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Biology and ecology of orthopteran species of natural and agricultural ecosystems: from outbreak management to biodiversity conservation

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Padua, 30 September 2021 Giacomo Ortis

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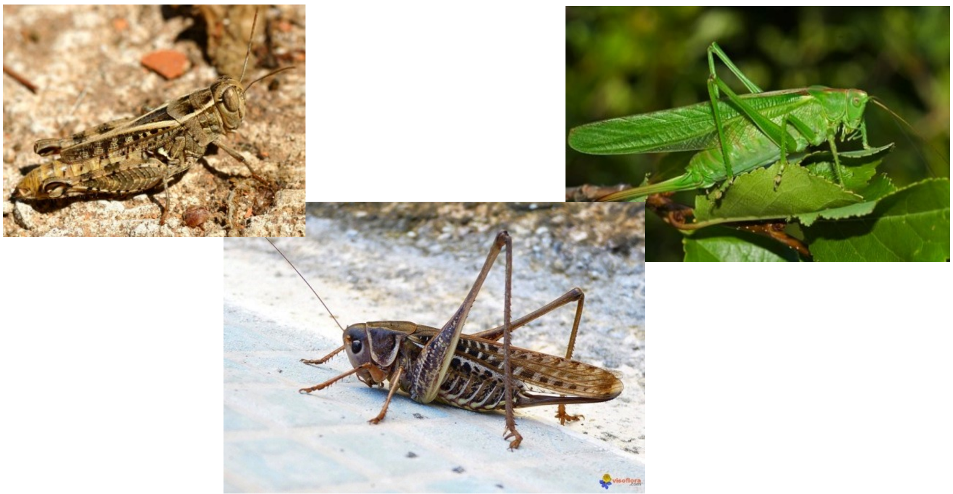
Summary

Orthopterans occur in most terrestrial habitats and their order includes the grasshoppers, locusts, crickets, and their relatives. They could have an ecological and economic importance, as pivotal elements in trophic food webs and as pests, causing devastating damages to agricultural and forest crops. There are two different management responses: reducing population levels in pest management and maintaining or increasing population levels in conservation management. While the phenomenon of outbreak in Orthoptera is well known worldwide especially for Caelifera suborder, less studied are the outbreaks of Ensifera. The recent and unexpected outbreaks of the bush-cricket *Barbitistes vicetinus* in the north-east of Italy, gave me the possibility to unravel some significant questions about this forest pest. Firstly, we confirmed the endemic origin of this species, suggesting that environmental factors rather than genetic factors could have a role in the demographic fluctuations of this orthopteran. Secondly, we clarified the role of temperature on survival and diapause, demonstrating how summer temperatures experienced by eggs after oviposition can affect their development, potentially shifting life-cycle from pluriannual to annual. Morevoer, we discovered a native egg parasitoid providing biological, taxonomical and distributional data that could be useful regarding its potential use in biological control against *B. vicetinus.*

Another goal of this thesis was to implement conservation strategies to preserve different Orthoptera species with different ecological requirements. In particular, because agricultural intensification is widely considered a major threat to biodiversity, we investigated wether extensively managed perennial crops such as vineyards and olive groves could represent surrogate habitats for orthopterans. In addition, we focused on the endangered bush-cricket *Anonconotus italoaustriacus*, analysing genetic populations diversity among its small distribution area in the Alps to improve conservation strategies and prevent extinction.

Chapter 1

Introduction



Insect outbreaks usually involve important ecological and economic consequences for agriculture and forestry. Heavy damages are caused in particular by Lepidoptera, Coleoptera and Orthoptera orders (Lorch et al., 2005), usually repeatedly over time. Despite efforts were made to understand fluctuations in the abundance of insects, multiple factors that drive population dynamics and their interactions are still to be identified and ranked (Lockwood, 1997; Joern, 2000; Cigliano et al., 2002). Delayed density-dependent processes can lead to fluctuations of insect populations. In particular, regulation by natural enemies and host plants are the most effective competing hypotheses for what drives herbivore dynamics (Martinat 1987; Gherlenda et al., 2016). In addition, it has also been frequently hypothesized that climatic variations could be responsible in modulating the population density by direct effects on life-history traits − such as survival, reproduction, developmental rates and diapause (Andrewartha & Birch, 1954) − as well as by indirect effects acting on host-plants resistance.

The phenomenon of Orthoptera outbreaks is well known worldwide, especially for Caelifera suborder and in particular for the family of Acrididae (Showler, 1995). Periodically, some species show a huge increase of abundance, shifting their behaviour toward a gregarious phase, with changes in colour and body shape (polyphenism phase), and forming swarms able to move several thousands of kilometres across many regions (Cigliano et al., 2002; Rong et al., 2006). Since from last centuries two species, the African desert locust *Schistocerca gregaria* (Forskål, 1775) and the migratory locust *Locusta migratoria* (Linnaeus, 1758), outbreak regularly in some area of Africa and Middle East causing severe damages to wild and cultivated plants, creating also social problems related to food shortages (Tanaka & Zhu, 2005; Rong, 2006, Latchininsky et al., 2011). Multiple locust outbreaks were also reported in Australia from 1844 by the Australian plague locust *Chortoicetes terminifera* (Walker, 1870) and in the United States by the Rocky Mountain locust *Melanoplus spretus* (Walsh, 1866). Orthoptera species that show outbreak dynamics were recorded also in South America and Central America, the most notable being *Schistocerca cancellata* (Serville, 1838) and *Schistocerca piceifrons* (Walker, 1870), respectively (Hunter & Cosenzo, 1990). Beside these species representing the most dangerous ones, there are other species not exhibiting polyphenism phase that can also undergo outbreaks, as reported for *Calliptamus italicus* (Linnaeus, 1758), *Dociostaurus maroccanus* (Thunberg, 1815) and *Kisella irena* (Fruhstorfer, 1921) in some regions of Italy (Melis, 1934; Buzzetti et al., 2005; Massobrio et al., 2010 Baldacchino et al., 2012), or for many grasshopper species in the western United States (Branson et al., 2006). In the first half of the last century, locust problem caused mainly by *D. maroccanus* infestations in Sardinia began to assume devastating proportions, with intensive use of chemicals, risking toxic damage to public health and the environment (Molinu et al., 2004). One explanation of the possible causes leading locust outbreaks is related to higher survival of eggs under favourable environmental conditions (soil moisture levels, optimal temperatures), able to increase the hatchling success. Indeed, recent large locust outbreaks reported in the last century are closely related to long-term droughts and warm winters coupled with occurrence of high precipitation in spring and summer during hatching periods (Peng et al., 2020).

Whereas most of the studies refer to Caelifera, less attention has been paid to Ensifera species. The most renowned case referred to this suborder is represented by the Mormon cricket *Anabrus simplex* (Haldeman, 1852), reporting outbreaks across wide Western North American areas in the late 90', with densities of more than 100 individuals/m^2 causing important damages to crops (Lorch & Gwynne, 2000; Lorch et al., 2005; Sword et al., 2005; Bailey et al., 2007a, b; Srygley, 2014). Other outbreaks of Ensifera damaging crops were reported in Southern Africa, by the Armored bush crickets of the genus *Acanthoplus* Stål, 1873 (Wohlleber, 1996; Minja et al., 1999) and in South Korea, by the Ussur brown katydid *Paratlanticus ussuriensis* (Uvarov, 1926) (Jung et al., 2011). In southern Italy, infestations of *Decticus albifrons* (Fabricius, 1775) caused damages to cereal crops (Massa et al., 2012). A single case of outbreak in the Alps was recorded for *Polysarcus denticauda* (Charpentier, 1825), with the appearance of a melanic form, although the causes of this event are still unknown (Della Beffa, 1946). Also, occasional outbreaks in agro-forest ecosystems were reported over Europe in some species of bush-cricket of the genus *Barbitistes* (Charpentier, 1825), which includes 9 West-Palaearctic species. Among these, *Barbitistes ocskayi* (Charpentier, 1850), occurring in the whole Balkan region, is known to affect deciduous forests in the Karst of Trieste with periodical outbreaks (Stergulc et al., 2005). Another example is *B. constrictus* Brunner, 1878, a species of Central and Eastern Europe feeding on coniferous species, mainly pines and larch, and causing occasional forest outbreak, as reported for Czech Republic and Poland (Escherich, 1928; Laussmann, 1994; Holusa et al., 2006). In the last decade, large outbreaks of *B. vicetinus* Galvagni & Fontana, 1993 interested some areas of the North-East of Italy.

Barbitistes vicetinus is an endemic forest pest species spread almost exclusively in the Veneto region (NE Italy). This bush-cricket was described just in 1993 as a rare species, being found only in small confined hilly areas in the Lessinia region, Euganean Hills and Berici Hills (Galvagni & Fontana, 1993). Since 2008, the first known outbreaks of this tettigoniid occurred in small areas of the Euganean Hills and, in the subsequent years, affected the whole hillside area and also some locations in the Berici Hills. During outbreak, *B. vicetinus* causes severe defoliations specially to forest plants (trees and bushes) and neighbouring crops such as vineyards, olive grove and cherry orchards (Mazzon et al., 2011; Cavaletto et al., 2015). A recent study highlights that forest trees more strongly defoliated were *Fraxinus ornus*, *Ostrya carpinifolia*, *Quercus* spp. and *Corylus avellana*, whereas the species with the lowest damage was *Robinia pseudoacacia* (Cavaletto et al., 2019). In particular, habitat modification and presence of *R. pseudoacacia*, a non-host alien tree species, reduce defoliation intensity at both the local and landscape scale (Cavaletto et al., 2019). During these outbreaks, from data obtained by emergence traps, Cavaletto et al. (2018) estimated a nymph density of over 1 million individuals/ha. Moreover, during outbreaks *B. vicetinus* changes its ethology showing a tendency to gather in large numbers in small spaces. Morphological features are modified too, with the appearance of a melanic form having aposematic coloration. This phenomenon has been reported also in other Ensifera (Lorch & Gwynne, 2000; Lorch et al., 2005; Sword et al., 2005; Bailey et al., 2007a, b), but its biological or ecological significance still remains debated, although some studies have highlighted a correlation between the melanin content and the increased immune response to pathogens (Bailey, 2011; Barbosa et al., 2012).

Like many tettigonids of temperate climates, *B. vicetinus* is a univoltine species overwintering as egg laid approximately 1-2 cm deep in the ground. Hatching generally begins at the end of March, but it could be anticipated or postponed depending on elevation and spring climatic conditions (Cavaletto et al., 2019). First instar nymphs feed mainly on bush and shrubs (in particular *Rubus* spp.) while subsequent instars climb up to the trees where they defoliate the canopy. Analysis conducted on the adult frass confirmed that the species may also eat insects (such as moths and aphids) and mites (Magello, 2001). Oviposition mainly occurs in June in soil of forest areas; adults die after oviposition (Cavaletto et al., 2018). Eggs of *B. vicetinus* could remain in diapause in the soil for several years (Magello et al., 2003), but there is a lack of knowledge about diapause duration, patterns, and the influencing factors, which could affect also the demo-ecological activity of this pest.

Even though some orthopterans can outbreak causing devastating consequences, other species are threatened with extinction or are already extinct. In this respect, there are two different management responses: reducing population levels in pests and maintaining or increasing population levels in rare species. Orthopterans are important members of nearly all terrestrial ecosystems and represent ideal organisms to study the effects of land-use changes and climate change on biodiversity (Bazelet & Samways, 2011). Among the about 40.000 described species, some ones show huge population variability, often becoming local and temporary keystone species, while communities of these insects may be essential to ecosystem functioning over long periods of time (Samways & Lockwood, 1998). Indeed, orthopterans are pivotal elements in trophic food webs as they are consumers in grassland and forest ecosystems, and constitute important food for many taxa, such as birds, reptiles, and mammals (Belovsky & Slade, 1993; Ingrisch & Kohler, 1998). A clear understanding of the ecological needs of Orthoptera species and the factors affecting their survival could contribute to implement conservation strategies able to support both single and multiple species at the community level.

Human activities are among the major threats identified for Orthoptera survival. Because the majority of European Orthoptera (*ca* 74%) occurs in grassland and shrubland habitats, their presence depends on specific management policy adopted (Otte & Naskrecki, 2001). In general, Orthoptera species richness is higher in grassland maintained by livestock grazing than in mown grasslands, as the structural heterogeneity of the vegetation is higher in pastures allowing species with different ecological requirements to co-occur. In particular bush-cricket species prefer higher vegetation, while grasshoppers usually need patches of bare ground for oviposition and basking (Marini et al., 2008).

Along with the different management of grasslands, the most important threat to Orthoptera is agricultural land use change. Transformation of grasslands and shrubland into cultivated crops completely destroys the habitat of certain species. Besides, in intensive agriculture, the frequent use of pesticides and fertilizers as well as ploughing, rarely allow Orthoptera species to reproduce. Also, the repeatedly use of insecticides in crops to control other pests affects Orthoptera species that colonize marginal habitats, such as shrubs between arable fields or road margins. Nevertheless, research on effects at population level is still lacking.

Together with agricultural intensification, also meadows and pastures abandonment has been shown to cause negative effect especially for species associated with semi-natural grasslands (IUCN, 2021). In fact, the encroachment of shrubs and trees causes a replacement of open habitats with forests, which do not represent a suitable environment for most Orthoptera species. Moreover, the recent general tendency to have fewer but larger farms, have led some areas to be overgrazed and other areas to be abandoned, with subsequent overgrowth of shrubs and trees.

An increase in the frequency of wildfires, induced by global warming and field abandonment, has also been identified as a threat to many Orthoptera species. Wildfires are mainly of human origin, but they are also facilitated by the increasing number of droughts caused by climate change. On one hand, the creation of new patches of bare ground is an important component of the habitat of many grasshopper species. On the other hand, bush-crickets are more sensitive to fires as they are flightless and live in shrubs or other woody vegetation.

Furthermore, climate change is believed to be a major driver of biodiversity loss, as droughts and extreme weather could trigger decline of species related to wetland habitats and for species that lay eggs on plants. For example, in mountain environments, it is well documented that plants are shifting their ranges moving upwards and therefore it is very likely that the habitats of Orthoptera will also shift, particularly for species restricted to mountain tops. For these reasons, many of the threatened Orthoptera species are confined to a small geographical area and they are highly threatened by anthropogenic impacts affecting their small ranges (Samways and Lockwood, 1998).

About the threatened Orthoptera, *Anonconotus* is a genus of endemic Ensifera occurring in the Alps and the Apennines. In Italy there are several *Anonconotus* species mostly located in the western Alps, and only one in the eastern Alps: *Anonconotus italoaustriacus* Nadig, 1987 (Galvagni and Fontana, 2004; 2005). This species lives only above 1800 m and up to 2700 m in mountain heliophilous grasslands characterized by the presence of *Juniperus*, *Rhododendron*, and *Erica* (Fontana et al., 2002; Massa et al., 2012). To date, only four populations have been reported in the Italian territory, on the Belluno Dolomites (Vette Feltrine, Busa delle Vette, BL), Scilliar Group (Alpe di Siusi, BZ), San Candido (Monte Elmo, BZ), and the Baldo Group (Monte Altissimo, TN) (Massa et al., 2012). Moreover, other populations of *A. italoaustriacus* are spread in the High Tauren range in Austrian territory (Illich et al., 2010; Zuna-Kratky et al., 2017). *Anonconotus italoaustriacus* is a medium-sized species with 17 mm long males and 21 mm long females, characterized by green to brown colours. This species is micropterous and typically consists of small populations with low density of individuals.

Because of its restricted range 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).

Aims

During my PhD, I focused my study on the unexpected outbreaks of *B. vicetinus* occurred in the Veneto region as well as on conservation of Orthoptera, with the aim to provide strategies for pest control and conservation management. In particular, I investigated different aspects of *B. vicetinus* bio-ecology: first, I analysed the genetic diversity of the populations coming from different outbreak areas. Second, I studied the effect of temperatures on diapause and overwinter survival. Third, I described a new parasitoid of *B. vicetinus*, because regulation by natural enemies could be an important factor in modulating population density.

Moreover, I conducted two studies focused on the conservation strategies of different Orthoptera species. These topics are exposed in the following chapters as follows:

Chapter 2 aims, with a genetic approach, to study the origin of outbreak populations of *B. vicetinus* through analysis of two mitochondrial regions from the two disjunct ranges (Euganean and Berici Hills). Furthermore, the study aims to answer the question about the native origin of the bush-cricket.

Chapter 3 studies the effect of field temperatures on diapause of *B. vicetinus*, focusing on how summer temperatures can trigger embryos to develop in the same year of oviposition and how winter temperatures affect egg survival. For comparative purposes, effects of summer and winter temperatures on diapause and survival were also tested under laboratory conditions.

Chapter 4 provides data on taxonomy, distribution and biological traits of a newly detected parasitoid of *B. vicetinus*, suggesting its potential impact in the Euganean Hills as a control agent.

Chapter 5 investigates if extensively managed perennial crops as olive groves and vineyards could support orthopterans as grasslands, providing key ecological information to suggest conservation strategies supporting both Ensifera and Caelifera sub-groups.

Chapter 6 focus on the endangered bush-cricket *Anonconotus italoaustriacus*, analysing genetic population diversity through amplification of different mitochondrial regions, with the aim of using the acquired knowledge for the management of this threatened species.

Chapter 2

Studying genetic population structure to shed light on the demographic explosion of the rare species *Barbitistes vicetinus* (Orthoptera, Tettigoniidae)

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Abstract

Insect outbreaks usually involve important ecological and economic consequences for agriculture and forestry. The short-winged bush-cricket *Barbitistes vicetinus* Galvagni & Fontana, 1993 is a recently described species that was considered rare until ten years ago, when unexpected population outbreaks causing severe defoliations across forests and crops were observed in north-eastern Italy.

A genetic approach was used to analyse the origin of outbreak populations. The analysis of two mitochondrial regions (Cytochrome Oxidase I and II and 12S rRNA-Control Region) of 130 samples from the two disjunct ranges (Euganean and Berici Hills) showed high values of haplotype diversity and revealed a high geographical structure among populations of the two ranges. The high genetic variability observed supports the native origin of this species. In addition, results suggest that unexpected outbreaks are not a consequence of a single or few pestiferous haplotypes but rather the source of outbreaks are local populations which have experienced an increase in each area. The recent outbreaks have probably appeared independently of the genetic haplotypes whereas environmental conditions could have affected the outbreak populations. These findings contribute to a growing understanding of the status and evolutionary history of the pest that would be useful for developing and implementing biological control strategies for example by maximizing efforts to locate native natural enemies.

Introduction

Herbivorous insect outbreaks usually involve important ecological and economic consequences for agriculture and forestry. In most cases, it concerns the accidental introduction of an exotic species into a new geographical area due to international trade and human movement (Wermelinger et al., 2008; Calabria et al., 2012; Battisti et al., 2014). In this scenario, the species is not under biological control by natural enemies, leading to a quick spread of the population. Instead, the insect outbreak of an indigenous species is less common but more enigmatic, due to some alterations of the biotic and abiotic components (Kobayashi et al., 2011).

Until a decade ago, among bush-crickets belonging to the genus *Barbitistes*, (Orthoptera, Tettigoniidae), occasional outbreaks were reported only for *B. constrictus* Brunner von Wattenwyl, 1878 in conifer forests of central and eastern Europe, for *B. ocskayi* (Charpentier, 1850) in broadleaf forests of north-eastern Italy (Holusa et al., 2006; Cavaletto et al., 2018) and for *B. serricauda* (Fabricius, 1798) in vineyards of north Italy. In spring 2008, the first outbreak of the species *B. vicetinus* Galvagni & Fontana, 1993 was recorded in a restricted area of north-eastern Italy (Euganean Hills) (Cavaletto et al., 2018). Since then, within two disjunct distribution ranges, the Euganean Hills first and the Berici Hills later, the outbreak areas have progressively enlarged causing heavy damage to forests and neighboring crops (mainly grapes and olives). Severe defoliations have been recorded in the most serious infestations, rising to nearly 90% of canopy loss (Mazzon et al., 2011; Cavaletto et al., 2019). Moreover, the outbreaks are also a source of annoyance to people living close to the attacked areas due to the tendency of bush-crickets to invade streets and gardens (Cavaletto et al., 2015, 2018).

Interestingly, the bush-cricket was first described just in 1993 as a rare endemic species of north-eastern Italy, being found only in small confined hilly areas (Galvagni & Fontana, 1993). From then on, all the records of *B. vicetinus* have been reported only in this area. Only since 2005 this species started to be more common in some localities and easy to be found also without bioacoustics technics. The recent discovery of the species (Galvagni & Fontana, 1993) and increase in these north-eastern areas affected by outbreaks during the past two decades has sparked a debate about the possible exotic origin of *B. vicetinus*. Recently, as a consequence of the outbreaks, some authors have thoroughly studied important aspects of the biology and ecology of *B. vicetinus* (Cavaletto et al., 2018, 2019, 2020; Ortis et al., 2020) but no data regarding population genetics is so far available. Genetic studies based on the use of high-resolution DNA markers allow to examine the structure of insect populations, identify haplotypes, reconstruct current or past patterns of gene flow and provide information on the origin and expansion routes of the insect (Bossart & Prowell, 1998). This information could be useful for improving the knowledge concerning the eruptive species, such as colonizing capacity, adaptability, behaviour and demographic history of a population (Brown et al., 2010; Simberloff et al., 2013; Maynard et al., 2017). Several population genetics studies have been conducted with the aim of highlighting and discovering the sources and colonization routes of outbreak populations of alien pests (Fonseca et al., 2000; Olazcuaga et al., 2020). However, studies on the genetic structure of native pests have generally received less attention (Kobayashi et al., 2011; Mock et al., 2007).

Among the genetic tools to be exploited, maternally-inherited mtDNA is widely used since it is relatively conserved compared to some nuclear genes, and is thus suitable when searching for historical processes (Avise, 2000). In this study, we used a combination of two mitochondrial markers, namely Cytochrome Oxidase I and II (COI-tRNALeu-COII) and 12S rRNA-Control Region (12S-CR) to study the genetic structure and demographic history of *B. vicetinus* populations from the two disjunct ranges where it currently outbreaks (Euganean Hills and Berici Hills, northern Italy). The COI and COII are considered slowly evolving genes of the mitochondrial protein-coding genes (Simon et al., 1994). The non-coding control region is in contrast, the most variable segment in the maternally inherited mtDNA (Wolstenholme, 1992; Boore, 1999) and its use in genetic analysis can alleviate biases of coding regions (Bronstein et al., 2018). Both markers have been widely used to conduct population genetic surveys in Orthopteran species including the Tettigoniidae family (Bailey et al., 2005; Fenn et al., 2007; Vandergast et al., 2007; Eweleit et al., 2015; Kaňuch et al., 2015, 2017).

By studying the genetic structure of the bush-cricket this study aims to answer three main questions:

(i) is there any evidence for an alien origin of the bush-cricket?

(ii) are *B. vicetinus* populations genetically distinct?

(iii) are either of the recent outbreaks caused by a single outbreak population followed by a spread pattern or have they derived from multiple local populations?

This information can help understand the factors related to the outbreak events of *B. vicetinus* and could provide insight to assist management of this outbreak pests.

Materials and methods

Study area

The study area was located in north-eastern Italy (Veneto Region) and included the two disjunct ranges where outbreaks have occurred (Euganean Hills and the Berici Hills) and a third range where no outbreaks have been reported to date and the species is rare (Lessini Mountains). The three ranges are separated from each other by cultivated and inhabited alluvial plains devoid of woody vegetation (Fig. 1B).

The Euganean Hills cover an elliptical area of approximately 180 km^2 and comprise approximately 100 hills of volcanic origin emerging from the alluvial sedimentary plain, with the highest elevation of approximately 600 m above sea level. They are characterised by numerous narrow and deep valleys, steep hills with different sun exposures and many microclimatic conditions that influence the vegetation. The annual mean temperature is approximately 12 °C and precipitation ranges from 700 to 900 mm (Kaltenrieder et al., 2009) even if they are extremely variable due to the inner geomorphological variability.

The Berici Hills are an isolated plateau situated on the southern Vicenza plain, they cover almost 160 km^2 with a maximum elevation of approximately 450 m above sea level (Sommaggio, 2017). The climate of the area is characterised by an annual rainfall of 958 mm, and average daily temperature of -1 °C in January and 23 °C in July (Borin et al., 2005).

The Lessini Mountains are a triangular-shaped tableland, which occupies some 800 km^2, at the transition between the Fore-Alps and the River Po Plain (Zampieri, 2000). The mountain group reaches over 2000 m above sea level and is characterised by multiple valleys and long ridges that descend towards the plain.

Insect sampling

Adults of *B. vicetinus* were collected, using a sweep net, throughout the spring of three successive years (2015, 2016, and 2017). Specimens from the north, centre and south part of both outbreak areas (the Euganean and Berici Hills) as well as from two sites on the Lessini Mountains were collected from bush and tree canopies on marginal side of the forests (Table 1). To avoid sampling relatives, bush-cricket specimens were collected by sweeping an area at least 100 m^2 at each sampling site. After capturing, samples were immediately kept in 95% ethanol and taken to the laboratory where they were morphologically identified and stored in individual vials at −20 °C until DNA extraction.

Table 1. Collection sites of *B. vicetinus* populations analysed and descriptive statistics of each population.

		Site							
		ID	**Latitude**	**Longitude**	**N. samples**	**Haplotypes**	**N. haplotypes**	**H**	**π (%)**
Euganean Hills	North	1	45°20'46.4"N	11°44'4.2"E	1	A6(1)	7	0.64 +/- 0.12	0.04
		2	45°22'30.6"N	11°40'38.7"E	10	A6(7), F6(2), A9(1)			
		3	45°21'54.3"N	11°38'52.7"E	8	A1(1), A6(4), A8(2), H6(1)			
		4	45°20'59.9"N	11°41'52.8"E	1	G6(1)			
	Center	5	45°19'14.8"N	11°45'57.8"E	1	A6(1)	7	0.72 +/- 0.09	0.09
		6	45°17'38.1"N	11°37'22.5"E	2	A6(2)			
		7	45°18'14.6"N	11°40'26.8"E	1	D7(1)			
		8	45°18'9.07"N	11°40'51.7"E	9	A6(1), A11(1), A12 (2), B6(1), E4(1), E6(3)			
		9	45°18'12.4"N	11°43'46.6"E	3	E6(1), A6(2)			
		10	45°17'45.6"N	11°39'35.3"E	3	A6(3)			
		11	45°18'9.7"N	11°46'48.5"E	1	A6(1)			
	South	12	45°14'53.8"N	11°44'39.5"E	12	A6(8), A10 (2), C6(2)	4	0.50 +/- 0.13	0.05
		13	45°14'41.1"N	11°40'6.2"E	2	A6(1), A13(1)			
		14	45°14'55.1"N	11°44'39.3"E	3	A6(3)			
Berici Hills	North	15	45°28'20.7"N	11°35'27.5"E	16	I1(10), I2(1), I3(1), I15(2), J6(1), L9(1)	9	0.80 +/- 0.06	0.10
		16	45°26'41.2"N	11°34'42.1"E	1	J6(1)			
		17	45°27'59.7"N	11°32'52.9"E	8	I2(2), J1(1), J5(1), J6(3), K2(1)			
	Center	18	45°25'31.5"N	11°33'02.8"E	1	I5(1)	8	0.90 +/- 0.04	0.13
		19	45°24'00.3"N	11°31'39.7"E	15	A1(1), I1(3), I5(1), I6(3), J1(3), J5(1), J8(2), J16(1)			
	South	20	45°25'14.1"N	11°26'59.0"E	17	I5(17)	3	0.28 +/- 0.12	0.02
		21	45°24'54.8"N	11°28'59.9"E	3	I1(2), I14(1)			
Lessini Mountains		22	45°29'12.3"N	11°02'14.6"E	7	N17(4), O1(3)	3	0.71+/- 0.07	0.24
		23	45°36'14"N	11°25'18"E	5	I1(5)			

DNA extraction, PCR amplification and sequencing

The genomic DNA was extracted from tissue samples taken from the hind femora of each specimen separately, according to a previously described salting-out protocol (Patwary et al., 1994). Two mitochondrial DNA regions were chosen for amplification: a fragment including the 3’ region of Cytochrome Oxidase I, tRNA-Leu and the 5’ region of Cytochrome Oxidase II (COI-tRNALeu-COII) and a fragment of the 12S rRNA and the Control Region (12S-CR). Two specific primers were designed for amplifying the COI-tRNALeu-COII fragment: Vicetinus_P7F (5’-ACCTGTTCTTGCAGGAGC-3’) and Vicetinus_P4R (5’-TCCACAGATTTCTGAACATTG-3’). The universal primers SR-J14610 (5’-ATAATAGGGTATCTAATCCTAGT-3’) and T1-N18 (5’-CTCTATCAARRTAAYCCTTT-3’) (Simon et al., 2006) were used to amplify the 12S-CR fragment. Amplifications were performed in 20 µl reactions (1x PCR Go Taq Flexi buffer - Promega, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 µM for each primer, 0.5 U of Taq polymerase - Promega, 2 µl DNA template). Thermal cycling conditions for the fragment including the COI-tRNALeu-COII were 5 min at 96 °C followed by 35 cycles at 96 °C for 1 min, 56 °C for 1 min, 72 °C for 1:30 min, and a final extension of 72 °C for 5 min. For the 12S-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 sec, and extension step of 60 °C for 3 min, with a final extension of 72 °C for 7 min.

PCR products were checked through electrophoresis on 1.0% agarose gels stained with SYBR® (Invitrogen), purified using Exonuclease and Antarctic Phosphatase (GE Healthcare) and sequenced at the BMR Genomics Service (Padua, Italy). Primers used for amplification were also used for sequencing.

Data analysis

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

Haplotype and nucleotide diversity, as well as the pairwise genetic distances between populations, were calculated with Arlequin 3.5 (Excoffier et al., 2010) using a Kimura 2-parameters model. The presence of population differentiation was also tested by conducting exact tests of population differentiation with 100,000 steps in Markov chain, with 10,000 dememorization steps. To compare the partition of genetic variability among sampled populations, an analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed using Arlequin.

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 & Templeton, 1993; Crandall, 1994).

The study of the 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, 1952). Populations at demographic equilibrium or decreasing in size should provide significant positive D and Fs values with a multimodal distribution of pairwise differences, whereas populations that have undergone a sudden demographic expansion usually show significant negative D and Fs values with a unimodal distribution (Slatkin & Hudson, 1991; Rogers & Harpending, 1992). The sudden expansion model was tested through analysis of the sum of square deviations (SSD) and raggedness index (r) representing the modality of distribution, obtaining the corresponding P values with a parametric bootstrap approach (10,000 replicates).

For those populations that did not deviate from sudden expansion ($p > 0.05$) the time since expansion was calculated considering the relationship $\tau = 2*u*t$ (where τ = age of expansion measured in units of mutational time, u = mutation rate per sequence and per generation, and t = number of generations since the expansion; (Rogers, 1995). The expansion time was then obtained by dividing the estimate of τ by the product of the sequence length in base pairs and the mutation rate per nucleotide (twice the per-lineage substitution rate; $u = 2\ \mu k$) in percentage per year. Two substitution rates were used: the classical rate of 2.3% divergence per Myr (Brower, 1994) frequently used in Orthoptera (Allegrucci et al., 2009; Voje et al., 2009; Husemann et al., 2013; Kaya et al., 2013; Sağlam et al., 2014) and the global mtDNA rate of 2.6% divergence per Myr proposed by Papadopoulou et al. (2010).

Results

Sampling

A total of 152 individuals were collected from the three natural ranges of *B. vicetinus* (Euganean Hills, Berici Hills and Lessini Mountains). Unfortunately, only 12 specimens were collected from the Lessini Mountains due to the rarity of the species in this range (Tab. 1).

Data analysis

The two fragments of the mitochondrial genes, COI-tRNALeu-COII and 12S-CR were successfully amplified and sequenced in 130 samples, with an average of 19.6 specimens for each of the three areas within the two main ranges (north, centre, and south of both the Euganean and Berici Hills). After quality assessment and trimming the sequences, high-quality sequences 799 bp long for the COI-tRNALeu-COII and 898 bp long for the 12S-CR fragment were obtained. Sequences of the COI-tRNALeu-COII fragment weretranslated with Transeq (EMBOSS: http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) to exclude the presence of any nuclear mitochondrial pseudogenes. This bioinformatics tool translates nucleic acid sequences to their corresponding peptide sequences and identifies stop codons. Since these pseudogenes (NUMTs) are characterized 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 to exclude presence of NUMTs.

A total of 13 variable sites, including 8 parsimony informative sites and 5 singleton sites, were identified by the alignment of COI-tRNALeu-COII sequences while the 12S-CR alignment showed 6 parsimony informative sites and 7 singleton sites (13 variable sites in total). Sequences of each haplotype of COI-tRNALeu-COII and 12S-CR obtained in this study are available through GenBank accession numbers MW405351- MW405381.

Partition homogeneity test confirmed that COI-tRNALeu-COII and 12S-CR fragments bear a homogeneous signal (P= 0.28), allowing data to be pooled for further analyses. A final dataset including 130 concatenated sequences of 1697 bp was obtained. Further analyses were conducted considering the combined dataset.

Genetic variability and population structure

The diversity indexes for the concatenated dataset ranged between 0.28 and 0.90 for the haplotype diversity (H) and between 0.02 and 0.24 for the nucleotide diversity (π). Among populations from both the main outbreak areas, those from the north and the centre part of the Berici Hills showed the highest H and π values (H = 0.92, π = 0.13 and H = 0.80, π = 0.10 respectively) while populations from the south part of the Berici Hills showed the lowest variability. The distribution of haplotypes among all populations analysed and other summary statistics are shown in Table 1.

The presence of population differentiation was confirmed by the tests of population differentiation ($P>0.001$). When *B. vicetinus* genotypes were grouped based on the six main collection sites (north, centre, and south of both Euganean and Berici Hills), the locus-by-locus AMOVA revealed a significant geographic structure between populations of the two main disjunct ranges (P= 0.0014) (Euganean vs Berici Hills). To avoid bias as a result of the small sampling size of Lessini

Mountains, populations from this area were not included in this analysis. The analysis showed that 40% of the variation was explained by differences among groups (Euganean and Berici Hills), whereas about 48% of genetic variation was explained within populations (Table 2).

Table 2. Analysis of molecular variance AMOVA for the combined data set (COI-tRNALeu-COII and 12S-CR).

Structure	Source of variation	Variance %	Fixation indices	*P-value*
Grouping by geographical range (Euganean and Berici Hills)	Among groups	40.32	F_{CT} =0.403	0.0014
	Among populations within groups	11.75	F_{SC} =0.197	<0.001
	Within populations	47.92	F_{ST} =0.520	<0.001

Amova was calculated among populations of *Barbitistes vicetinus* divided according to the outbreak areas.

TCS Network

TCS Network of the combined dataset revealed the presence of 32 haplotypes of which 15 were exclusive to samples from the Euganean Hills, 13 were exclusive to the Berici Hills and 2 regarded samples only from the Lessini Mountains (Fig. 1). Only 2 haplotypes were shared by samples from different ranges: A1 included samples from the Euganean and Berici Hills, and J1 samples from both the Berici Hills and Lessini Mountains. The network evinced a geographical separation among haplotypes, with samples from the Euganean Hills showing a star-like pattern and samples from the Berici Hills connected among them (Fig. 1A).

Going deeper into the network's characteristics, the most common haplotype, A6, included 34 samples coming from all three parts of the Euganean Hills (north, centre and south). Ten rare haplotypes including samples exclusively from the Euganean Hills were connected, separated by only one mutational step, to A6 in a star-shape. Five of them (A9, A11, B6, G6, and H6) were represented by only one sequence, six (C6, A10, F6, A8) by two sequences, and one (E6) by 4 sequences. In addition, haplotype A1, which included samples from both the Euganean and Berici Hills, was also connected to A6 separated from it by only one mutational step.

The second most common haplotype, I1, was composed of 20 sequences:15 belonged to samples from the three geographical populations of Berici Hills (north, centre and south) while 5 were represented by samples from site 23 (Lessini Mountains). This haplotype (I1) was separated from the most dominant haplotype (A6) by two mutational steps. A star-like pattern could also be observed in this part of the network, with haplotype I1 surrounded by nine haplotypes, eight of which (I2, I3, I5, I6, I14, I15, K2 and L9) exclusively represented by samples from the Berici Hills. Among them, haplotype I5 was shared by 19 samples from the southern and central populations of the Berici

Hills while haplotypes I2, I3, I15, K2 and L9 were rare (3 sequences each at most) and included only samples for the north part of the Berici Hills. Two haplotypes, O1 and N17, were exclusive to samples from site 22 (Lessini Mountains) the furthest site from the outbreak areas (Fig. 1).

The parsimony networks of the single markers (COI-tRNALeu-COII and 12S-CR) showed a similar pattern, with most haplotypes including exclusively samples from only one disjunct distribution range (Euganean Hills or Berici Hills or Lessini Mountains) (S2 Fig. and S3 Fig.).

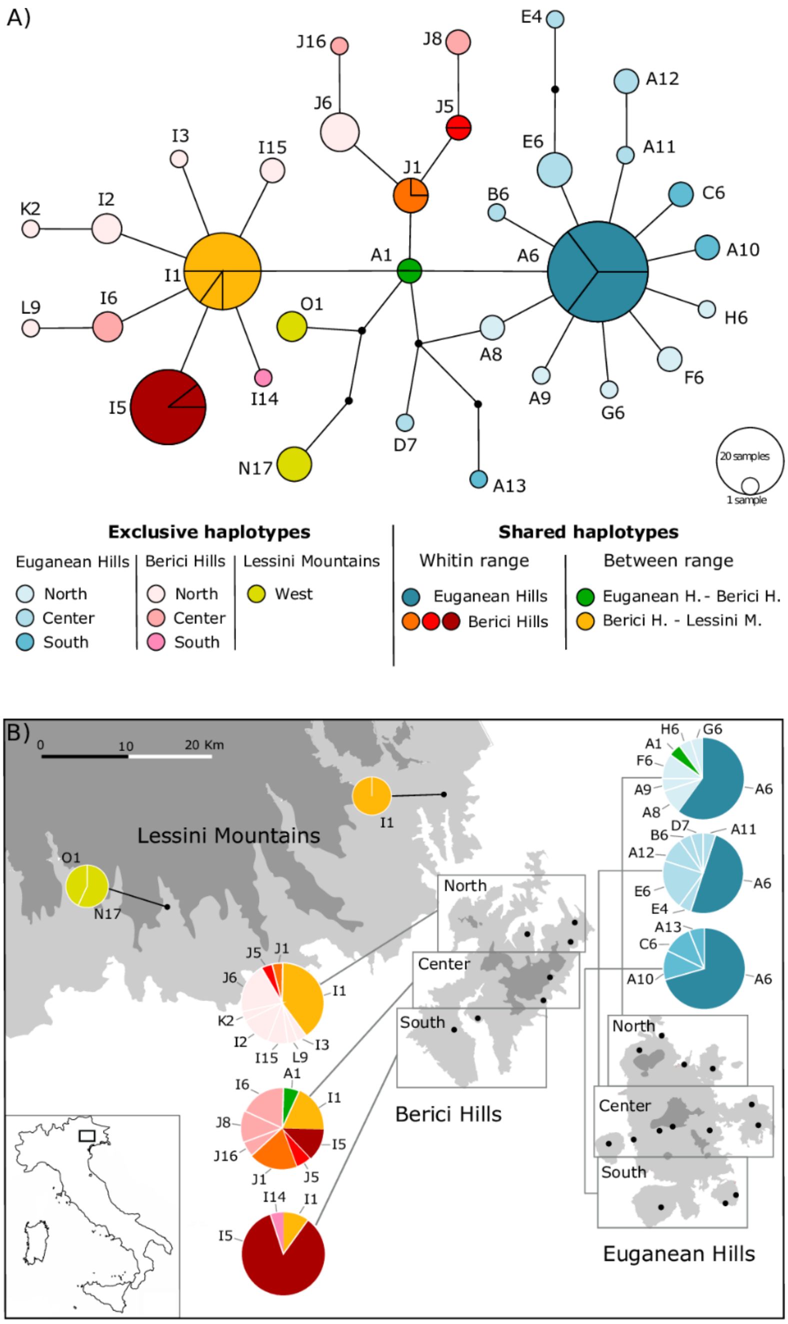

Fig 1. Parsimony network and geographic distribution of *B. vicetinus* haplotypes. A) Haplotypes network based on the combined dataset (COI-tRNALeu-COII and 12S-CR). Each haplotype is represented by a circle, and the area of the circle is proportional to its frequency. Lines within haplotypes circles indicate the proportions shared between

collection areas. The colours represent differences in geographic distribution, and small black dots symbolize missing intermediate or unsampled haplotypes. Codes indicate the haplotype ID, reported in Table 1. B) Map showing the proportional geographic distribution of bush-cricket haplotypes across sampled populations. Map republished from (Bensasson et al., 2001) under a CC BY license, with permission from [Regione del Veneto–L.R. n. 28/76 –Formazione della Carta Tecnica Regionale], original copyright [2020].

Past demographic events

Tajima's D and Fu's Fs tests were applied in populations from the Euganean and Berici Hills in order to check for past demographic events. The null hypothesis of neutrality was rejected in populations from the Euganean Hills (D= -2.05; P= 0.02 and Fs= -12.51; P < 0.001), suggesting a past population expansion after a period of low effective sample size (Table 3). Berici populations showed only significant negative Fu's Fs value (Fs= -6.62; P = 0.004), suggesting that *B. vicetinus* populations of this hilly area did not conform to the theory of neutral evolution (Table 3).

Table 3. Statistic summary of the past demographic events analysis of *Barbitistes vicetinus* populations.

Area	**Tajima's D**	**Fu's Fs**	**SSD**	**r**	**τ** (confidence interval 95%)	**Expansion time (ka)** 2,3% subst.rate	**Expansion time (ka)** 2,6% subst.rate
Euganean Hills	-2 .05*	-12 .51*	0 .009	0.069	0.96 (0.34-1.67)	12.4 (4.4-21.6)	10.7 (3.8-18.7)
Berici Hills	-0 .23	-6 .62*	0 .002	0.030	2.14 (0.54-3.62)	27.6 (7-46.7)	24 (6.1-40.3)

Tajima's (D) and Fu's neutrality test (Fs) mismatch distribution analysis under a sudden expansion model and time since expansion calculated for mitochondrial populations of the Euganean and Berici Hills considering the combined data base. SSD: sums of squared deviations; r: raggedness index, τ: age of expansion measured in units of mutational time. Expansion time shows as 1,000 years ago (ka). * Significant at $p < 0.05$.

The mismatch distribution plots of both ranges were smooth and unimodal, revealing that these populations were undergoing population expansion (S4 Fig.). They were characterised by the following observed means: 1.25 and 1.91 for the Euganean and Berici Hills, respectively. Moreover, for both the Berici and Euganean populations, the computed SSD and raggedness index values did not reject a sudden expansion model (S4 Fig.), and in particular raggedness values were low (Table 3).

Estimation of the expansion time showed that populations from the Euganean Hills lineage started to expand about 10,700 years ago (with a 2.6% substitution rate) and 12,400 years ago (with a 2.3% substitution rate) (Table 3). Populations from the Berici Hills probably expanded from about 24,000 to 27,600 years ago with 2.6% and 2.3% substitution rates, respectively (Table 3).

Discussion

This study presents the first population genetic analysis of the species *B. vicetinus* in its outbreak areas and reveals the presence of a high geographical structuring among populations of the two outbreak ranges analysed (Euganean and Berici Hills).

Populations from both ranges showed high values of haplotype diversity, a typical characteristic of ancestral populations (Barrett & Kohn, 1991; Hewitt et al., 2004; Wares et al., 2005), supporting that *B. vicetinus* is a native species. Conversely, populations of invasive alien species are traditionally thought to have reduced genetic variation relative to their source populations because of genetic founder effects linked to small population size during the introduction and establishment phases of an invasion (Handley et al., 2011). Furthermore, the genetic variability found clustered according to geographical ranges is in contrast with the possibility that *B. vicetinus* may be an invasive species accidentally introduced.

Even though outbreaks have been reported on both the Euganean and Berici Hills, different haplotypes have been found in these two distribution ranges. Ninety-four percent of haplotypes were exclusive to a single distribution range (i.e. either Euganean or Berici Hills) and only one haplotype, scarcely represented, was found in samples from both hilly areas. These results suggest that outbreaks are not a consequence of a single or few pestiferous haplotypes but rather that the source of outbreaks is due to local populations which experienced a demographic increase in each area. Thus, it seems that outbreaks have appeared independently from the genetic origin, as also found in some studies which indicate that outbreak events are often more affected by environmental conditions than by genetic characteristics of the local population (Kobayashi et al., 2011; Pureswaran et al., 2018; Serrana et al., 2019; Ward & Aukema, 2019).

The geographical separation between populations of the Euganean and Berici Hills, observed in the network and confirmed by population differentiation test and AMOVA, indicates a limited gene flow among populations. 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; Lesieuret al., 2016). The limited dispersal ability of this flightless species could have favoured the lack of gene flow among its distribution ranges. *B. vicetinus* has been reported to show a low dispersal ability (Cavaletto et al., 2019), as well as other ground-dispersing species that move only relatively short distances, such as 100-200m during their whole life (e.g. *Pholidoptera griseoaptera*, (Diekötter et al., 2005). In addition, the mostly lowland areas between the Euganean and Berici Hills, with the presence of agricultural fields and the absence of woody vegetation, might be hostile areas for bush-cricket survival and could have acted as a geographical barrier limiting the effective dispersal of the species. Furthermore, spatial configuration of habitats is another factor influencing genetic structure, that affects mainly species with limited dispersal ability (Lange et al., 2010). In *B. vicetinus* outbreak areas, it has been observed that habitat loss and the presence of patchy areas of the non-host alien tree *Robinia pseudoacacia* play an important role in reducing population density and dispersion (Cavaletto et al., 2019). These factors, coupled to the low mobility of the pest, could have a synergic action that might explain the high level of differentiation observed among populations across its distribution ranges.

High haplotypic diversity, low levels of sequence divergence (nucleotide diversity) and a star-like phylogeny were observed in populations of *B. vicetinus* from the Euganean Hills and to a lesser extent from the Berici Hills (Fig. 1; Table 1). This pattern is consistent with what is expected for populations that have experienced past demographic expansions (Avise et al., 1984; Slatkin & Hudson, 1991). These results were highlighted by the neutrality tests and the unimodal mismatch distribution in both hilly areas supporting that, besides the current and ongoing outbreaks, populations from the Euganean and Berici Hills underwent an expansion after a period of low effective sample size (S4 Fig.). The bush-cricket population of the Euganean Hills experienced a postglacial expansion starting approximately 10,700-12,400 years ago after the Last Glacial Maximum (LGM; 21,000 years ago) whereas the expansion process in populations on the Berici Hills could have occurred at the end of the LGM. During this glacial period, climate effects were minimal in the Euganean Hills, with thermophilic vegetation serving as a refuge for several species (Mazzetti, 1992; Gubler et al., 2018). Accordingly, *B. vicetinus* populations could have survived during the climatic oscillations, and exploited these hilly areas with potentially suitable environmental conditions throughout the late Pleistocene. Once climatic conditions were favourable, at the end of the last ice age, populations might have experienced an expansion, shaping the present genetic structure of the species. The slow movement of the species and lack of host plants in the lowlands during these periods probably prevented dispersal of the species between the two hilly areas, favouring differentiation of the mitochondrial haplotypes. Thus, both historical (e.g., expansion after LGM) and recent changes (e.g. habitat loss) have contributed to determining the genetic structure and diversity of the bush-cricket in its outbreak range, resulting in genetically structured populations.

Further studies increasing the sampling size, mostly in areas where the species is rare (e.g. Lessini Mountains), combined with analysis of other markers could help obtain a better picture of *B. vicetinus* population structure.

The current findings contribute to a growing knowledge of the status and evolutionary history of the pest. Here, by removing doubt about the origin of the *B. vicetinus*, we have achieved an important step in understanding this native species. Shedding light on the origin of a species is also important for its biological control. Given that natural enemies and their host tend to coevolve, biological control programmes often rely on the use of parasitoids present in the native areas. Failure to identify the correct origin of a pest may lead to the use of unsuitable species as biocontrol agents with negative effects on control programmes (Hoddle et al., 2015; Lesieur et al., 2016). Maximizing efforts to locate native natural enemies (native parasitoids), as the egg parasitoid reported in Ortis et al. (2020) will be useful for control programmes. Finally, although *B. vicetinus* is currently in outbreak status, control programme strategies should consider the intrinsic vulnerability of an endemic species. Its low dispersal ability and small and fragmented distribution range could lead this native species to quickly shift from outbreaking to endangered.

Supplemental material

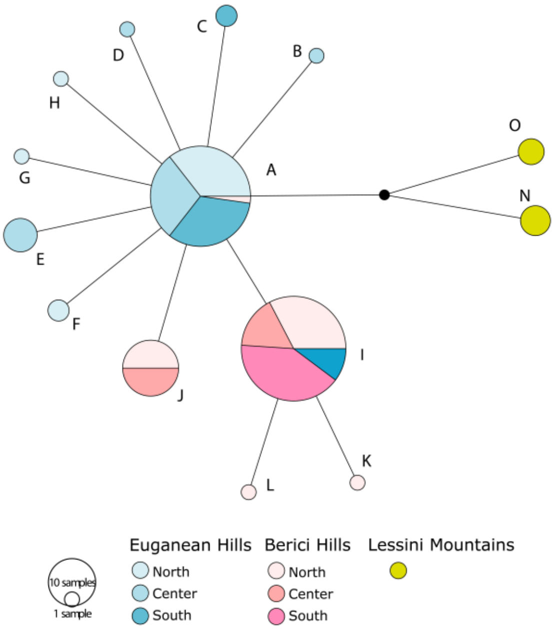

Fig. S2. Parsimony network and geographic distribution of *Barbitistes vicetinus* haplotypes. Network obtained with the COI-tRNALeu-COII data set.

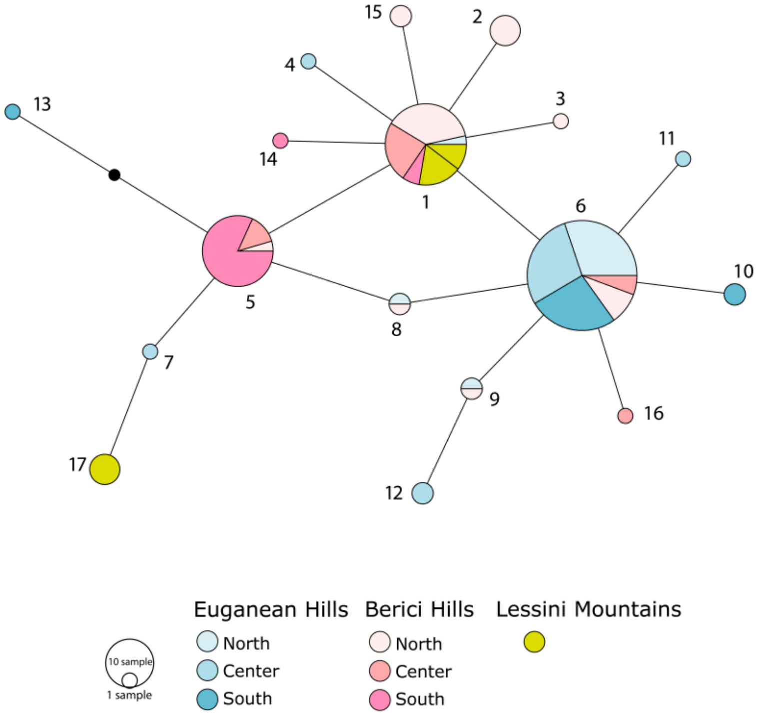

Fig. S3. Parsimony network and geographic distribution of *Barbitistes vicetinus* haplotypes. Network obtained with the COI-tRNALeu-COII data set. B) Network obtained with the 12S-CR data set. Network obtained with the 12S-CR data set.

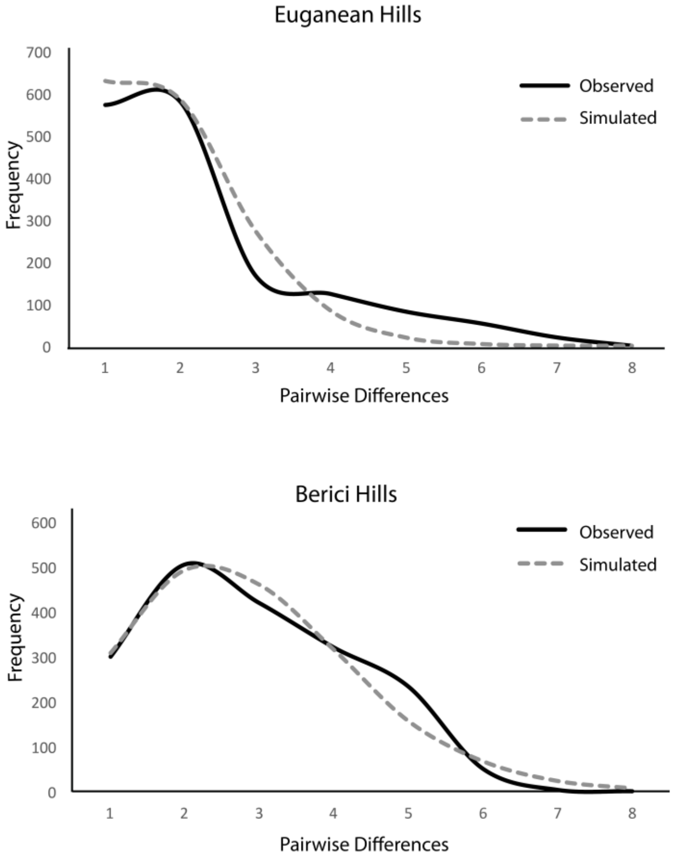

Fig. S4. Mismatch distribution under the population expansion model of *Barbitistes vicetinus* populations in the disjunct ranges.

Chapter 3

Can temperature affect diapause and overwinter survival of the outbreak species *Barbitistes vicetinus* (Orthoptera, Tettigoniidae)?

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Manuscript ready for submission

Abstract

Outbreaks of insect populations are common in the pest species while demographic explosions of rare species are less frequent and poorly studied. Among drivers able to affect insect fitness, an important role is played by temperature, able to alter development and survival, thus influencing phenological activity and abundance. *Barbitistes vicetinus* Galvagni & Fontana was described in 1993 as an endemic bush-cricket of north-east Italy and was considered rare until 2008, when repeated outbreaks occurred in ever-increasing surface areas. This species has become a forest pest, causing severe damages to forests and crops, mainly vineyards and olive groves. Despite the occurrence of a multi-year diapause of eggs was observed, there is a lack of knowledge about how temperatures could influence diapause and phenological activity. In this study, we explored the effect of field temperatures on diapause of *B. vicetinus*, focusing on how summer temperatures can trigger embryos to develop in the same year of oviposition and how winter temperatures affect survival after diapause termination. The effect of temperature on diapause and overwinter survival was also tested in the laboratory, exposing eggs to constant temperatures. *B. vicetinus* showed a strong thermal sensitivity, being capable to develop in large numbers within a specific thermal range. Field observations showed that the proportion of embryos that completed development at the end of summer ranged from zero to nearly 90%, suggesting that more than half embryos of *B. vicetinus* can develop when mean temperatures are above 20°C. A substantial shift in rates of development from 20 to nearly 80% occurred in a thermal range of about 1°C, from 19.5°C to 20.5°C. However, treatment of eggs at constant temperatures allowed lower number of embryos to develop during summer. Overall, overwinter survival was high (90%) for eggs exposed to natural temperatures but eggs incubated at constant temperatures reported 70% of survival and a prolonged hatching period.

Introduction

Common expectations from climate warming suggest that increasing temperatures will shape distribution and abundance of terrestrial arthropods (Bale et al., 2002, Deutsch et al., 2008). Because temperature is the main driver affecting fitness of many insects, warmer temperatures are expected to affect their reproduction, movement, survival and development (Hughes, 2000; Altermatt, 2010). Among these key functions, development is an essential component for building phenological models and to predict emergence times, voltinism and outbreaks in a climate change context (Colinet et al., 2015; Tougeron, 2019).

In temperate climates, many insects have evolved seasonal strategies to face recurrent environmental changes, being physiologically adapted to develop only over a limited range of temperatures (Dixon, 2009). One strategy developed by insects to overcome adverse meteorological conditions is entering diapause, enabling them to survive in unfavourable conditions and to maintain high numbers in an environment which might otherwise support only a low population (Andrewartha, 1952). Diapause is a state of arrested development, genetically determined, induced and also terminated in response to environmental stimuli as temperature, photoperiod, light and water or moisture (Denlinger, 2002).

Among Orthoptera, there is evidence that some Western Palaearctic Tettigoniids species could shift from annual to multi-year life-cycle due to differences in diapause patterns, spreading generations over different years (Ingrish, 1986a). Among factors affecting diapause in tettigoniids such as parental photoperiod, parental age and drought (Ingrish, 1984, 1985,1986b; Srygley, 2014, 2020) temperature could represent one of the main drivers producing a strong effect on embryonic development in a large number of species. Because in temperate climates the oviposition period of tettigoniids can start as early as July or as late as October (Ingrish, 1986a), different temperatures experienced by eggs before winter could trigger embryonic development until the completion of final stages before hatching.

Because most eggs of Orthoptera species remain in the ground for at least one winter, a possible explanation of increase in population abundances in certain years has been attributed to warmer temperatures during cold periods, able to affect egg survival and hatchling success. Some studies reported winter mortality at sub-zero temperatures from eggs of certain Acridids (Mukerji & Braun, 1988; Branson, 2020), in particular when exposed to prolonged chill periods without high temperature intervals (Jing et al., 2005). Moreover, recent large locust outbreaks reported in the last century are closely related to long-term droughts and warm winters coupled with occurrence of high precipitation in spring and summer during hatching periods (Peng et al., 2020). While more attention has been paid to the sub-order Caelifera, the role of winter temperatures affecting survival in Ensifera is still unknown.

Since 2008, repeated outbreaks of the endemic tettigoniid *Barbitistes vicetinus* (Cavaletto et al., 2015) occurred in small hilly areas of north-east Italy. This bush-cricket has become a pest, spreading quickly among multiple locations of its small geographic range and causing severe damages to forest plants and crops, mainly vineyards and olive groves (Cavaletto et al., 2015). Like many tettigoniids of temperate climates, *B. vicetinus* is a univoltine species overwintering as egg laid under the surface of the ground. Oviposition occurs in June mainly in the soil of forest areas (Cavaletto et al., 2018). In spite of the presence of recent extensive studies about genetic population, ecology and life traits of *B. vicetinus* (Cavaletto et al., 2015; 2018; 2019; Ortis et al., 2020; Martinez et al., 2021) there is a lack of knowledge about the factors that influence the egg development. Although most of the studies on orthoptera phenology try to predict the timing of egg hatch during the season, further knowledge of environmental factors influencing development during all the year is necessary to understand diapause patterns and to organize effective control strategies. Most of the studies on orthopteran development are usually performed under laboratory constant temperatures, known to poorly predict performance of many insects compared to fluctuating natural temperatures (Colinet et al., 2015), thus, testing effects of field temperatures on diapause could lead to a better understanding of the phenomenon. In this study, we want to clarify if summer temperatures may affect the number of eggs able to hatch in the next spring and how winter temperatures affect egg survival. For comparative purposes, effects of summer and winter temperatures on embryonic development and survival were also tested under laboratory conditions. Both factors could have contributed to generating outbreaks of *B. vicetinus* during the last decade.

Materials and methods

Bush-cricket rearing

Adults of *B. vicetinus* were collected at the beginning of the mating season from a single site located in the Euganean Hills on 1 June 2020 (45°21'47.2"N - 11°43'46.9"E). Individuals of both sexes were reared in a greenhouse with natural light and temperature fluctuating between 19°C and 35°C and relative humidity cycling between 70 and 80%. Bush-crickets were split in 5 cages (150 X 50 X 50 cm) with a wooden frame and walls made of nylon net. Each cage contained a tray filled with washed sievable sand for egg laying. Adults were fed with branches of four different species of plants (*Rubus* sp., *Ostrya carpinifolia, Fraxinus ornus* and *Prunus* sp.) replaced every two days. Each cage interior was sprayed with water once per day to provide moisture. On the 15th June, eggs were sifted from the sand and randomly assigned for each treatment. After oviposition, a young embryo can arrest development in a state called initial diapause, in which can remain for several years before resuming development, or begin to develop in the same year reaching a state called final diapause, where the embryogenesis is complete and the embryo occupies the whole egg space (Warne, 1972; Bailey and Rentz, 1990). When final diapause is reached, the mature embryo overwinters for one winter before resuming development and hatching when conditions are favourable again.

Field experiments

In order to understand the effect of temperatures on embryonic development and overwinter survival of egg, two sets of experiments were performed under natural conditions: i) during the summer period 2020 and ii) during the winter period 2020/2021. We selected two distinct areas in Veneto Region (Italy) to obtain different thermal conditions both for the summer and winter experiments: one in the outbreak area of the Euganean Hills (Table 1) and one at Monte Cavalletto in the pre-alpine area (Table 1), selected for the presence of an altitudinal gradient of over 1000 m above sea level.

Table 1. Characteristics of experimental sites. Black dots indicate if site was used for the summer and/or winter field experiment.

Site ID	Location	Latitude-Longitude	Elevation (m)	Summer	Winter
26	Euganean Hills	45°18'44.96"N - 11°43'59.68"E	37	●	
19	Euganean Hills	45°15'2.50"N - 11°41'25.90"E	39	●	
4	Euganean Hills	45°19'22.60"N - 11°45'19.70"E	44	●	●
15	Euganean Hills	45°15'41.34"N - 11°40'51.24"E	48	●	
2	Euganean Hills	45°20'17.54"N - 11°39'16.71"E	66	●	
1	Euganean Hills	45°18'12.42"N - 11°43'46.56"E	99	●	
31	Euganean Hills	45°19'32.78"N - 11°39'51.68"E	124	●	
3	Euganean Hills	45°19'19.68"N - 11°43'20.67"E	140	●	
22	Euganean Hills	45°20'15.17"N - 11°39'40.49"E	159	●	●
18	Euganean Hills	45°19'1.36"N - 11°46'13.37"E	168	●	●
30	Euganean Hills	45°19'59.64"N - 11°40'8.47"E	208	●	
5	Euganean Hills	45°19'2.99"N - 11°40'37.03"E	261	●	●
6	Euganean Hills	45°21'0.80"N - 11°39'35.90"E	295	●	●
12	Euganean Hills	45°19'3.99"N - 11°41'20.10"E	456	●	●
310	Monte Cavalletto	45°46'0.49"N - 11°33'7.52"E	315	●	
B	Monte Cavalletto	45°46'31.00"N - 11°31'50.72"E	415	●	
GA	Monte Cavalletto	45°46'43.02"N - 11°32'21.20"E	487	●	
C	Monte Cavalletto	45°46'30.47"N - 11°31'21.29"E	493	●	●
D	Monte Cavalletto	45°46'53.39"N - 11°30'51.13"E	633	●	●
BET	Monte Cavalletto	45°47'6.89"N - 11°32'19.15"E	700	●	
E	Monte Cavalletto	45°47'7.36"N - 11°31'7.33"E	745		●
F	Monte Cavalletto	45°47'11.87"N - 11°30'41.99"E	850		●
G	Monte Cavalletto	45°47'17.36"N - 11°30'58.92"E	960		●
H	Monte Cavalletto	45°47'32.83"N - 11°30'55.92"E	1060	●	●
I	Monte Cavalletto	45°47'50.25"N - 11°30'28.95"E	1150		●

Experiment with summer temperatures

To detect the effect of summer temperature on embryonic development we selected 21 sites (including 14 sites on the Euganean Hills and 7 sites on the Monte Cavalletto) along an elevation gradient (Table 1). In each site, 200 eggs were divided equally in 5 soil-filled plastic cups (10x10x5 cm) covered with a nylon net of 0.9 mm mesh in order to prevent damage by predators. The bottom of each cup was removed and replaced with a nylon net to allow water flow. All the cups were buried approximately 2 cm beneath the surface and spaced about 10cm from each other. In each site, temperature was recorded every 15 minutes by one datalogger (Extech TH10) buried approximately 2 cm under the surface in the proximity of the cups. A total of 4200 eggs were placed. Cups and dataloggers were placed together in forest soil on the 22nd of June 2020 and recovered on the 20th of September 2020. At the end of this period, in laboratory, we counted for each cup i) the number of eggs with embryonic development in progress (i.e. final diapause), ii) the number that did not develop (i.e. initial diapause), and iii) the number that were strongly flattened or damaged (Figure 1). Eggs in final diapause can be easily recognised with stereoscope by their turgescence *sensu* Warne (1972) and for the typical green coloration of the embryo visible by increasing the transparency of the chorion with water.

To clarify how many eggs in initial diapause can develop after two years from oviposition, we buried in the ground, as described above, 770 eggs in initial diapause coupled with a datalogger. Eggs were placed in forest soil on the 20nd of October 2020 and recovered on the 20nd of September 2021 and counted again.

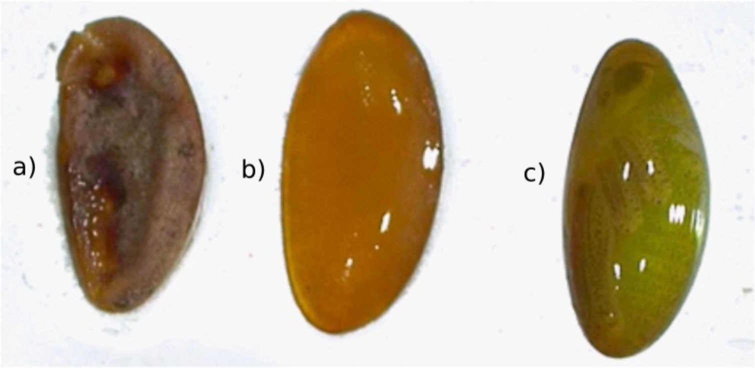

Figure 1. Typologies of eggs. a) unviable egg; b) egg in initial diapause; c) egg in final diapause.

Experiment with winter temperatures

To detect the effect of winter temperatures on egg survival, we selected 6 sites in the Euganean Hills and 7 sites at M. Cavalletto. Again, 5 cups coupled with datalogger were placed in each site. Each cup contained 30 eggs with evidently developed embryo (in final diapause), randomly assigned from all eggs retrieved at the end of the summer experiment described above. A total of 1950 eggs were placed in field. Cups were settled in the field on 18 November 2020 and recovered on 4 March 2021, shortly before the hatching period (Cavaletto et al., 2020). Eggs were extracted from the soil of the cups and placed outside the laboratory in a shady place in petri dishes on moist filter paper to allow hatchings. All the eggs were checked every day until the end of the hatching period.

Laboratory experiments

To verify the effect on embryonic development and survival of constant different temperatures we placed eggs of *B. vicetinus* in climatic chambers (Pol-eko Aparatura, ST 3) during the same periods of the field experiments.

Summer treatment

For the summer period (22nd June to 20th September) we choose six constant temperatures along a thermal gradient from 19 to 24 °C at 1° interval. In each chamber 240 eggs were randomly split in 6 petri dishes on moist filter paper and provided with water weekly during the whole period to prevent dehydration (Ingrisch, 1983). At the end of summer period, eggs were counted and divided as described above

Winter treatment

For the winter period (18th November to 4th March 2021) we chose four constant temperatures along a thermal gradient: 1°C, 3°C, 4°C, 5°C. In each chamber, 100 eggs in final diapause randomly assigned from the summer field experiment were split in 5 petri dishes and provided with water weekly. At the end of the winter period, eggs were placed outside the laboratory in a shaded place to allow hatchings together with eggs from winter field treatment.

Statistical analyses

We related the proportion of eggs that reached final diapause on total viable eggs (final diapause + initial diapause), for both field and laboratory trials of the summer period, with the mean temperature registered by dataloggers in the period between 22 June to 31 August and constant temperatures in the chambers, respectively. Trends were described as fitting two non-symmetrical sigmoid Weibull curves. To compare the two Weibull distributions fitted with two different datasets, we created a dataset with pooled data from field and laboratory experiments. Therefore, we compared the log-likelihood of the pooled model to the sum of the log-likelihoods of the separate models.

We tested the relation of survival and mean temperatures recorded during winter field experiment by a binomial model using “glm” function. To better visualize difference of hatchings from field and laboratory winter experiments, cumulated hatching percentages were calculated summing all hatchings in each day for all temperatures tested in each experiment.

All analyses were performed using R-4.0.3 (R Core Team, 2020).

Results

Summer experiment

Overall, the choice of experimental sites in the field arranged along an altitudinal gradient made it possible to record an average summer temperature range between approximately 15 and 22°C. From the 4200 eggs placed in the ground, we recovered 3383 eggs. Our results showed that the different summer temperatures to which the eggs were exposed significantly influenced the rate of embryonic development. In particular, at low summer temperatures in the field (17 to 19°C) a little proportion of eggs (less than 20%) reached final diapause, whereas more than 90% of eggs reached final diapause when mean temperatures were over 21°C (Table 2). The field temperature dependent model estimated that 50% of eggs will reach final diapause when mean summer temperature is close to 20°C (Table 4, parameter e).

The model based on laboratory data showed that the incubation of eggs at constant temperatures produced a different effect compared to field temperatures ($p<0.001$). A lower number of embryos were able to reach final diapause at constant temperatures compared to field conditions (Figure 2, Tables S2, S3). The shape of the curve was however similar to the curve of field conditions, suggesting a strong relationship between rates of development and increasing temperatures.

After two years from oviposition, from the 770 eggs placed in the ground in initial diapause, 499 eggs reached final diapause, 167 remained in initial diapause and 104 were not viable.

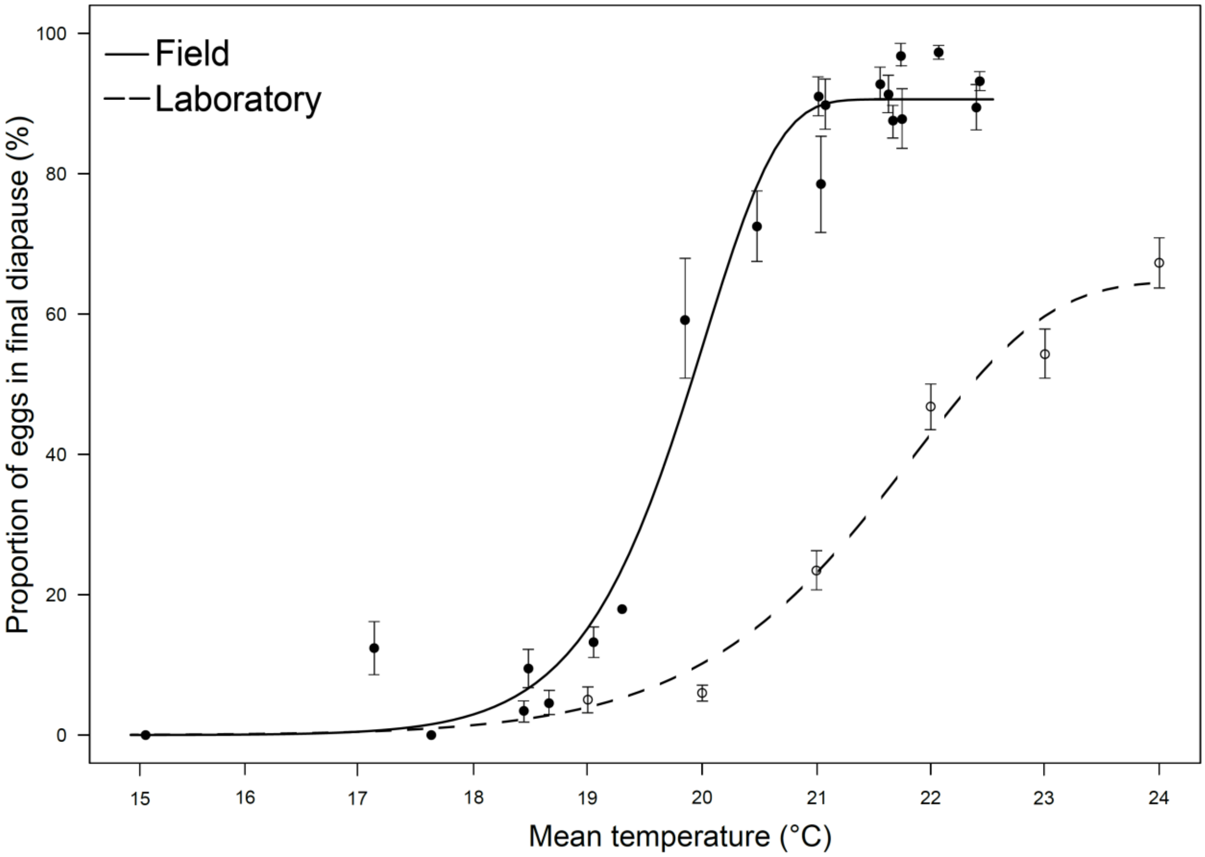

Figure 2. Temperature-dependent rate (%) of eggs that reached final diapause at the end of summer period (22 June-31 August) in field and laboratory experiments. Standard error is calculated on sub-replicates for each site. Observed (points) and predicted (lines).

Table 4. Parameter estimates of Weibull curves from summer experiments. b=slope at the inflection point; d=maximum asymptote; e=inflection point (x-axis).

Parameter	Estimate	t-value	p-value	Residual standard error
Field				5.93
b	31.76	5.51	< 0.001	
d	90.59	44.33	< 0.001	
e	20.03	157.28	< 0.001	
Laboratory				4.90
b	19.38	3.85	0.030	
d	64.62	10.86	0.002	
e	21.90	63.37	< 0.001	

Winter experiments

Overall, average winter temperatures recorded ranged from approximately 1 to 7°C. However, the different winter temperatures experienced by the eggs in the field did not affect hatching number (p=0.585). From the total 1950 eggs in final diapause, 1744 hatched during spring. The overwinter survival rate was high (approximately 90%) in all the temperatures considered (Table S5) and differed from survival of eggs incubated at constant temperatures (approximately 70%) (Table S6). The hatching period for eggs coming from field winter treatment lasts until 11 April whereas hatching period for eggs incubated at constant temperatures lasts until 22 April (Figure 3).

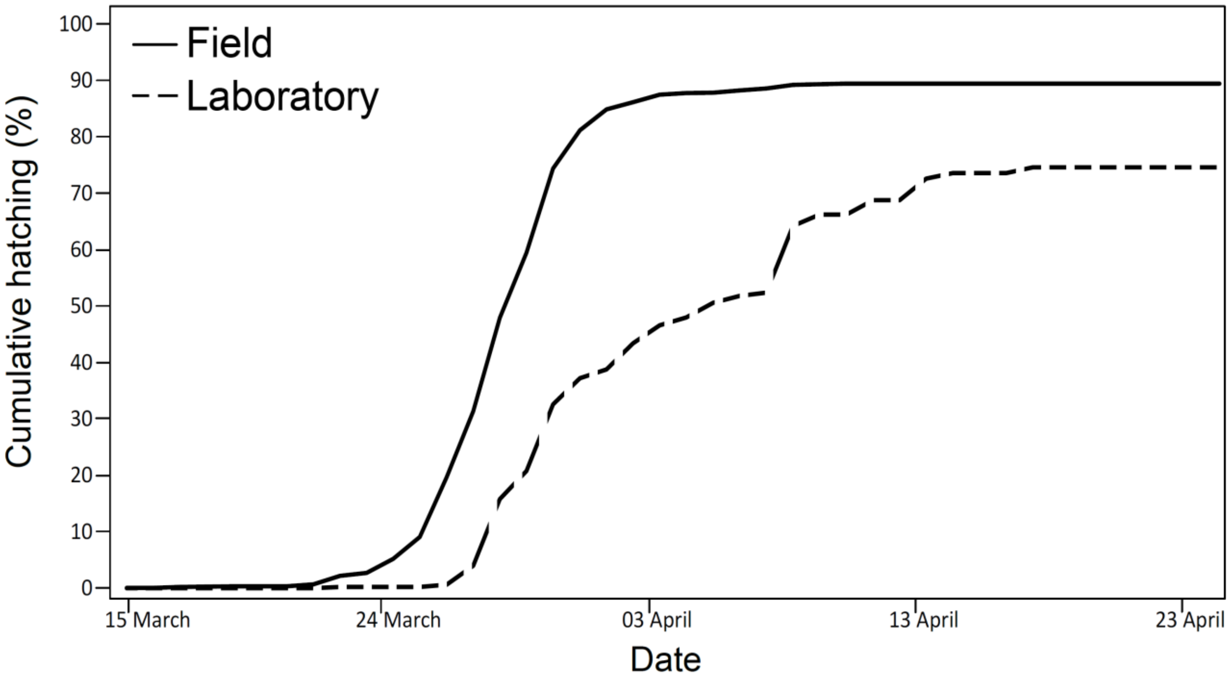

Figure 3. Cumulated hatching percentage during the whole hatching period for field and laboratory experiments. Percentages were calculated on total eggs from winter field experiment (1950 eggs) and winter laboratory experiment (500 eggs) respectively.

Discussion

This work demonstrates that summer temperature strongly affected embryonic development of *B. vicetinus* while winter temperatures did not influence egg mortality. From our results, less than 20% of embryos were able to develop even if field temperatures were under 19.5°C, suggesting that those temperatures represent the lowest limit where inception of embryonic development could occur only marginally. Besides, a substantial shift in rates of final diapause from 20 to nearly 80% occurred in a thermal variation of about 1°C between 20 and 21°C. Probably, mean temperatures above 21°C already represent suitable thermal conditions, allowing the species to maintain high numbers of individuals in the year following the oviposition. However, even with temperatures higher than 21°C, a portion of eggs did not develop. This survival strategy is well documented among other Tettigoniid species (Hartley and Warne, 1972), where variability of initial diapause would prevent premature development, providing species with another chance for development in the subsequent years. Because our experiment tested temperatures in soil, we did not expose eggs to extreme hot temperatures. However, it seems unlikely that eggs could experience such extreme temperatures unless directly exposed to sunlight. The embryonic developmental response in this range of temperatures is particularly relevant considering a global warming scenario. Having recurrent years with temperatures above those thermal conditions could potentially lead to a demographic population increase by shifting life cycle from pluriannual to annual.

Trend obtained from laboratory experiment supported the hypothesis of a strong influence of temperature on embryonic development of *B. vicetinus*. However, the incubation of eggs at constant temperatures produced a different effect on embryos compared to fluctuating field temperatures. A possible explanation is the effect of fluctuating temperatures that could improve fitness and thermal tolerance of insects compared to those exposed to constant temperatures (Kingsolver et al., 2009; Srygley & Senior, 2018). Fluctuating temperatures that extended within the thermal range of a species can result in diverse responses, probably depending on the thermal mean that is used and its proximity to developmental thresholds (Colinet., 2015).

To our knowledge, this is the first study reporting egg survival of a tettigoniid species under field winter temperatures. Although some studies reported winter mortality at sub-zero temperatures from eggs of certain Acridids (Mukerji & Braun, 1988; Jing et al., 2005; Branson, 2020) in our study we found very high survival rates (>90% of hatchings) for all winter temperatures. The cold temperatures tested (average from 1 to 7°C) were beyond the coldest temperature limit of species range distribution (Cavaletto et al., 2020). It could be relevant to note that even if air temperatures reached sub-zero for several days during the winter period, we did not detect such extreme temperatures with dataloggers, thus, embryos could have benefited from the higher temperatures maintained in forest ground.

Although for field experiment overwinter survival was high (>90%) among all temperatures recorded, we detected lower survival rates (70%) for eggs incubated at constant temperatures. It was already reported how interrupting prolonged cold exposure with warm periods could promote fitness of many insect species, allowing physiological changes that are not otherwise possible (Nedved et al., 1998; Jing et al., 2005; Yocum et al., 2012). In our case, probably, prolonged exposure of eggs to constant cold temperatures could have damaged embryos and lengthen the period of embryonic maturation after diapause inducing mortality and a prolonged hatching period. A similar prolonged post-diapause development was obtained exposing eggs of *B. vicetinus* at progressively colder temperatures at the end of the winter period (Cavaletto et al., 2020).

While outbreaks of bush-crickets reported in the last century were supposed to be related to climatic alterations, the possible role of temperature in influencing demographic fluctuations of bush-crickets is still unknow. As well as confirming that eggs of *B. vicetinus* can hatch after two years from oviposition, we clarify that the occurrence of a facultative multi-year diapause is related to summer temperatures experienced by eggs after oviposition.

The early phenology of *B. vicetinus* and the strong thermal sensitivity of embryonic development to summer temperatures could have favoured the abundance of this species compared to other Tettigoniids of temperate climate. Future research focused on embryonic development of orthoptera should take into account the effect of realistic field temperatures on diapause, in that little variations of temperature over the thermal range of each species are potentially able to shift their multi-year life cycle. Our methodology would be useful to improve knowledge about biology and ecology of different species and to predict their outbreak risk.

Supplemental materials

Supplementary Table 2. Mean and standard error of the number of each egg type at the end of the summer field experiment. Values were calculated on cups recovered for each site.

Site	Mean temperature (°C)	Final diapause	Initial diapause	Unviable
26	22.07	36.00±1.04	1.00±0.31	1.20±0.48
19	21.75	28.75±2.01	4.00±1.47	4.75±1.31
4	21.02	18.02±5.54	1.08±0.80	7.80±3.12
15	22.40	31.75±1.88	3.75±1.10	3.50±1.19
2	21.59	33.60±1.20	3.20±0.96	2.00±0.44
1	21.65	32.40±1.16	4.60±0.92	3.60±1.50
31	21.74	18.00±5.55	0.60±0.60	1.80±1.20
3	21.08	24.60±6.12	2.80±1.24	7.20±2.75
22	22.43	24.60±6.02	1.80±0.20	11.60±3.50
18	21.58	25.60±5.53	2.00±0.31	8.40±5.90
30	21.04	27.80±3.49	7.60±1.86	1.60±0.60
5	20.48	27.40±2.24	10.40±1.72	1.80±1.06
6	19.30	7	32	1
12	19.85	19.40±2.63	13.40±2.83	3.60±0.92
310	18.44	1.20±0.48	33.60±1.46	3.00±1.14
B	19.05	3.60±0.74	23.60±2.83	8.60±2.44
GA	18.48	2.20±0.58	21.00±2.12	16.00±2.36
C	18.66	1.00±0.30	21.00±3.83	14.60±3.32
D	17.63	0.00±0.00	20.20±7.53	14.00±5.85
BET	17.13	3.00±0.89	21.20±3.91	8.60±3.88
H	15.13	0.00±0.00	9.60±3.00	27.40±2.01

Supplementary Table 3. Mean and standard error of the number of each egg type at the end of the summer laboratory treatment.

Mean temperature (°C	Final diapause	Initial diapause	Unviable
19	1.80±0.65	34.50±1.17	3.60±0.76
20	2.30±0.42	36.05±1.08	1.16±0.83
21	8.80±1.04	28.80±1.16	2.30±0.42
22	17.10±1.10	19.50±1.56	3.10±0.87
23	19.00±1.73	16.00±1.34	4.50±1.23
24	23.30±1.40	11.30±1.33	5.30±1.28

Supplementary Table 5. Mean and standard error of the number of eggs hatched at the end of the winter field treatment. Percentages of hatchings are reported in brackets. Values were calculated on 5 cups (30 eggs per cup) for each site.

Site	Mean temperature (°C)	Hatchings
I	1.37	23.40±3.05 (78%)
G	2.46	27.40±1.40 (91%)
H	2.79	28.40±0.74 (94%)
E	4.04	26.60±1.16 (88%)
22	4.25	28.20±0.96 (94%)
12	4.48	28.02±1.15 (94%)
F	4.54	24.80±1.49 (82%)
5	4.75	28.02±0.37 (94%)
D	4.80	28.25±0.85 (79%)
6	4.95	27.80±1.01 (92%)
C	5.46	28.02±0.86 (94%)
4	5.65	29.00±0.94 (96%)
18	7.20	27.40±3.27 (82%)

Supplementary Table 6. Mean and standard error of the number of eggs hatched at the end of the winter laboratory treatment. Percentages of hatchings are reported in brackets. Values were calculated on 5 cups (20 eggs per cup) for each site.

Mean temperature (°C)	Hatchings
1	13.40±1.69 (67%)
3	13.60±0.92 (68%)
4	15.80±1.52 (79%)
5	14.00±1.87 (70%)

Chapter 4

Taxonomic identification and biological traits of *Platystethynium triclavatum* (Donev & Huber, 2002), comb. n. (Hymenoptera, Mymaridae), a newly recorded egg parasitoid of the Italian endemic pest *Barbitistes vicetinus* (Orthoptera, Tettigoniidae)

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Abstract

The little known fairyfly (Hymenoptera, Mymaridae), *Platystethynium* (*Platystethynium*) *triclavatum* (Donev & Huber, 2002), comb. n. from *Pseudocleruchus* Donev & Huber, 2002, is newly recorded as an egg parasitoid of *Barbitistes vicetinus* Galvagni & Fontana, 1993 (Orthoptera, Tettigoniidae). This bush-cricket is endemic to northeastern Italy (mainly Euganean Hills of Veneto Region), where it has recently become an economically significant agricultural and forest pest. Data on discovery, distribution, and some remarkable biological traits of this gregarious egg parasitoid are presented. Its identification and availability of many well-preserved fresh specimens have made possible to re-define *Pseudocleruchus* Donev & Huber, 2002 syn. n., with type and the only described species *Pseudocleruchus triclavatus* Donev & Huber, 2002, as a synonym of *Platystethynium* Ogloblin, 1946 and its nominate subgenus, *P.* (*Platystethynium*), and also to describe the brachypterous male of *P.* (*Platystethynium*) *triclavatum*. It is the first known male for the entire genus. Enlarged mandibles of the megacephalous males are used to chew holes in the hard chorion of the host egg, allowing fully winged females, whose mandibles are strongly reduced and do not cross over, to emerge after mating with the males inside it. Up to 136 individual parasitoids (about 77 on average) can hatch from a single egg of *B. vicetinus*, with their sex ratio being strongly female biased (80–97% females per egg).

Introduction

Occasional outbreaks have been reported in some species of bush-crickets (Orthoptera, Tettigoniidae), causing severe damage to crop and forest plants (Escherich, 1928; Laussmann, 1994; Holuša et al., 2006). During these demographic fluctuations, the population density rises to values much higher than those usually presented by a species (Lorch & Gwynne, 2000; Bailey et al., 2007a, 2007b; Srygley, 2014). A significant impact on a particular life-history stage of Tettigoniidae could be associated with the activity of predators and other natural enemies (Gwynne, 2001). There are stressors, such as parasitoids (Polak, 1997b; Hunt & Allen, 1998; Umbanhowar & Hastings, 2002), that could adversely affect both fitness and developmental stability of a pest species. The behaviour of individual parasitoids in response to an increasing prey density is thought to be an important attribute related with parasitoid success (Fernández & Corley, 2003). To date, interactions between parasitoids and bush-crickets during years of demographic explosion have been virtually unknown. In a large number of temperate species the egg stage persists for longer than a year, being adapted to withstand all the seasonal and annual adversities (Bailey & Rentz, 1990). Although Orthoptera eggs are available to natural enemies for such long periods, few of them are recorded at the egg stage, probably because finding and collecting eggs in the soil is a lengthy and difficult task (ElSayed et al., 2016). The best collection methods, such as by Malaise traps, yellow pan traps, sweep netting or Berlese funnels, are not suitable to detect these parasitoids in associations with their hosts, especially for egg parasitoids that search for their hosts in the ground (Chiappini & Huber, 2008). Most of the studies were conducted on egg-pods of Celifera, and they identified members of the family Scelionidae (Hymenoptera, Platygastroidea) as the most important parasitoids of grasshopper eggs (Grissell, 1997; Ghahari et al., 2009). In addition, some chalcidoid wasps (Hymenoptera, Chalcidoidea) were also reported to parasitize eggs of Tettigoniidae (Gupta, 2015).

The occurrence of severe outbreaks (since 2008) in the endemic area of northeastern Italy (Euganean Hills of Veneto Region) of the recently described bush-cricket *Barbitistes vicetinus* Galvagni & Fontana, 1993 (Orthoptera, Tettigoniidae), prompted interest in studying its biology; this bush-cricket is a pest which causes severe defoliation across sub-Mediterranean forests and neighbouring crops (Cavaletto et al., 2018; Cavaletto et al., 2019). Like many other Tettigoniidae, this species is univoltine and overwinters as eggs laid in the forest soil. Egg hatching generally begins at the end of March, and the life cycle lasts until the first half of July when the last adults die after oviposition. The eggs of *B. vicetinus* can remain in the soil for one or more years in a diapause state, but since the outbreaks occurred only in the last decade, no data are currently available (Cavaletto et al., 2019). Damage in forests and crops caused by the outbreaks, and the environmental impact caused by conventional control strategies have stimulated search for alternative control methods such as potential use of natural enemies.

Here we taxonomically identify the newly recorded egg parasitoid of *B. vicetinus* and provide data on its biological traits and geographical distribution data.

Materials and Methods

Study area

The study was carried out during 2018-2019 in the Euganean Hills (Province of Padua, Veneto Region, northeastern Italy). The area is characterized by about 100 volcanic hills covering an elliptical area of approximately 22000 ha and with elevation ranges from sea level to 600 m a.s.l. This area corresponds with a small part of *B. vicetinus* distribution range, most affected by the outbreaks of the pest. The climate is characterized by an annual average temperature of about 12°C and 700–900 mm of precipitation (Kaltenrieder et al., 2009). The landscape is fragmented and consists of forest patches, interspersed with crop fields and rural settlements.

Eggs of *B. vicetinus* sampling procedures

During autumn 2018, we collected eggs laid by *B. vicetinus* in the soil from 40 outbreak forest sites across the Euganean Hills, where the nymph density had been monitored in the previous study (Cavaletto et al., 2019). In each site, we identified six circular areas (sub-replicates) of 15 cm radius and 5 cm deep, free from branches, roots, stones and coarse litter. The six circular areas were spaced by no less than 1 meter from each other. In total, from each site, the soil was collected from a surface of 0.42 square meters. The soil sample for each sub-replicate was collected with a small shovel and carried to the laboratory in sturdy plastic bags. Each sample of soil was washed in a hydraulic sieving-centrifugation device with different sieves of progressively smaller mesh (weaves decreasing from 20 to 1 mm) to facilitate separation of the eggs from organic and inorganic matter. Eggs were washed carefully from the remaining soil with running water, collected using soft tweezers, and placed in Petri dishes.

Eggs of *B. vicetinus* analysis

The eggs (length: 4,5 mm; width: 2,50 mm) (Galvagni & Fontana, 1999) were then recognized under a stereomicroscope and divided for each sub-replicate in i) hatched eggs, ii) flattened eggs, iii) eggs with small holes in the chorion, and iv) fertile eggs. The hatched eggs displayed a rupture in correspondence of the hatching line. Small pieces of chorion were not included in the count. The flattened eggs had no broken chorion and were recognized by the flat profile probably due to the lack of yolk inside. Eggs with small circular emergence holes, across the surface of the chorion, were assumed to be parasitized in the past seasons. The apparently fertile eggs, recognized by their turgescence *sensu* Warne (1972), were kept outdoors, in Petri dishes on filter paper and moistened weekly, to allow overwintering. The impact of parasitoids in the previous seasons was assessed, for each site, by counting eggs with small holes. Moreover, to evaluate efficacy of the egg parasitoid, the index of "discovery efficiency", proposed by Bin & Vinson (1990), was applied. For each site, discovery efficiency was calculated as the number of sub-replicates discovered by the parasitoid over the 6 sub-replicates sampled. A sub-replicate was considered discovered when it included at least one egg with holes. Sub-replicates not containing any typology of eggs were not included in the count.

To describe the relationship between activity of the parasitoid and density of the bush-cricket, we related, for each site, the number of the previously parasitized eggs with the sum of all collected eggs.

Identification of the egg parasitoid and taxonomic studies

Morphological terms, mounting and photography techniques, measurements (in mm), and abbreviations (F = funicle segment of female antenna or flagellomere segment of male antenna; mps = multiporous plate sensillum or sensilla) follow those described in Triapitsyn et al. (2019). Specimens sent to the second author were labeled including a UCRC ENT database number (unique identifier for each individual). Most voucher specimens of the parasitoids were deposited at DAFNAE; additional specimens were deposited in CNC (Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Ontario, Canada) and UCRC (Entomology Research Museum, Department of Entomology, University of California, Riverside, California, USA), as indicated under "Material examined" section below.

Statistical analysis

All analyses were conducted using R (version 3.6.2) (R Core Team). To relate the activity of the parasitoid during the past with the oviposition density of the bush-cricket we used a linear model (LM). For each site, the number of total eggs (hatched eggs, flattened eggs, fertile eggs, eggs with holes) was included in the model as fixed effect, while the response variable (number of eggs with holes) was log-transformed to improve linearity.

The impact of parasitoids was assessed, for each site, as the number of previously parasitized eggs divided by the total number of eggs collected. In addition, we calculated the sex ratio by dividing, for each parasitized egg, the number of hatched females with the total number of individuals.

Results

Distribution and biology of the egg parasitoid

From the 40 sampling sites considered, in a total of 240 samples of soil (sub-replicates), we collected and analyzed 0.847 cubic meters of soil. A total of 24329 eggs of *B. vicetinus* were identified from which 18965 were recognized as hatched eggs, 3132 flattened eggs, 1669 fertile eggs and 559 eggs with small emergence holes. The average number of hatched eggs of *B. vicetinus* per site was 474 ± 85 SE, while that of the other Orthoptera was 10 ± 3 SE. We recorded activity of the parasitoids in 31 of the 40 sites studied. From 231 sub-replicates containing eggs of the bush-cricket, eggs with small holes were found in 113 sub-replicates (index of discovery efficiency = 48%) (Table S1) (Fig. 1).

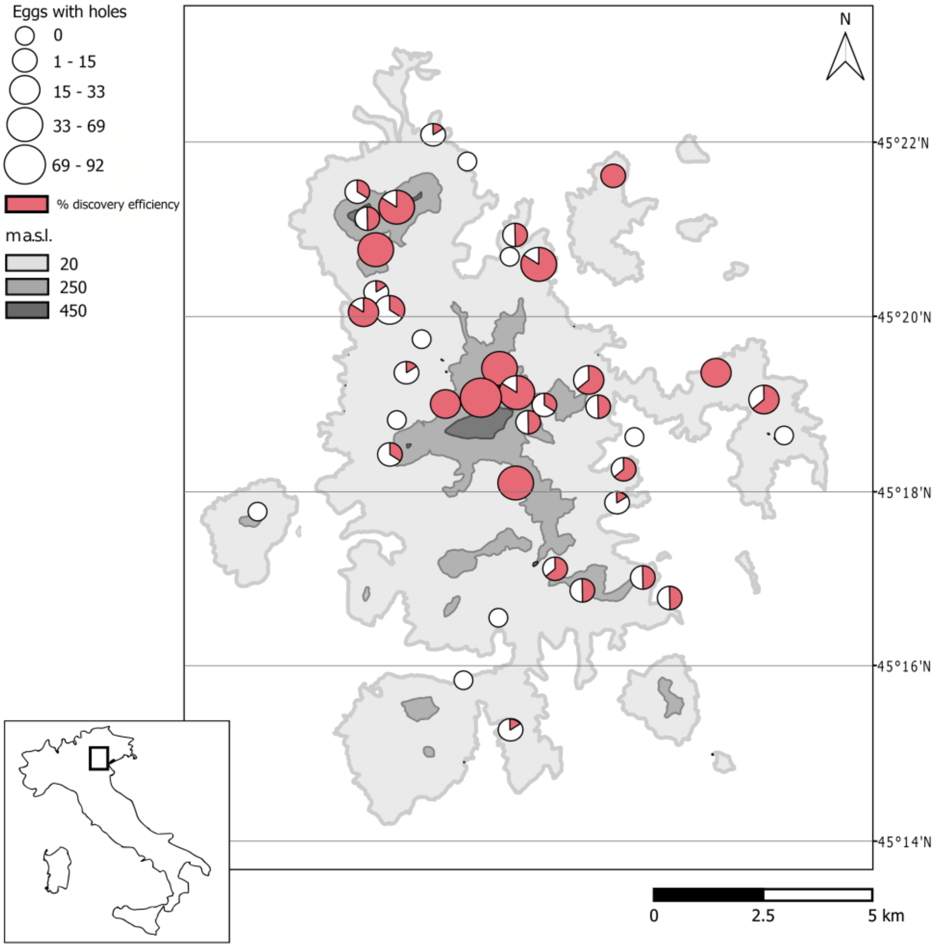

Figure 1. Distribution of *Platystethynium triclavatum* in the sampling sites of Euganean Hills, based on the presence of eggs of *Barbitistes vicetinus* with emergence holes. The abundance is indicated by white circles, elevation is indicated by gray color. The discovery efficiency for the six sub-replicates is represented by red color. Latitude is indicated by gray lines. Map Source: Curve di livello della Regione Veneto, IODL 2.0.

The number of eggs with emergence holes was considered as number of the previously parasitized eggs. This number was positively related to the total number of eggs collected for each site, i.e. higher number of eggs with emergence holes was found in sites where the oviposition density of the host was elevated (Fig. 2) (LM; R-squared=0,35; df=38, p-value=4.41e-05).

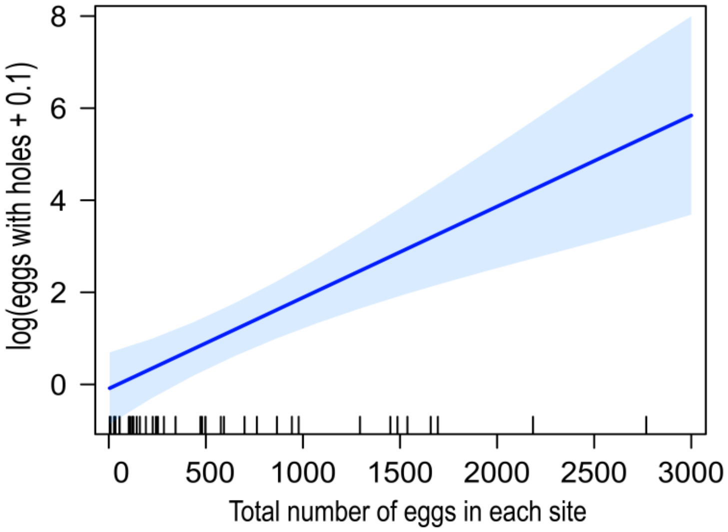

Figure 2. Relation between previously parasitized eggs and the amount of eggs in each site.

At the end of spring, a portion of about 70 fertile eggs, which were not hatched, was monitored to verify the presence of parasitoids. Approximately the 20% of these eggs was randomly chosen and dissected. Among them, eight eggs showed numerous parasitoids in the embryonic or larval stages (Fig. 3). Subsequently, presence of parasitoids inside the eggs was detected without dissection, exploiting transparency of the chorion, increased by the treatment with xylene as described by Warne (1972) (Fig. 3).

Figure 3. Eggs of *Barbitistes vicetinus*. (A) Fertile egg treated with xylene to enhance the transparency of the chorion; (B) dissected egg with embryos of the parasitoid; (C) larval stages of the parasitoids visible in transparency in an egg treated with xylene; (D) emergence holes of Platystethynium triclavatum; (E) eggs of *B. vicetinus*.

All these eggs were monitored and placed individually within single transparent plastic vials covered on top with cotton, provided with water weekly, and placed in a climatic chamber (T 26 ± 1 ° C, RH 75 ± 5%, photoperiod 12:12). In June, we obtained a total of 537 samples of parasitoid wasps which emerged from seven eggs across an average number of holes per egg of 2.5. On average, 76.7 parasitoids emerged per egg. Up to 136 individuals hatched from a single egg (Table 1).

Table 1. Number of individuals of *Platystethynium triclavatum* hatched from each single egg of *Barbitistes vicetinus.*

Site	Latitude-Longitude	Elevation (m)	Holes	Total	F	M
12	45°19'3.99"N - 11°41'20.10"E	447	4	136	132	4
12	45°19'3.99"N - 11°41'20.10"E	447	2	124	121	3
12	45°19'3.99"N - 11°41'20.10"E	447	2	120	115	5
12	45°19'3.99"N - 11°41'20.10"E	447	2	95	89	6
6	45°21'0.80"N - 11°39'35.90"E	295	4	40	35	5
3	45°19'19.68"N - 11°43'20.67"E	140	3	11	9	2
13	45°21'37,05"N - 11°39'56,92"E	367	2	11	10	1
3	45°19'19.68"N - 11°43'20.67"E	140	dissected	6	na	na
4	45°19'22.60"N - 11°45'19.70"E	44	dissected	50	na	na
17	45°19'17.1"N - 11°41'33.3"E	459	dissected	50	na	na
4	45°19'22.60"N - 11°45'19.70"E	54	dissected	30	na	na
4	45°19'22.60"N - 11°45'19.70"E	54	dissected	30	na	na
4	45°19'22.60"N - 11°45'19.70"E	54	dissected	10	na	na
4	45°19'22.60"N - 11°45'19.70"E	54	dissected	13	na	na

Note:
Holes: number of exit holes; Total: total number of hatched individuals; F: number of females; M: number of males.

We estimated a mean of previously parasitized eggs of 2.3% ± 0.5 SE among the 40 sites. The sex ratio of the parasitoid was strongly female biased, with a mean of 92% ± 2.4 SE of females per egg.
A stereomicroscope with an eyepiece micrometer was used to measure and compare the maximum diameter of the parasitoid emergence holes observed in these fertile eggs and in the previously parasitized eggs (Fig. 4A). No differences were found between the diameter means of the two groups (t-test, n=19, n=32, p-value=0.66). The average diameter of holes was 0.33 mm ± 0.15 SE. The emerged parasitoids were preserved in 96% ethanol awaiting to be identified. All the samples of parasitoid wasps emerged were initially identified by the second author as *Pseudocleruchus triclavatus* Donev & Huber, 2002 (Hymenoptera, Mymaridae).

Figure 4. Emergence holes and females of *P. triclavatum*. (A) Eggs of *Barbitistes vicetinus* with numerous emergence holes of *Platystethynium triclavatum*; (B) newly emerged females of *Platystethynium triclavatum* preserved in ethanol.

Identification and taxonomy of the egg parasitoid

Identification. Females of the egg parasitoid (Figs 4B, 5) were compared morphologically with the original description and illustrations of *Pseudocleruchus triclavatus* from Bulgaria (Donev & Huber, 2002) and with the illustrations of the non-type specimens from Czech Republic (Samková et al., 2020), and also with taxonomic notes on the females of the two undescribed *Pseudocleruchus* spp., one from Romania (Pricop, 2011) and the other (examined by the second author) from Finland (Samková et al., 2020). The reared females from Italy were found to be almost identical to those of *Pseudocleruchus triclavatus* from Bulgaria, besides the slight differences in having the fore wing slightly wider (the length:width ratio is slightly lower) and having a few more rows of discal microtrichia; that might be due to the fact that the Italian specimens are from a much lower elevation whereas the Bulgarian ones came from higher elevations and thus might have a slight degree of brachyptery. The length:width ratio of the scape of the female antenna is either the same or slightly higher in the Bulgarian specimens (as described), but that could be probably due to the way specimens of the type series of *P. triclavatus* were slide-mounted by Atanas Donev (in a somewhat different orientation). Even if these minor differences were correctly assessed, they are, undoubtedly, due to intraspecific variation, likely both geographical and host-induced. Indeed, because *B. vicetinus* is endemic only to the two hill areas of Veneto Region in Italy (Eugenean and Berici Hills), *Platystethynium* (*Platystethynium*) *triclavatum* (Donev & Huber, 2002), comb. n. from *Pseudocleruchus* Donev & Huber, 2002, has to have different tettigoniid hosts (likely bush-crickets) in Bulgaria and Czech Republic, which are yet to be identified. Like many other fairyflies, it is unlikely that *P. triclavatum* is very narrowly host specific; rather, it might be able to parasitize eggs of several, likely more or less related, genera of Tettigoniidae that lay eggs in soil. A molecular comparison and analysis of specimens morphologically identified as *P. triclavatum* could clarify the topic, but unfortunately no other properly preserved specimens of this species from other countries in Europe are currently available for DNA extraction.

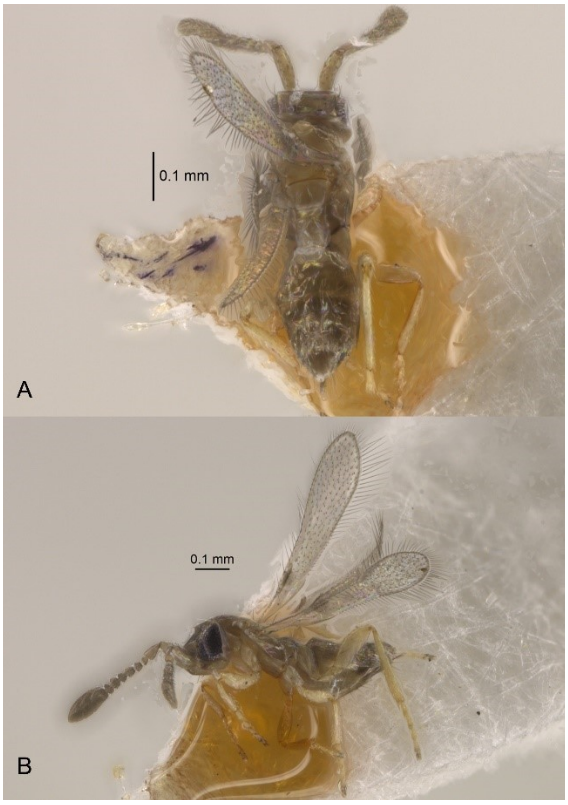

Figure 5. Dry-mounted female of *Platystethynium triclavatum*. (A) Habitus in dorsal view; (B) habitus in lateral view.

Taxonomy

Genus *Platystethynium* Ogloblin, 1946

Platystethynium Ogloblin, 1946: 290. Type species: *Platystethynium onomarchicidum* Ogloblin, 1946, by original designation.

Platypatasson Ogloblin, 1946: 293. Type species: *Platypatasson fransseni* Ogloblin, 1946, by original designation. Synonymy by Donev & Huber, 2002: 118; given subgeneric status as *Platystethynium* (*Platypatasson*) by Huber et al., 2020: 289–292.

Pseudocleruchus Donev & Huber, 2002: 118–120. Type species: *Pseudocleruchus triclavatus* Donev & Huber, 2002, by original designation. **Syn. n.**

Pseudocleruchus: Pricop, 2011: 25–26 (comparison with the nominal subgenus of *Cleruchus* Enock, 1909, updated diagnosis); Samková et al., 2020: 205 (key to females), 207 (key to males).

Platystethynium: Sankararaman et al., 2019: 10 (key to world species).

Platystethynium (*Platypatasson*): Huber et al. 2020: 289–292 (diagnosis, discussion, illustrations).

Brief diagnosis of the subgenus *Platystethynium* (*Platystethynium* Ogloblin). Female: head in lateral view rectangular, at least a little longer than high, with face usually strongly projecting anteriorly; mandibles strongly reduced, not crossing over; funicle 6-segmented, with at least some segments transverse; clava 3-segmented; only macropterous individuals are known; frenum longitudinally divided medially; gaster sessile; tarsi 4-segmented. Male (so far known only for one species, *P.* (*Platystethynium*) *triclavatum*): megacephalous (Figs 6C, 7), with enlarged, 3-toothed mandibles crossing over (Figs 8C, 9B); antenna much shorter than body, with flagellum normally 10-segmented (Figs 7B, 8A, 8B); strongly brachypterous.

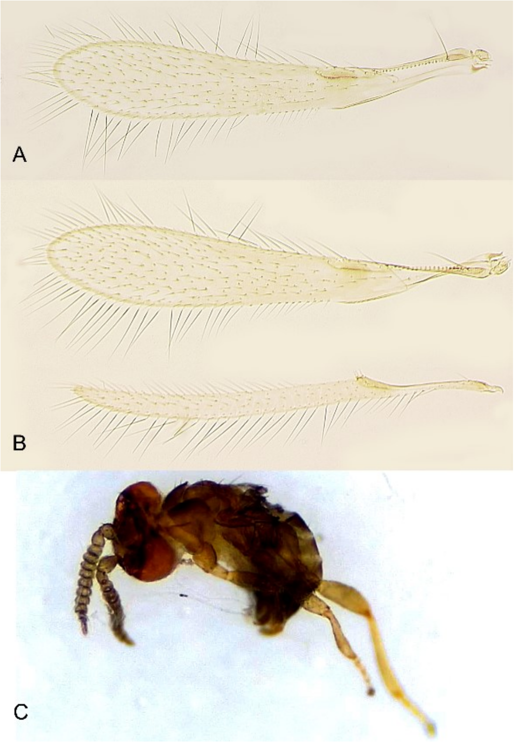

Figure 6. ***Platystethynium triclavatum*.** (A) Female fore wing; (B) female fore and hind wings; (C) habitus of male in lateral view (preserved in ethanol upon emergence).

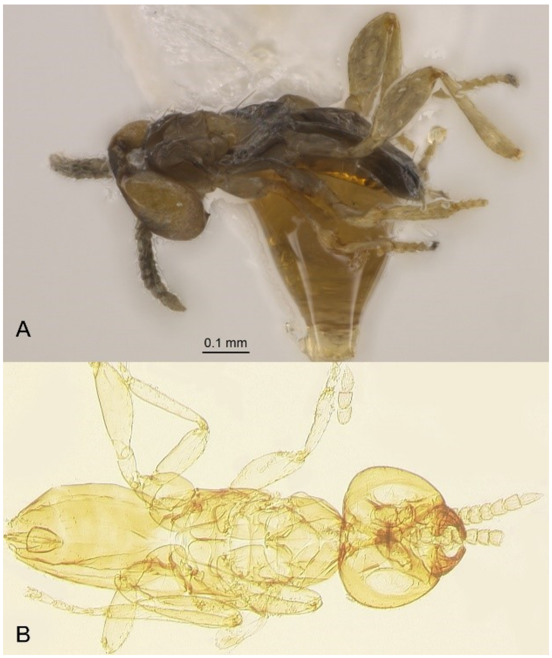

Figure 7. Male of *Platystethynium triclavatum*. (A) Habitus in dorsolateral view (dry-mounted specimen); (B) body (slide-mounted specimen).

Hosts. Orthoptera, Tettigoniidae (Ogloblin, 1946).

Comments. *Platystethynium* belongs to the *Cleruchus* group of genera (Huber et al., 2020). Its nominate subgenus occurs only in the Old World, while *P.* (*Platypatasson* Ogloblin, 1946) occurs in both New and Old Worlds. In Europe, females of the genus and its nominate subgenus (as *Pseudocleruchus*) can be identified using a key in Samková et al. (2020), who incorrectly guessed (p. 207, in the key to males of the European genera of Mymaridae), that males of *Pseudocleruchus*, then unknown, could be macropterous like conspecific females.

Recently, three species were transferred from *Cleruchus* to *Platystethynium* (Donev & Huber, 2002; Triapitsyn, 2014), so the genus currently contains six described, valid species worldwide including *P. triclavatum* (Sankararaman et al., 2019; Huber et al., 2020). Among them, three species belong to the subgenus *P.* (*Platypatasson*): *P.* (*Platypatasson*) *fransseni* (Ogloblin, 1946) from Indonesia, *P.* (*Platypatasson*) *terebrator* (Ogloblin, 1959) and *P.* (*Platypatasson*) *vagatus* (Ogloblin, 1959) from Argentina. Supporting molecular evidence, which is now lacking, would be needed to confirm the subgeneric concepts within *Platystethynium*, as suggested by Huber at al. (2020).

Pseudocleruchus is synonymized herein under *Platystethynium* and its nominate subgenus *P.* (*Platystethynium*) because all of their important generic-level morphological features, including the reduced female mandibles, are very similar, and the known host of *P.* (*Platystethynium*) *onomarchicidum* Ogloblin, 1946 is also a bush-cricket, *Onomarchus uninotatus* (Serville, 1838) (Tettigoniidae) (Ogloblin, 1946). Females of the European species of the former genus

Pseudocleruchus (1 described and 2 undescribed species, which for the time being, until genetic data becomes available, can be grouped in the informal *triclavatum* species group), have shorter heads, less flattened and shorter bodies, and wider fore wings than the two described Oriental species of the nominate subgenus of *Platystethynium*, *P.* (*Platystethynium*) *onomarchicidum*, known from Indonesia (Ogloblin, 1946), and *P.* (*Platystethynium*) *glabrum* Jin & Li, 2016, known from China (Jin & Li, 2016) including Taiwan (Triapitsyn, 2018), and also from India (Sankararaman et al., 2019). The latter two nominal species can be grouped in the informal *onomarchicidum* species group and are possibly synonymous (Triapitsyn, 2018), although both Jin & Li (2016) and Sankararaman et al. (2019) indicated some of their apparent differences, but their validity requires further study and confirmation. A key to females of the described species of *Platystethynium* (*Platystethynium*) follows.

Key to species groups and described species of the subgenus *Platystethynium* (*Platystethynium* Ogloblin, 1946), females

1 Head in dorsal view at least as long as wide; body strongly flattened (*onomarchicidum* species group) …2

– Head in dorsal view wider than long; body at most a little flattened (*triclavatum* species group) …*P.* (*Platystethynium*) *triclavatum* (Donev & Huber, 2002), **comb. n.**

2 (1) Ovipositor about 3.0 times length of metatibia … *P.* (*Platystethynium*) *onomarchicidum* Ogloblin, 1946

– Ovipositor about 1.8 times length of metatibia … *P.* (*Platystethynium*) *glabrum* Jin & Li, 2016

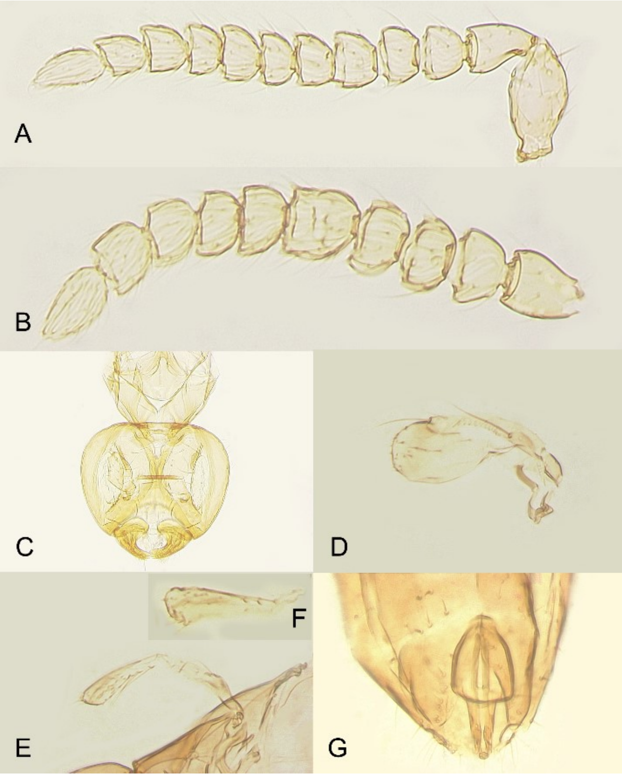

Figure 8. Male of *Platystethynium triclavatum*. (A) Antenna (flagellum 10-segmented); (B) antenna (flagellum nine-segmented); (C) head; (D) fore wing; (E and F) hind wings; (G) genitalia.

***Platystethynium* (*Platystethynium*) *triclavatum* (Donev & Huber, 2002), comb. n.**

(Figs 4B, 5, 6, 7, 8, 9A, 9B, 10)

Pseudocleruchus triclavatus Donev & Huber, 2002: 121–122. Type locality: 1470 m, mountain hostel Martsiganitsa (Martsiganitsa Chalet), Rhodope (Rodopi) Mountains, Plovdiv Province, Bulgaria. Holotype female, in private collection, currently with Mrs. Doneva, Dr. Atanas Donev's widow, Asenovgrad, Bulgaria (not examined); it will be donated to the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria (Peter S. Boyadzhiev, personal communication to S. V. Triapitsyn).

Pseudocleruchus triclavatus: Procop, 2011: 26–27 (compared with *Pseudocleruchus* sp. from Romania); Samková et al., 2020: 226 (distribution in Czech Republic).

Material examined. Italy, Veneto, Euganean Hills: 45°19'19.68"N 11°43'20.67"E, 140 m, parasitized egg of *Barbitistes vicetinus* collected in soil 20.x.2018, G. Ortis: parasitoids emerged 7.vi.2019 (5 females, CNC, UCRC [UCRC ENT 541215–541219]); host egg dissected 1.vii.2019 (5 males, UCRC [UCRC ENT 541220–541224]). 45°19'3.99"N 11°41'20.10"E, 448 m, parasitized egg of *B. vicetinus* collected in soil 20.x.2018, G. Ortis, parasitoids emerged 3–7.vi.2019 (25 females, CNC, UCRC [UCRC ENT 542125–541249]). Also numerous additional, unmounted, specimens of both sexes (DAFNAE).

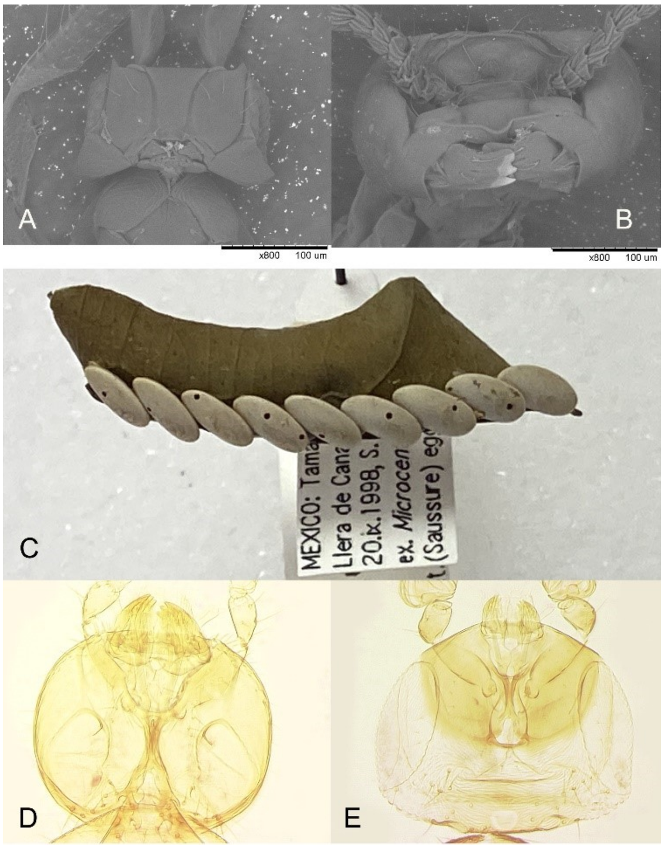

Figure 9. ***P. triclavatum*** **compared to** ***B. mexicana*****.** (A and B) Mandibles of *Platystethynium triclavatum* (scanning electron micrographs): (A) female; (B) male; (C) eggs of *Microcentrum rhombifolium* with exit holes of *Burksiella mexicana*; (D and E) mandibles of *Burksiella mexicana*: (D) male; (E) female.

Redescription. FEMALE. Body length of critical point dried, point-mounted specimens 0.53–0.66 mm; of slide-mounted specimens 0.8–0.88 mm. Body and appendages (Figs 4B, 5) mostly light brown to brown except pronotum and apex of gaster darker. Ocelli present, on a well-defined stemmaticum; subantennal grooves prominent (Fig. 10A). Mandibles strongly reduced, not crossing over (Fig. 9A). Antenna (Figs 10A, 10B) with short radicle fused to the rest of scape, scape smooth, 2.8–3.2 times as long as wide; pedicel longer than any funicular segment; F1 slightly shorter than F2, F1–F3 each about as long as wide (but sometimes F3 clearly longer than wide), F3 the longest funicular segment, F4–F6 each wider than long, mps only on F3 (2) and F5 (1); clava either about as long as or a little shorter than funicle, 2.3–2.5 times as long as wide, with 7 mps (2 on first, 2 on second, and 3 on third claval segments). Body (Fig. 10C) slightly compressed dorsoventrally. Mesosoma shorter than metasoma, smooth; pronotum longitudinally divided; midlobe of mesoscutum with a pair of adnotaular setae close to posterior margin; scutellum wider than long; frenum subquadrate. Macropterous; fore wing (Figs 6A, 6B) 5.5–5.8 times as long as wide (0.6–0.65 mm long), disc notably infuscate throughout, with about 10 rows of microtrichia in the broadest part; longest marginal seta 0.8–0.9 times greatest width of wing. Hind wing (Fig. 6B) 17–18 times as long as wide; longest marginal seta 2.5–3.0 times greatest width of wing; disc slightly infuscate and with about 3 rows of microtrichia. Legs (Fig. 10C) with femora not as prominently enlarged as in the male (Figs 7A, 7B). Ovipositor (Fig. 10C) about 0.3 mm long, occupying about 0.6 length of gaster, 1.9–2.0 times length of metatibia, slightly exserted beyond gastral apex (by at most 0.06 times own length).

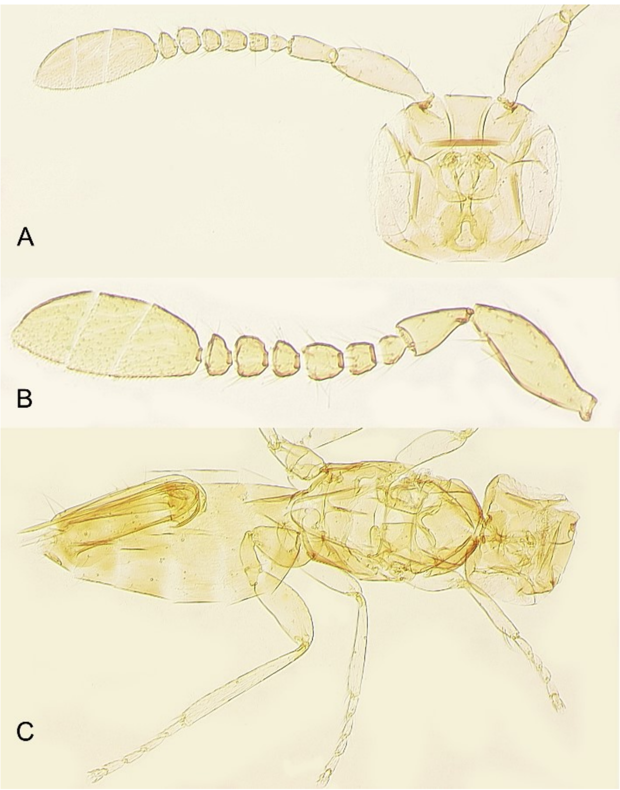

Figure 10. Female of *Platystethynium triclavatum*. (A) Head and antenna; (B) antenna; (C) body.

Description. MALE. Body length of air-dried, point-mounted, shriveled specimen (Fig. 7A) 0.66 mm; of slide-mounted specimens 0.99–1.08 mm. Body mostly brown to dark brown except lower face, eyes, mesoscutum, axillae and frenum light brown; antenna brown, legs light brown. Larger than female, megacephalous (Figs 6C, 7), head 1.5 times wider than mesosoma, with mandibles 3-toothed, strongly enlarged and crossing over (Figs 7B, 8C, 9B). Antenna (Fig. 7B) much shorter than body (length of antenna of one slide-mounted specimen 0.47 mm), with scape 2.1–2.2 times as long as wide; flagellum normally 10-segmented (Fig. 8A) but occasionally with F4 and F5 fused, so then antenna 9-segmented (Fig. 8B); flagellar segments without a distinct clava, either subquadrate or transverse except F9 slightly longer than wide and F10 notably so (the longest flagellomere); all flagellar segments with several mps. Strongly brachypterous, only short stubs with very little membrane remain of the wings, these do not extend beyond posterior margin of propodeum; fore wing (Fig. 8D) in one specimen 2.9 times as long as wide; hind wing length varies (Figs 8F, 8E) from about as long as that of fore wing or notably shorter, or, occasionally, slightly longer; genitalia (Fig. 8G) length 0.13–0.15 mm, with phallobase rather wide, aedeagal apodemes almost extending to its base, and with volsellar digiti straight.

Distribution. Bulgaria (Donev & Huber, 2002), Czech Republic (Samková et al., 2020), and Italy (new record).

Host. Orthoptera, Tettigoniidae: *Barbitistes vicetinus* Galvagni & Fontana, 1993 (new record).

Discussion

In this study we reported for the first time the discovery of a parasitoid wasp *Platystethynium triclavatum* belonging to the family Mymaridae, from eggs of *B. vicetinus.* The species, initially described as *Pseudocleruchus triclavatus* Donev & Huber, 2002, was previously known only from Bulgaria (Donev & Huber, 2002) and Czech Republic (Samková et al., 2020), while the genus *Pseudocleruchus* was also known from Romania (Pricop, 2011) as well as Finland and Sweden (Samková et al., 2020). The high number of individuals hatched from field-collected host eggs gave us the opportunity to evaluate the genus placement of the parasitoid and propose the synonymy of *Pseudocleruchus* with *Platystethynium*, to redescribe the female of *P. triclavatum*, and also to describe its previously unknown male. In fact, these are the first known males for the entire genus. That is not that surprising given their peculiar biology, as described herein. Only fully winged females of this species can be collected by the traditional methods such as sweeping with a net or by various traps. Even those are very rarely collected in Europe, and prior to this study have never been collected in Italy. The strongly brachypterous, megacephalous males with enlarged mandibles can only be found inside the host eggs in the ground, while females are fully winged, with normal sized heads, and with strongly reduced mandibles. Thus, females depend on the males to be able to hatch from host eggs; as common for the minute egg parasitoids, males are likely to hatch first and wait for their sisters to emerge; mating occurs inside the egg.

However, we still do not know when and how oviposition of the egg parasitoid takes place; one of the reasonable assumptions is that it occurs during oviposition of a female bush-cricket host, while the egg chorion is still relatively soft. An interesting oviposition behaviour was described in the gregarious *Ufens* spp. (Hymenoptera, Trichogrammatidae), egg parasitoids of *Homalodisca* spp. sharpshooters (Hemiptera, Cicadellidae, Cicadellinae, Proconiini) in North America (Al-Wahaibi et al., 2005). We suspect that a generally similar, albeit likely a somewhat different ovipositional strategy (given the fact that host eggs are laid in soil) might be in *P. triclavatum*, when females of the egg parasitoid, aggregating at the ovipositional sites of their hosts (probably at ground surface in this case) would respond rapidly and aggressively to the chemical or other, unknown, cues by the ovipositing female bush-crickets, landing on their ovipositors and then walking (squeezing) down to parasitize host eggs. That would also explain why parasitized eggs increase with the higher density of the host bush-crickets: their response time to the narrow opportunity for oviposition would be much shorter.

We assume that *P. triclavatum* is a gregarious, arrhenotokous species because gregarious behavior during oviposition could explain high number of the embryos found inside the host egg (Chiappini & Huber, 2008); however, egg production in most mymarid species probably does not exceed 100 (Sahad, 1984; Cronin & Strong, 1990; Huber et al., 2006). If this parasitoid is indeed a gregarious species, it is likely that one mated female (who, as many other fairyflies, is likely to be born with a full content of mature eggs and thus ready for ovipositing immediately, without additional feeding) would be able to parasitize just one host egg, using her entire supply of eggs. However, we do not exclude a possibility that more than one female could oviposit in a single host egg. Unmated females of the egg parasitoid would produce only males in the progeny. Otherwise, albeit unlikely because this species seems to be arrhenotokous, polyembryony could be suspected, but to our knowledge it has never been reported to occur in this family.

Apparently a partially similar trait, regarding sexual morphological dimorphism necessary for chewing exit holes through a very hard egg chorion of the katydid host, is evident in the recently described egg parasitoid of an unidentified species of Tettigoniidae, *Burksiella mexicana* Ávila-Rodríguez & Myartseva, 2011 (Hymenoptera, Trichogrammatidae) from Tamaulipas, Mexico (Ávila-Rodríguez et al., 2011). In its original description it is only mentioned that males of this gregarious species are larger, lighter colored than females, and megacephalous, but without noticing the striking peculiarity of the male mandibles. In fact, while the mandibles of the females are of normal size for the genus (Fig. 9E), those of the conspecific males are enlarged (Fig. 9D); these are used to chew out 1 or 2 round holes in each host egg (Fig. 9C) through which numerous smaller females emerge. Unlike in *Platystethynium triclavatum*, however, in *Burksiella mexicana* female mandibles can cross over, and males are fully winged. A similar sexual dimorphism occurs in *Burksiella benefica* (Dozier, 1932), originally described from Haiti; both (along with several undescribed species from the New World in UCRC) belong to the *benefica* species group of the genus *Burksiella* De Santis, 1957, as defined by Pinto (2006), members of which parasitize eggs of those Tettigoniidae that lay egg masses openly on leaves and possibly other substrates above the ground. The following non-type specimens of *Burksiella mexicana* were identified and examined by the second author: Mexico, Tamaulipas: Ciudad Victoria, 1999 or 2000, S. N. Myartseva, from eggs of an unidentified katydid (Tettigoniidae) on citrus leaf (3 females and 2 males, UCRC [UCRC_ENT 00536185, 00536187–00536190]); Llera de Canales, Rancho La Purísima, 20.ix.1998, S. N. Myartseva, from eggs of the broad-winged katydid, *Microcentrum rhombifolium* (Saussure, 1859), on leaf of a lemon (222 females and 22 males, UCRC [UCRC_ENT 00541280–00541370]). Thus, in the latter sample from Llera de Canales, males constituted about 9% of the total number of *B. mexicana* individuals which emerged from 8 host eggs (those with parasitoid emergence holes); about 30 wasps of both sexes emerged per host egg on average.

Data on this parasitoid were obtained through a study to assess the egg density of *B. vicetinus* after the oviposition period. Eggs of *B. vicetinus* were collected 10 years after the first outbreak reported in the Euganean Hills; during this period of time, this forest and agricultural pest has spread throughout the area of study. In this work we found that the diameter of the emergence holes of the eggs collected in the forest soil is comparable with diameter of the holes from which *P. triclavatum* emerged in the laboratory. Therefore, we assumed that all the eggs with these emergence holes across the surface of the chorion collected in the forest soil belong to this mymarid wasp. Our reconstruction of the distribution area of *P. triclavatum* is based on eggs with holes, showing that this species is spread in almost all the sites sampled and along all the elevation gradients. Since the index of discovery efficiency reached almost 50% among all the sub-replicates, fitness of this parasitoid could be related to the ability to find eggs in the soil. It is important to note that higher rates of discovery efficiency were found in sites with greater total amount of eggs.

During outbreaks of *B. vicetinus*, the presence of other Orthoptera is in overwhelming minority, particularly in the canopy. As we reported, the numbers of eggs belonging to other species were much lower compared to those for the eggs of *B. vicetinus.* From this point of view, the different abundance of this parasitoid among sites could be the result of the local level of infestation of the pest during the years. A higher number of the previously parasitized eggs can be explained by the oviposition density of the bush-cricket. Our results indicate that this wasp could take a reproductive advantage when large numbers of host eggs of *B. vicetinus* are present in the area. Probably, a major volume of eggs could be fundamental for the searching behavior, identification and later acceptance of the host (Moreau et al., 2009). Because outbreaks occurred only in the last decade, the low parasitism rate reported in this study probably is the effect of a time lag in the parasitoid-host system.

A portion of eggs collected in this study could date back to the first period of the outbreaks, when the abundance of this parasitoid was lower.

Although *P. triclavatum* hatched from numerous eggs of *B. vicetinus*, we cannot rule out that this wasp could parasitize other hosts. The parasitoid has been already recorded, even though without any host data, also in other European countries, where the geographical distribution area of other species of the genus *Barbitistes* Charpentier, 1825 overlaps (e.g. *B. serricauda* Fabricius, 1798, *B. ocskayi* Charpentier, 1850, *B. constrictus* Brunner von Wattenwyl, 1878) (Massa et al., 2012), suggesting a possible host-specificity with bush-crickets of this genus, at least. It will be important, in perspective, to explore host specificity of this parasitoid both on *B. vicetinus* eggs and those of other species and genera of bush-crickets.

The activity of this mymarid could be increased by the repeated outbreaks of *B. vicetinus*, whose eggs represent suitable hosts. The high number of individuals that can develop from a single host egg demonstrates that this host provides enough nutritional material for the development of more than one hundred larvae of the parasitoid. Despite the low parasitism rate found in this study, the total number of *P. triclavatum* individuals that can hatch from a single egg can better reflect the potential impact of this discovered parasitoid in the Euganean Hills.

Our results provide new insights into the biology of *P. triclavatum* that could be useful regarding the potential role in biological control against the pest *B. vicetinus*. Undoubtedly, our findings will be also useful for discovering the currently unknown males and determining hosts of some other described members of *Platystethynium*, particularly of the two known but still undescribed species from Europe.

Supplemental material

Table S1. Mean and standard error for each category of eggs found in each site. The mean and standard error for each site is calculated on the 6 sub-replicates. The mean indicated in the last row is calculated on the sum of eggs of the 6 sub-replicates for each site.

Site	Latitude-Longitude	Elevation (m)	Hatched eggs	Flattened eggs	Fertile eggs	Eggs with holes	Total	Discovery efficiency (%)	Hatched eggs of other species
1	45°18'12,42"N 11°43'46,56"E	99	147.7±39.8	1.8±0.5	5.7±1.6	1.8±0.6	157±41.3	83	0
2	45°20'17,54"N 11°39'16,71"E	66	178.8±48.6	45.8±13.7	20±7.6	3.2±1.6	247.8±66.6	83	4.6±2.7
3	45°19'19,68"N 11°43'20,67"E	152	65.7±13.2	1.5±0.6	8.7±2	2.8±1.4	78.7±14.5	67	0
4	45°19'22,6"N 11°45'19,7"E	54	81.3±26.7	2.3±1.3	9.8±4	5.2±1.8	98.7±32.6	100	8.8±4.4
5	45°19'2,99"N 11°40'37,03"E	261	173.5±38.2	16.7±3.6	21.2±4.4	4.3±1.6	215.7±40.7	100	6.9±2.1
6	45°21'0,84"N 11°39'35,85"E	294	119.2±32.2	20.5±15.8	11.8±5.5	11.5±2.8	163±52.3	100	11.2±1.6
7	45°18'36,36"N 11°46'12,67"E	167	126±37.7	1.2±0.7	0	0	127.2±37.4	0	4.2±1.6
8	45°19'7,58"N 11°41'33,19"E	481	77.2±27.7	5±2.6	10.8±5.6	3.2±1.4	96.2±36	83	0
9	45°21'10,91"N 11°41'58,6"E	129	27.2±5.7	12.2±7.9	0.2±0.2	1±0.5	40.5±8.5	50	13.7±5.9
10	45°18'10,09"N 11°42'1,36"E	337	308.3±47	26.3±4.1	20.3±5.5	9.2±2.8	364.2±54.3	100	0
11	45°20'53,03"N 11°42'21,6"E	144	212.3±50.2	13±6.7	6.8±3.1	9.2±3.4	241.3±58.8	83	1.2±0.5
12	45°19'4"N 11°41'20,1"E	456	97.2±33.4	8.3±2.8	9±4.7	15.3±5.7	129.8±46	100	2±2
13	45°21'37,05"N 11°39'56,92"E	369	214.3±83.8	26.7±12.2	9.8±4.8	5.5±1.7	256.3±99.5	83	0
14	45°16'46,72"N 11°44'8,68"E	111	189.8±48.5	77.8±41.2	13.3±6.5	1.5±1	282.5±94.7	50	16.3±2.5
15	45°15'41,34"N 11°40'51,24"E	48	19.7±4.9	1.3±0.9	0.2±0.2	0	21.2±4.8	0	5.8±1.9
16	45°22'30,57"N 11°40'38,67"E	71	19.7±4.4	10.7±3.9	0.2±0.2	1.3±1.3	31.8±8.9	17	7.5±1.9
17	45°19'17,1"N 11°41'33,3"E	467	379.8±140.3	55.7±13.7	18.7±8.3	7.2±1.7	461.3±161.3	100	20.3±2.9
18	45°19'1,36"N 11°46'13,37"E	168	88.5±29	24.3±7.9	3.3±1.8	0.3±0.2	116.5±36	67	0
19	45°15'2,5"N 11°41'25,9"E	39	4.5±1.2	0	1±0.7	0.2±0.2	5.7±1.8	16	9.3±1.7
20	45°16'55,49"N 11°42'35,17"E	268	34.3±7.1	19.3±7.2	2±1.1	1.7±0.6	57.3±13.3	83	6.2±1.8
21	45°21'26,55"N 11°39'23,97"E	462	33.8±12.1	1.5±0.6	0.7±0.7	1.7±0.7	37.7±13.4	50	0
22	45°20'15,17"N 11°39’40,49”E	159	173.5±57.4	71.7±26	30.8±19.3	0.3±0.2	276.3±78.8	67	32.2±12.8
23	45°20’24,7”N 11°39'30,78"E	72	32.8±14.1	2±0.7	7.2±2.6	0.2±0.2	42.2±14.4	17	17.2±5.3
24	45°17'50,17"N 11°37'30,17"E	193	16.7±3.8	2.8±1.2	4.5±2.6	0	24±6.7	0	2±0.9
25	45°19'0,06"N 11°42'7,02"E	311	54.2±25.3	21.7±14.5	6.7±1.9	0.5±0.2	83±41	50	6.7±3.3
26	45°18'44,96"N 11°43'59,68"E	37	5.8±3.3	0.2±0.2	3.3±3.3	0±0	9.3±6.5	0	4.8±1.7

27	45°16'38,85"N 11°44'27,98"E	12	24.5±3.3	0	1.7±0.9	0.5±0.2	26.7±2.6	50	9±1.7
28	45°20'59,99"N 11°41'52,79"E	113	14.2±6.3	5.8±3	0	0	20±8.6	0	2.7±1.1
29	45°16'48,24"N 11°43'0,93"E	331	10.5±2.4	2.5±1.5	4±2.8	1.2±0.8	18.2±5.4	50	17.3±7.8
30	45°19'59,64"N 11°40'8,47"E	208	4.3±1.7	0.5±0.5	0.5±0.3	0	5.3±1.8	0	0.8±0.5
31	45°19'32,78"N 11°39'51,68"E	131	3.7±0.7	0	0.7±0.7	0.2±0.2	4.5±0.8	16	0.7±0.3
32	45°21'48,97"N 11°39'24,3"E	220	14.7±4.5	3.2±1.9	2±1	0.5±0.3	20.3±7	33	2.5±0.8
33	45°19'5,18"N 11°42'8,11"E	371	31±11.9	0.8±0.3	8.5±5.3	0.3±0.2	40.7±16.1	33	5.5±2.9
34	45°18'30,26"N 11°39'43,3"E	233	40.5±17.9	3.2±1.9	3.3±3.3	0.3±0.2	47.3±21.2	33	11.5±5.4
35	45°18'56,25"N 11°39'48,75"E	155	12.5±3.1	0.3±0.2	4.3±3.9	0±0	17.2±6	0	2.5±0.4
36	45°17'48,73"N 11°43'47,36"E	82	6.2±3.1	2.2±0.7	0.7±0.7	0.2±0.2	9.2±3.6	20	6.8±3.1
37	45°22'14,54"N 11°41'15,33"E	41	1±0.6	0	0.5±0.3	0	1.5±0.8	33	0.3±0.2
38	45°21'52,24"N 11°43'49,69"E	160	97.5±13.4	29.5±7.8	14.8±4	2.5±0.8	144.3±20.6	100	11.3±2.6
39	45°19'0,54"N 11°43'16,32"E	182	63±44.5	5.8±3	10.7±7.1	0.5±0.2	80±53.4	50	3.7±1.4
40	45°16'21,7"N 11°41'34,77"E	61	0.3±0.3	0	0.5±0.3	0	0.8±0.5	0	0
	Mean per site		**474±85**	**78±19**	**42±7**	**14±3**	**608±106**		**10±3**

Chapter 5

Can extensively managed perennial crops serve as surrogate habitat for orthopterans typical of dry calcareous grasslands?

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Abstract

Both agricultural intensification and abandonment are considered among the main drivers of biodiversity loss, especially for species typical of semi-natural grasslands. In the Mediterranean regions, semi-natural grasslands are often abandoned or replaced by perennial crops such as vineyards and olive groves with potential negative consequences on the associated diversity. However, when these crops are managed under low-intensity practices, their inter-row grass cover can potentially provide suitable habitats for grassland specialists. Here, we analysed the effects of land use on orthopterans by sampling 67 sites belonging to four different habitats (vineyards, olive groves, semi-natural grasslands and abandoned semi-natural grasslands) along a landscape composition gradient. Overall, species richness was highest in abandoned grasslands, while vineyards presented the lowest diversity. Semi-natural grasslands and olive orchards presented intermediate and similar species richness. However, this effect was significant only for Ensifera species, while species richness of Caelifera did not differ between the four habitats. Increasing forest cover within a scale radius of 500 m also increased Ensifera species richness probably providing undisturbed refugia at the margins. Orthopteran abundance was higher in semi-natural grasslands than in abandoned grasslands and vineyards. Despite the differences in species richness, we observed large overlap in species composition between olive groves and semi-natural grasslands suggesting that perennial crops managed under traditional, non-intensive practices can provide suitable habitats for orthopterans typical of semi-natural grasslands. In particular, olive groves appeared to be a better surrogate habitat than vineyards for the conservation of both Ensifera and Caelifera.

Introduction

Both agricultural intensification and abandonment are considered among the main drivers of biodiversity loss, especially for species associated with semi-natural grasslands (Henle et al., 2008; Kleijn et al., 2009; Fumy et al., 2020). These habitats are now facing degradation and fragmentation as a result of changes in land use (Uchida & Ushimaru, 2014; Löffler et al., 2019). While the intensive exploitation of agricultural lands mostly affect grasslands located on flat areas, hilly and mountain regions are more frequently influenced by the abandonment and reforestation (MacDonald et al., 2000; Lambin et al., 2003). The negative effects of the abandonment of semi-natural habitats have been extensively studied in particular for plants and invertebrates. Besides habitat restoration (Winsa et al., 2015), we still lack alternative management solutions to support species associated to semi-natural grasslands.

In the Mediterranean regions, semi-natural grasslands are often interspersed with perennial crops such as olive groves, citrus orchards and vineyards. These agro-ecosystems are usually characterized by a perennial grass cover between the crop rows that could potentially provide suitable habitats for grassland specialists. The recent expansion of these crops across Mediterranean landscapes and their location in warm and dry sites, especially in economically-marginal and peripheral rural areas (Marchi et al., 2018), arouse a debate of whether these crops can represent suitable sites for species of conservation concern usually inhabiting natural and semi-natural habitats.

Recently, research efforts about biodiversity and its related ecosystem services in vineyards and olive groves have increased, focusing on the diversity of plants and animals and their response to local management and landscape features (Sauberer et al., 2004; Isaia, 2006; Bruggisser et al., 2010; Nascimbene et al., 2012; Gonçalves et al., 2018; Paiola et al., 2020). Although most of these studies considered arthropods to detect changes in biodiversity, only a few have examined the potential role of these two types of crops in maintaining the diversity of Orthoptera compared to semi-natural habitats (but see Adu-Acheampong et al., 2016; Sisterson et al., 2020). Orthopterans represent ideal organisms to study the effects of land-use changes on biodiversity (Bazelet & Samways, 2011). They are pivotal elements in trophic food webs as they are consumers in grassland ecosystems, and constitute important food for many taxa, such as birds, reptiles, and mammals (Belovsky & Slade, 1993; Ingrisch & Kohler, 1998). Most of the previous research has focused on comparing grasslands along management intensity gradients finding that low cutting frequency and fertilization usually enhance Orthoptera diversity and abundance (Kruess & Tscharntke, 2002; Marini et al. 2008, 2009; Chisté et al., 2016; Uchida et al., 2016; Bonari et al., 2017). However, there is a need to understand if similar, extensive practices applied in traditional vineyards or olive groves could also potentially support Orthoptera species typical of semi-natural grasslands. This question has important implications for conservation since semi-natural grasslands are often abandoned and replaced by these perennial crops. In this study, we compared orthopteran communities among four different habitats characterized by a low-intensity management in the Mediterranean region. The following research questions were addressed: (i) Can extensively managed agro-ecosystems (vineyards and olive groves) provide a surrogate habitat for orthopterans usually associated with extensive managed or recently abandoned semi-natural grasslands? (ii) How does orthopteran species composition shift across different habitats? The results will provide key ecological information to suggest conservation strategies to support both Ensifera and Caelifera sub-groups that differ in their ecological requirements.

Material and Methods

Study area

The study was located in the Berici Hills in the northern Mediterranean basin (north-east of Italy). The geological substrate was mainly composed of carbonate sedimentary rocks. The study area covers almost 200 km^2 with a maximum elevation of approximately 450 m above sea level. The mean annual temperature is 12.8°C (sea level) and the mean annual rainfall of c. 1000 mm/year. In the last decades, agricultural activities related to dairy farming progressively decreased, reducing the presence of semi-natural grasslands maintained by traditional and non-intensive farming practices (Marini et al., 2011; Cariolato et al., 2014). The landscapes have rapidly changed and there has been an intense process of spontaneous reforestation. The woody vegetation is dominated by thermophilous oak woods on the south-eastern side whereas *Quercus spp.*, *Carpinus spp.* and *Castanea sativa* woods prevail on the higher elevations and on the north-facing slopes. Grasslands are interspersed with forest patches, crop fields, and urban elements. All grasslands were dry belonging to the *Festuco-Brometalia* vegetation (Tasinazzo, 2001; Sommaggio, 2017) and were managed extensively. At the same time, olive and vineyard cultivation increased due to the higher profitability of these farming systems.

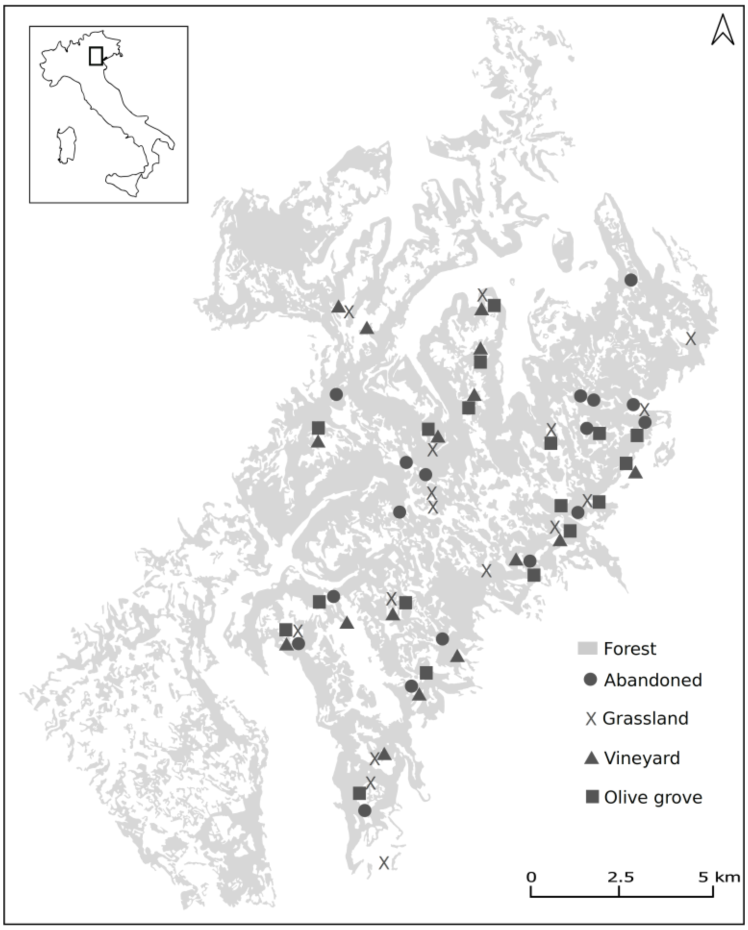

Fig. 1. Map of the study area and location of the 67 sites divided in the four habitats.

Sampling design

The study was carried out in 2020. To assess the role that different habitats for the conservation of Orthoptera, four types of habitats were considered: vineyards, olive groves, semi-natural grasslands, and abandoned semi-natural grasslands. A completely randomized sampling design was chosen because it was impossible to find all four habitats in a same site (e.g. block). We selected 17 sites for abandoned grasslands, 18 sites for olive groves and 16 sites for semi-natural grasslands and vineyards (Fig. 1). Each site comprised small or medium-sized fields (overall sites:1590 m^2±100SE). The minimum distance between sites of a same habitat was 500 m.

Vineyards and olive groves were all characterized by small patches conducted under low-intensive management (Table 1). A spontaneous perennial vegetation cover was present in all the crop fields, and it was usually cut in spring and during summer depending on the crop. Herbicides and fertilizers were not applied in the selected fields. Canopy application of fungicides and insecticides followed the guidelines of integrated pest management (IP: www.regione.veneto.it). In vineyards, to contain *Scaphoideus titanus* one treatment was applied, whereas in olive groves one treatment was applied against *Bactrocera oleae* in late summer. Dry grasslands were mown once or twice during the year and were not fertilized. The abandoned sites were all fallow grasslands established on former mown meadows that were abandoned for less than 5-10 years (i.e. early stages of succession).

Orthoptera surveys

Orthoptera (Ensifera and Caelifera) were sampled by visiting the sites thrice, during the periods of maximum activity and density of the species. The first period was in the mid of July (13th-19th), the second at the beginning of August (27th July-12th August) and the third at the end of August (24th-31st) to ensure detection of species with different phenology. The first sampling was conducted before any mowing event. Surveys took place between 10.00 and 18.00 on warm sunny days that did not follow a day of rainfall. To better detect effects caused by different management types among cultivated habitats and semi-natural habitats on orthopteran communities, we quantified the abundance of each species. Orthopteran species abundance was determined using a “timed counts” method (Gardiner et al., 2005). Within each site, orthopteran species and their relative abundance were recorded during 20 minutes avoiding edges (Bieringer & Zulka, 2003). Species were recorded by a combination of visual sighting, manual catching and sweep netting. Species identification was performed in the field, mainly by morphological characteristics using Iorio et al. (2019). Specimens that could not be identified in the field were collected and identified later in the lab. Although the different vegetation structure among habitats did not allow a standardized estimation of species abundance, we visually estimated the relative abundance of each species in situ using a five-grade ordinary scale (1: 1-2 individuals, 2: 3-4 individuals, 3: 5-10 individuals, 4: 11-30, 5: >30 individuals). For each site, overall abundance of each species was calculated by summing the score values (1-5) from the three periods of sampling. Total orthopteran abundance for each site was calculated by summing the scores of all species. Nomenclature of orthopterans followed Iorio et al. (2019).

Table 1. a) Management practices applied in the four habitats: numbers indicate average number of treatments performed during 2020 and b) differences (mean ± SE) of the five classes of habitats in a buffer area of 500 m radius.

	Abandoned grasslands (n=17)	**Grasslands (n=16)**	**Olive groves (n=18)**	**Vineyards (n=16)**
Size of patch (m²)	1313±189	1570±235	1801±110	1665±258
a) Management practice				
Fungicides	no	no	no	6-7
Mowing	no	1-2	1-2 (inter-row)	3-4 (inter-row)
Herbicide applications	no	no	no	no
Insecticide applications	no	no	1 (late summer)	2
Fertilizer applications	no	no	no	no
Tillage (n° treatments)	no	no	no	under-row (1-2)
b) Landscape composition				
Proportion of forest (%)	73±1.9	65±4.5	65±2.3	60±3.9
Proportion of grassland (%)	15±1.3	21±2.7	19±2.4	20±3
Proportion of olive grove (%)	2±1	2±1.1	4±1.4	4±1.1
Proportion of vineyard (%)	2±0.7	3±1.4	3±0.8	6±1.3
Proportion of urban element (%)	5±0.6	7±2.3	5±0.8	8±1.6

Landscape composition

Because landscape context could also influence orthopteran diversity (Batáry et al., 2007; Marini et al., 2008), we calculated the area of different habitats surrounding each site. Habitat patches in the landscape were measured within a circular buffer of 1000 m, 500 m, 250 m and 100 m radius around the geographical centre of each site using QGIS. The landscape variables were obtained from a vector-based land-use map from 2018 (ARPAV, Agenzia Regionale Prevenzione e Protezione Ambientale Veneto). We identified five habitats: (i) urban elements; (ii) grasslands; (iii) forests; (iv) vineyards; (v) olive groves. The proportion of the different habitats within the buffers were calculating using

QGIS (Table 1). In the statistical analyses, we focused on the cover of forests and grasslands as landscape variables as these represented the dominant habitats in our study area. Because forest cover was negatively correlated with grassland cover (Buffer 500 m: Spearman's rank correlation index=-0.43, n=65, P<0.01), the latter was excluded from further analyses. In preliminary analyses, we found that forest cover had always a better fit than the cover of grasslands.

Statistical analyses

In order to relate Orthoptera species richness and abundance to the explanatory variables, we used generalised linear models (GLM) with two fixed effects, i.e. one categorical factor (habitat type) and one continuous variable (proportion of forest in the landscape) and their interaction. When the interaction was not significant, we removed it and refitted the model with only the main effects. To test the effect of landscape variables at different spatial scales, four different GLMs were fitted (100, 250, 500, and 1000 m). Separate models were fitted with the same predictors for the total orthoptera species, and for Caelifera and for Ensifera, separately.

We tested for potential multicollinearity between predictors using the variation inflation factor (VIF). All VIFs were below 1.53 indicating no collinearity issues. Heteroscedasticity, and normal distribution of residuals were tested using diagnostic plots of model residuals. Potential spatial dependence of model residuals was tested using a Moran's I (Legendre & Legendre, 2012). No spatial autocorrelation was found in the model residuals.

The species composition of Orthoptera was also analysed using non-metric multidimensional scaling (NMDS; Oksanen et al., 2016), based on Orthoptera abundance (sum of the abundance scores across the three rounds). For Orthoptera, we used the Bray-Curtis index as distance metric, with a maximum number of 20 random starts in the search for stable solutions. A post hoc multilevel pairwise analysis was performed using the pairwise.adonis function in the pairwiseAdonis package (Martinez Arbizu, 2020). All analyses were performed using R-4.0.3 (R Core Team, 2020).

Results

In the 67 sites, we observed a total of 46 Orthoptera species, 27 of which were Ensifera and 19 were Caelifera species. The most frequent species were *Calliptamus italicus* (frequency=94%), *Euchorthippus declivus* (92%), *Pezotettix giornai* (82%), *Ruspolia nitidula* (79%), *Platycleis grisea* (62%) and *Phaneroptera nana* (59%). In the 17 abandoned meadows, 37 species were found (4 exclusively), 31 were found in grasslands (2 exclusively) and 33 in olive groves (1 exclusively), while 29 (1 exclusively) in vineyards.

We found a significant effect of habitat type on orthopteran species richness and abundance (Table 2, Fig. 2). Overall species richness was highest in abandoned grasslands, while vineyards presented the lowest diversity. However, a contrasting response was found between Ensifera and Caelifera species. While the number of Caelifera species did not differ between the four habitats, the number of Ensifera species was highest in the abandoned patches, significantly differing from vineyards. Orthoptera abundance also differed among the habitats and sub-orders. Caelifera species were more abundant compared to Ensifera species that were present at low and similar density among all the habitats. Caelifera abundance decreased in the four habitats in the following order: managed grasslands > olive groves > vineyards > abandoned grasslands. Number of species was also affected by the proportion of forest within a scale radius of 500 m (Fig. 3, Table 2). However, forest cover had a significant positive effect only on Ensifera species richness and abundance. This trend was also true for a radius of 1000 m (Table S1) while no effect was found at 250 and 100 m.

Table 2. Table model results for species richness and abundance with percentage of forest calculated in a buffer of 500 m radius.

	DF	SS	MS	F	P-value
Species richness					
a) All species (R^2=0.13)					
Habitat	3	62.43	20.809	3.1778	0.030
% of forest	1	33.6	33.604	5.1316	0.027
b) Caelifera (R^2=0.03)					
Habitat	3	1.147	0.38224	0.19	0.901
% of forest	1	3.012	3.01238	1.4977	0.226
c) Ensifera (R^2=0.16)					
Habitat	3	52.234	17.4115	4.3536	0.008
% of forest	1	16.494	16.4937	4.1241	0.047
Abundance					
a) All species (R^2=0.12)					
Habitat	3	1210.5	403.49	3.7596	0.015
% of forest	1	200.7	200.68	1.8699	0.176
b) Caelifera (R^2=0.09)					
Habitat	3	1046.8	348.94	3.7499	0.015
% of forest	1	7.1	7.09	0.0761	0.784
c) Ensifera (R^2=0.2)					
Habitat	3	416.7	138.901	5.1688	0.003
% of forest	1	133.89	133.892	4.9824	0.029

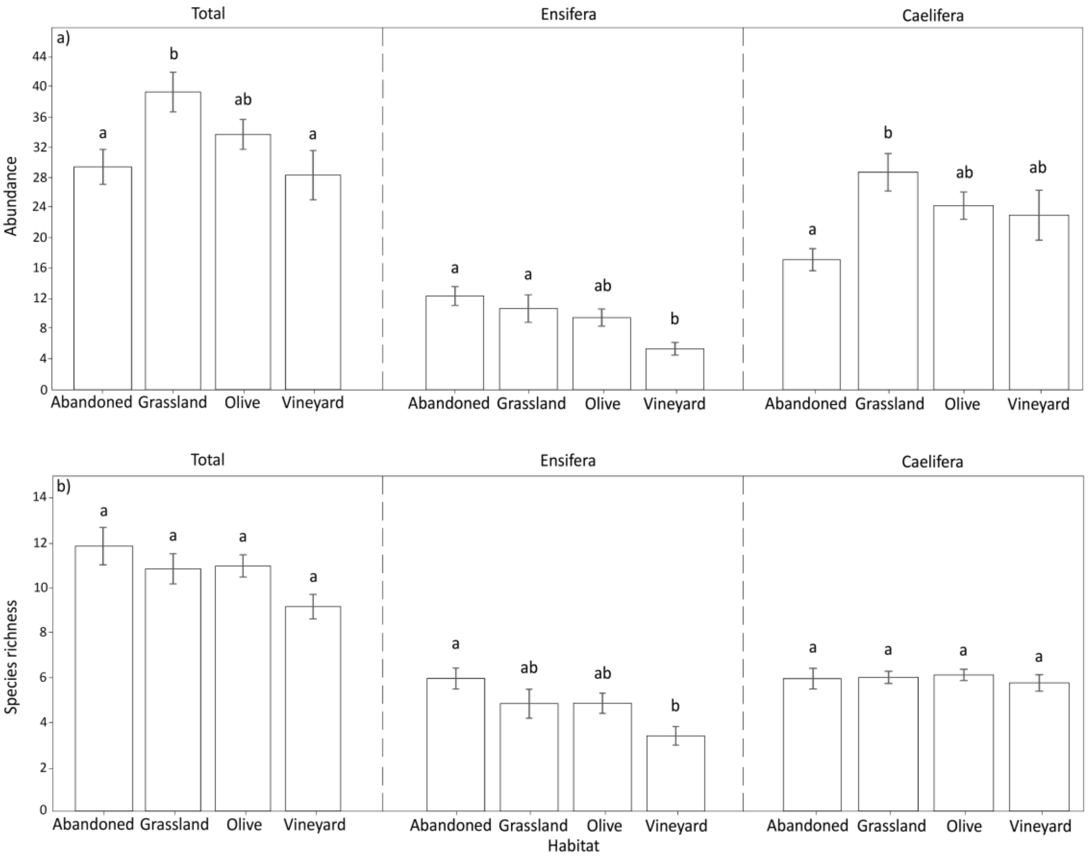

Fig. 2. Differences (mean ± SE) in (a) abundance and (b) species richness of all, Ensifera and Caelifera species between the four land-use types. Significant differences between the land-use types were calculated using GLM with % of forest as continuous variable. Different letters indicate significant differences according to Tukey's multiple comparison test.

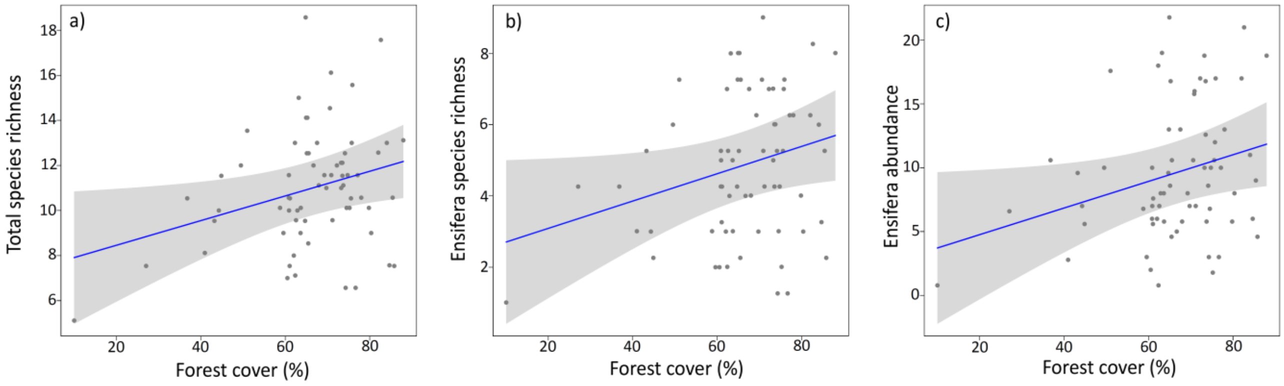

Fig. 3. Effect of forest cover within a scale radius of 500 m (%) on a) total species richness, b) Ensifera species richness, c) Ensifera abundance. Separate GLMs were fitted with the same predictor variables for the total orthoptera species and Ensifera. Shaded lines indicate intervals of confidence (95%), grey dots represent partial residuals. Only significant results are showed.

NMDS ordination (stress: 0.23, two dimensions) showed that the species composition of abandoned grasslands differed from the three others habitats, which did not show differences at the community level (Fig. 4, Table 3).

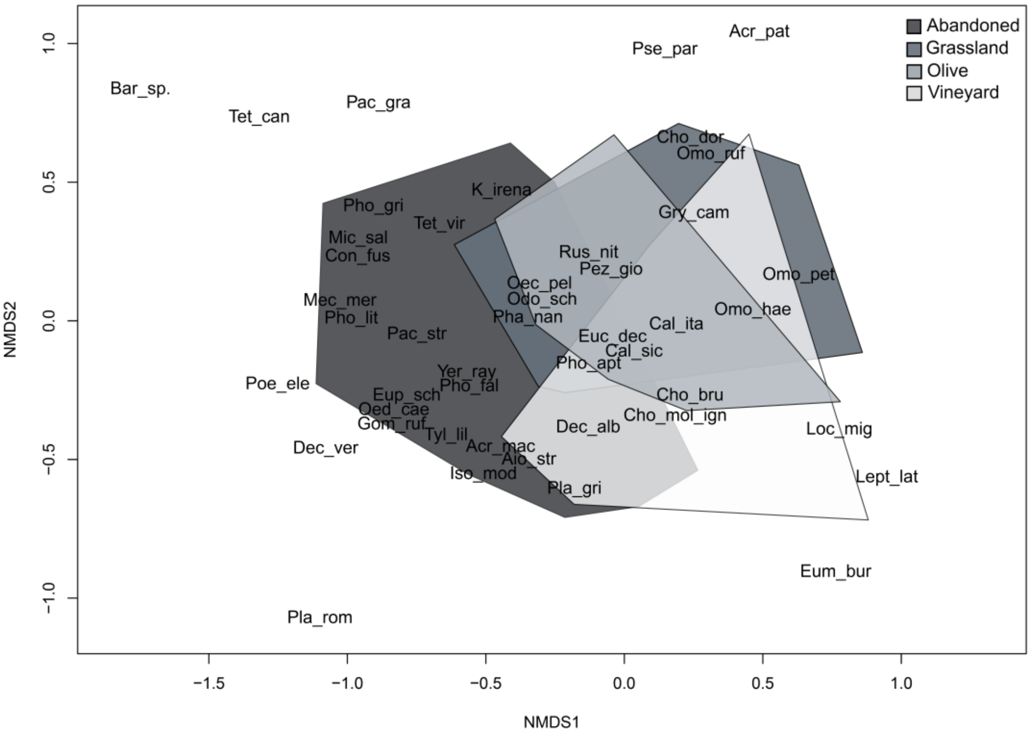

Fig. 4. NMDS plot based on the abundance of all Orthoptera species. The four polygons represent the different habitats. For abbreviations of species see Table S2.

Table 3. Results from pairwise comparison adonis of the NMDS. P-value <0.05 indicate that the species composition between the two habitats differed.

Pairwise comparison	**SS**	**F**	**P-value**
Abandoned vs grasslands	0.705	4.81	0.006
Abandoned vs olive groves	0.498	3.65	0.006
Abandoned vs vineyards	0.636	3.75	0.006
Grasslands vs olive groves	0.247	2.17	0.258
Grasslands vs vineyards	0.418	2.86	0.060
Olive groves vs vineyards	0.234	1.71	0.480

Discussion

Although usually land-use changes caused by agriculture have been demonstrated to impact biodiversity negatively (Tscharntke et al., 2005), this study indicated that small patches of vineyards and olive groves managed with low intensity practices supported the same number of species and rather similar species composition as semi-natural grasslands. Only abandoned grasslands were characterized by different orthopteran communities and exhibited the highest number of species (Table S2). These results are in contrast with other studies that have reported higher species richness for semi-natural habitats (e.g. grasslands, fynbos) than cultivated crops (arthropods: Magoba & Samways, 2012; parasitoids: Gaigher et al., 2015; grasshoppers: Adu-Acheampong et al., 2016). However, even if the same number of Orthoptera species was found in perennial crop fields compared to grasslands, their abundance was still higher on managed grasslands. Because abundance could be an important indicator of population viability (Löffler & Fartmann, 2017), this result confirms the importance of these semi-natural habitats for supporting higher number of individuals of different species.

Approximately 44% of the total species sampled in this study were found in all four habitat types. Although there was large overlap in species composition between habitats the total orthopteran richness and abundance changed among the habitats, with a contrasting response between Caelifera and Ensifera. While the number of Caelifera species did not differ between the four habitats, their abundance was higher in grasslands than in abandoned sites. This could be explained by the sward management adopted in grasslands that can strongly affect vegetation structure and microclimate with cascading effect on orthopteran communities (Joern, 2005; Kruess & Tscharntke, 2002; Chisté et al., 2016). High temperatures created after mowing events probably foster the above-ground stages of the life cycle of Caelifera species (Uchida & Ushimaru, 2014), affecting physiological processes (e.g. oviposition and feeding) and creating suitable habitat conditions able to maintain viable populations (Marini et al., 2009). In addition, low temperatures created by dense vegetation could not be suitable for embryonic development in the egg stage of the most thermophilus Caelifera species (Bieringer & Zulka, 2003). Most of the individuals observed in the managed habitats belonged to *C. italicus* known for its association with bare ground for oviposition and basking (Buzzetti et al., 2005). Its abundance in vineyards and olive groves can be explained by the occurrence of open and bare ground within rows which permit high sunlight penetration necessary for basking.

In contrast, although Ensifera species were all found at low densities, their abundance and richness decreased with management intensity. Vineyards, that represent the most disturbed habitat in the area, exhibited the lowest number of species and abundance compared to abandoned sites. This is in line with other studies that report how Ensifera can persist more than Caelifera in transitional habitats characterized by the presence of trees and shrubs (Bieringer & Zulka, 2003; Marini et al. 2009). These species are often related to cooler microclimates created by the shading of dense vegetation. Therefore, multiple cuts often applied in vineyards could create unsuitable microclimate for Ensifera species. As reported in several studies, cutting frequency higher than once a year can drastically reduce orthopteran diversity (Marini et al., 2008; Gardiner & Dover, 2008; Nuhlíčková et al., 2021) due to high direct mortality related to mowing operation. Nevertheless, the low cutting regimes in olive groves coupled with the shelter provided by the cover of trees could help to support more Ensifera species. Our study shows that olive groves can be compared to grasslands for both abundance and species richness suggesting that their extensive cultivation could represent a valid alternative for the conservation of orthoptera.

Although forest cover in the landscape could have a positive effect on the abundance and number of species at small spatial scales (e.g. 95 to 250 m buffer radius) (Marini et al., 2009; Weking et al., 2016), our study indicates the importance of forests at slightly larger scales. The positive effect is probably related to the establishment of ecotonal habitats (forest edges, hedgerows and bushes) suitable for the life cycle of many Ensifera species (Marini et al., 2009; Iorio et al., 2019). In contrast, the forest cover did not affect Caelifera species probably because most of these orthopterans are related to open grasslands and do not use forest for reproduction and development. Based on these considerations, overall abundance and richness found in sites surrounded by forest in our study area suggest that Orthoptera can persist in small and isolated patches when extensive managed conditions are adopted. Similar conclusions were reported in some studies that underline how orthoptera can persist also in small patches if habitat quality is high enough (Schouten et al., 2007; Poniatowski et al., 2018).

Low-intensity practices adopted in the crops, as partial ground management, few mechanical cuts and low pesticide applications could have contributed to increase within-field habitat quality (heterogeneity), known to be a key element to support biodiversity both in semi-natural habitats and crops (Benton et al., 2003; Fahrig, 2003; Löffler & Fartmann, 2017). The spontaneous vegetation cover present in vineyards and olive groves could have a positive effect on orthoptera, mirroring the influence of cover crops on abundance and richness of arthropods (Danne et al., 2010; Sáenz-Romo et al., 2019) compared to more intensive practices such as tillage (Bruggisser et al., 2010; Sanguankeo & León, 2011; Masoni et al., 2017). Furthermore, the presence of small vegetation patches not affected by mowing due to difficult physical conditions (e.g. stones, tree stems, subsidence, steep slopes) could serve as refuge sites, potentially preserving species with different ecological requirements.

Conclusions

Based on the results of our study, olive groves and secondarily vineyards could represent surrogate habitats for orthopterans, hosting species communities similar to those found in managed semi-natural grasslands. However, these findings should not be generalized to other crop habitats managed more intensively. As also suggested in other studies, maintaining traditional mowing practices is essential to support abundance and diversity of orthopterans. A spontaneous vegetation cover should be maintained in the inter-row and sectors of tall and dense vegetation should be left as a shelter for orthopterans during the cuts. The different species communities hosted in abandoned grasslands suggests that conservation strategies should also focus on preserving different types of habitat across the landscape considering the differential response of the two sub-orders. Finally, the effect of grassland abandonment and the positive effect of forest cover on Ensifera suggest that landscapes dominated by natural elements can help maintaining orthopteran communities with higher diversity.

Supplemental materials

Table S1. Table model results for a) species richness and b) abundance with percentage of forest calculated in a buffer of 1000 m radius.

	DF	SS	MS	F	P-value
a) Species richness					
a) All species (R^2=0.18)					
Habitat	3	61.13	20.378	3.2645	0.027
% of forest	1	53.87	53.874	8.6304	0.005
b) Caelifera (R^2=0.01)					
Habitat	3	1.15	0.38346	0.1874	0.905
% of forest	1	0.818	0.81752	0.3994	0.530
c) Ensifera (R^2=0.25)					
Habitat	3	50.922	16.974	4.6909	0.005
% of forest	1	410418	41.418	11.4463	0.001
b) Abundance					
a) All species (R^2=0.09)					
Habitat	3	1210.5	403.49	3.6502	0.017
Percentage of forest	1	1.2	1.18	0.0107	0.918
b) Caelifera (R^2=0.12)					
Habitat	3	1046.8	348.94	3.8599	0.013
Percentage of forest	1	171.5	171.74	1.8967	0.173
c) Ensifera (R^2=0.25)					
Habitat	3	416.7	138.901	5.4627	0.002
Percentage of forest	1	223.54	223.543	8.7916	0.004

Table S2. **Table showing presence of species in each habitat (gray fill).** Numbers indicate the proportion (%) of species records on the total sites sampled for each habitat. Species abbreviations are given in brackets.

Species	**Habitat**			
Caelifera	**Abandoned grasslands (17)**	**Grasslands (16)**	**Olive groves (18)**	**Vineyards (16)**
Acrotylus patruelis (Acr_pat)				6
Aiolopus strepens (Aio_str)	82	19	39	38
Calliptamus italicus (Cal_ita)	88	93	94	100
Calliptamus siciliae (Cal_sic)	47	44	39	31
Chorthippus brunneus (Cho_bru)	35	44	72	63
Chortippus dorsatus (Cho_dor)	29	75	61	31
Chorthippus mollis ignifer (Cho_m_ign)	6	13	6	
Omocestus petraeus (Omo_pet)			11	13
Euchorthippus declivus (Euc_dec)	94	100	100	75
Gomphocerippus rufus (Gom_ruf)	12			6
Kisella irena (Kis_ire)	12	13	17	13
Locusta migratoria cinerascens (Loc_mig)		6	6	
Micropodisma salamandra (Mic_sal)	35	19		
Odontopodisma schmidti (Odo_sch)	12		6	
Oedipoda caerulescens (Oed_cae)	18		6	6
Omocestus haemorrhoidalis (Omo_hae)			17	38
Omocestus petraeus (Omo_pet)			11	13
Omocestus rufipes (Omo_ruf)	41	69	39	63
Pezotettix giornai (Pez_gio)	71	75	94	88
Pseudochorthippus parallelus (Pse_par)	12	31	6	6
Ensifera				
Acrometopa macropoda (Acr_mac)	24			
Barbitistes sp. (Bar_sp)	6			
Conocephalus fuscus (Con_fus)	6		11	
Decticus albifrons (Dec_alb)	30	50	33	38
Decticus verrucivorus (Dec_verr)		6		
Eumodicogryllus burdigalensis (Eum_bur)			6	6
Eupholidoptera schmidti (Eup_sch)	35	13	17	13
Gryllus campestris (Gry_cam)		13	6	13
Isophya modestior (Iso_mod)		6		
Leptophyes laticauda (Lep_lat)			6	
Meconema meridionale (Mec_mer)	12	6	11	
Oecantus pellucens (Oec_pel)	29	25	50	19
Pachytrachis gracilis (Pac_gra)	18	6		6
Pachytrachis striolatus (Pac_str)	29	38	44	19
Phaneroptera nana (Pha_nan)	88	50	61	38
Pholidoptera aptera (Pho_apt)	6			6
Pholidoptera fallax (Pho_fal)	24	19	17	12
Pholidoptera griseoaptera (Pho_gri)	24	13	11	
Pholidoptera littoralis (Pho_lit)	12			
Platycleis grisea (Pla_gri)	70	56	67	56
Platycleis romana (Pla_rom)	6			
Poecilimon elegans (Poe_ele)	12	13		
Ruspolia nitidula (Rus_nit)	59	88	89	81
Tettigonia cantans (Tet_can)	12	6		
Tettigonia viridissima (Tet_vir)	47	68	39	25
Tylopsis lillifolia (Tyl_lil)	35	6	11	694
Yersinella raymondii (Yer_ray)	6		6	

Chapter 6

Genetic diversity in the fragmented populations of the endangered Eastern Alpine Bush-cricket *Anonconotus italoaustriacus* (Tettigoniidae) and implications for conservation

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Manuscript ready for submission

Abstract

Information regarding the genetic diversity of species populations is critical for initial conservation assessments, and subsequently for successful long-term population recovery and maintenance. With the recent decline and deterioration of semi-natural grasslands some species face decline or extinction. The eastern alpine bush-cricket, *Anonconotus italoaustriacus* Nadig, 1987, is a grassland species with a restricted range in north-eastern Italy and High Tauern in Austria. The species 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). The present study aimed to get genetic information of this bush-cricket to establish reasonable conservation strategies. The genetic variability and population structure of the four Italian populations as well as one population from Austria were studied through the analysis of four fragments of the mitochondrial DNA. A final data set of 48 concatenated sequences 2019 bp long was obtained. Results revealed the presence of high geographical structuring among populations, indicating absence of gene flow. This lack of genetic exchange may pose an important threat for the persistence of *A. italoaustriacus* and suggests that populations should be protected. Conservation efforts focused on monitoring, preserving and enlarging the existing habitat patches are proposed.

Introduction

The genetic variability of species populations is considered a basic precondition to keep the reproductive fitness, react to environmental changes and guarantee long-term survival of many species (Saccheri et al., 1998; Frankham et al., 2002). Even apparently healthy populations can be at long-term risk if they lack genetic diversity (Saccheri et al., 1998; Frankham et al., 2002; Keller & Waller, 2002; Reed & Frankham, 2003; Schmitt & Hewitt, 2004; Spielman et al., 2004a, b). Understanding genetic variability and population genetic structures has become an integral component of many management plans concerning endangered species (Moritz, 1994; Meffe & Carroll, 1997; Palsbøll et al., 2007). Thus, information regarding the genetic diversity of species populations is critical for initial conservation assessments, and subsequently for successful long-term population recovery and maintenance (e.g. Rasplus et al. 2001; Geist & Kühn 2005; Pabijan et al., 2005; Gradish et al., 2015).

Many semi-natural grassland species, with the recent decline and deterioration of their habitats, are now facing decline or extinction (Isselstein et al., 2005; Uchida & Ushimaru, 2014; Bubová et al., 2015). Small and fragmented populations are prone to bottlenecks and thus susceptible to genetic degradation caused by increased rates of inbreeding or loss of genetic diversity due to genetic drift, both reducing long-term fitness (Frankham et al. 2002). In addition, the habitat fragmentation is one of the main threats to the survival of organisms with limited dispersal abilities (Hanski & Gilpin 1997; Hanski 2005).

Anonconotus is a genus of endemic Ensifera spread in the Alps and the Apennines. In Italy there are several *Anonconotus* species mostly located in the western Alps, only one present in the eastern Alps (Galvagni & Fontana, 2004; 2005). 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 & Fontana, 2004; 2005; 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, characterized by the presence of *Juniperus* sp., *Rhododendron* sp., and *Erica* sp. (Fontana et al., 2002; Massa et al., 2012). It prefers steep sun-exposed slopes with a southeast to southwest exposition. 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 restricted range 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 and the number of mature individuals seem to be declining, mainly due to abandonment of extensive grazing and mowing (Zuna-Kratky et al., 2016).

Analysis focusing on the genetic structure of its populations are therefore advisable to assess its current situation and to guarantee their conservation. The present study aimed to shed light in the genetic contour of the endangered species to establish reasonable conservation strategies. For this purpose, four fragments of the mitochondrial DNA were used to investigate the genetic variability and population structure of the four Italian populations as well as one population from Austria.

Material and Methods

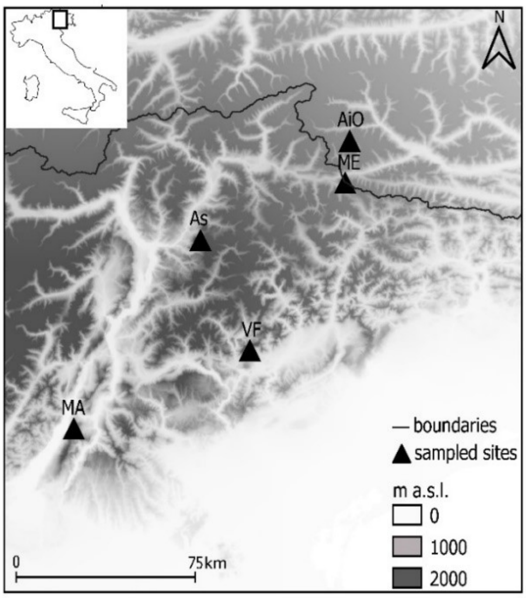
Sample collection

The study focus in all the small fragmented ranges known for the species. In particular, the study area was located in north-eastern Italy and included 4 Italian areas where the species have been historically reported from the literature (Galvagni and Fontana, 2005): Monte Elmo, Alpi di Siusi, Monte

Altissimo, and Vette Feltrine. In addition, a site within the Austrian distribution range of Hight Tauern (Heinkaralm) was also considered for sample collection. The mean geographic distance between all sample sites was 88 km (14±SE) ranging from 50.5 km to 167.1 km. In all the sites the sampling is carried out at altitudes between 2000 and 2300 m asl (Fig. 1).

Adults of *A. italoaustriacus* were collected, using a sweep net, throughout the summer of two successive years (2018 and 2019). Due to its endangered condition no more than 20 samples were collected for each site and only one leg was removed. To avoid sampling relatives, bush-cricket specimens were collected by sweeping randomly in different areas at each sampling site. After capturing, sampled legs were immediately kept in 95% ethanol and taken to the laboratory where they were morphologically identified and stored in individual vials at −20°C until DNA extraction.

Furthermore, 4 related species: *A. alpinus*, *A. ghilianii*, *A. occidentalis* and *Barbitistes vicetinus* (Tettigoniidae) were used as outgroups to encompass a representative sample for the following molecular analysis (Fig. 1).



Species	**Site**			
		Latitude	**Longitude**	**Elevation**
Anonconotus italoaustriacus	Monte Elmo (ME)	46° 42' 49.1"N	12° 23' 12.7"E	2200-2300
	Alpi di Siusi (As)	46° 30' 41.1"N	11° 35' 22.6"E	2200-2300
	Monte Altissimo (MA)	45° 48' 36.8"N	10° 53' 17.0"E	2000
	Vette Feltrine (VF)	46° 5' 34.9"N	11° 50' 38.7"E	2000
	Heinkaralm (AiO)	46° 52' 12.5"N	12° 25' 9.2"E	2100 - 2150
Anonconotus alpinus	La Thuille	45° 42' 29.1"N	6° 55' 5.5"E	1831
Anonconotus ghilianii	Lago Moncenisio	45° 13' 40.2"N	6° 57' 44.8"E	2006
Anonconotus occidentalis	Colle degli Agnelli	44° 41' 41.7"N	6° 58' 44.0"E	2440
Barbitistes vicetinus	Monselice	45°14'53,82"N	11°44'39,53"E	70

Figure 1. Sampled sites of *A. italoaustriacus* across all its distribution range. In the table are reported also the 4 species used as outgroups in the molecular analyses.

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 indication provided by the manufacturer. Before starting with the lysis step, each sample was manually homogenated 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 *cox1*, the *nad4*, the 5'of *12S rRNA* and the adjacent part of *Control Region* (*CR*) and a second part of *CR* with the primes listed in table (Tab. S1). The amplification thermal protocol for the fragment including the *cox1* and the *nad4* involved following conditions: denaturation at 95°C x 5 minutes; followed by 35 cycles at 95°C x 30 sec, 45-56°C x 30 sec and 72°C x 30sec, and a final extension at 72°c for 10 minutes. For the *12S rRNA-CR* amplification, the thermal profile followed the conditions described by Eweleit et al. [33] 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 sec, and extension step of 60˚C for 3 min, with a final extension of 72˚C for 7 min. Each reaction was carried out at the following final concentrations: 1x 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, while low-quality sequences were not included in the analysis.

Protein coding genes, were aligned using TranslatorX software with MAFFT (http://translatorx.co.uk/) (Abascal, Zardoya, & Telford, 2010. Not coding protein genes, were aligned by 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). Then, the subsets of alignments were combined in a unique concatenated alignment (here after 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). Exact tests of population differentiation with 100,000 steps in Markov chain, with 10,000 dememorization steps was conducted to test the presence of population differentiation. To assess significance among sampled populations, an analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed using the same software.

A haplotype parsimony network with a probability cut-off of 95%, was reconstructed using the TCS 1.21 software (Clement e al., 2000) and PopART 1.7 (Leigh & Bryant, 2015) and used for depicting the geographical relationships among haplotypes.

Demographic history

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

Phylogenetic analysis

Maximum Likelihood phylogenetic analysis (ML), were performed with Iqtree v1.6.12 software (Nguyen, Schmidt, von Haeseler, & Minh, 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, von Haeseler, & Minh, 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 analyses 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, Chernomor, von Haeseler, Minh, & Vinh, 2018; Minh, Nguyen, & von Haeseler, 2013). The tree was rooted on the outgroup species *Barbitistes vicetinus*. Furthermore, combining the maximum parsimony (MP) approach implemented into Mesquite 3.70 software (Maddison & Maddison, 2019), to the haplotype phylogenetic relationships previously calculated, we inferred the ancestral range distribution of the *A. italoaustriacu*s among 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 through time (Allegrucci et al., 2009; Drummond et al., 2006; S. Y. Ho & Larson, 2006; Ho, et al., 2008;Ho, 2007; Papadopoulou et al., 2010). The strategy involved a Bayesian analysis Drummond, Nicholls, Rodrigo, and Solomon (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 *Anonconotus*, we decided to infer the DTE using the empirical mutation ranging from 2% and 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-Sanudo et al., 2021; Papadopoulou et al., 2010; Rogers, 1995). To assess the convergence across independent runs, we conduct 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, Suchard, Xie, & Rambaut, 2012). The log files were investigated with the Tracer v1.7.1 package to detect problems within the analyses (Nylander, Wilgenbusch, Warren, & Swofford, 2008; Rambaut, Drummond, Xie, Baele, & Suchard, 2018), and the trees were summarized with TreeAnnotator through Maximum clade credibility option (Drummond & Rambaut, 2007). Then annotated trees were combined using LogCombiner software, and the output file was visualized with FigTree v1.4.4 (Bouckaert et al., 2014; Drummond & Rambaut, 2007). Posterior probabilities (pp) were considered significant at 95%.

Results

Sample collection

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

Data analysis

The obtained sequences were aligned gene-by-gene, after quality assessment and trimming, producing 5 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 COI 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 already been deposited.

The mitochondrial fragments *12S rRNA* and the first part of *CR* of the output species *Anonconotus occidentalis* were not successfully amplified. Then, the respectively portions in the concatenated alignment were coded as missing data.

Genetic variability and population structure

The diversity indexes for the concatenated dataset ranged from 0.63 (AS) to 0.93 (AiO) for the haplotype diversity (H) and between 0.14 (AS and VF) and 0.21 (AiO) for the nucleotide diversity (π). The distribution of haplotypes among all populations analysed and other summary statistics are shown in Table 1.

The exact test of population differentiation was significant ($P < 0.05$) for all the pairwise comparisons, indicating a non-random distribution of haplotypes among populations. AMOVA revealed a significant geographic structure among populations ($P < 0.01$).

The haplotype network of the combined dataset revealed the presence of 24 haplotypes, all of them exclusive of a single population (Figure2-network). No haplotype was shared by samples from two (or more) populations. Samples from Monte Elmo (ME) were represented by 5 haplotypes connected among them. Similarly, samples from Alpi di Siusi (AS) were included in 5 grouped haplotypes. Sequences of the Austrian samples (AiO) were included in 6 haplotypes scattered throughout the network and connected with haplotypes represented by samples from ME or AS (Tab. 1; Fig. 2). The Vette Feltrine (VF) samples were represented by a high numer of haplotypes (8), connected among them and separated from the other populations (Me, AS and AiO) by 19 mutational steps with missing intermediate haplotypes (Fig. 2).

Table 1. Descriptive statistics of each population and summary of the past demographic events analysis.

Site	ID	Haplotypes	N. hapl.	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	H7(3), H8(9), H9(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	H1(1), H2(1), H3(1), H4(2),H5(2), H6(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)

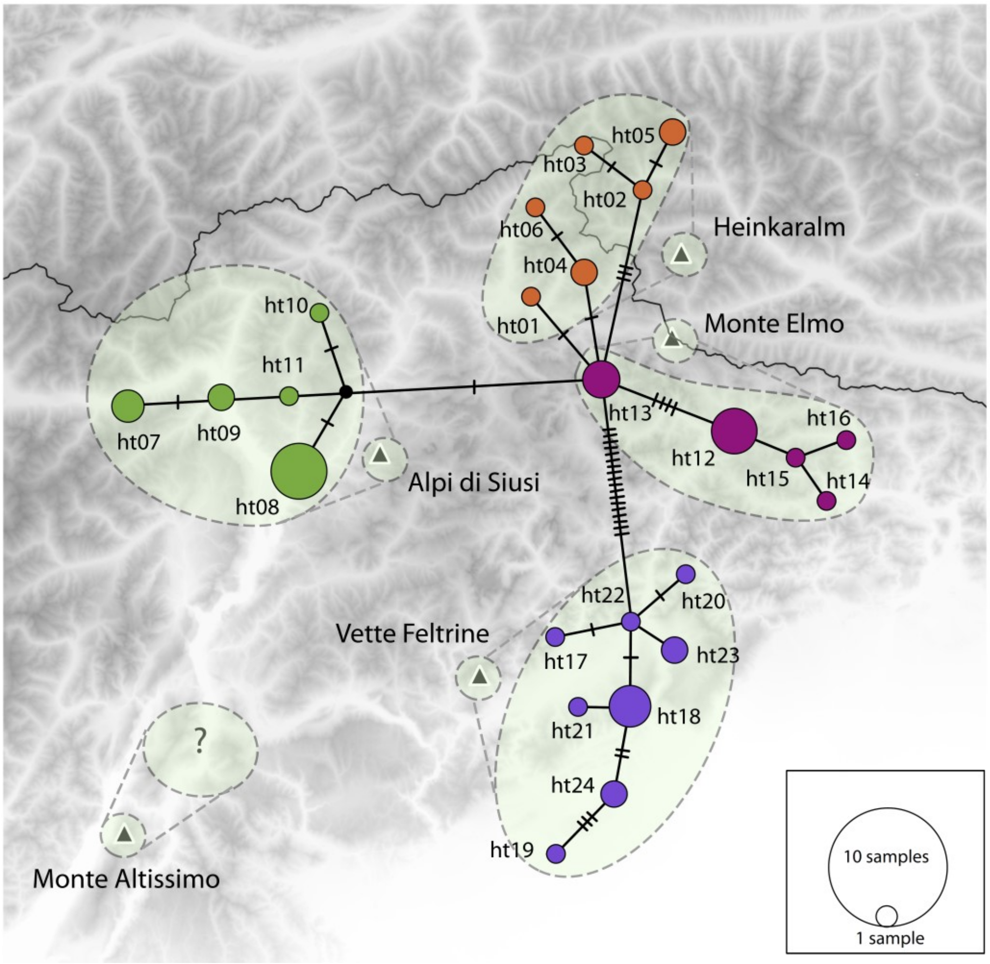


Figure 2. Network and geographic distribution of *A. italoaustriacus* haplotypes.

Demographic history

Neutrality test (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 (Tab. 1) 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 (Fig. S1). In contrast, mismatch distribution of Vete Feltrine (VF) was unimodal All populations showed SSD and raggedness index values that did not reject a sudden expansion model. In particular population from VF showed not significant low r values (<0.05) suggesting a population expansion event (Tab. 1). In addition, although r index was not significant, populations from ME and AS high showed high r values (>0.05) typical of stationary populations (Tab. 1).

Phylogenetic and Divergence time estimation analyses

The most likelihood trees (-ln= 6075.7194) obtained from the ML analyses (Fig. 3) were calculated using the following models: TIM2+F+G4 for *cox1*, TPM2u+F+I for *nad4*, HKY+F+R2 for the not coding protein genes (*12S rRNA + CR*), according to the BIC score. Both the BI and ML analyses retrieved fully congruent trees, besides some nodes, are not supported. The analysis yielded a phylogenetic tree grouping *A. italoaustriacus* in a monophyletic group, with specimens from different populations clustered in statistically supported monophyletic groups, except for one ME specimen (Fig. 3).

Figure 3. 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 divergente time estimated in milions of years ago (mya). Pies on these nodes show the ancestral distribution through MP analisis. ●=VF; ●=ME; ●=AiO; ●=AS.

The DTE was calculated for all the nodes, but only selected fully supported nodes by both pp, UFB, and SH-alRT will be discussed (Fig. 3). The basal node one set at the split between *A. italoaustiacus* and the congeneric species (a) shows a separation occurred 1.6 Mya (95% HPD interval 0.9 to 2.3). Within *A. italoaustriacus* species, the VF clade (b) is equally supported in all tests with the separation between other clades calculated 1.5 Mya (95% HPD interval 0.8 to 2.2). Similarly, the split and the paraphyly of the ME clade (c) prepense 1.0 Mya (95% HPD interval 0.5 to 1.5). The node between AS and AiO clades (d) was supported only by ML analysis with divergence calculated at 0.9 Mya (95% HPD interval 0.4 to 1.4). Other strongly supported nodes within clades are not further discussed considering, there are no physical barriers to divided specimens inside their populations. Finally, the ancestral range distribution analysis suggests that anciently the species was homogenously distributed from AiO to VF and, subsequently, they undergo to significant contractions probably as the effect of the climate instability occurred ca 1.5 Mya (Fig. 3 node d) (Hansen et al., 2013; Lisiecki & Raymo, 2005).

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.

No specimens of *A. italoaustriacus* could be found in the study areas of Monte Altissimo. The last available report dates back to 1909 (Krauss, 1909) which was observed in the mountaintop. Several efforts were made later by Galvagni and other colleagues (Galvagni & Fontana, 2004; 2005) to retrieve the bush-cricket in this area but as well as in our case, repeated attempts were unsuccessful, suggesting the extinction of the species in these areas. Galvagni & 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 lead population to extinction. In addition, the species was retrieved by Krauss in the mountain top of Monte Altissimo (Krauss, 1909). The increase in temperatures in the last decades may have further contributed to the decline of populations, as the species had no possibility of moving to higher elevations and find more suitable (colder) sites.

Samples found in the other four study areas showed high values of haplotype diversity within each population and all the haplotypes retrieved were exclusive to a single population. The population differentiation test and AMOVA indicated a strong geographical separation among populations. These results suggest absence of gene flow among populations. 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 family Tettigoniidae, move only relatively short distances during their whole life (e.g. *Pholidoptera griseoaptera*, *Barbitistes vicetinus*) (Diekötter et al., 2005; Cavaletto et al., 2019). This limited dispersal ability together with the great distances separating its distribution ranges with geographical barriers (e.g. valleys) and the tendency to live in fragmented patches have probably favoured the lack of gene flow among Italian populations. This lack of genetic exchange may pose an important threat for the persistence of *A. italoaustriacus* and suggests that populations could be protected by securing from anthropogenic disturbances or increasing the size of existing habitat patches.

The haplotype network was characterized by the presence of several rare haplotypes with almost no high frequency haplotype (Fig. 2). This pattern is consistent with what is expected for populations in equilibrium as highlighted by both the neutrality test (Tajima D and Fu's) 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. Due to the endangered status of the species the study was purposely limited to a small sampling size. Further analyses increasing number of samples could help draw better conclusions about past demographic events.

Populations from VF were genetically very different from the other alpine populations, as showed by both the network and the phylogenetic tree. This high level of sequence divergence suggests a long-term isolation of VF populations respect to the remaining populations. It was confirmed by the divergence time estimation (DTE), which indicated that separation of VF population from other clades could occur approximately 1.5 Mya (0.810- 2.200 Mya), during the early Pleistocene. After surviving low temperatures of the late Pliocene and once climatic conditions were favourable, VF populations might have experienced an expansion during the interglacial Pleistocene (1.2-1.7 Mya) that led to a genetic differentiation.

In addition, on the basis of our results, 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. those living in refugial areas) leading to genetic differentiation, occurred first in ME and later in AiO and AS populations. Then, extant populations must have persisted over successive glacial cycles. As suggested for other species, the genetic divergence of isolated refugial populations was further maintained and favoured by its discontinuous distributions and the limited dispersal capability, (Conroy & Cook 2000; Whorley et al. 2004).

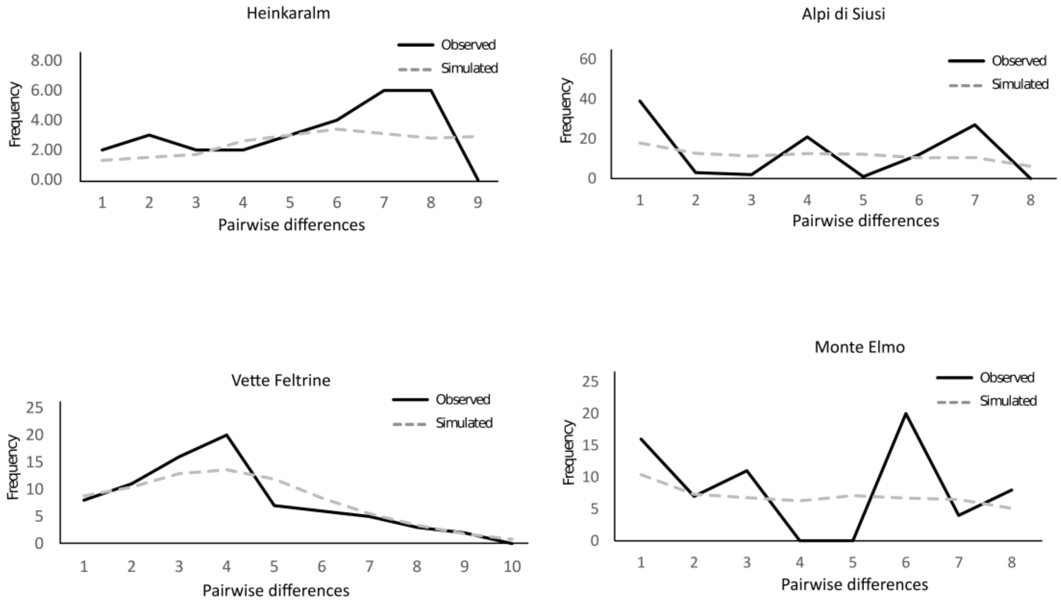
Overall, our study highlights that populations of the endangered *A. italoaustriacus* are genetically isolated across the distribution range. As with other poor dispersers, the absence of gene flow and the low geographical dispersion could make the species more prone to extinction and, therefore conservation strategies are needed. Since establishing species in new habitats could be a limited solution because the bush-cricket may not colonise new patches readily, a suitable management option might be to preserve and enlarge existing habitat fragments. In addition, results show that despite the strong fragmentation of species inhabiting the small habitat patches, bush-cricket populations have maintained genetic diversity over time, even in the absence of gene flow. Thus, the protection of their habitats should be a paramount measure for conserving the genetic diversity of *A. italoaustriacus* as well for other species. Furthermore, these differentiated populations appear to have evolved independently and they should be recognised as demographically independent. Therefore, conservation efforts should consider every population as a management unit and try to maintain as many populations as possible, also in relation to the host plants present in each site. Future studies aimed at getting this information will favour, with appropriate actions, the presence of these botanical species in the bush-cricket range.

Regarding Monte Altissimo, where *A. italoaustriacus* was not retrieved, the convenience and possibility to reintroduce the bush-cricket should be assessed by evaluating i) whether the habitat is still suitable for the species and ii) which are the appropriate populations and haplotypes to use for the area reintroduction.

Supplemental materials

Supplemental table S1. Information on the five fragments chosen for amplification.

Mitochondrial region	**Primers**	**Sequence**	**Source**
cox1	LCO-1490	5´-GGTCAACAAATCATAAAGATATTGG-3´	Folmer et al., 1994
	HCO-2198	5 -TAAACTTCAGGGTGACCAAAAAATCA-3´	Folmer et al., 1994
nad4	NAD4_intF2	5’-ACCCCAGAATAAATAGCTCCATG-3’	This study
	NAD4_Rev2	5’-TGGGGATATCAACCAGAGCG-3’	This study
12S rRNA	SR-J14610	5’-ATAATAGGGTATCTAATCCTAGT-3’	Simon et al., 2006
	T1-N18	5’-CTCTATCAARRTAAYCCTTT-3’	Simon et al., 2006
Control Region	SR-J14610	5’-ATAATAGGGTATCTAATCCTAGT-3’	Simon et al., 2006
	T1-N18	5’-CTCTATCAARRTAAYCCTTT-3’	Simon et al., 2006



Supplemental Figure S1. Mismatch distribution under the population expansion model of *Anonconotus italoaustriacus* populations in the four sites sampled.

Chapter 7

General conclusions

The main aim of this thesis was to improve biological and ecological knowledge of some Orthoptera species, exploring the influence of genetic, biotic and abiotic factors able to affect their life-history, behaviour and population dynamics. In particular, the recent and unexpected outbreaks of the bush-cricket *B. vicetinus* gave the possibility to highlight the role of different factors during demographic fluctuations of this pest. Firstly, because this species was unknown until 1993 (Galvagni & Fontana, 1993) and outbreaks occurred in two separated geographical areas (Euganean and Berici Hills), we investigated if genetic factors could have a role in the origin of the outbreaks. From our results we found high values of haplotype diversity, a typical characteristic of ancestral populations, supporting the hypothesis that *B. vicetinus* is a native and endemic species. In addition, results suggest that outbreaks are not a consequence of a single or few haplotypes but environmental factors rather than genetic factors could have affected outbreaking populations.

In this respect, we clarified the role of one environmental factor in the outbreak dynamics in the Chapter 3, testing the effect of temperature on egg development and survival, as these two aspects could affect species abundance. Although we didn't detect an effect of winter temperatures on egg survival (hatchings), we found that summer temperatures experienced by eggs after oviposition can affect their development, potentially shifting life-cycle from pluriannual to annual, reducing the mean egg mortality suffered by diapausing eggs and increasing the probability to have outbreaking populations.

In Chapter 4 we explored the role of the egg parasitoid *Platystethynium triclavatum* in the outbreak dynamics of *B. vicetinus.* This parasitoid is present in the whole area of the Euganean Hills and it increases in density according with the host density. Our results suggest that *P. triclavatum* co-evolved with its host and it could be useful in biological control programs applied against *B. vicetinus*. In this respect, the new insights on the bio-ecology of this parasitoid represent the first step of a biological control protocol that should be preferred to classical chemical control, often not feasible in natural and forest areas where outbreaks occur.

Some possible strategies to conserve rare orthopteran species were also explored by two different approaches. Chapter 5 shows, by an ecological approach, how crops as olive groves and vineyards managed with low intensity practices could support orthopterans as grasslands. The managed habitats not only have similar values of species richness and abundance, but also similar species composition compared to grasslands. These results highlight how traditional mowing practices and natural habitats are essential practices to support abundance and diversity of orthopterans.

With a genetic approach, Chapter 6 provides molecular results on the known populations of the endangered bush-cricket *A. italoaustriacus*. Results revealed that populations are genetically different and geographically separated. The fragmented distribution ranges, limited gene flow and low dispersal ability makes necessary to apply conservation strategies to prevent extinction of the endangered species. Suitable conservation options might focus on securing existing habitat patches from anthropogenic disturbances and increasing their size.

In conclusion, this thesis successfully employs some different and novel approaches to answer questions about the origin of outbreaks, how they could be related to environmental conditions and which strategies could be adopted to control harmful species and conservation of rare Orthoptera. When testing the effect of environmental drivers such as temperature, field experiments should be preferred to laboratory conditions as constant temperatures could often not produce a reliable result. The methodology applied in this study would be a useful model to study diapause patterns of other Tettigoniids in relation to environmental factors such as temperature or moisture. Because different Orthoptera species could have a facultative or obligatory diapause and different developmental thresholds, understanding these characteristics would be useful to improve knowledge about biology and ecology of different species and to predict their outbreak risk. Furthermore, the interaction between multiple factors should be taken in consideration in future studies.

Another important priority for future research should consider the potential role of *P. triclavatum* as agent of biological control. Although our results demonstrate that this parasitoid is present in all the studied area, further genetic analysis will shed light on the origin of this mymarid wasp and on its host specificity. Indeed, further experiments are necessary to clarify if this is a specialized or generalist parasitoid. To date, we still don’t know if it can parasitize different Orthoptera species or even other insect orders. Besides, periodic samplings during different outbreak years could help to detect variations in density of this parasitoid. Both this information, together with other biological data (e.g. hatching period, fecundity, life span) would be useful to improve the basic knowledge of these poorly known genus of parasitoid wasps and maximize the effectiveness of biological control strategies.

Finally, control strategies should have to consider the intrinsic vulnerability of the endangered species, usually having low dispersal ability and small and fragmented distribution ranges. In this view, analyses aimed to understand genetic diversity could be useful regarding the application of conservation strategies, as species reintroduction. Indeed, the survival chances of a new population will depend on the intraspecific genetic diversity and the adaptation of specific haplotypes to certain habitats.

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