v6 (Rozas et al., 2017). The subsets of alignments were then combined in a unique concatenated alignment (hereafter 4.mtDNA).

Bioinformatic analyses

Genetic variability and population structure

Haplotype and nucleotide diversity, as well as the pairwise genetic distances between populations, were calculated with Arlequin 3.5 (Excoffier & Lischer, 2010) and MEGA X. A haplotype parsimony network with a probability cut-off of 95% was reconstructed using the TCS 1.21 software (Clement et al., 2000) and PopART 1.7 (Leigh & Bryant, 2015) and used for depicting the geographical relationships among haplotypes. Ambiguous connections (loops) were resolved using approaches from coalescent theory based on three criteria: frequency, network location and geography (Crandall, 1994; Crandall & Templeton, 1993).

Demographic history

Past demographic history of the species was inferred using Arlequin 3.5 through the Tajima's D and Fu's Fs tests (Fu, 1997; Tajima, 1989), and the mismatch distributions of the pairwise genetic differences (Rogers & Harpending, 1992).

Phylogenetic analysis

Maximum likelihood (ML) phylogenetic analyses were performed with Iqtree v1.6.12 software (Nguyen et al., 2015) on 4.mtDNA.us dataset. Firstly, the dataset was analysed using the edge-proportional partition model implemented in Iqtree to choose the best evolutionary model considering the partitions (Chernomor et al., 2016; Nguyen et al., 2015). Then the phylogenetic analysis was run independently 10 times to select the best tree according to the log-likelihood score. The Robinson–Foulds distance (Robinson & Foulds, 1981) was computed between the 10 trees, but the result was not conclusive due to the high variation within terminal taxa. The selected tree was fixed on the final ML analysis to infer the SH-like approximate likelihood ratio tests (SH-aLRT) on branches and the ultrafast bootstrap (UFB) on the

nodes using 10,000 replicates for each test (Hoang et al., 2018; Minh et al., 2013). The tree was rooted on the outgroup species *Barbitistes vicetinus*. Furthermore, combining the maximum parsimony (MP) approach implemented in Mesquite 3.70 software (Maddison & Maddison, 2019), with the previously calculated haplotype phylogenetic relationships, we inferred the ancestral range distribution of *A. italoaustriacus* along the Alps.

Divergence time estimation

The divergence time estimation (DTE) requires information to calibrate the analyses, using known dated fossil records, palaeogeographical events or substitution rates, which commonly show a degree of variability among genes and lineages over time (Allegrucci et al., 2009; Drummond et al., 2006; Ho, 2007; Ho et al., 2008; Ho & Larson, 2006; Papadopoulou et al., 2010). The strategy involved a Bayesian inference (BI) analysis (Drummond et al., 2002) implemented in BEAST2 software (Bouckaert et al., 2014) available on CIPRES Portal (Miller et al., 2010) with the following setting. The study was run under random local clock (RLC) using HKY substitution model (according to the ML-analysis: see Results section), which was set to estimate the frequencies on the 4.mtDNA dataset. The Bayesian tree search was performed with the Yule model. Due to the unavailability of molecular data about the species of *Anonconotus*, we decided to infer the DTE using the empirical mutation ranging from 2% to 3% (mean 2.5%, equal to 1.25×10^{-8} substitutions/site/year) according to the literature (Allegrucci et al., 2009; Brower, 1994; Chang et al., 2020; Martinez-Sañudo et al., 2021; Papadopoulou et al., 2010; Rogers, 1995). To assess the convergence across independent runs, we conducted the RLC_HKY analyses twice, each one set for 50 million generations and sampling trees every 1000 generations with a final burn-in of 25%. Settings were coded using BEAUti2 (Drummond et al., 2012). The log files were investigated with the Tracer v1.7.1 package to detect problems within the analyses (Nylander et al., 2008; Rambaut et al., 2018), and the trees were summarised with TreeAnnotator through Maximum clade credibility option (Drummond & Rambaut, 2007). Annotated trees were then combined using LogCombiner software, and the output file was visualised with FigTree v1.4.4 (Bouckaert et al., 2014; Drummond & Rambaut, 2007). Posterior probabilities (pps) were considered significant at 95%.

RESULTS

Sample collection

Samples of *A. italoaustriacus* were found in four of the five sites chosen for the study on the basis of the literature: Monte Elmo (ME), Alpi di Siusi (AS), Vette Feltrine (VF) and Heinkaralm (AiO). In the 2 years of samplings, no specimens were retrieved in Monte Altissimo (MA).

Data analysis

The obtained sequences were aligned gene-by-gene, after quality assessment and trimming, producing five alignments spanning, respectively 675 nt for COX1, 477 nt for NAD4, 233 nt for 12S rRNA, and 315 + 422 nt for the portions of CR. A final dataset including 48 concatenated sequences of 2019 nt was obtained. Further analyses were conducted considering the combined dataset.

The presence of pseudogenes in the coding sequences was excluded by Transeq. The COX1 sequences were compared with sequences of the BoldSystem database. A similarity of >99% with genus *Anonconotus* was obtained since to date, no sequences of the species *A. italoaustriacus* have been deposited.

The mitochondrial fragments 12S rRNA and the first part of CR of the outgroup species *Anonconotus occidentalis* were not successfully amplified. The respective portions in the concatenated alignment were then coded as missing data into the 4.mtDNA dataset.

Sequences of each haplotype of *A. italoaustriacus* obtained in this study are available through GenBank accession numbers OL364192-OL364215. Outgroup sequences have been submitted under the accession numbers OL580778-OL580785 (NAD4), OL471011-OL471014 (COX1) and OL470896-OL470898 (12S rRNA-CR).

Genetic variability and population structure

The diversity indexes for the concatenated dataset ranged from 0.63 (AS) to 0.93(AiO) for haplotype diversity (H) and between 0.14 (AS and VF) and 0.21(AiO) for nucleotide diversity (π). The distribution of haplotypes among all populations analysed and other summary statistics are shown in Table 2.

The haplotype network of the combined dataset revealed the presence of 24 haplotypes, all of them exclusive to a single population (Figure 1). No haplotype was shared by samples from two (or more) populations. Samples from ME were represented by five connected haplotypes. Similarly, samples from AS were included in five grouped haplotypes. Sequences of the AiO samples were included in six haplotypes scattered throughout the network and connected with haplotypes represented by samples from ME or AS (Table 2 and Figure 1). The VF samples were represented by a high number of connected haplotypes ($n = 8$) and separated from the other populations (Me, AS and AiO) by 19 mutational steps with missing intermediate haplotypes (Figure 1). Genetic distances of the COX1 marker between VF individuals and specimens from the other populations showed values ranging from 0.009 to 0.01.

Since the control region is known to be highly variable in insects and sometimes even contains microsatellites (Sureshan et al., 2021; Taylor et al., 1993), a haplotype network was constructed by removing CR sequences to avoid any possible bias. Even if the number of haplotypes decreased, the topology of the network was maintained, as well as the presence of haplotypes exclusive to a single population. Regarding the VF population, the individuals were still included in

TABLE 2 Descriptive statistics of each population and summary of the past demographic events analysis

Site	Haplotypes	Number of haplotypes	H	π (%)	Tajima's D	Fu's Fs	SSD	*r*
Monte Elmo (ME)	h12(5) h13(4) h14(1) h15(1) h16(1)	5	0.76 ± 0.09	0.15	0.55 ($p > 0.1$)	0.91 ($p > 0.1$)	0.1 ($p = 0.14$)	0.22 ($p = 0.15$)
Alpi di Siusi (AS)	h07(3) h08(9) h09(1) h10(1) h11(1)	5	0.63 ± 0.12	0.14	0.62 ($p > 0.1$)	1.11 ($p > 0.1$)	0.11 ($p = 0.07$)	0.28 ($p = 0.09$)
Vette Feltrine (VF)	h17(1) h18(4) h19(1) h20(1) h21(1) h22 (1) h23(2) h24(2)	8	0.90 ± 0.07	0.14	−0.41 ($p > 0.1$)	−2.30 ($p > 0.5$)	0.01 ($p = 0.59$)	0.04 ($p = 0.62$)
Heinkaralm (AiO)	h01(1) h02(1) h03(1) h04(2) h05(2) h06 (1)	6	0.93 ± 0.08	0.21	0.47 ($p > 0.1$)	−0.76 ($p > 0.1$)	0.02 ($p = 0.45$)	0.06 ($p = 0.84$)

Note: In the haplotypes column, numbers within brackets represent the number of specimens showing this haplotype.
Abbreviations: H, haplotype diversity ± standard deviation; π, nucleotide diversity; SSD, sum of square deviations; r, raggedness index.

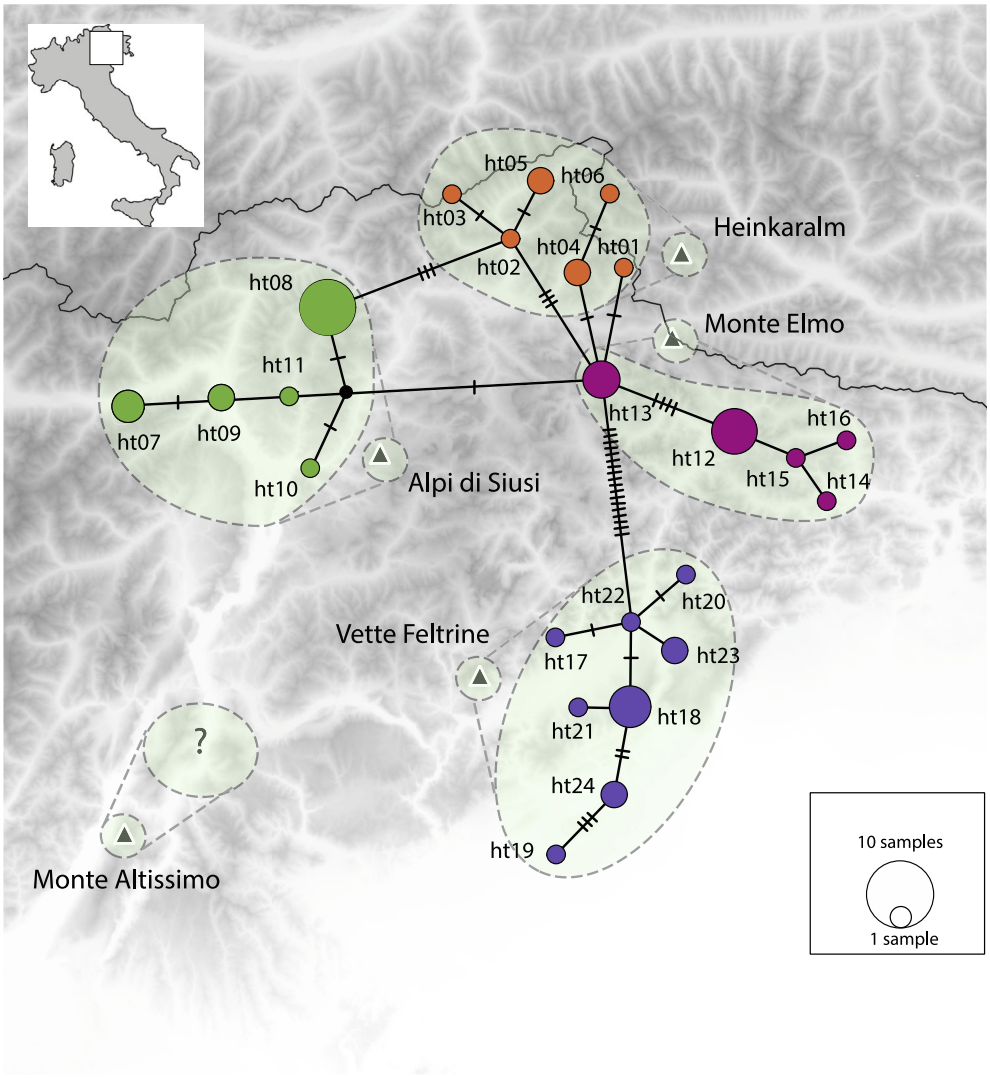

FIGURE 1 Haplotype network based on the combined dataset of mitochondrial DNA of *Anonconotus italoaustriacus* Nadig, 1987 showing geographical distribution of haplogroups. The haplotypes are represented by circles whose sizes are proportional to their frequencies. Colours represent the different populations where haplotypes are present. Each node or little bar represents one mutational step.

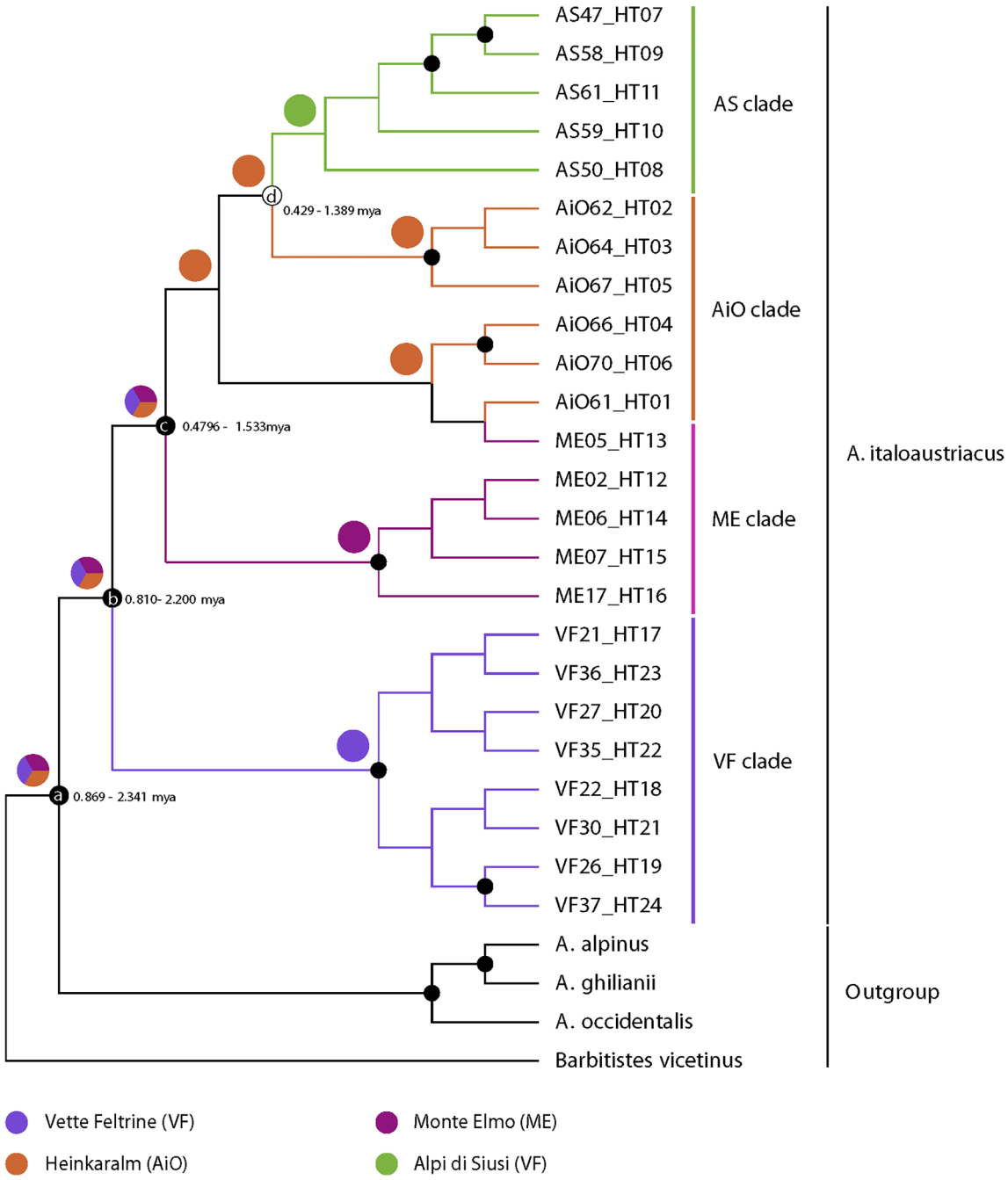

FIGURE 2 Phylogenetic tree and estimation of *Anonconotus italoaustriacus* clades divergent time, based on ML and BI analyses. ●, indicate fully supported nodes by posterior probabilities (BI), ultrafast bootstrap and SH-aLRT branch support (ML); ◦, indicate a node supported only in ML analysis. Numbers reported on selected nodes indicate the divergent time estimated in millions of years ago (mya). Pies on these nodes show the ancestral distribution through MP analysis.

haplotypes separated by some mutational steps from the other population haplotypes (Figure S1).

Demographic history

Neutrality tests (Tajima's D and Fu's Fs) were applied in order to check for past demographic events. The null hypothesis of neutrality was not rejected in any of the populations analysed (Table 2) suggesting population equilibrium, thus neither expansion nor selection in these populations. The mismatch distributions of populations AS and ME were broadly multimodal, as well as the one of AiO population albeit to a lesser extent, as expected under a model of relative constant population size. In contrast, mismatch distribution of VF was unimodal (Figure S2). All populations showed SSD and raggedness index values that did not reject a sudden expansion model. In particular, the

population from VF showed not significant low *r* values (<0.05) suggesting a population expansion event (Table 2). In addition, although *r* index was not significant, populations from ME and AS high showed high *r* values (>0.05) typical of stationary populations (Table 2).

Phylogenetic and DTE analyses

ML tree was computed (−ln = 6075.7194) with the optimal partitioning scheme and the best-fitting evolutionary models according to BIC scores (TIM2 + F + G4 for COX1, TPM2u + F + I for nad4, and HKY + F + R2 for 12S rRNA and CR) and displays a stable backbone topology. *Anonconotus italoaustriacus* forms a monophyletic group with specimens clustered into well-supported clades mainly based on collection sites, with the exception of ME05_HT13, assigned to the AiO cluster. Furthermore, ML and BI analyses obtained with comparable evolutionary models retrieved trees with congruent topology and supports, except for node d, which is supported only by ML (Figure 2). DTE analysis set the separation of *A. italoaustriacus* (a) as occurring around 1.6 Mya (95% HPD interval 0.9–2.3). Within *A. italoaustriacus* species, the VF clade (b) separated from other clades around 1.5 Mya (95% HPD interval 0.8–2.2). Similarly, the split and the paraphyly of the ME clade (c) tend to 1.0 Mya (95% HPD interval 0.5–1.5). The node between AS and AiO clades (d) was supported only by ML analysis with divergence estimated at 0.9 Mya (95% HPD interval 0.4–1.4). Other strongly supported nodes within populations are not further discussed, given that there are no physical barriers to specimens dividing inside their population.

DISCUSSION

This study presents, for the first time, detailed information regarding population genetic structure of the endangered species *A. italoaustriacus* and reveals the presence of a high geographical structuring among populations. Overall, even though the sampling size was limited due to the endangered status of the species, our findings allow us to confirm the genetic isolation of the four *A. italoaustriacus* populations.

No specimens of *A. italoaustriacus* could be found in the study areas of Monte Altissimo. The species was retrieved by Krauss on the top of this mountain (Krauss, 1909). Several efforts were later made by Galvagni and other colleagues (Galvagni, 2005; Galvagni & Fontana, 2004) to locate the bush-cricket in this area but as in our case, repeated attempts were unsuccessful, suggesting the extinction of the species in this area. Galvagni and Fontana (2004) proposed that alterations in the habitat during the First World War and subsequent building interventions in the limited mountain area could have drastically reduced populations in Monte Altissimo and even led to population extinction.

Samples found in the other four known distribution sites of *A. italoaustriacus* showed high haplotype diversity within each population and all the haplotypes retrieved were exclusive to a single population. In phytophagous insects, dispersal capacity, geographical or reproductive barriers, host plant and habitat fragmentation are reported as the main drivers of genetic structure (Bertheau et al., 2013; Bon et al., 2015; Lesieur et al., 2016). *A. italoaustriacus* is a flightless species and similarly to other ground-dispersing species of the Tettigoniidae family (e.g. *Pholidoptera griseoaptera* and *Barbitistes vicetinus*) (Cavaletto et al., 2019; Diekötter et al., 2005), moves only relatively short distances during its lifetime. This limited dispersal ability together with the great distances separating the populations with geographical barriers (e.g. valleys) and the tendency to live in fragmented patches have favoured the lack of gene flow.

The analysed populations were characterised by the presence of several rare haplotypes with almost no high-frequency one. This pattern is consistent with what is expected for populations in equilibrium as highlighted by both the neutrality test (Tajima's D and Fu's Fs) and the multimodality of the mismatch distribution curves, especially in ME, AS and AiO populations (even though SSD and raggedness index did not confirm it). Clues of past demographic expansions could be inferred for VF population based on its unimodal mismatch distribution curve and the not significant SSD and raggedness values which cannot reject the hypothesis of population expansion.

The strong differentiation of the VF population deserves a little bit more attention. Both the network (with and without CR sequences) and the phylogenetic tree showed that VF were genetically different from the other Alpine populations. This high level of sequence divergence suggests a long-term isolation of VF populations with respect to the others and it was also confirmed by the DTE. Furthermore, genetic distances of the COX1 gene between VF individuals and the other populations (i.e. around 0.01) were at the limit of the range generally found between subspecies (0.01–0.02) (Avise, 2000) suggesting that this population could be treated as a subspecies. *A. italoaustriacus* was first recovered in the Vette Feltrine by Marcuzzi (1961). Although it is a population with high density, it appears extremely restricted to the area of the glacial circus "Busa delle Vette" (Dolomiti Bellunesi National Park) about 24 km of the SE limit of the Alps, and thus particular attention should be given to this population, so that it may be appropriately protected. Further studies, combining genetic, morphological and/or behavioural surveys, should be conducted to determine whether the VF population can be considered a different taxonomic status. The DTE indicated that separation of VF population from other clades could have occurred approximately 1.5 Mya (0.810–2.200 Mya), during the early Pleistocene. After surviving low temperatures of the glacial periods and once climatic conditions were favourable, VF populations might have experienced an expansion during the interglacial periods that shaped the present genetic structure of the population. The ancestral range distribution analysis suggests that anciently the species was homogeneously distributed from AiO to VF (including AS) and, subsequently, they underwent significant contractions probably as an effect of the climate instability occurring ca 1.5 Mya (Figure 2 node d) (Hansen et al., 2013; Lisiecki & Raymo, 2005). In addition, results suggest that populations from ME and AiO separated approximately 1 Mya. We could hypothesize that other *A. italoaustriacus* populations inhabited the Alps during

the interglacial period of the early Pleistocene. With the arrival of the glaciation (Günz: 0.9–1.2 Mya) only some populations managed to survive (e.g. in refugia areas or ice-free mountain tops, 'nunataks') leading to separation, occurring first in ME and later in AiO and AS populations. The presence of refugia in south-eastern and central Europe during the Pleistocene have been reported for several plant and animal species, including other cold-tolerant and low dispersal species (Pauls et al., 2006; Pinceel et al., 2005). In particular, refugial areas on the southern and eastern Alps and ice-free mountain tops in inner Alpine regions have been proposed as alternative sources for species survival during glaciations (Pan et al., 2020; Schönswetter & Schneeweiss, 2019; Von Reumont et al., 2012). Further genetic studies using deeper sequencing methods, such as microsatellites, will shed light on recent historical events, overcoming some mtDNA data limitations.

As with other poor dispersers, the absence of gene flow and low geographical dispersion could make *A. italoaustriacus* more prone to extinction, so conservation strategies are therefore needed. Due to the geographical distance and low dispersal ability of the species, it is not feasible to enhance genetic connectivity among populations. The arrival of immigrants from other populations to the isolated areas where the species lives is not likely and so these populations require adequate conservation strategies to avoid extinctions. Furthermore, promoting gene flow between these strongly differentiated populations could even have deleterious effects such as outbreeding depression (Ralls et al., 2013). Therefore, crossing populations is not advisable in this case. However, results show that, despite the strong fragmentation of species inhabiting the small habitat patches, the bush-cricket populations have maintained genetic diversity over time, even in the absence of gene flow.

Given the deep divergence among *A. italoaustriacus* populations, to preserve the full genetic variation within the species, we propose to consider these populations as ESUs in future conservation management strategies. Although the species is well known as a taxonomic entity (Galvagni & Fontana, 2004; Zuna-Kratky et al., 2016), little is known about its biology. The presence of the species in alpine and pre-alpine grasslands and dwarf shrub heaths of *Juniperus* sp., *Rhododendron* sp., and *Erica* sp. has been reported (Fontana, 2002; Massa et al., 2012), but the real need in terms of host plants is still unknown. The species could use them as food plants and/or for microclimatic advantages. Therefore, the conservation actions of the management units should be preceded by ecological and biological studies of the species. Paramount measures for conserving *A. italoaustriacus* could be focused on the protection of their habitats, for example, by limiting human disturbances and grazing with fences.

A management action might be the translocation of adults from the known populations to nearby apparently suitable areas. Translocation is a measure of common use in conservation actions that may result in the founding of new populations (Seddon et al., 2007). *A. italoaustriacus* inhabits small habitat patches and, on the basis of our knowledge close suitable habitats for the species should be available. The reasons why these apparently appropriate habitats are unoccupied are not clear. We could hypothesise that these areas, which were once unsuitable, have been recently restored, becoming suitable (e.g. decrease in grazing pressure). Thorough studies focused on the habitat features of every single population site will be necessary before any translocation action. Conducting translocations of individuals of one population to nearby patches without any geographical barriers, even areas 600 m away from the population main core, would allow genetic connectivity, favouring the establishment of new larger populations. Nevertheless, any action of translocation needs thorough planning and management. Previous detailed studies are required on population dynamics, dispersal abilities, habitat suitability and other poorly understood factors such as principal food plants of the bush cricket.

Finally, establishing the species in Monte Altissimo, where it was not retrieved, or in new suitable sites identified in the eastern Alps could be a limited solution because the bush cricket may not readily colonise new patches. A thorough analysis of the threats on Monte Altissimo (e.g. habitat uses, climatic conditions) will be necessary. Furthermore, genetic analyses of potential museum specimens collected in Monte Altissimo as well as other Austrian populations (e.g. Pöllatal population) would provide crucial information for the choice of genotypes to be used.

In conclusion, in accordance with the genetic results, we proposed to consider *A. italoaustriacus* populations as ESUs and management units (MUs). The main focus should be on expanding these populations in their areas to preserve the genetic diversity of the species over time. These populations will probably be able to conserve their diversity even without gene flow. Finally, the protection of these habitats by safeguarding from human disturbances and increasing their size may be the best measure for conserving the genetic diversity.

AUTHOR CONTRIBUTIONS

Isabel Martinez-Sañudo: Conceptualization (equal); data curation (lead); formal analysis (lead); methodology (lead); supervision (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal). **Andrea Basso:** Data curation (supporting); formal analysis (equal); methodology (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal). **Giacomo Ortis:** Formal analysis (supporting); investigation (equal); validation (equal); writing – original draft (supporting). **Federico Marangoni:** Conceptualization (supporting); formal analysis (supporting); investigation (equal); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Gionata Stancher:** Conceptualization (equal); resources (equal); validation (equal); writing – original draft (supporting). **Luca Mazzon:** Conceptualization (equal); funding acquisition (lead); project administration (equal); resources (equal); supervision (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

ORCID

Andrea Basso https://orcid.org/0000-0001-6784-7271
Luca Mazzon https://orcid.org/0000-0002-8459-893X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1 Haplotype network based on the combined dataset including the mitochondrial regions: CO1, NAD4 and 12S rRNA of *Anonconotus italoaustriacus* Nadig, 1987. The haplotypes are represented by circles whose sizes are proportional to their frequencies. Colours represent the different populations where haplotypes are present. Each node or little bar represents one mutational step.

Figure S2 Mismatch distribution under population expansion model of *Anonconotus italoaustriacus* in the in the four sites sampled.

Table S1 Primer sequences used for target amplification and sequencing in this study.

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