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Ph.D. COURSE IN: BIOSCIENCES

CURRICULUM: EVOLUTION, ECOLOGY AND CONSERVATION

SERIES XXXII

EXPLORING THE DIVERSITY OF A NEGLECTED GROUP OF SOIL INVERTEBRATES (CHILOPODA) ACROSS THE SOUTH-EASTERN PREALPS

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CONTENTS

ABSTRACT

Despite their documented importance in maintaining ecosystem functioning, many groups of soil-dwelling invertebrates have been inadequately studied with regards to many facets of their biology. In this thesis, I focussed my attention on two poorly understood aspects of the diversity of soil-dwelling invertebrates: (i) local values of species richness and (ii) species boundaries. As a study system, I used one of the most neglected groups of soil invertebrates, i.e. the centipedes (Myriapoda: Chilopoda), and I carried out my research in the area of the Southeastern Prealps, which are known to harbour a large amount of biodiversity.

The present work is in the form of a paper collection and contains a general introduction, two chapters and a conclusive paragraph. An original research article, dealing with the aforementioned topics, is presented at the end of each chapter.

In chapter I, I addressed the dearth of knowledge on the local species richness of centipede communities. I applied statistical models to estimate the actual species richness of 10 centipede communities in the Southeastern Prealps and to compare richness values between these communities. I demonstrated that up to 27-28 species of centipedes can coexist in syntopy in temperate forests of the Southeastern Prealps, and that richness values can vary significantly among communities.

In chapter II, I addressed the issue of species delimitation in poorly-vagile endogeic invertebrates, for which traditional morphology-based taxonomy has proven ineffective in identifying species boundaries. In particular, I applied a rigorous integrative approach, in order to test for the existence of species boundaries in a set of populations traditionally referred to a single species of endogeic centipede, i.e., *Clinopodes carinthiacus* (Chilopoda: Geophilomorpha). Taking advantage of the integration between different lines of evidence (sequences from three DNA loci and morphological characters) and different methods for species discovery (ABGD, GMYC, PTP and Expectation Maximisation cluster analysis with v-fold cross-validation), I found evidence for the existence of at least two candidate species within the investigated population system.

INTRODUCTION

Soil organisms have been shown to play a key-role in the functioning of terrestrial ecosystems (Bardgett & van der Putten, 2014), and biodiversity loss in soil biota due to environmental changes and degradation have been repeatedly recognized as potential threats for ecosystem sustainability (Wagg *et al.*, 2014; Wall *et al.*, 2010). Despite this, soil-dwelling fauna has received far less attention than above-ground animal communities. This disparity is the result of the low awareness of the general scientific community and public on the magnitude of diversity and the importance of the soil biota (Decaëns, 2010); besides, this may be explained by the difficulties in studying soil-dwelling taxa due to their low accessibility (Wurst *et al.*, 2012), the dearth of specialists (e.g., Jeffrey *et al.*, 2010), and the inadequacy of standard sampling methods and designs (André *et al.*, 2002). As a consequence of this, our knowledge of soil biodiversity patterns, both at local and global scale, is fragmentary (see the review by Decaëns, 2010), as it remains basic information on species biology, ecology and distribution patterns (Decaëns *et al.*, 2008). One of the major issues in the research on soil biodiversity is the lack of adequate knowledge on the taxonomic diversity of many animal groups (Decaëns *et al.*, 2006), where a large part of species diversity has yet to be discovered and described (Orgiazzi *et al.*, 2016).

Among the components of the soil fauna, invertebrates (i.e., animals traditionally included in the meso- and macrofauna; see Petersen & Luxton, 1982; Anderson, 1988; Coleman *et al.*, 2017) represent an extremely heterogeneous group. This includes a wide range of animal species, belonging to many distantly related clades (e.g., annelids, gastropods, insects and myriapods) and performing different ecological functions in the soil ecosystems (Lavelle *et al.*, 2006).

Some groups of soil invertebrates have been better studied than the others in terms of species diversity, taxonomic classification and community ecology. In the case of carabid beetles, for example, this knowledge leverages their widespread use as bioindicators for habitat disturbance and environmental changes (Rainio & Niemelä, 2003). However, the majority of soil-dwelling invertebrate groups share some features that make investigations on their biodiversity patterns challenging. At the community level, they display remarkably high levels of species diversity at local scale (ranging from centimetres to a few meters). In fact, the high level of heterogeneity of soil habitats and the degree of specialisation in trophic ecology and life history strategies of many soil inhabitants allows a marked niche differentiation and, thus, the coexistence of a large number of species (Bardgett, 2005).

Endogeic animals are generally characterized by low vagility and low dispersal ability. As a consequence, populations can easily remain isolated from each other in very restricted areas, such as single mountain massifs, giving rise to micro-endemic patterns of speciation (e.g., Wesener & Conrad, 2016). However, the assignment of differentiated populations to unique species is not straight-forward: morphological differentiation between isolated populations can be subtle or even cryptic, while they can accumulate a large amount of genetic variation between each other (e.g.,

Boyer *et al*., 2007). Similar to the case of other groups of poorly studied organisms (e.g., meiofauna, see Fontaneto *et al*., 2015), an "integrative approach" has been advocated as the best practice to assess species boundaries. This consists of the integration of different lines of evidence in a multidisciplinary framework, in order to draw the most likely species delimitation hypothesis in a given set of populations (Padial *et al*., 2010; Schlick-Steiner *et al*., 2010). However, the use of integrative protocols for species delimitation is unevenly applied across different animal groups: for some groups, e.g., spiders (e.g., Hendrixson *et al*., 2013; 2015; Satler *et al*., 2013), an integrative approach has already been repeatedly applied with success, whereas for other groups it has been used only sporadically or not at all.

In this thesis, I used centipedes (Myriapoda: Chilopoda) inhabiting the Southeastern Prealps (Southern Europe) as a study system to explore aspects of the biodiversity of the soil invertebrates that remain poorly known by means of two different methodologies: (i) application of a rigorous statistical toolkit addressing incomplete detection to estimate local species richness and (ii) application of an integrative protocol for testing species boundaries in a system of populations at a small spatial scale. The thesis comprises two articles (presented in Chapter I and Chapter II, respectively), each one addressing one of the aspects listed above.

1. Centipedes

Centipedes are one of the major groups of soil predators, with over 3300 species recognized as valid (Edgecombe & Giribet, 2007). It includes five main evolutionary lineages: the Geophilomorpha, Lithobiomorpha, Scolopendromorpha and Scutigeromorpha are widely distributed across all continents (with the exception of the Antarctica), whereas the Craterostigmomorpha are restricted to Tasmania and New Zealand (Bonato & Zapparoli, 2011). Centipedes are distributed in many types of habitats, including deserts and caves; however, the highest abundance and diversity is found in the soil and the leaf litter of forest habitats (Voigtländer, 2011). In these environments, they contribute significantly to the total biomass and density of soil invertebrates (e.g., Petersen & Luxton, 1982; Scheu *et al*., 2003).

Based on their morphology and life-style, centipedes can be roughly divided into three main ecomorphotypes (Manton, 1977; Voigtländer, 2011): (i) the "borrowing" type, with elongated bodies and several adaptations to the endogeic life (e.g., blindness, shortened appendages, weak or no pigmentation) is represented mainly by Geophilomorpha, that is also the most diverse among the known lineages (ca. 1250 described species, most of them endogeic; see Bonato *et al*., 2011 for a taxonomic overview on centipedes); (ii) the "running" type is mainly represented by the vast majority of the Lithobiomorpha (another largely diverse group, with ca. 1100 described species), which actively move above-ground; (iii) the "intermediate" type includes part of the Scolopendromorpha (ca. 700 described species), which are able both to dig into the soil and run quickly on the surface, with different degrees of specialisation for the above-ground or below-

ground environments; for example, those belonging to the family Cryptopidae are all blind and adapted to a more endogeic life-style, with respect to those belonging to the family Scolopendridae.

All centipedes are very vulnerable to desiccation, since they lack a wax layer on the epicuticle (Rosenberg *et al.*, 2011). Consequently, even those actively moving on the surface avoid direct exposure to the external environment during the daytime, when they usually remain inactive in crevices or under temporary shelters such as stones, logs or barks. Because of the heterogeneity of life-forms among centipedes, their overall low detectability and since no attractive is known to efficiently trap them, collecting them requires a larger amount of effort in comparison with that required for many other above-ground invertebrate taxa, both in terms of dedicated time and number of different methodologies employed.

Despite their abundance and diversity, their role as top invertebrate predators among soil fauna and their consequent impact in shaping and maintaining the functioning of soil ecosystems, centipedes remain one of the most neglected groups of soil-dwelling animals. In fact, when compared to other important groups of soil predators (e.g., carabid beetles and spiders), research on Chilopoda lags far behind. In particular, some basic aspects of centipede biology are still poorly understood, such as the diversity of species and many facets of their ecology, including community structure and niche differentiation. Similar to other groups of soil invertebrates, research is hindered by the lack of taxonomic knowledge and identification tools, and the consequent difficulties in identifying the specimens. For the European fauna during the last ca. 150 years, many new taxa were described by different authors (e.g., R. Latzel, K.W. Verhoeff and C. Attems), mostly at sub-specific level (i.e., subspecies, varieties etc.). These were often on the basis of a few specimens or even a single one. Primary taxonomic literature is not readily available, and few systematic reviews have been published in recent times. Besides, many characters used to diagnose species in the traditional morphology-based taxonomy of centipedes have been heavily criticised by modern authors (e.g., Lewis, 2009; Bonato & Minelli, 2014).

2. South-Eastern Prealps

The study area of the present work includes the main marginal (i.e., prealpine) mountain groups belonging to the South-Eastern Alps area according to the SOIUSA classification of the Alps (Marazzi, 2005). Therefore hereafter, we refer to the area as the Southeastern Prealps. Only one of the study sites (see Chapter II) is located out of this range, in the Northern Dinarides, at the border with the alpine system.

The landscape of the Southeastern Prealps is characterized by several carbonatic plateaus modelled by karstic phenomena and partially by glaciations; indeed, the main lithogenetic processes in this area are of a sedimentary nature, whereas volcanic and metamorphic ones are of secondary importance. Main depositional events include the formation of thick carbonatic shelves

(e.g., the Dolomites) in shallow coastal marine environments by corals and other organisms, as well as the formation of clay, marl and flint in pelagic environments (e.g., in the Brescia and Garda Prealps); nevertheless, volcanic rocks formed by local magmatic intrusions are also present (e.g., the Adamello Group). Compared to other sectors of the Alps, Southeastern Prealps have a pretty complex orography: a multitude of confluent valleys with different orientation separate distinct mountain groups and isolated massifs. The valleys network harboured a large system of interconnected glaciers during the Pleistocene ice ages (Carton, 2005).

Besides areas that were used for agriculture or kept clear for pasture, the Southeastern Prealps are mainly covered by broadleaf forests. The montane zone (approximately 800-1600 m a.s.l.) is dominated by beech woodlands (*Fagus sylvatica*), which are sometimes mixed with conifer species, such as the Norway spruce (*Picea abies*) and the silver fir (*Abies alba*). Pure coniferous forests are rarer, often originated by replanting of Norway spruce. In the lower altitude zones, woodlands are composed of more thermophile tree species, such as the European hop hornbeam (*Ostrya carpinifolia)*, the common hornbeam (*Carpinus betulus*), the manna ash (*Fraxinus ornus*) and several species of oaks (*Quercus* spp.), which form mixed stands of different composition depending on the local climate, aspect and the nature of the substrate (Blasi *et al*., 2010; San-Miguel-Ayanz *et al*., 2016).

Southeastern Prealps are characterized by the presence of a high species diversity, which has been documented for several animal and plant taxa at different levels. Species richness at the regional scale is among the highest in the entire Europe for many taxa (e.g., orthopterans); moreover, some areas in the Southeastern Prealps and Northern Dinarides have been documented to harbour remarkably diverse communities, with high values of local species richness (e.g., Brandmayr & Pizzolotto, 1989). Another remarkable feature of this area is the presence of many local endemics (e.g., Minelli *et al*., 2006; Graf *et al*., 2014), and several biogeographic and phylogeographic studies, focused mostly on epigeic and relatively mobile animal taxa, have highlighted the contact between formerly divergent lineages of the same species within this area (e.g., Stefani *et al*., 2012; Bonato *et al*., 2018; Štundlová *et al*., 2019).

Many factors have contributed to shaping the biogeography and phylogeography of all the organisms inhabiting this area. These include major changes in structural features of the landscape and climatic oscillation during the Pleistocene (Schmitt, 2007), the high heterogeneity of habitats and climate conditions and the merging of biotas belonging to different biogeographic units, i.e., the Italian and the Balkan peninsulas.

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CHAPTER I

1. Introduction to Article I

Following Magurran (2004), species diversity can be viewed as the variety and abundance of species in a defined unit of study.

Measuring the diversity of species in a community, where the latter is intended as a system of species occurring at specified place and time (Fauth *et al*., 1996), is a focal point of most ecology studies. The main interest relies in the possibility of comparing patterns of species diversity in space (assessing whether communities are different from each other) and time (assessing whether communities change over time) (Magurran, 2004). This information can be used in addressing an array of related ecological problems, e.g., to explain how communities respond to biotic and abiotic factors, or for planning conservation policies (Fleishman *et al.,* 2006). Several metrics can be used to investigate different aspects of species diversity, like richness, evenness and rarity (Maurer & McGill, 2011). In most measures of species diversity all species are treated as equivalent, which means that the presence of any given species has the same weight. However, in other cases species relatedness or specific features of the species, like their ecological function, can be taken into consideration. Actually, communities with the same species diversity (simply intended as variety and abundance) can differ significantly in phylogenetic or functional diversity (Gotelli & Chao, 2013).

Species richness is actually one of the most intuitive and widespread measures of species diversity. It is defined as the number of species in a community and it is a widespread metric in many theoretical and practical fields of biology, including community ecology (e.g., Magurran 2004). Nonetheless, the mathematical simplicity and intuitiveness of the concept of species richness hides a major shortcoming when it is put into practice: for many groups of organisms it is usually very difficult, and often virtually impossible, to obtain a complete and exhaustive count of the species that form a community. The main reason for this is the impracticable amount of sampling effort required, which is also often difficult to quantify. The amount of effort depends on many factors, such as the size and the features of the study area, the size of the investigated taxon, the abundance distribution among the species and the accessibility of the habitat in which they live (e.g., Bonar *et al*., 2011). Moreover, the detectability can be significantly different among the species of the same community (Buckland *et al*., 2011), to the point that some species could remain undetected in field surveys. However, an array of statistical methods has been developed, in order to estimate the actual number of species of a community and to compare the species richness between communities on the basis of samples of individuals (Colwell *et al*., 2012; Chao & Chiu, 2016). Despite being available from a relatively long time, these methods have been scarcely employed in some little studied soil animals, such as centipedes, for which basic knowledge on species diversity is still lacking (Bardgett & van der Putten, 2014).

In the paper presented in this chapter, we used the most widespreadly used and best established statistical models to estimate the species richness in Southeastern Prealps centipede communities.

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2. Article I

Peretti E. & Bonato L. (2018) How many species of centipedes coexist in temperate forests? Estimating local species richness of Chilopoda in soil coenoses of the South-Eastern Prealps. *European Journal of Soil Biology* 89: 25-32.
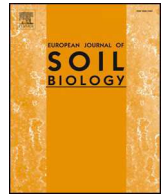
How many species of centipedes coexist in temperate forests? Estimating local species richness of Chilopoda in soil coenoses of the South-Eastern Prealps

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ABSTRACT

Centipedes (Chilopoda) are widespread and abundant predators in temperate forest soils, but reliable estimates of the actual number of species living in syntopy and interacting with each other are virtually nil. We estimated the local species richness of 10 centipede communities in the South-Eastern Prealps by sampling within small (ca. 1800 m^2) and uniform forest sites, employing complementary methods (pitfall traps and hand extraction of centipedes from standard volumes of soil) throughout two consecutive years and applying non-parametric statistical methods (Chao-1 and Abundance-based Coverage Estimator) to account for incomplete species detection. We recorded a maximum of 26 syntopic species of centipedes and estimated that up to 27–28 species may coexist in a single site. By comparing the communities investigated, we found that species richness is not strictly associated with species composition, it does not strongly correlate with average density of centipedes, and communities with the same species richness may differ significantly in functional richness. Additionally, our results suggest that temperate forests of the South-Eastern Prealps, along with those in the Northern Dinarides, harbour the richest known communities of centipedes in the world.

1. Introduction

Centipedes (Chilopoda) are a widespread and diverse component of the soil fauna [1,2]. Their ecological importance in temperate forest ecosystems has been repeatedly stressed (e.g. Ref. [3]) as they represent one of the most abundant groups of soil predators, both in terms of biomass and density of individuals (e.g. Refs. [4–6]). However, in comparison with other major groups of soil predators (notably carabid beetles and spiders), many facets of the diversity of centipede communities in temperate forests are almost unknown. Even though published results of field surveys suggest that temperate forest soils could harbour the most diverse centipede communities in terms of species richness (see also [5,7]), precise and reliable estimates of the actual number of species living in syntopy in these habitats are still lacking.

As a matter of fact, estimating how many species of centipedes can strictly cohabit – and potentially interact with competition for prey – remains a hard task.

In the first instance, species assemblages of poorly vagile invertebrates in the soils are strongly influenced by local environmental conditions (e.g. Ref. [8]), and can change substantially within a few meters, as it has been documented in other soil predators such as

carabid beetles (e.g. Ref. [9]) and spiders (e.g. Ref. [10]). As a consequence, also the species richness may vary within a very short spatial scale. Notwithstanding, only a few studies of centipede communities have been rigorously and explicitly based on narrowly circumscribed sample areas, hampering the possibility to control for beta-diversity.

Moreover, centipede communities of temperate forest soils comprise species of very different body sizes – from a few mm in length for juveniles up to ca. 2 dm for adults – and strictly endogeic species invariably coexist with species that perform regular epigeic activity [7,11]. Such inter-species differences in body size and behaviour also result in significant differences in catchability, depending on sampling methods. An integration of different methods is required in order to effectively sample the entire community [12]. Nevertheless, many published surveys rely only on a single method and are thus likely to result in incomplete inventories of species.

In addition, almost all published assessments of species richness in centipede communities merely reported on counts of detected species, which is hardly expected to approximate to the actual local species richness. In fact, samples obtained from field surveys regularly include a remarkable proportion of "rare" species (e.g. Refs. [4,13,14]), which suggests that centipede communities usually include species with

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relatively low "probability of detection" and may be expected to escape sampling. To obtain reliable estimates of the actual number of species in a local community, data should be analysed with available statistical tools that account for "incomplete detection". Suitable methods have been developed for some time [15,16] and are regularly applied in surveying different components of soil coenoses (e.g. Refs. [17–19]) but have been almost completely unexploited for centipede assemblages.

Our aim was to obtain some first estimates of the local species richness of centipede communities in temperate forest soils by applying a thorough sampling plan and by accounting for incomplete detection of species by different statistical models. In detail, we applied two alternative estimators (i.e., Chao-1 and ACE) that are based on different information on the abundance of rare species in samples and were proven to provide reliable estimates of species richness when applied to a number of other taxonomic groups. Furthermore, we explored whether and how the local species richness correlates with other major features of the centipede community such as species composition, density of individuals and diversity of functional traits.

2. Materials and methods

2.1. Study sites

A total of 10 sites were investigated within a 37 km long sector of the South-Eastern Prealps between the Vette Feltrine and the Schiara mountain groups in the Dolomiti Bellunesi Italian National Park (Table A1, see supplementary data). The minimum distance between any two sites was ca. 530 m. They were selected within areas that had remained continuously covered with forests during the last centuries and soils were not affected by human usages other than wood harvesting. Sites were chosen to sample the diversity of pedological conditions and forest vegetation within the area, and are different in slope, aspect and altitude (Table A1, see supplementary data). Each site was defined as a circular area with diameter 48 m, uniform in vegetation structure and ground features and at least 10 m away from forest edges, ecotonal zones and roads.

2.2. Sampling protocol

In order to sample species with different probability of detection, we used two complementary standard sampling methods (e.g. Ref. [12]) in each site: (i) extraction of all centipedes occurring in a standard volume of soil and leaf litter, by means of direct search; (ii) pitfall traps. The former method is proven to effectively sample the strictly endogeic part of the centipede community (in temperate forests: most of the Geophilomorpha, the Scolopendromorpha belonging to the family Cryptopidae and a few small Lithobiomorpha) but is not very efficient for larger Lithobiomorpha, which are unevenly distributed when non active as they usually aggregate under larger stones or wood logs (e.g. Ref. [20]). Conversely, the second method is proven to effectively sample the regularly epigeic part of the community (in temperate forests: most of the Lithobiomorpha and some Geophilomorpha) but is inadequate in detecting the strictly endogeic species.

Samples of soil and leaf litter (square surface area: 25 × 25 cm; depth: whenever possible 10 cm) were taken within each site by means of randomly generated coordinates. Each sample was immediately disposed on to a white plastic sheet and examined accurately by eye (spending ca. 2 h per sample) by one of the authors (EP), sometimes assisted by the other one (LB), to detect and extract all centipedes by hand.

A linear array of 6 pitfall traps, spaced 8 m apart, was arranged inside each site along the diameter at the same altitude. Traps consisted of plastic containers (upper diameter: 84 mm; height: 120 mm), filled alternatively along the array with 4% formaldehyde + wine vinegar (as, e.g., in Ref. [21]) or 4% formaldehyde + water (as, e.g., in Ref. [22]), as we found no information on the relative effectiveness of the

two alternative solutions in sampling centipedes. Each trap was covered with a plastic roof suspended by screws at about 8 cm above ground level, in order to prevent rain and leaf litter from falling into the trap.

One standard volume of soil was examined and all traps were emptied within each site every 28 days (with a few exceptions: 25–31 days) in two consecutive years, i.e., 2015 (1.VI-23.X) and 2016 (2.V-30.VI). Pitfall traps were not active during the winter between the two years.

2.3. Species identification

Centipedes were fixed in 70° ethanol and identified to species level. All Geophilomorpha and the smallest specimens of Lithobiomorpha and Scolopendromorpha were examined using a Leica DMLB microscope with magnification up to 400× after mounting on temporary microscopic slides [23]. Larger Lithobiomorpha and Scolopendromorpha were examined using a Leica MZ12.5 stereo-microscope with magnification up to 100×. Whenever necessary, anatomical parts (head in Geophilomorpha and Scolopendromorpha, female gonopods in Lithobiomorpha) were dissected.

Geophilomorpha were identified mainly with ChiloKey [24], Lithobiomorpha mainly following Koren (1992) [25] and Stoev et al. (2010) [26], Scolopendromorpha mainly following Brolemann (1930) [27], Pichler (1987) [28] and Lewis (2011) [29]. To facilitate species identification, a preliminary list of expected species was assembled from all the available published records for the entire South-Eastern Prealps (summarized by Zapparoli & Minelli, 2005 [30]). For taxonomy and nomenclature, we followed ChiloBase 2.0 [31].

All collected specimens were identified with the exception of: (i) anomalous or damaged specimens lacking anatomical parts bearing indispensable diagnostic characters (e.g., lithobiomorphs lacking both 15th legs); (ii) all specimens of Lithobiomorpha in the anamorphic stages or in the early epimorphic stages before developing species-diagnostic characters (e.g., arrangement of coxal pores, presence/absence of the "Trvm" spine on 15th legs and "plectrotaxy" of 15th legs; see Ref. [32] for terminology).

2.4. Species richness estimation

To estimate the number of species in each site, we employed the following two non-parametric estimators, pooling together all specimens sampled by the two methods: the Chao-1 estimator, which is based on the proportion between the number of species collected once and the number of those collected twice [33] and the ACE (Abundance-based Coverage Estimator), which is based on the frequency of "rare" species [34]. These estimators allow one to overcome the known limitations of parametric estimators and extrapolation of fitted parametric functions [15,16,35].

The bias-corrected Chao-1 estimator was computed by means of EstimateS 9.1.0 [36]. PAST 3.16 [37] and the *vegan* package in R [38] were also employed and gave the same results, differing only slightly because of the approximation. The 95% confidence intervals were computed both by the log-linear method implemented in EstimateS and the bootstrap method in PAST.

ACE was calculated using *vegan*. The cutoff between "rare" and "common" species was set at 10 individuals as default [35].

In order to compare species richness among sites with different numbers of collected specimens, we used two alternative methods: (i) integration of rarefaction and extrapolation from the observed value of species richness, with 95% confidence intervals based on "unconditional" variance, as proposed by Colwell et al. [39]; (ii) rarefaction analysis with 95% confidence intervals based on "conditional" variance [35].

For the former method, species richness was extrapolated up to the double of the sample size [16,40], based on Chao-1 estimates of asymptotic richness. The analysis was performed using both EstimateS,

Table 1
Species of Chilopoda found in 10 communities in the South-Eastern Prealps (see also Table A.2, see supplementary data).

	Number of sites	Number of specimens	
		soil samples	pitfall traps
Geophilomorpha			
Clinopodes carinthiacus (Latzel, 1880)	9	48	0
Clinopodes flavidus C.L. Koch, 1847	6	19	1
Dicellophilus carniolensis (C.L. Koch, 1847)	8	29	20
Eurygeophilus pinguis (Brölemann, 1898)	1	1	0
Geophilus alpinus Meinert, 1870	7	20	0
Geophilus electricus (Linnaeus, 1758)	1	1	0
Geophilus pygmaeus Latzel, 1880	1	71	1
Geophilus sp. [a]	4	16	0
Henia montana (Meinert, 1870)	5	15	18
Henia vesuviana (Newport, 1845)	3	7	6
Pleurogeophilus mediterraneus (Meinert, 1870)	4	6	0
Schendyla carniolensis Verhoeff, 1902	9	117	1
Schendyla tyrolensis (Meinert, 1870)	9	38	0
Stenotaenia linearis (C.L. Koch, 1835)	4	9	0
Strigamia acuminata (Leach,1815)	10	20	34
Strigamia crassipes (C.L. Koch, 1835)	10	16	33
Strigamia transsilvanica (Verhoeff, 1928)	2	2	3
Lithobiomorpha			
Eupolybothrus grossipes (C.L. Koch, 1847)	8	1	246
Eupolybothrus tridentinus (Fanzago, 1874)	10	5	338
Harpolithobius anodus (Latzel, 1880)	5	2	6
Lithobius borealis Meinert, 1868	1	0	1
Lithobius carinthiacus Koren, 1992	10	140	5
Lithobius castaneus Newport, 1844	10	3	33
Lithobius dentatus C.L. Koch, 1844	9	13	129
Lithobius forficatus (Linnaeus, 1758)	6	0	13
Lithobius lapidicola Meinert, 1872	6	11	22
Lithobius latro Meinert, 1872	1	0	1
Lithobius cfr. *mutabilis* L. Koch, 1862 [b]	1	0	2
Lithobius nodulipes Latzel, 1880	4	0	6
Lithobius pelidnus Haase, 1880	7	0	7
Lithobius tenebrosus Meinert, 1872	4	0	6
Lithobius tricuspis Meinert, 1872	10	15	76
Lithobius validus Meinert, 1872	7	1	51
Scolopendromorpha			
Cryptops anomalans Newport, 1844	4	4	2
Cryptops hortensis (Donovan, 1810)	5	13	2
Cryptops parisi Brolemann, 1920	10	157	8

[a] Putative undescribed species belonging to the genus *Geophilus*.
[b] Uncertain identification because only female specimens were collected.

which calculates confidence intervals following Colwell et al. [39], and the *iNEXT* package in R [41], which employs the bootstrap method proposed by Chao et al. [40]. For *iNEXT*, the parameters were set at default, except for the number of bootstrap replications, which was set to 1000.

The rarefaction analysis with 95% confidence interval based on "conditional" variance was performed with PAST.

2.5. Species composition

Differences in species composition between sites were evaluated with the Jaccard similarity index (e.g. Ref. [42]), using PAST, and were further investigated by means of a Correspondence Analysis [43] performed separately on the data obtained by soil samples and those obtained from the pitfall traps. In order to account for minor variation in the time duration of trapping sessions and for occasionally damaged traps, counts obtained by any pitfall trap in any session were divided by the actual number of days of effective trapping activity. The analysis was performed using the *FactoMineR* package in R [44,45] and the results were visualized through the contribution biplots [46] using the *factoextra* package in R [47].

2.6. Density

As is common practice for soil invertebrates and standard for Chilopoda [7], the average density of centipedes within each site was calculated referring to the surface area (individuals/m^2), based on the counts obtained from all the soil samples, including not identified specimens.

2.7. Functional diversity

We selected 6 functional traits that could be measured or scored for all species: (i) maximum body length (approximated to ± 5 mm); (ii) degree of thickness of the cuticle (estimated on an ordinal scale of three levels); (iii) number of ocelli; (iv) degree of projection (either protruding or sinking) of the anterior margin of the forcipular coxosternite (Fig. A1, see supplementary data); (v) elongation of the forcipular trochanteroprefemur (Fig. A1, see supplementary data); (vi) percentage of individuals caught by pitfall traps with respect to the total number of specimens collected with both methods. Traits i-iii were selected among those proposed by Moretti et al. [48] for terrestrial invertebrates. All traits were defined on adults only, but trait i may be considered also as a proxy of the intraspecific trait variability between adults and different instars, as the maximum body length is expected to be correlated with
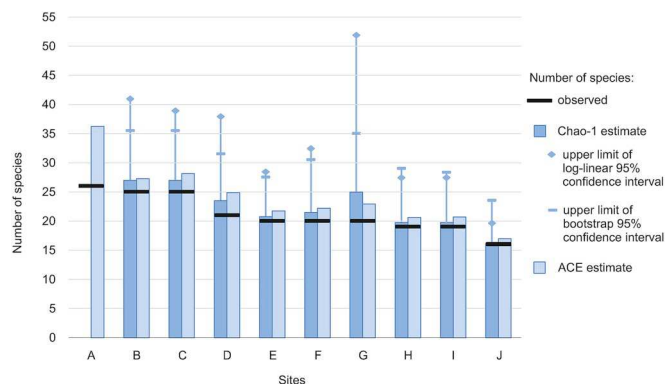
Fig. 1. Observed and estimated species richness in 10 communities of Chilopoda in the South-Eastern Prealps. For site A, the bias-corrected Chao-1 was not calculated (coefficient of variation of the abundance distribution = 0.628) and the uncorrected Chao-1 is not reported (see par. 3.1).

the range of variation of size-related traits: individuals of larger species grow up more during their development and consequently the population shows a higher intraspecific variability in size-related traits. Traits iv-v were chosen to represent morphological differences in the forcipules (which are used to grab the prey and inject venom; e.g. Ref. [49]) and were found to catch most of the shape variation in a preliminary survey on selected species of Geophilomorpha [50]. Trait vi was taken as a proxy for the frequency of epigeic activity performed by the species.

All traits were evaluated or measured either on drawings from the literature (mainly [25,27,51]) or directly on collected specimens.

Functional richness, i.e., the amount of niche space occupied by the species within a community (see Ref. [52] for a review), was used as a descriptor of functional diversity [53]. In order to measure functional richness we used the FRic multidimensional index proposed by Villéger et al. [53]. It was calculated by the *FD* package in R [54]. The maximum number of principal coordinates t was set to meet the condition $2^t \leq$ number of species. Since the species x species distance matrix was still not Euclidean after the default square root correction, the correction method by Cailliez [55] was applied. FRic values were standardized by the values obtained considering all the species so that they were constrained from 0 to 1. As the functional richness is expected to

be strongly correlated with species richness [56], it was compared only between sites without significantly different species richness according to the rarefaction and extrapolation analysis.

3. Results

A total of 2219 centipedes were collected and 1871 of these (84%) were identified to species level, for a total of 36 species (Table 1; Table A.2, see supplementary data).

3.1. Species richness

Between 16 and 26 species were detected in each of the ten centipede communities (Fig. 1; Table A.3, see supplementary data): 19–21 in most of the sites, only 16 in a single site and 25–26 in three sites. In most of the sites, estimates of actual species richness (Chao-1 and ACE) exceeded slightly the observed number of species: 1 to 5 species likely remained undetected, but with a broad degree of uncertainty (Fig. 1; Table A.3, see supplementary data). In the site with the lowest number of observed species (site J), both estimators suggested that the survey may have been exhaustive or at most one species remained undetected (up to 8 considering the 95% confidence intervals for Chao-1). In contrast, in the site with the highest number of observed species (site A), ACE suggested at least another 10 undetected species, whereas it was not possible to calculate the bias-corrected Chao-1 because the coefficient of variation of the abundance distribution was > 0.5 (see User's guide of EstimateS [36]) and the uncorrected estimate resulted unreliable.

Comparing sites by integrating rarefaction and extrapolation of species richness with 95% confidence intervals based on "unconditional" variance (Fig. 2A), a statistically significant difference in species richness was found between the poorest site (site J, with 16 detected species) and the three richest ones (sites A, B, C, with 25–26 detected species). Confidence intervals computed with the method of Colwell et al. [39] were slightly different but concordant (results not shown).

The rarefaction analysis with 95% confidence intervals based on "conditional" variance suggested statistically significant differences also between other sites (Fig. 2B). In particular, the three richest sites were significantly richer than most of the others, with the possible exception of the two least sampled sites (G and H). Additionally, among the three richest sites, site A (with 26 detected species) resulted
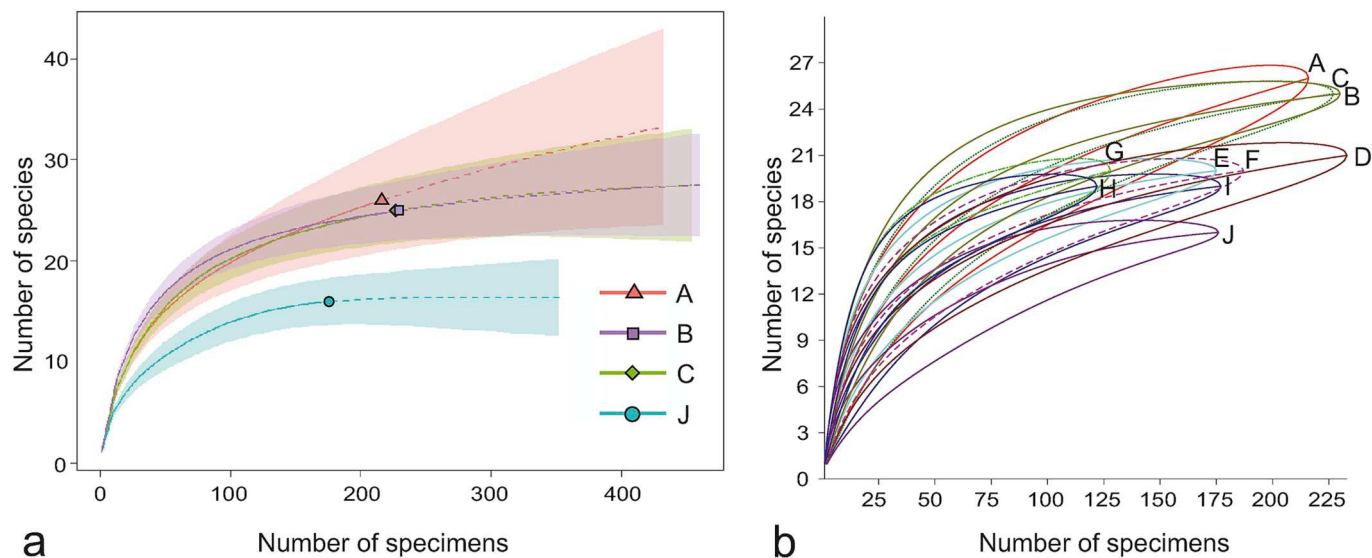
Fig. 2. Comparison of estimated species richness between 10 communities of Chilopoda in the South-Eastern Prealps. **a**: integration of rarefaction (solid line) and extrapolation (dashed line), 95% confidence intervals based on unconditional variance (coloured areas surrounding the curves) computed following Chao et al. [40]. Only four sites are shown for reasons of clarity. **b**: rarefaction analysis, 95% confidence intervals based on conditional variance.
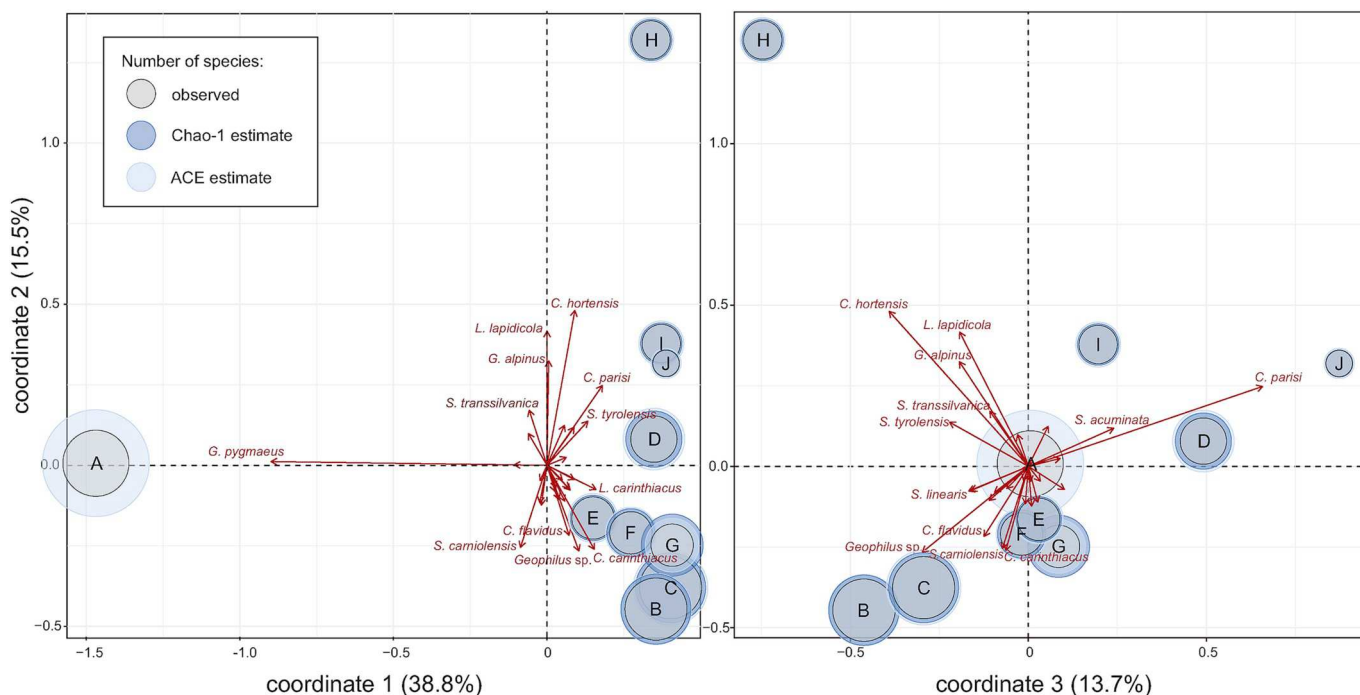
Fig. 3. Contribution biplots of the Correspondence Analysis performed on data obtained from soil samples. Only the 12 most contributing species were labelled. The diameter of circles is proportional to the species richness of the sites.
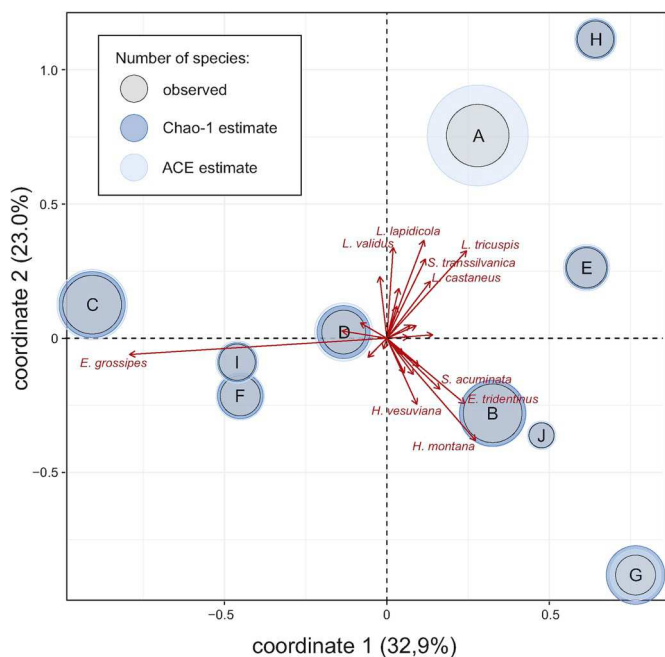

Fig. 4. Contribution biplot of the Correspondence Analysis performed on data obtained by pitfall trapping. Only the 10 most contributing species are labelled. The diameter of the circles is proportional to the species richness of the sites.

significantly richer than sites B and C (both with 25 detected species).

3.2. Relations between species richness and other community features

3.2.1. Species composition

Considering the species detected in the ten communities, the pairwise values of the Jaccard similarity index ranged between 0.44 and 0.71 with a mean value of 0.58 and a standard deviation of 0.07 (Table A.4, see supplementary data). Both minimum and maximum similarity

in species composition were found between communities with similar intermediate values of species richness (19–21 detected species). Intermediate values of similarity were found between the three richest sites, from 0.55 (between sites A and C) to 0.67 (between sites B and C).

Considering the strictly endogeic component of the centipede communities, the Correspondence Analysis performed on the standard soil samples produced three main coordinates, accounting for 39%, 16% and 14% of the total variation (Fig. 3). Of the three richest sites, site A turned out to be remarkably different in the composition of strictly endogeic species with respect to sites B and C, which instead were found to be quite similar to each other. Also most sites with intermediate values of species richness showed relatively uniform composition of strictly endogeic species, with the exception of site H.

Considering the regularly epigeic component of the centipede communities, the Correspondence Analysis performed on the samples collected by pitfall traps produced two main coordinates, accounting for 33% and 23% of the total variance respectively (Fig. 4). All the three richest sites (sites A, B and C) turned out to be quite different in the composition of regularly epigeic species. Also the sites with intermediate species richness were found very different for this component.

3.2.2. Abundance

The average density of centipedes estimated within each site ranged from 94 to 296 ind./m^2, with an overall average among sites of 175 ind./m^2 and a standard deviation of 61 ind./m^2 (Table A.3, see supplementary data). Species richness and average density resulted only weakly positively correlated and with little statistical support (Spearman's $\rho = 0.59$, $p = 0.071$, with observed number of species, Fig. 5; $\rho = 0.50$, $p = 0.138$, with ACE; $\rho = 0.28$, $p = 0.452$, with Chao-1). The site with the highest richness (A) harboured also the highest density of centipedes (average ± standard deviation: 296 ± 140 ind./m^2), largely due to the high density of a single species (142 ± 81 ind./m^2 for *Geophilus pygmaeus*). Among the sites with moderate species richness, a comparably high density was found in a single site (E, with 220 ± 153 ind./m^2), while the community of site I was the least dense, with only 94 ± 94 ind./m^2. The poorest site (J) had an intermediate value of density (168 ± 105 ind./m^2).
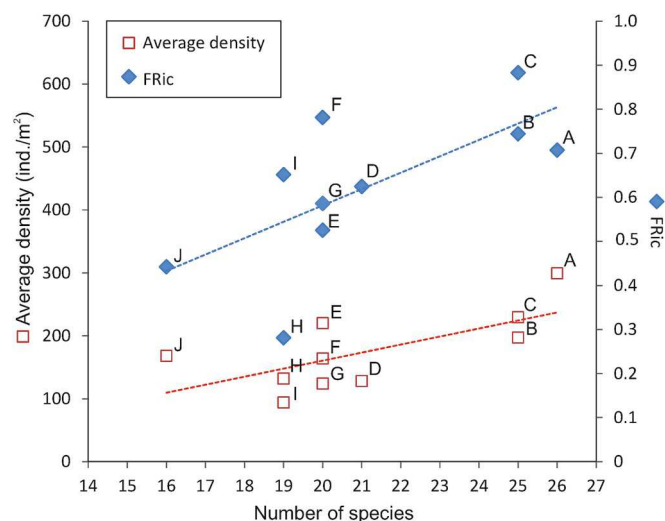
Fig. 5. Average density of centipedes and functional richness (FRic) in relation to species richness.

3.2.3. Functional diversity

Considering six traits related to body size, thickness of integument, vision, shape of predatory appendages and behaviour, the quality of the reduced-space representation of the functional richness was 0.62 and standardized values of FRic were in the range 0.28–0.88 (Table A.3, see supplementary data). The expected positive correlation between species richness and FRic [53,56] was moderate (Spearman's ρ = 0.68; p = 0.03; Fig. 5). The richest communities (sites A, B, C) showed values of FRic among the highest ones (0.71–0.88). Instead, the sites with intermediate values of species richness showed broadly variable values of FRic, between 0.28 (the lowest value overall, site H) to 0.78 (site F).

4. Discussion

Our results could be partially affected by some methodological constraints that were not possible to overcome. Especially the estimates of relative abundance between species may be biased not only by different probabilities of detection between species (see par. 1), but also by the fact that it was impracticable to sample deeper soil levels (see par. 2.2) and to identify most juveniles of Lithobiomorpha (par. 2.3). Moreover, the functional richness was necessarily estimated from adult morphologies only, therefore not accounting properly for the expected intraspecific variability of size-related traits (e.g. Ref. [52]). Additionally, because of the intense and time-consuming sampling effort devoted to any single site (par. 2.2), it was impracticable to extend the investigation to a larger number of sites or to a larger number of repetitions within sites. As a consequence, it was not possible to explore how richness, composition and structure of local centipede communities are affected by environmental variation. Nevertheless, our results provide original insights on the level of species richness of centipede communities in temperate forests and how it correlates with other major community features.

4.1. Richness estimates

Our estimates, although with broad confidence intervals, showed that 20 or more species of centipedes —reasonably up to 27–28 — can regularly live in syntopy in the temperate forests of the South-Eastern Prealps.

Searching the literature, we found counts and estimates of local species richness for 378 other temperate forest sites in the world. However, most of these sets of data are hardly comparable for different reasons. Firstly, only for less than 20% of the sites had the surveys been

rigorously and explicitly performed within small (less than 2 ha) and reasonably uniform areas. Moreover, less than 35% of the surveys had been carried out with effective sampling methods for both strictly endogeic and regularly epigeic species of centipedes (see par. 2.2). Furthermore, to the best of our knowledge, considering only temperate forests, an attempt to estimate the actual species richness accounting for incomplete detection through statistical methods was made only in seven sites, all in the Northern Dinarides, by applying a Jacknife estimator [57,58]. However, in this case centipedes were collected only by extraction from soil samples, which we found a poorly effective method for larger and regularly active species (Table 1), and the Jackknife estimation was performed only separately on samples collected in different seasons.

Additionally, investigations have been strongly biased towards Europe. In fact, we found only 13 published counts of local species richness from non-European temperate forests, and specifically from North America [59–64].

Despite the above mentioned limits of comparability, the values for species richness we found in the South-Eastern Prealps (16–26 species, within 1800 m²) are higher than almost all other values reported from other temperate forests, with the possible exception of four beechwood sites in the Northern Dinarides (24–28 species, but within 2500–5000 m² [57]). To the best of our knowledge, along with the latter records, our values of species richness represent the highest at a global scale for centipede communities not only for temperate forests but also among other habitats.

Because of the variable probability of detection between different centipede species, it is expected that the actual species richness in our studied communities could be even higher, as suggested by the statistical estimators (Fig. 1). However, in the site with the highest number of observed species (site A), the estimation through non-parametric methods was hindered by the peculiar structure of the local community, as most of the species were collected with low frequencies (i.e., less than 10 specimens) and many of them were even represented by single specimens in our samples.

4.2. Relations between species richness and other community features

Relations between species richness and composition in centipede communities had remained previously unassessed, with just a few exceptions (e.g. Refs. [65,66]). In the forest soils of a relatively limited area (spanning 37 km), we found centipede communities with a very similar number of species but a remarkably different composition both for the regularly epigeic species and the strictly endogeic ones.

In particular, when comparing the six sites with the most similar values of species richness (sites D-I: 19–21 observed species, up to 25 estimated species; Fig. 1), a major difference in the strictly endogeic component (Fig. 3) was found between site H and the others, due to higher abundance of multiple species including *Cryptops hortensis*, *Lithobius lapidicola* and *Geophilus alpinus*. Additionally, major differences in the regularly epigeic component (Fig. 4) were found between site C and the others, mainly due to the relative abundance of *Eupolybothrus grossipes*, and between communities with *Lithobius lapidicola*, *Lithobius validus*, *Lithobius tricuspis* and *Strigamia transsilvanica* on one hand (e.g. site H) and communities with *Henia montana*, *Henia vesuviana*, *Eupolybothrus tridentinus* and *Strigamia acuminata* on the other hand (e.g. site G). Great diversity in species composition was found also among the three richest communities (sites A-C: 25–26 observed species, at least 27 estimated species; Fig. 1): site A was differing from the other two sites in both the strictly endogeic and the regularly epigeic components of the community (Figs. 3–4), especially for the higher abundance of *Geophilus pygmaeus* among the strictly endogeic species; conversely, sites B and C were similar to each other in the strictly endogeic component (Fig. 3) but not so in the regularly epigeic component (Fig. 4).

We also found that the number of locally coexisting centipede

species correlates only weakly with the overall abundance of centipedes. When excluding the somehow anomalous site with the maximum species richness and individual density (site A), the statistical support for such correlation further decreases (Spearman's $\rho = 0.50$; $p = 0.171$; $N = 9$). Also when considering published data for forest sites of the Northern Dinarides [57,58,67], the number of recorded species does not correlate significantly with the estimated average density of individuals (Spearman's $\rho = 0.34$; $p = 0.171$; $N = 18$).

Finally, we found that the number of locally coexisting centipede species correlates with their functional richness. This is expected because the FRic index is defined as the amount of functional space occupied by the community and the selected traits (describing body size, thickness of integument, vision, shape of predatory appendages and behaviour) are not strictly associated across species (see Ref. [56]). Nevertheless, different communities comprising a similar number of ca. 20 species showed remarkably different levels of functional richness for the traits analysed, thus weakening the expected correlation.

4.3. Coexistence of multiple species

By reporting that more than 20 species of centipedes regularly coexist in temperate forest soils, our results demand further investigation of the interactions between such a plethora of potentially competing predator species and on the processes allowing their coexistence. The similar remarkable species richness observed in some forest sites of the Northern Dinarides (see above) has been explained by reference to glacial refugia harbouring old communities comprising coevolved species [57]. However, the autoecology of most centipede species is insufficiently known to evaluate hypotheses of differentiation of ecological niche between species. Arguably, given the fine-scale heterogeneity of the forest ground, some separation between species at a small spatial scale (even as low as metres or decimetres) could be allowed by behavioural and microhabitat differences. Actually more subtle differentiation should be invoked to explain the syntopy of species that share a very similar morphology and behaviour, such as *Schendyla carniolensis* and *Schendyla tyrolensis*, which we found to coexist in 9 of the 10 investigated sites.

Since almost all centipede species living in temperate forests are long-lived and have slow rates of reproduction (e.g. Ref. [11]), we can exclude "temporal" niche shifts between species. On the other hand, both horizontal (e.g. Ref. [57]) and vertical (e.g. Ref. [68]) migrations could play a role by reducing direct competitive interactions. In reality very little is known about the vertical distribution of the species within the soil, as only the most superficial layers of forest soils (up to 10–15 cm depth) are usually sampled.

Even some differentiation of trophic niche may be expected between species but diet preferences are almost unexplored in centipedes when compared to other soil predators (e.g. Ref. [69]). Molecular investigations of prey DNA in gut contents [70,71] and comparative analyses of the functional diversity of the feeding apparatus [50] have begun to address this question only recently.

Declaration of interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejsobi.2018.10.001.

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Appendix A: Supplementary Data for the article:

How many species of centipedes coexist in temperate forests? Estimating local species richness of Chilopoda in soil coenoses of the South-Eastern Prealps

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Table A.1: Geographic features of the investigated sites.

Site	Locality	Latitude (°N)	Longitude (°E)	Altitude (m)	Aspect	Slope (%)	Dominant tree species
A	Costagranda: Ponte dei Ross	46.26002	12.20625	800	N	40	*Abies alba*
B	Val del Mis: California	46.20549	11.97414	720	N	30	*Acer pseudoplatanus, Fraxinus excelsior*
C	Maragno	46.06848	11.86533	830	SE	40	*Fagus sylvatica, Fraxinus ornus, Ostrya carpinifolia*
D	Monte Tamberella	46.12082	11.93320	710	N-NE	40	*Fagus sylvatica, Ostrya carpinifolia*
E	Pian d'Avena	46.05016	11.83886	880	SE	30	*Fagus sylvatica*
F	Lago della Stua	46.13376	11.94566	730	E	50	*Fagus sylvatica*
G	Val Pegolera	46.23665	12.09629	545	N	20	*Pinus sylvestris*
H	Caiada: Casera d'Igoli	46.22750	12.24450	1265	E	20	*Fagus sylvatica, Picea abies*
I	Maragno	46.07223	11.86890	785	NE	35	*Fraxinus ornus, Ostrya carpinifolia*
J	Le Boscaie	46.07182	11.84264	1300	S	45	*Fagus sylvatica*

Table A.2: Number of individuals found for each species within each site. See also Table 1.

Order	Species	A	B	C	D	E	F	G	H	I	J
Geophilomorpha	*Clinopodes carinthiacus*	1	9	9	3	5	6	10	0	3	2
	Clinopodes flavidus	1	3	7	1	5	0	3	0	0	0
	Dicellophilus carniolensis	8	12	0	11	3	4	5	0	3	3
	Eurygeophilus pinguis	0	0	1	0	0	0	0	0	0	0
	Geophilus alpinus	3	1	0	1	5	2	0	7	0	1
	Geophilus electricus	0	1	0	0	0	0	0	0	0	0
	Geophilus pygmaeus	72	0	0	0	0	0	0	0	0	0
	Geophilus sp.	0	11	3	0	0	1	0	0	1	0
	Henia montana	3	8	0	0	7	0	12	0	0	3
	Henia vesuviana	0	0	0	2	0	3	8	0	0	0
	Pleurogeophilus mediterraneus	0	0	1	2	0	1	0	0	2	0
	Schendyla carniolensis	27	6	15	6	31	20	8	2	3	0
	Schendyla tyrolensis	1	7	2	4	3	2	9	8	2	0
	Stenotaenia linearis	0	1	4	0	0	3	0	1	0	0
	Strigamia acuminata	3	10	4	3	1	4	8	3	3	15
	Strigamia crassipes	6	6	4	4	3	12	3	1	8	2
	Strigamia transsilvanica	1	0	0	0	0	0	0	4	0	0
Lithobiomorpha	*Eupolybothrus grossipes*	5	14	80	43	0	45	2	0	56	2
	Eupolybothrus tridentinus	20	52	8	60	31	34	20	10	45	63
	Harpolithobius anodus	2	3	1	0	0	0	1	0	1	0
	Lithobius borealis	0	0	0	0	0	0	1	0	0	0
	Lithobius carinthiacus	12	26	29	7	12	16	6	11	10	16
	Lithobius castaneus	2	2	4	1	9	2	1	6	6	3
	Lithobius dentatus	0	27	19	29	11	15	13	12	5	11
	Lithobius forficatus	3	0	4	1	2	1	0	2	0	0
	Lithobius lapidicola	6	4	2	0	2	0	0	13	6	0
	Lithobius latro	1	0	0	0	0	0	0	0	0	0
	Lithobius cfr. *mutabilis*	0	0	2	0	0	0	0	0	0	0
	Lithobius nodulipes	1	0	0	2	0	0	0	2	1	0
	Lithobius pelidnus	1	1	1	0	1	0	1	1	0	1
	Lithobius tenebrosus	1	0	1	0	2	0	0	0	0	2
	Lithobius tricuspis	9	5	7	13	20	4	8	16	5	4
	Lithobius validus	14	3	7	19	0	0	1	6	0	2
Scolopendromorpha	*Cryptops anomalans*	1	3	0	1	1	0	0	0	0	0
	Cryptops hortensis	0	2	2	0	0	1	0	8	2	0
	Cryptops parisi	12	13	10	20	21	11	8	9	15	46

Table A.3: Observed and estimated values of species richness, average density of individuals and functional richness (FRic) in 10 communities of Chilopoda in the South-Eastern Prealps.

	Site									
	A[a]	B	C	D	E	F	G	H	I	J
Observed richness	26	25	25	21	20	20	20	19	19	16
Estimated richness by Chao-1	-	26.99	26.99	23.49	20.75	21.49	24.96	19.74	19.75	16.17
Upper limit of the log-linear 95% confidence interval of Chao-1 index	-	40.95	38.91	37.92	28.41	32.42	51.91	27.40	27.41	19.58
Upper limit of the 95% confidence interval of Chao-1 index (9999 bootstrap replicates)	-	35.5	35.5	31.5	27.5	30.5	35.0	29.0	28.3	23.5
Estimated richness by ACE	36.26	27.29	28.13	24.89	21.73	22.20	22.92	20.61	20.68	16.96
Average density (ind./m^2) ± St. Dev.	296 ±140	194 ±101	226 ±112	128 ±81	220 ±153	164 ±76	124 ±54	132 ±139	94 ±94	168 ±105
FRic	0.71	0.74	0.88	0.62	0.53	0.78	0.59	0.28	0.65	0.44

[a] For site A, the bias corrected Chao-1 was not calculated (coefficient of variation of the abundance distribution = 0.628) and the uncorrected Chao-1 is not reported (see par. 3.1).

Table A.4: Jaccard index of similarity among sites.

| Site	A	B	C	D	E	F	G	H	I	J
A	1.00	0.65	0.55	0.62	0.70	0.44	0.59	0.55	0.50	0.56
B	0.65	1.00	0.67	0.59	0.67	0.61	0.67	0.57	0.63	0.58
C	0.55	0.67	1.00	0.53	0.55	0.61	0.55	0.57	0.63	0.46
D	0.62	0.59	0.53	1.00	0.64	0.71	0.64	0.54	0.60	0.54
E	0.70	0.67	0.55	0.64	1.00	0.54	0.60	0.56	0.50	0.64
F	0.44	0.61	0.61	0.71	0.54	1.00	0.54	0.56	0.70	0.50
G	0.59	0.67	0.55	0.64	0.60	0.54	1.00	0.44	0.56	0.64
H	0.55	0.57	0.57	0.54	0.56	0.56	0.44	1.00	0.52	0.46
I	0.50	0.63	0.63	0.60	0.50	0.70	0.56	0.52	1.00	0.46
J	0.56	0.58	0.46	0.54	0.64	0.50	0.64	0.46	0.46	1.00
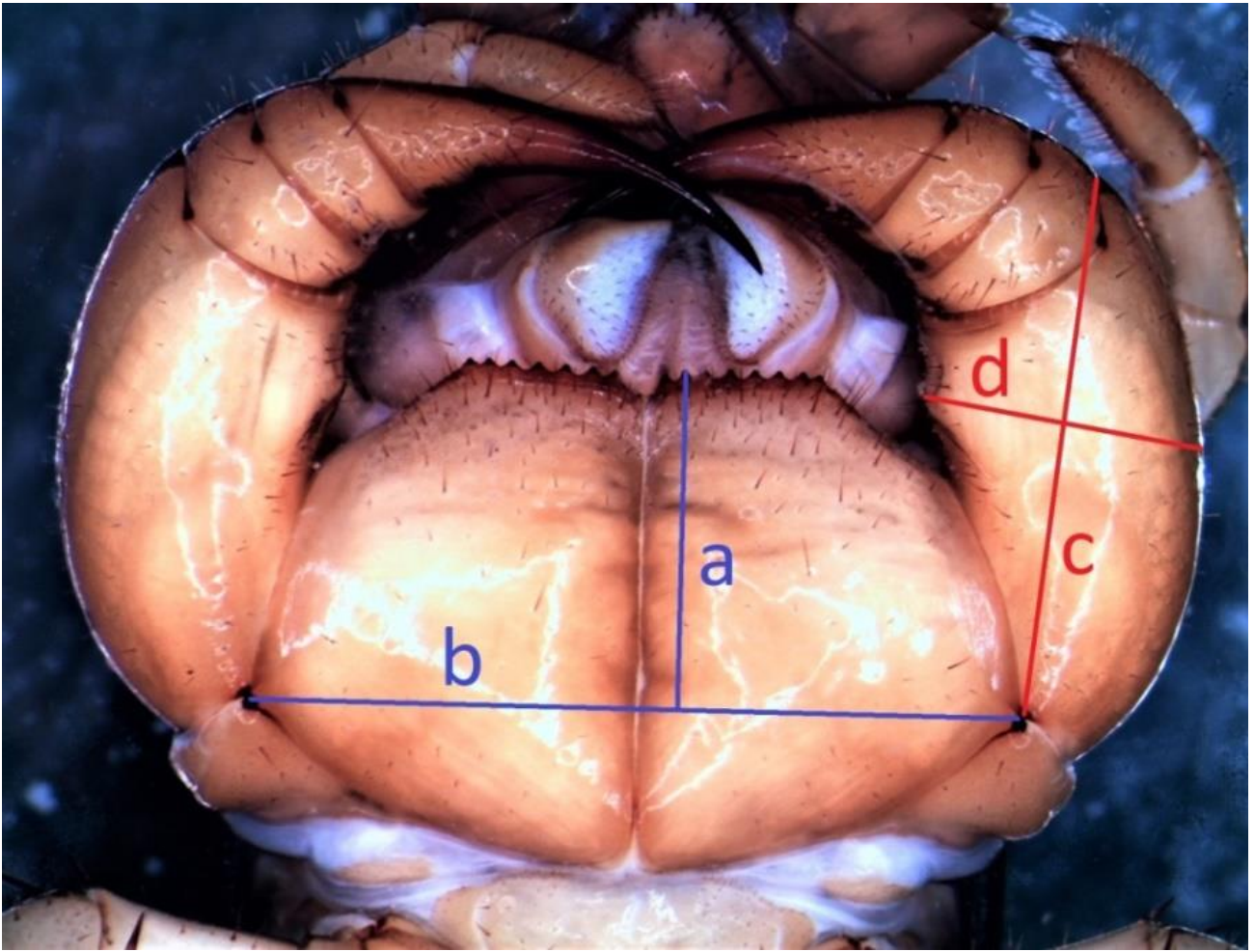
Figure A.1: Measures taken on the forcipular apparatus of centipedes to assess functional traits iv and v. **a/b** = projection of the anterior border of the coxosternite (iv); **c/d** = elongation of the trochanteroprefemur (v).

CHAPTER II

1. Introduction to Article II

The species is a fundamental unit in many questions of biology, from functional morphology, to evolution, ecology and conservation biology (e.g., Agapow *et al.*, 2004; De Queiroz, 2005). However, the problem of what a species actually is has fuelled a large debate in the last century, giving rise to the so-called "species problem" (Pigliucci, 2003; Barberousse & Samadi, 2010): a plethora of alternative species concepts has been proposed in the scientific literature, often with the aim to provide the most appropriate definition of species (Hey, 2001). Among the alternative species concepts, some of the most popular are the Biological Species Concept (e.g., Mayr, 1942), the Evolutionary Species Concept (e.g., Wiley, 1978), the Ecological Species Concept (Van Valen, 1976), yet many others are available. Nevertheless, since De Queiroz (1998), most of the contemporary alternative definitions of species refer, explicitly or implicitly, to a broader concept of species as "segments of population level evolutionary lineages" (i.e., successions over time of all and only the populations belonging to a single evolutionary lineage), and most of the differences between the so-called alternative species concepts are indeed related to the operational criteria to identify and delimit species taxa (e.g., reproductive isolation, in the case of the Biological Species Concept; unique evolutionary history and expected fate, in the case of Evolutionary Species Concept; distinct ecological niche, in the case of the Ecological Species Concept). In this view, all these alternative definitions can be reconciled under the so-called *General Lineage Concept of Species* (De Queiroz 1998; 1999; 2007), which identifies species as segments of separately evolving metapopulation lineages. In this definition, a metapopulation is intended as a system of interbreeding subpopulations, while lineages are intended as single lines of ancestry and descent. Under this unified concept, the criteria to identify and delimit species used in the constellation of alternative species concepts are seen as secondary defining properties of the species, which can emerge at different times and in a variable order during the process of speciation. Accordingly, these secondary defining properties can be used as lines of evidence to assess species separation (De Queiroz, 2007).

In recent years, a consensus has emerged towards the application of this conceptual framework to the exploration of biological diversity at the species level (i.e., species delimitation as defined by Carstens *et al.*, 2013). In particular, the integration of different kinds of evidence for speciation through a multidisciplinary and multisource approach has been recognized as the most effective way to address the question of species delimitation. This approach, known as *integrative taxonomy* (Dayrat, 2005), has two main advantages with respect to traditional morphology-based taxonomy and also with respect to the more recent DNA-based taxonomy: (i) it overcomes the underestimation of species-level diversity in systems where morphology alone is not informative, as well as the frequent diversity overestimates deriving from genetically defined species; and (ii) it acknowledges the complexity of the evolutionary processes of speciation (Schlick-Steiner *et al.*,

2010). Many different approaches are available for integrating different types of data and methods (e.g., using species discovery and validation methods; see Carstens *et al.*, 2013). However, as best practice, Schlick-Steiner *et al*. (2010) suggested the investigators to integrate many data types and analyses, looking for congruence among the results, in order to draw the most likely delimitation hypothesis, and to provide an evolutionary explanation for disagreements among lines of evidence and methods.

In the manuscript presented in this chapter (Article II) we used an array of species delimitation methods that apply on different sources of data (i.e., molecular and morphological characters) to test alternative species delimitation hypotheses in populations of endogeic centipedes across the Southeastern Prealps. The manuscript is in the form of a research article to be submitted to an international journal in the field of systematic and evolutionary zoology.

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2. Article II

Peretti E. & Bonato L. (in prep.) Testing the existence of species boundaries in the endogeic centipede *Clinopodes carinthiacus* (Chilopoda: Geophilomorpha) in the Southeastern Prealps through an integrative approach.

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Testing the existence of species boundaries in the endogeic centipede *Clinopodes carinthiacus* (Chilopoda: Geophilomorpha) in the Southeastern Prealps through an integrative approach

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Abstract

The use of an integrative approach to taxonomy has proven successful for investigating species boundaries in soil-dwelling animals with low dispersal ability because the integration of different sources of evidence outperforms both the traditional morphology-based taxonomy and pure molecular taxonomy. This approach has been successfully adopted for some groups of soil-dwelling animals, but for some other major groups, like endogeic centipedes, it has never been applied. In this research, we used a rigorous integrative approach to test for the existence of overlooked species boundaries in a system of populations of endogeic centipedes that is considered belonging to a single species. By integrating evidence from three molecular loci (mitochondrial 16S and COI, and nuclear 28S) through different species discovery methods (ABGD, GMYC and PTP), and evidence from morphological characters through a clustering algorithm without a-priori hypotheses (Expectation Maximisation Cluster Analysis with v-fold cross-validation), we demonstrated that a set of 27 populations of *Clinopodes carinthiacus* distributed across the Southeastern Prealps actually belong to at least two different species. We found concordance between all the sources of information indicating the existence of a candidate species broadly distributed across the investigated area, and another one restricted to the Carnic and the Julian Prealps. As a further evidence for this delimitation scheme, the two candidate species were found to coexist in syntopy in one of the sampling sites.

1. Introduction

Integrative taxonomy (Dayrat, 2005; Will *et al*., 2005) consists of the integration of multiple lines of evidence for speciation in order to shed light on species boundaries (e.g., morphological, genetic, biochemical, ecological or behavioural differentiation, syntopy of organisms diverging in one or more of these features). From a theoretical point of view, it relies on a unified concept of species: species are separately evolving metapopulations and their differentiation in morphology, DNA sequences and other features can emerge at different times during the process of speciation (De Queiroz, 2007). This modern view is becoming increasingly widespread among taxonomists and is receiving large support as an appropriate framework for addressing problems related to species delimitation (e.g., Padial *et al*., 2010; Schlick-Steiner *et al*., 2010). In particular, it has proven useful in organisms where morphology-based taxonomy is inefficient in detecting differences between species due to either intrinsic properties of the organisms (e.g., conservation of morphological features after speciation) or operational limitations for the investigator (e.g., artefacts due to samples preparation). In the same way, taxonomical studies based on DNA alone (e.g., phylogenetic analyses and DNA barcoding applied to species discovery) have been found often inconclusive (e.g., Will et al., 2005; Hickerson *et al*., 2006), especially because it can be hard to disentangle expected intraspecific variation and phylogeographic structure from among-species genetic divergence.

The integrative taxonomy is turning into a cornerstone for exploring species diversity especially for small soil-dwelling animals (e.g., Parmakelis *et al*., 2013; Satler *et al*., 2013; Inäbnit *et al*., 2019). Due to their low vagility and dispersal ability, especially the endogeic animals are expected to display a remarkable genetic structuring among populations, resulting in micro-allopatric diversity with fragmented populations representing genetically divergent lineages. Molecular investigations may overestimate the real species-level diversity in these animals, whereas morphology-based taxonomy often underestimates it (Bond & Stockman, 2008).

Among small soil-dwelling animals, centipedes represent one of the major groups of predators, including to date ca. 3300 species recognized as valid by taxonomists (Edgecombe & Giribet, 2007) and many others expected to be yet discovered and named. Nevertheless, knowledge on their species-level diversity still lags behind. As a matter of fact, the exploration of species boundaries by means of a multidisciplinary approach is still in its infancy (Edgecombe & Giribet, 2019): the first few attempts towards an integrative species delimitation often contradicted the current species taxonomy, suggesting the existence of cryptic or simply overlooked diversity within different groups of centipedes (e.g., Joshi & Karanth, 2012; Joshi & Edgecombe, 2013; 2018; Voigtländer *et al*., 2017). However, the majority of taxonomic studies still rely on morphology alone and without rigorous statistical assessment, and the exploration of species boundaries by means of molecular tools is often not integrated with other lines of evidence in a multidisciplinary framework (see, e.g., Del Latte *et al*., 2015; Wesener *et al*., 2016). In particular, a rigorous integrative

approach has never been employed for assessing the species-level diversity in strictly endogeic, non-vagile centipedes. Hence, we explored the potential of integrative taxonomy focussing on a representative traditional species of strictly endogeic centipede broadly inhabiting forests along the Southeastern Prealps. This region is known to harbour a large amount of diversity, both in terms of local species richness, as it has been shown also for centipedes (Peretti & Bonato, 2018), and in terms of differentiation of cryptic intraspecific lineages and species (e.g., Stefani *et al*., 2012; Štundlová *et al*., 2019).

We focused our research on the geophilomorph centipede *Clinopodes carinthiacus* (Latzel, 1880). This taxon has been hitherto considered as a single species distributed in the Southeastern part of the Alps and possibly also in the western and southern part of the Balkan Peninsula (Bonato *et al*., 2011). While the real identity of the Balkanic populations is still unclear and poorly studied, populations from the Southeastern Prealps were universally considered as belonging to the same species in the scientific literature. As a consequence, we focussed on this system of populations to investigate putative intra-specific variation and to test for the existence of previously undetected species boundaries.

As it is common for small endogeic animals, published records and specimens of *C. carinthiacus* in zoological collections are from a little number of scattered and unevenly distributed localities, with often imprecise localization. Moreover, specimens were often collected decades ago and preserved in 70° ethanol, resulting unsuitable for molecular analyses. To cope with this, on the base of the few known records, we actively sampled populations in a dense set of evenly spaced sites.

With this research, we investigated the genetic and morphological variation among populations of *C. carinthiacus* across the Southeastern Prealps, in order to test for the existence of species boundaries within this population system by applying an integrative protocol for species delimitation.

2. Material and methods

2.1. Field sampling, specimen identification and preparation

As a preliminary step, in order to obtain information on the known distribution, we collected, evaluated and georeferenced all published records of *C. carinthiacus* across the study area. We also obtained unpublished records from the Bonato-Minelli collection of Chilopoda (University of Padova).

We searched for *C. carinthiacus* in 55 sites, ranging from the Brescia and Garda Prealps in the West to the Pohorje in the East. Sampling sites were selected on the basis of the known distribution and the presumed habitat requirements of the species. The sites were at 600-1500 m a.s.l., have remained covered by forest in the last centuries and were not affected by activities other than wood harvesting. For each site we searched within an area of at most 1 ha, entirely

covered by uniform forest vegetation and uniform also in major features of the ground, at least 10 m far from forest edges, roads and other human artefacts. Each site was visited once to four times, in different days in 2017-2019.

During each visit, centipedes were searched by hand in the soil and in the leaf litter, as well as under stones, barks and other shelters by 1 to 5 people working simultaneously. All geophilomorphs were collected, stored in 70° ethanol and then identified in the lab. All specimens were identified at species level using ChiloKey (Bonato *et al.*, 2014).

We obtained specimens of *C. carinthiacus* from 27 sites (Tabs. 1 and S1; Fig. 1). All these sites were separated by areas with unsuitable habitats for *C. carinthiacus* (i.e., lowlands, rivers, valleys deforested since centuries), so that we referred to them as currently populations.

A total of 66 specimens were selected for the analyses (Tab. 1). As far as possible, we selected specimens with developed or partially developed gonopods and no damages or anomalies on the anterior and the posterior parts. For each specimen, the middle portion of the body trunk was used for DNA extraction, whereas the remaining parts were examined for morphological characters.

2.2. DNA extraction, amplification and sequencing

We used DNeasy Blood and Tissue kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). We selected a set of three genes that were successfully employed for other molecular studies in Chilopoda (e.g., Murienne *et al.*, 2010): the mitochondrial genes 16S rRNA and cytochrome c oxidase subunit I (COI), and a fragment of the nuclear gene 28S rRNA. 16S was amplified using primer pair 16Sa/16Sb (Edgecombe *et al.*, 2006) and COI using the primer pair LCO1490/HCO2198 (Folmer *et al*., 1994). For the fragment of 28S we designed the new primer pair 28SIClinoFor (AGTCGTAGGGTCTGCTTCC) and 28SIClinoRev (ATGTCCGTGCTTCAATCC) specifically for *C. carinthiacus*. PCRs were performed in 20 µl reactions containing 4.0 µl of 5X Flexi Buffer, 0.4 µl of 10mM dNTPs, 0.8-1.0 µl of 25 mM MgCl$_2$, 0.5 µl of 100% DMSO, 1.0 µl of each 10 µM primer, 0.1 µl of 5U/µL GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 1 µl of template DNA and purified water. The reaction was carried out as follows: one first step at 95°C for 5 min; then 25-38 cycles consisting of 1 min at 94°C, 1 min at 40-59°C and 1 min 30 s at 72°C; one final step at 72°C for 7 min. PCR products were purified using a MinElute PCR purification kit (Qiagen) and sequenced on both strands with the same primer pairs as used for amplification. Sanger sequencing was performed by Eurofins MWG Operon (Munich, Germany). Empirical chromatograms were visually checked for signal intensity and quality using Finch TV 1.4.0 (Geospiza, PerkinElmer). Forward and reverse sequences of the same gene of each specimen were then aligned with Clustal W2 (Larkin *et al*. 2007) and combined in a single sequence.

2.3. Sequence alignment

16S, COI and 28S genes sequences were aligned with MAFFT (Katoh & Standley, 2013): for 16S

and 28S sequences we used the Q-INS-i algorithm (Katoh & Toh, 2008), which accounts for the secondary structure of the RNA; for COI sequences we used default L-INS-i algorithm (Katoh *et al.*, 2005). All parameters were set to the default settings.

The 16S and 28S alignments were subsampled using Gblocks (Castresana, 2002) with low stringency options as implemented in SeaView v4.7 (Gouy *et al.*, 2010), in order to remove ambiguously aligned regions while maintaining the short motifs of rRNA sequences (Castresana, 2000). The 28S alignment was visually inspected and manually corrected for one minor error in the automatic alignment of identical sequences (1 base shift). Since for 16S ambiguities could not be manually resolved, we used Gblocks with default high stringency options. As a result, all putative indels were removed from 16S alignment. Terminal regions of different length were also manually removed from the COI and 28S alignments.

2.4. Haplotype networks

We used haplotype networks produced with PopART (Leigh & Bryant, 2015) to preliminarily explore variation among the sequences.

For 16S and 28S genes, haplotype networks were produced with the Median-Joining algorithm (Bandelt *et al.*, 1999) as suggested by Fontaneto *et al.* (2015). For the COI alignment we used TCS network (Clement *et al.*, 2002), as the Median-Joining algorithm could not be computed by PopART due to calculation limitations.

2.5. Sequence distances

Pairwise distances between sequences were obtained with MEGA v.7.0.26 (Kumar *et al.*, 2016). They were computed as simple distances (p-distances) for 16S and 28S alignments. Instead, we used distances corrected by Kimura-2-parameter model (k2p distances) for the COI alignment because it contained much more substitutions, and k2p models nucleotide site substitution with multiple mutational hits. Indels were treated with pairwise deletion.

2.6. Species delimitation using sequence data

We employed three methods for species discovery and delimitation by means of sequence data: Automatic Barcode Gap Discovery (ABGD; Puillandre *et al.*, 2012), General Mixed Yule Coalescent (GMYC; Pons *et al.*, 2006) and the Poisson Tree Process (PTP; Zhang *et al.* 2013). The ABGD clusters sequences into candidate species based on the pairwise distances, by detecting an expected difference between lower intraspecific values and higher interspecific values (i.e., "barcoding gap"). The GMYC and the PTP are tree-based methods employing the coalescence model to distinguish between within-population and between-species processes. The GMYC estimates the expected threshold in the branching rate between intra-population base

substitutions (under the coalescent model) and speciation events (under the Yule model) on an ultrametric tree. The specimens are then assigned to candidate species, and a confidence interval for the total number of candidate species is estimated. The PTP is similar to the GMYC, as it relies on the coalescent model to distinguish between intraspecific and interspecific branching events, but it does not require an ultrametric tree. Moreover, PTP estimates Bayesian posterior probabilities for the candidate species.

2.6.1. ABGD

We ran the ABGD method in the online version: 1000 steps were considered in a range of prior values of maximum intraspecific divergence (P) of 0.001-0.12 for 16S, 0.001-0.21 tor COI and 0.001-0.07 for 28S. Upper values of P were selected in order to cover most of the range of variation of the distances calculated for each gene. We specified no prior minimum relative gap width (X).

2.6.2. Tree-based species delimitation methods

We employed the GMYC both in its single threshold (ST-) and multiple threshold (MT-GMYC; Monaghan *et al.*, 2009) implementations, and the PTP both in the original maximum-likelihood and in the Bayesian (bPTP) implementation, on the two mt-DNA loci (16S and COI) separately. In order to build gene trees, we produced alignments using MAFFT (Q-INS-i algorithm for 16S, L-INS-i as default for COI) using sequences from specimens of the related species *Clinopodes flavidus* C.L. Koch, 1847 as outgroup. The 16S was sequenced anew from a specimen from site ROS in the Venetian Prealps (PD-G 7129 in the Bonato-Minelli collection). The COI of *C. flavidus* was downloaded from GenBank (BC ZSM MYR 00485; Spelda *et al.*, 2011). We removed ambiguously aligned regions with Gblocks with high stringency options from the 16S alignment (see paragraph 2.3). We tested also more distantly related outgroups, namely *Pleurogeophilus mediterraneus* (Meinert, 1870) (PD-G 4755 from the Euganean Hills in the South-Eastern Prealps, stored in the Bonato-Minelli collection) and *Strigamia maritima* (Leach, 1817) (GenBank code AY288733.1). However, after deleting ambiguously aligned regions, the 16S alignments with both alternative outgroups resulted shorter, and therefore less informative.

We employed JModelTest in order to find the best fitting substitution models, following both the corrected Akaike Information Criterion (cAIC) and the Bayesian information criterion (BIC). PhyML was used to produce two ML trees per gene, by averaging the models selected by means of the cAIC and BIC respectively. For the species delimitation analyses we chose the cAIC-model-averaged tree for the 16S gene, because of the higher bootstrap node supports, and the BIC-model-averaged tree for the COI, because it contained no polytomies, at difference with the cAIC-model-averaged tree.

For the GMYC analyses, ultrametric trees (i.e., trees where the length of the branches is proportional to absolute or relative time, see paragraph 2.6), were obtained using Reltime (Tamura *et al*. 2012) in MEGA, which estimates a relative time calibration, from the same trees we used for the PTP analysis. For time calibrating procedure we used the most fitting substitution models according to cAIC for the 16S alignment (TrN+I+G) and the most fitting one according to BIC for the COI alignment (HKY+I+G).

GMYC was implemented in its online version (http://species.h-its.org/gmyc/), after removing the outgroup manually. PTP was also implemented in web server version (http://species.h-its.org/), setting all parameter at default; the outgroup was automatically removed by the software.

2.7. Species delimitation using morphological data

Variation in morphological characters was explored in a subsample of the specimens used for the molecular analysis (Tab. 1).

2.7.1. Character choice and definition

We selected and defined a set of 14 characters that encompass all the known morphological variation in adults inside the genus *Clinopodes* and so far reported as putative differential features between species or infraspecific nominal taxa, as well as the variation between populations of *C. carinthiacus* that we found from a preliminary analysis (Tab. 2). Distance measures were taken using a micrometer applied to the eye-piece of a light microscope, after mounting the specimens on a temporary slide. Definitions and operational details of each distance measure are given in Table S2. The body length (Character 1 in Tab. 2) was measured either by a ruler or by a micrometer applied to the eye-piece of the light microscope with the precision of 1 mm. We corrected the number of leg-bearing segments (character 2 in Tab. 2) in males by adding two segments to the counted number, in order to remove the expected variation due to sex dimorphism, since on average females have two pairs of legs more than males within a population (reviewed in Minelli & Koch, 2011). The terminology of morphological parts follows Bonato *et al*. (2010).

2.7.2. Cluster analysis of the specimens based on morphological characters

We carried out two analyses on two different data sets: (i) a broader one using all the characters but body length (character 1), for which we found that within-population variation is largely broader than differences between populations, and (ii) a subset of three characters, for which differences between some of the populations were larger than within-population variation. In order to delimit a set of candidate species, we employed the EM (Expectation Maximisation) Cluster Analysis (Witten & Frank, 2005) as implemented in the software Statistica (Statsoft). This method allows to cluster specimens based on continuous, meristic and categorical variables, without an a-priori assumption

of the number of clusters. The v-fold cross-validation algorithm was used to determine the most likely number of clusters in the dataset. As output, the EM algorithm returns the probability of each specimen to belong to a given cluster. We performed 10 runs of the EM Cluster Analysis, each with 100 iterations of v-fold cross validation, changing the seed number at every run. The v-fold cross-validation tested a number of clusters between 1 and 25. Continuous variables were standardized in order to have mean = 0 and standard deviation = 1. For the continuous variables we assumed a normal distribution, while for the meristic variables we assumed a Poisson distribution.

3. Results

3.1. Sequence distances and haplotype networks

For the 16S gene, we obtained a 421 bp alignment representing 16 different haplotypes, with a maximum pairwise distance of 11.6% (Fig. 2, Tab. S3). A group of similar haplotypes (coded 1-3) were found in the most western sector of the sampling area, from site PDP (Brescia and Garda Prealps) in the west to site BDD (western Venetian Prealps) in the east. In the central part of the investigated area, between site CIS (central Venetian Prealps) and site CUM (Carnic Prealps), we found two clearly divergent groups of haplotypes: one including haplotypes coded 4-8 and 10, the other one including haplotypes coded 9 and 11. Two deeply diverging haplotypes (9 and 10; distance = 11.2%), were found in different specimens in a single site in the Carnic Prealps (BRU). The haplotypes found in the most eastern part of the investigated area, from TRN in the Dinarides to POL in the Pohorje in the East (coded 12-16) have been found exclusively in single sites and are relatively more divergent between each other and from all the previously mentioned haplotypes.

For the COI gene, we obtained a 608 bp alignment representing a total of 25 haplotypes, with a maximum pairwise distance of 25.7% (Fig. 3, Tab. S3). The following clusters of closely related haplotypes can be recognized: a cluster including haplotypes 1-6 (all in the Brescia and Garda and western Venetian Prealps), one including haplotypes 7 and 9 (both in the central Venetian Prealps), one including haplotypes 10 and 11 (both from a single site in eastern Venetian Prealps), one including haplotypes 12 and 13 (in Carnic and Julian Prealps, respectively), one including haplotypes 17 and 18 (both from a single site in the Dinarides), one including haplotypes 21-23 (in Kamnik-Savinja Alps and Pohorje), and another one including haplotypes 24 and 25 (both from site POL in the Pohorje). Like for 16S, also for COI two very divergent haplotypes were found in the single site BRU (distance = 23.7%).

For the 28S gene we obtained a 685 bp alignment representing a total of 9 haplotypes, with a maximum pairwise distance of 7.2% (Fig. 4, Tab. S3). A single heterozygous specimen was found with two very similar alleles that collapsed in a single haplotype after alignment trimming. A group of closely related haplotypes (coded 1-3 and 7-9) was spread across the entire investigated area, whereas another group of haplotypes (coded 4-6) were found only in the Julian and the Carnic

Prealps. Two relatively diverging haplotypes (3 and 4; distance = 6.4%) were found in different specimens in the site BRU.

3.2. Species delimitation with sequence data

Results of species delimitation using sequence data with different methods are summarized in Figures 5 and 6.

3.2.1. ABGD

For 16S haplotypes, the ABGD analysis produced 4 alternative hypotheses of primary partition into 9, 4, 3, and 2 candidate species, assuming higher and higher maximum intraspecific distance (P), before indicating a single candidate species at P>0.0285 (Fig. S1).

For COI haplotypes, the primary partition of the ABGD produced a stable hypothesis of 11 candidate species in the range P=0.001-0.044, but only 7 and 2 candidate species for higher values of P, then a single candidate species for P>0.080. The recursive application of the method produced several additional splits, before converging to the 11-species hypothesis and then indicating 7 and 3 species (Fig. S2).

For 28S haplotypes, the ABGD produced two very stable hypotheses of either 3 or 2 candidate species, before indicating a single candidate species for P>0.064 (Fig. S3).

An hypothesis of 2 species, one spread all across the investigated area and the other one inhabiting only a small area between the Carnic and the Julian Prealps, was recovered consistently by the three loci. The two candidate species were found in syntopy the site BRU, in the Carnic Prealps. Other hypotheses of higher numbers of candidate species were incongruent in the specimen composition between the three loci.

3.2.2. Tree-based species delimitation methods

After applying Gblocks, the alignment of 16S gene sequences including the outgroup was 416 bp long and contained 16 haplotypes of *C. carinthiacus*. We obtained a ML tree with all node supports ranging 81-100%. An hypothesis of 14 candidate species (confidence interval: 8-14) was indicated by the ST-GMYC, while the existence of 9 candidate species (confidence interval: 8-9) was suggested by the MT-GMYC. A much lumper hypotheses of two candidate species resulted from the PTP (in both its implementations): the composition of these two candidate species is in agreement with the 2-species hypothesis suggested by the ABGD (see paragraph 3.2.1). The Bayesian support was 0.52 for the candidate species distributed all across the study area, and 0.93 for the species restricted to the Carnic and Julian Prealps.

The COI gene alignment was identical to the one used for the haplotype network analysis (25 haplotypes of *C. carinthiacus*, 608 bp long) even after adding the outgroup. We obtained a ML tree with all node support ranging 71-100%. Up to 18 candidate species (confidence interval: 17-18)

were suggested by ST-GMYC, 15 (confidence interval: 12-16) by the MT-GMYC and 13 by the PTP (in both its implementations). Bayesian supports for the PTP-delimited species were in the range 0.74-1.0.

3.3. Species delimitation with morphological characters

The EM Cluster Analysis with v-fold cross-validation on all 13 characters suggested the existence of no species boundaries in the population system.

Considering only the three characters with higher inter-population differences in comparison with within-population variation (number of leg-bearing segments, relative length of the denticles on the coxosternite and proportion between the size of the ultimate leg-bearing segment and the forcipular segment; i.e., characters 2, 6 and 12 in Tab. 2), 2 clusters were recovered in all the 10 runs, and each specimen was invariantly assigned to the same cluster in all the runs. In detail, all specimens were confidently assigned to a cluster with probability >0.997, with the single exception of a specimen from the site GUI, in the Venetian Prealps, which was assigned to one of the candidate species with a probability ranging 0.58-0.70.

One of the two candidate species differed from the other for a higher modal number of leg-bearing segments, more elongate denticles on the anterior margin of the coxosternite on average and a slightly larger ultimate sternite in proportion to the forcipules on average. With the exception of the above-mentioned specimen from site GUI, the partition of the specimens into the two candidate species is fully matching the partition into two species supported by the molecular data (see paragraph 3.2.1).

4. Discussion

Considering the information from three different molecular loci and from a set of morphological characters, we obtained several hypotheses of species delimitation within *C. carinthiacus*, most of them in contrast with the established current taxonomic opinion of a single species distributed all across the Southeastern Prealps and the Northern Dinarides.

Unfortunately, despite the sampling effort was successful in detecting and sampling many populations, intra-population variation could not be extensively investigated because of the small number of specimens available per population (Tab. S3). Such shortcoming is common in studies of small soil-dwelling animals (e.g., Hendrixson *et al.*, 2013) and is due to the limited efficacy of the sampling methods available to date for many endogeic species including centipedes.

However, consistency emerged between the different lines of evidence and methods, in indicating the existence of at least two candidate species, with agreement on their composition in terms of sample populations. The same 2-species hypothesis emerged from the 3 loci in the less splitter results of the ABGD and in the PTP on 16S sequences. The same hypothesis was suggested by the EM cluster analysis on a restricted set of morphological characters, i.e., those that were found

to be less affected by body size. Even if the number of sampling sites and specimens used for the different analyses was slightly different, congruent results provide evidence for the existence of two morphologically and genetically distinct species. As a further evidence, the two candidate species were even found to coexist in syntopy in one site (BRU), suggesting the existence of reproductive incompatibility between them (Fig. 7).

The syntopy of two genetically divergent lineages differing also in morphological features allows to rule out confidently the alternative hypothesis of a single species, where populations may be diverging by distance but still potentially interconnected with each other by gene flow. The traditional 1-species hypothesis was actually consistent with the results of the EM cluster analysis using all morphological characters. However, overall variation of characters is expected to be affected by growth allometry and intra-population variation, which introduce noise in the signal of inter-population (and interspecific) variation.

Species delimitation methods applied to mitochondrial loci suggested the existence of many other species boundaries within the investigated population system. In particular, with the exception of PTP on 16S sequences, tree-based species delimitation methods were prone to oversplit the datasets of 16S and COI sequences in a very high number of candidate species. Regarding the GMYC, the oversplitting could be explained by an intrinsic property of this method highlighted by Talavera *et al*. (2013): when there is a disproportion between the extension of the study area of two investigated species due to a sampling bias, the intraspecific distances are unbalanced between species and this could result in an oversplitting of the species that is sampled from a wider area. If the 2-species hypothesis suggested by the other methods is true, one of the species was sampled from a much wider area (i.e., the entire Southeastern Prealps) with respect to the other one (the Carnic and Julian Prealps) and this could have resulted in oversplitting the most widespread species. In the case of MT-GMYC for 16S and ST-GMYC for COI, the confidence intervals for the estimated number of species were pretty narrow, suggesting that there was no bias due to undersampling of one or more species (see Fontaneto *et al*., 2015). However, the GMYC is known to often oversplit the datasets, especially if compared to the PTP, and this has been usually explained with errors in time-calibration of the tree (Pentinsaari *et al*., 2017). Unfortunately, the Reltime method we used for time calibration was not included in the comparative analysis by Talavera *et al*. (2013) who tested alternative methods for dating maximum likelihood phylogenetic trees to be used for GMYC. As a consequence, the performance of the Reltime calibration method for the species delimitation purpose remains to be assessed.

For the 16S gene, the delimitation hypothesis of the ST-GMYC is likely inaccurate, given the broad confidence interval of the resulting number of candidate species. It is worth noting that the MT-GMYC produced a hypothesis of an identical number of putative species (n = 9) if compared to the ABGD; nevertheless, the haplotypes were clustered in different candidate species. In particular, in the 9-species hypothesis obtained with the ABGD all populations from the Brescia and Garda

Prealps, the western Venetian Prealps, the Kamnik-Savinja Alps and the Pohorje are separated into two allopatric candidate species, whereas in the 9-species hypothesis obtained with the MT-GMYC all those populations are grouped in a single candidate species. On the contrary, 3-, 4- and 9-species hypotheses resulting from the ABGD are in agreement with the MT-GMYC in separating a single population from the Western Slovene Prealps (site JAM) from all other candidate species.

A possible explanation of the discrepancies between the results of the ABGD and the tree-based species delimitation methods is that the latter ones are unable to detect species that are not monophyletic in the gene trees, as recently diverged species may have not yet reached reciprocal monophyly (Knowles & Carstens, 2007). This has been empirically observed in many cases across different groups of animals, including arthropods (Funk & Omland, 2003). Another possible explanation is the different amount of information contained in the alignments used for the different methods, because the alignment analysed with the ABGD is shorter (5 bp) than the alignment analysed with the GMYC and may have lost some motifs of the ribosomal structure because of the removal of ambiguously aligned regions.

For COI gene, in the delimitation hypotheses suggested by the ST- and the MT-GMYC specimens from the same site that bear haplotypes differing only for a few substitutions were splitted into separate candidate species. This would imply the existence of syntopic cryptic species sharing an almost identical mitochondrial DNA, which seems very unlikely. Moreover, these splits did not emerge for any other locus with any other method.

Instead, some of the candidate species inferred by the ABGD and the tree-based methods on the mitochondrial genes could actually represent intraspecific divergent genetic lineages, as a genetic structuring between populations at a very small spatial scale is expected, due to the low vagility of the animals under study. This could be the case, for example, of the lineage represented by the populations from the Brescia, Garda and western Venetian Prealps, which was consistently indicated by several methods (i.e., ABGD and PTP on both mitochondrial loci and GMYC on 16S). This does not necessarily imply that these lineages have to be considered as separate species, as mt-DNA divergence between populations is only one of the properties achieved by the populations towards speciation, while we need other evidence in order to confidently recognize them as separate cryptic species. In this view, we maintain a cautious and conservative approach, which is highly recommended in species delimitation practice, as it is preferable to fail in recognizing a lineage as a separate species than drawing non-existing species boundaries. On the other hand, differences in the results of methods employed are expected, as they rely on evolutionary models (i.e., simplifications of the parameter space) that are not overlapping in their assumptions (Carstens et al., 2013).

Summing up, this study represents a first contribution towards the understanding of species boundaries in a species complex of endogeic small animals. The integration of other lines of evidence for speciation (e.g., differentiation in ecological niche) and a broader sampling including

populations from the remaining part of the range of the species complex will be probably helpful in corroborating species boundaries with higher confidence.

Acknowledgements

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Tables

Table 1: sampling sites of *C. carinthiacus* and number of specimens used for each analysis. Mountain ranges refer to SOIUSA classification of the Alps (Marazzi, 2005). Coordinates and altitudes are given in Table S1 in supplementary material.

Site ID	Site	Mountain range	N. of specimens used for the analyses			
			16S	COI	28S	Morphology
PDP	M. Ario : W slope : Passo delle Piazze	Alps : Brescia Prealps	1	1	1	1
COL	M. Colombine : Valle Serramando : Ronco	Alps : Brescia Prealps	3	2	2	3
PAG	Corno Barzo : N slope : Paghera	Alps : Brescia Prealps	2	1	1	2
SOR	Val di Concei : Val Sorda	Alps : Garda Prealps	2	1	2	2
CAS	Val di Ledro : Val Casalino	Alps : Garda Prealps	2	3	3	2
REC	Lessini hills : Recoaro Mille	Alps : Venetian Prealps	1	1	1	1
BDD	Altopiano dei Sette Comuni : Bosco del Dosso	Alps : Venetian Prealps	4	4	4	4
CIS	M. Grappa : M. Cismon : NE slope	Alps : Venetian Prealps	2	2	2	2
MAR	Val di Lamen : Maragno	Alps : Venetian Prealps	4	4	4	3
STU	Val Canzoi : Lago della Stua	Alps : Venetian Prealps	1	1	1	0
CAL	Val del Mis : California	Alps : Venetian Prealps	3	3	3	3
GUI	M. Cesen : Val Caldanè, near Guia	Alps : Venetian Prealps	5	4	3	5
ROS	Val del Grisol : Ponte dei Ross	Alps : Venetian Prealps	3	2	3	2
CPR[a]	Cansiglio : Pian Rosada – Pich	Alps : Venetian Prealps	2	3	2	2
LES	Val Cellina : Bosco Lesis	Alps : Carnic Prealps	1	0	0	1
BRU	M. Valinis : Brusat	Alps : Carnic Prealps	6	7	6	7
ART	Rio Radina valley, near Arta Terme	Alps : Carnic Prealps	1	0	0	1
LUN	M. Sernio : Lunze	Alps : Carnic Prealps	1	0	0	0
SIM	M. San Simeone : Casera S. Simeone	Alps : Carnic Prealps	3	2	3	3
BER	Le Bernadia : M. Lédina : E slope	Alps : Julian Prealps	1	1	2	1
CUM	M. Cum : NW slope	Alps : Julian Prealps	2	3	3	3
TRN	Trnovski gozd : Trnovo-Nemci	Dinarides	1	2	2	2
RAV	Rodica : Kneža valley : Kneške Ravne	Alps: Julian Alps	0	2	1	2
JAM	Jelovica : Jamnik	Alps: Western Slovene Prealps	1	1	1	1
KOM	Komen : Bezovec	Alps: Kamnik-Savinja Alps	2	2	2	2
VEL	Veliki vrh : Kos	Alps: Pohorje	2	2	1	2
POL	Mala Polskava : Lobanškov kogel	Alps: Pohorje	2	2	1	2

Notes: [a] Since the sampling sites in the Cansiglio (Pian Rosada and Pich) were less than 6 km apart within a single mountain plateau, shared similar vegetation and ground features and no ecological barriers (e.g., valleys, rivers, interruptions of forest cover) existed between them, specimens collected in these sites were considered as belonging to the same population (site CPR).

Table 2: morphological characters selected for the species delimitation analysis. See Tab. S2 for operational definitions of the distance measurements.

ID	Name	Definition
1	Body length	Total body length (mm) measured from the anterior margin of the cephalic capsule to the posterior tip of the body (excluding the ultimate legs)
2	Number of leg-bearing segments	-

| 3	Elongation of the XIV articles of the antennae	Average between the ratios between the length and the maximum width of the articles XIV of the antennae. If one of the articles XIV was lacking or anomalous or damaged, only the other article was considered.
4	Proportion between the forcipular segment and the head	Ratio between the maximum width of the forcipular segment, and the maximum width of the cephalic capsule
5	Relative breadth of the forcipular coxosternite	Ratio between the maximum width and the mid-longitudinal length of the exposed part of the forcipular coxosternite
6	Relative length of the denticles of the coxosternite	Ratio between the length of the longest coxosternal denticle and the maximum width of the forcipular coxosternite
7	Elongation of chitin-lines	Chitin-lines reaching the condyles (yes/no)
8	Number of sensorial structures on the first sternite	Number of setae and all other projecting sensilla, as detected through light microscopy
9	Number of sensorial structures on the second sternite	See character 8
10	Elongation of the penultimate metasternite	Ratio between the length of the exposed part and the maximum width of the metasternite of the penultimate leg-bearing segment
11	Elongation of the pore-field of the penultimate metasternite	Ratio between the length of the exposed part of the metasternite of the penultimate leg-bearing segment and the length of the pore-field
12	Proportion between the sternite of the ultimate leg-bearing segment and the forcipular segment	Ratio between the maximum width of the sternite of the ultimate leg-bearing segment, and the maximum width of the forcipular coxosternite
13	Relative number of coxal pores	Residual of the linear regression of the maximum number of coxal pores between the two coxopleura on the length of the same coxopleuron
14	Relative length of the ultimate pair of legs, with respect to the penultimate pair	Ratio between the length of the longest ultimate leg of the ultimate pair, and the length of the longest leg of the penultimate pair

Figures
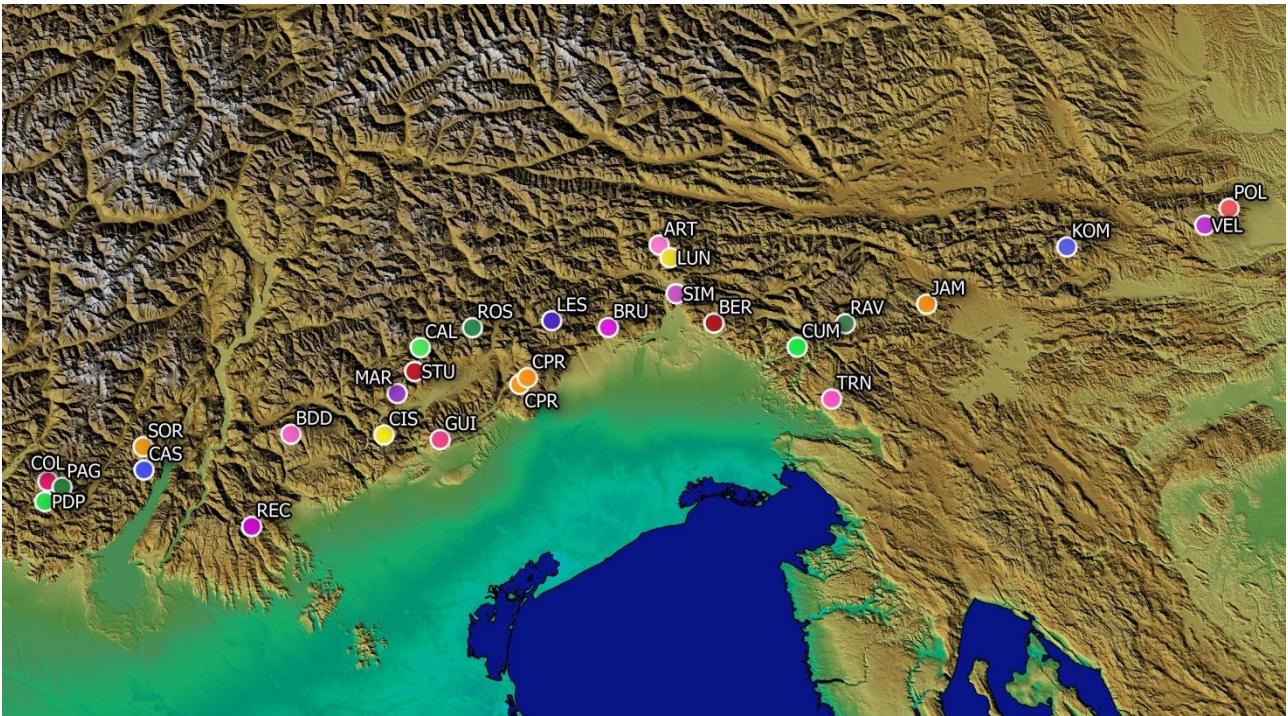
Figure 1: geographic distribution of the sampling sites. Site IDs refer to Table 1. Colours of the localities refer to those used in Figures 2, 3 and 4.
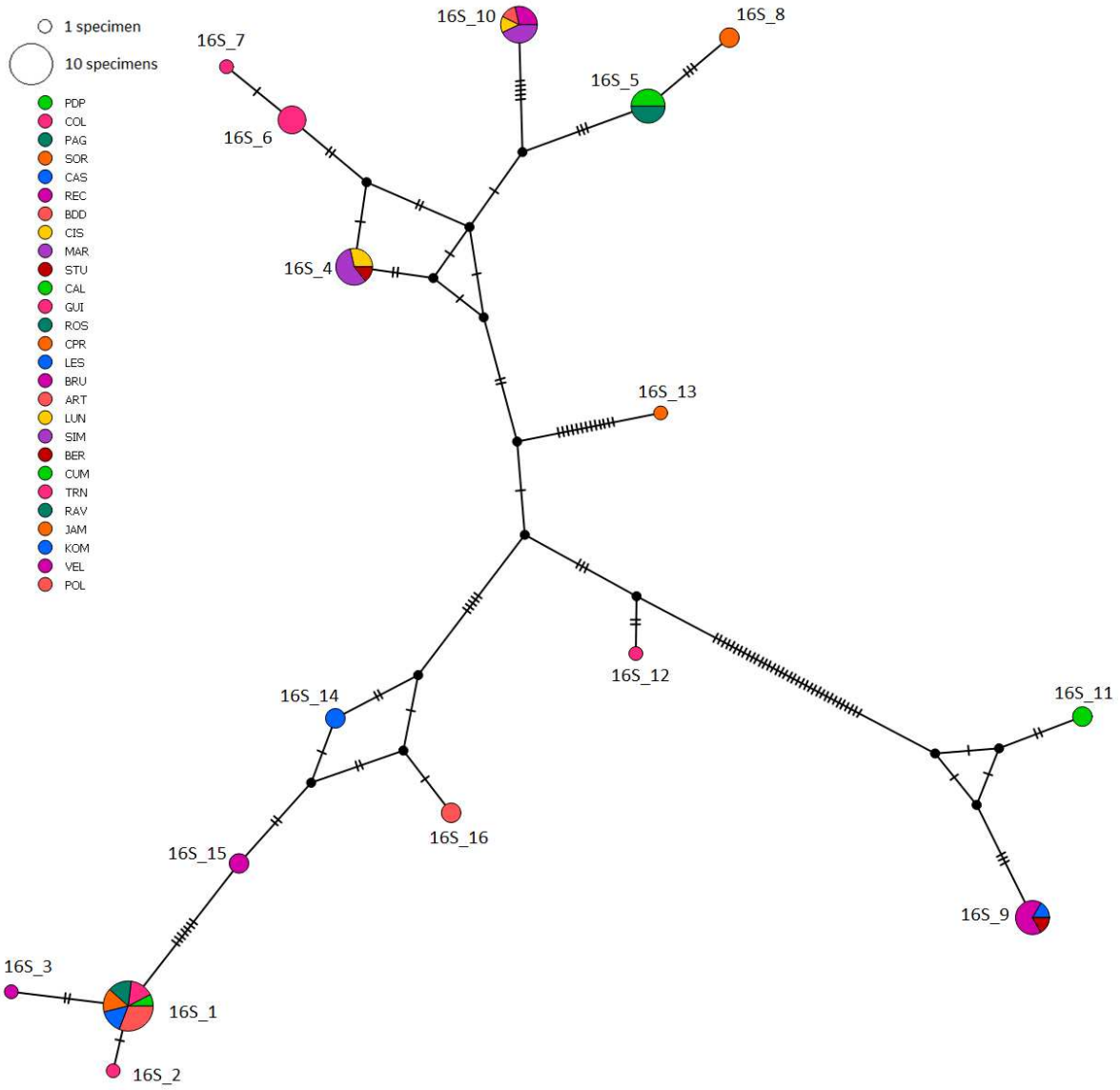
Figure 2: Haplotype network of the 16S gene sequences from 58 specimens from 26 sites.
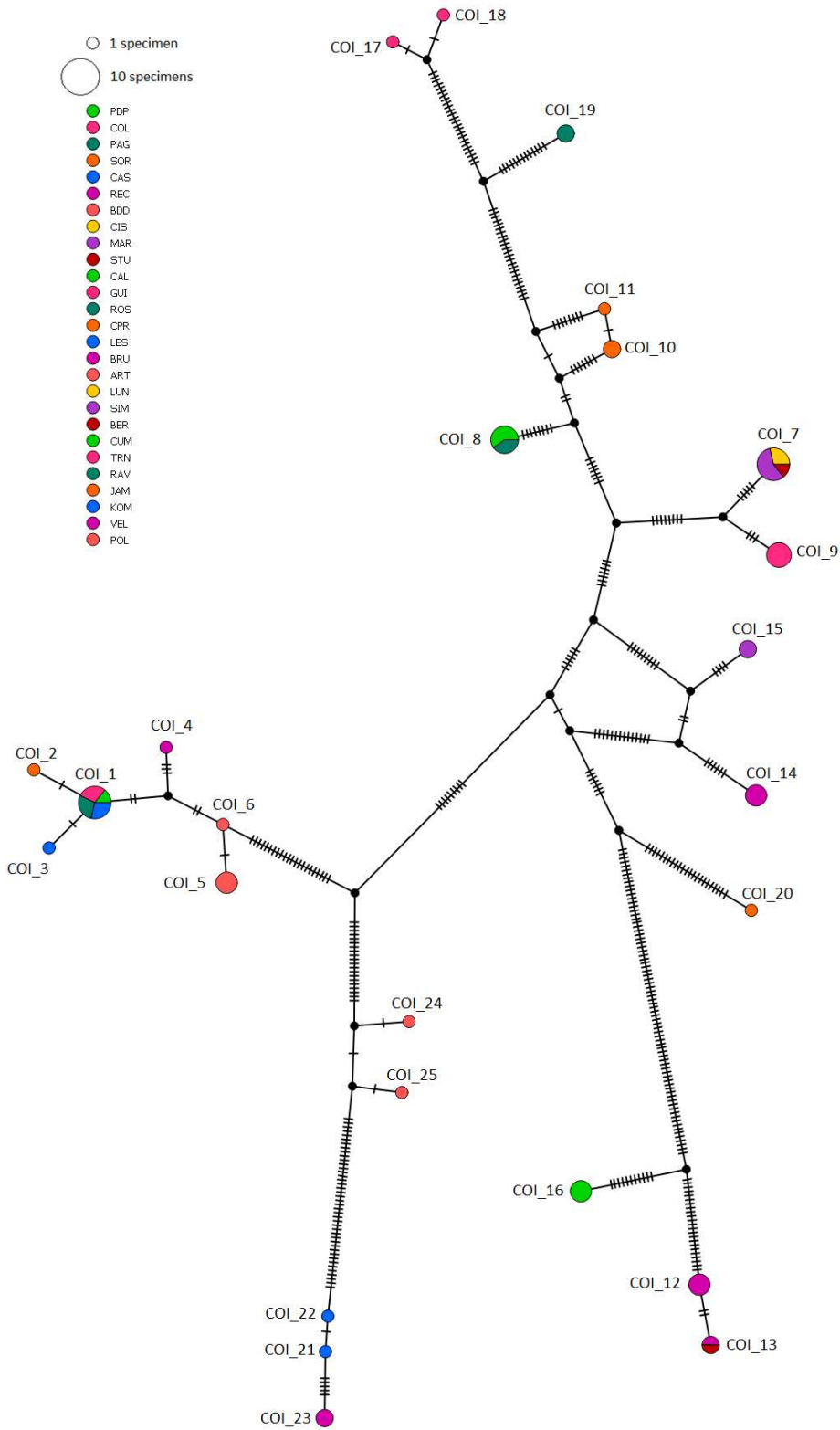
Figure 3: Haplotype network of the COI gene sequences from 56 specimens from 25 sites.
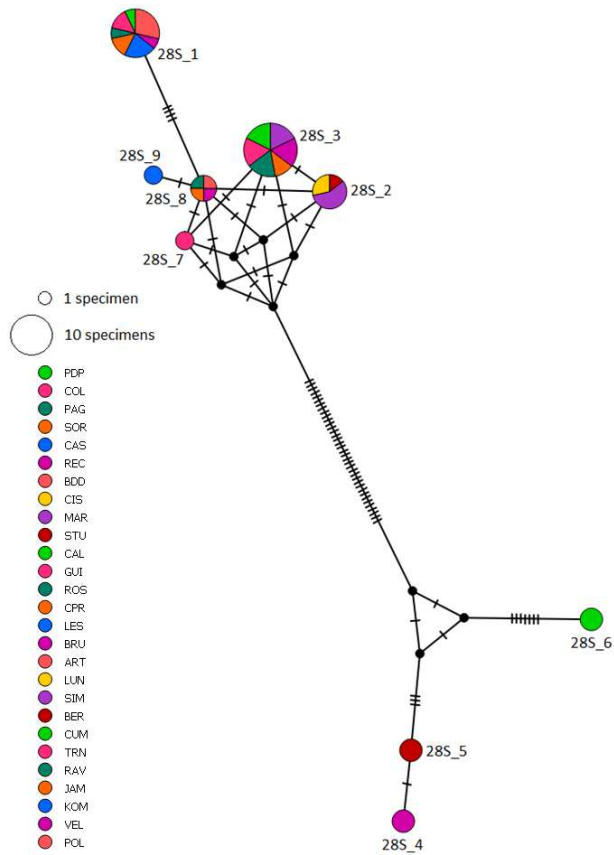
Figure 4: Haplotype network of the 28S gene sequences form 54 specimens from 25 sites.

Figure 5: Subdivision of the 16S haplotypes into candidate species according to different species delimitation methods employed. Mountain range in which the haplotypes were found is also given. The most specious hypothesis resulting from the ABGD method is omitted. Relationship among the haplotypes is represented by the ultrametric tree used for the GMYC analysis.

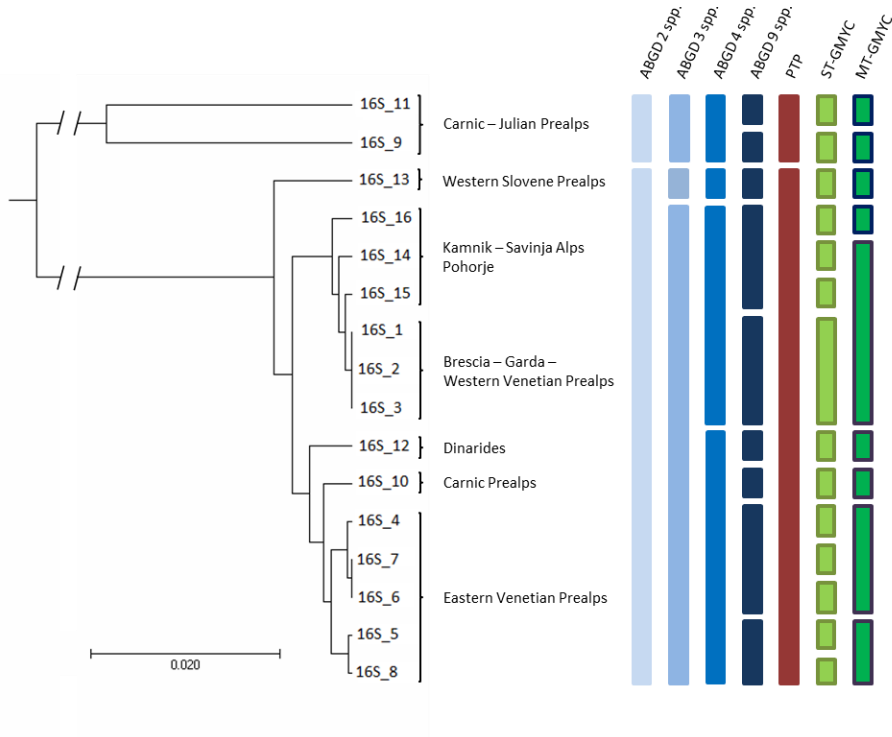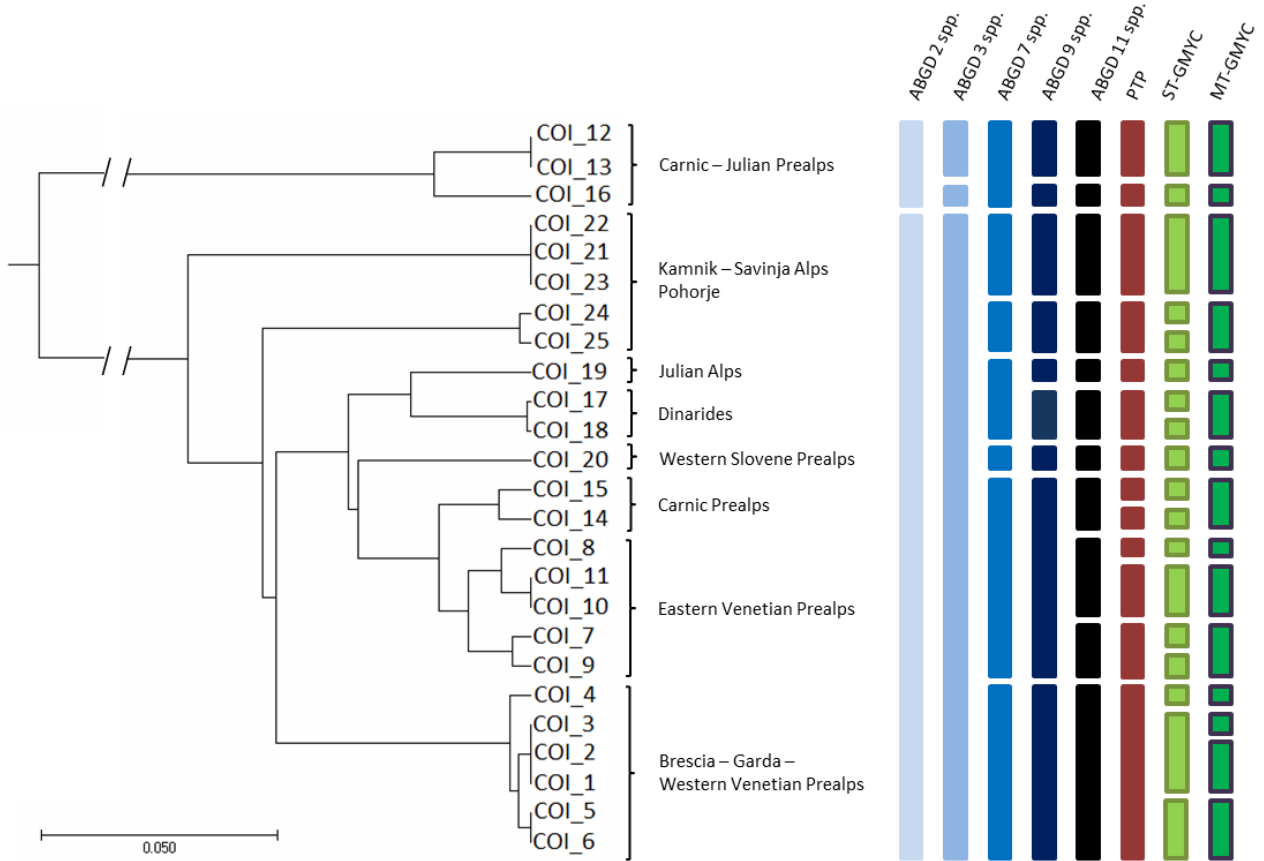Figure 6: Subdivision of the COI haplotypes into candidate species according to different species delimitation methods employed. Mountain range in which the haplotypes were found is also given. More specious hypotheses resulting from the ABGD method are omitted. The ABGD 3- and 9-species hypotheses were obtained through the recursive partitioning approach. Relationship among the haplotypes is represented by the ultrametric tree used for the GMYC analysis.

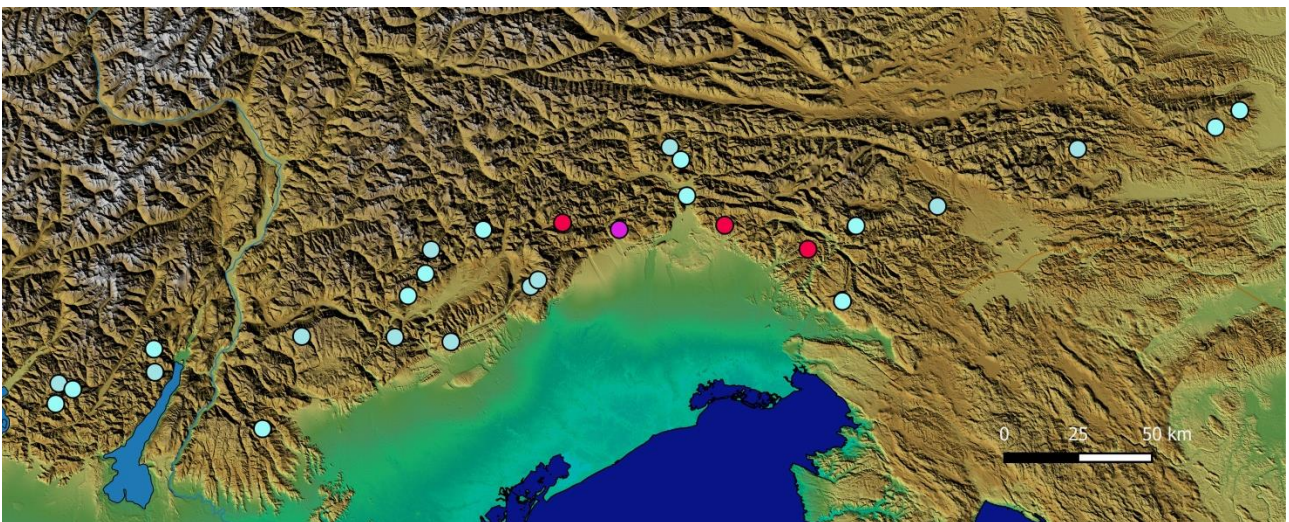Figure 7: Geographic distribution of the two candidate species (light-blue and red dots respectively) found in the South-Eastern Prealps. The purple dot represents the site (BRU) where they have been found in syntopy.

Supplementary material

Table S1: altitude (approximated to the nearest 10 m a.s.l.) and geographical coordinates expressed in decimal latitude and longitude (approximated at the fifth decimal digit) of the sampling sites.

Site ID	Site	Altitude (m a.s.l.)	Latitude (°N)	Longitude (°E)
PDP	M. Ario : W slope : Passo delle Piazze	1250	45.76789	10.31452
COL	M. Colombine : Valle Serramando : Ronco	1220	45.82963	10.32949
PAG	Corno Barzo : N slope : Paghera	1230	45.81104	10.39063
SOR	Val di Concei : Val Sorda	970	45.92889	10.74809
CAS	Val di Ledro : Val Casalino	740	45.85833	10.74982
REC	Lessini hills : Recoaro Mille	1040	45.67821	11.21260
BDD	Altopiano dei Sette Comuni : Bosco del Dosso	1470	45.95555	11.39489
CIS	M. Grappa : M. Cismon : NE slope	1120	45.94398	11.80191
MAR	Val di Lamen : Maragno	840	46.06852	11.86548
STU	Val Canzoi : Lago della Stua	750	46.13376	11.94566
CAL	Val del Mis : California	710	46.20549	11.97414
GUI	M. Cesen : Val Caldanè, near Guia	600	45.92365	12.04571
ROS	Val del Grisol : Ponte dei Ross	760	46.26024	12.20623
CPR	Cansiglio : Pich	1080	46.10071	12.43759
CPR	Cansiglio : Pian Rosada	1120	46.08173	12.40346
LES	Val Cellina : Bosco Lesis	760	46.27096	12.55563
BRU	M. Valinis : Brusat	760	46.24255	12.80281
ART	Rio Radina valley, near Arta Terme	890	46.48535	13.04375
LUN	M. Sernio : Lunze	900	46.44499	13.08919
SIM	M. San Simeone : Casera S. Simeone	1180	46.33462	13.10701
BER	Le Bernadia : M. Lédina : E slope	770	46.23943	13.26651
CUM	M. Cum : NW slope	700	46.15234	13.62498
TRN	Trnovski gozd : Trnovo-Nemci	860	45.98838	13.76218
RAV	Rodica : Kneža valley : Kneške Ravne	700	46.21464	13.84169
JAM	Jelovica : Jamnik	800	46.25824	14.20347
KOM	Komen : Bezovec	1070	46.40212	14.83525
VEL	Veliki vrh	Kos	1050	46.43497	15.44689
POL	Mala Polskava	Lobanškov kogel	860	46.48000	15.55937

Table S2: Definition of all the distance measures taken on specimens of *C. carinthiacus*. Distance measures are ordered according to the position of parts of the body, anterior to posterior.

Name	Magnification	Measurement error	Definition	Measured on paired structures
Length of the XIV article of the antenna	200x	± 5 µm	Mid-longitudinal length of the XIV article of the right antenna, from the anterior margin of the XIII article to the tip of the XIV article	Yes
Width of the XIV article of the antenna	200x	± 5 µm	Maximum transverse width of the XIV article of the right antenna	Yes
Width of the cephalic capsule	100x	± 10 µm	Maximum transverse width of the cephalic capsule	No
Length of forcipular coxosternite	100x	± 10 µm	Mid-longitudinal length of the forcipular coxosternite from the anterior edge of the sternite of the first leg-bearing segment to the mid-point of the medial embayement between the coxosternal denticles	No
Width of forcipular coxosternite	100x	± 10 µm	Maximum transversal width of the forcipular coxosternite	No
Length of coxosternal denticles	400x	± 2,5 µm	Mid-longitudinal length of the coxosternal denticle from the medial embayement between the denticles to the tip of the denticle	Yes
Length of penultimate sternite	200x	± 5 µm	Mid-longitudinal length of the metasternite of the penultimate leg-bearing segment, from the posterior margin of the preceding metasternite (or the posterior margin of the presternite of the measured sternite) to the posterior margin of the measured sternite	No
Width of the penultimate sternite	200x	± 5 µm	Transversal width of the metasternite of the penultimate leg-bearing segment, measured between the mesal margins of the coxae at their shortest distance	No
Length of pore-field of penultimate sternite	200x	± 5 µm	Mid-longitudinal length of the pore-field on the metasternite of the penultimate leg-bearing segment, from the posterior margin of the metasternite to the most anterior pore	No
Length of the telopodite of the penultimate leg	100x	± 10 µm	Total length of the telopodite of the right penultimate leg from the posterior margin of the coxa to the tip of the tarsus 2	Yes
Maximum width of the ultimate sternite	200x	± 5 µm	Maximum transverse width of the ultimate sternite	No
Length of the coxopleuron	200x	± 5 µm	Maximum longitudinal length of the right coxopleuron, from the tip to the posterior margin	Yes
length of the ultimate leg	100x	± 10 µm	Total length of the telopodite of the right ultimate leg from the posterior margin of the coxopleuron to the tip of the tarsus 2	Yes

Table S3: Specimens used for the analyses, site where they have been collected and haplotype of the three genes. Specimens are ordered approximately west to east.

| Specimen	Site ID	Site	16S haplotype	COI haplotype	28S haplotype	Morphometric analysis
PD-G 9084	PDP	M. Ario | W slope | Passo delle Piazze	16S_1	COI_1	28S_1	Yes
PD-G 9113	COL	M. Colombine | Valle Serramando | Ronco	16S_1	COI_1	28S_1	Yes
PD-G 9114	COL	M. Colombine | Valle Serramando | Ronco	16S_1	-	-	Yes
PD-G 9116	COL	M. Colombine | Valle Serramando | Ronco	16S_2	COI_1	28S_1	Yes
PD-G 9151	PAG	Corno Barzo | N slope | Paghera	16S_1	-	-	Yes
PD-G 9153	PAG	Corno Barzo | N slope | Paghera	16S_1	COI_1	28S_1	Yes
PD-G 8414	SOR	Val di Concei | Val Sorda	16S_1	COI_2	28S_1	Yes
PD-G 8420	SOR	Val di Concei | Val Sorda	16S_1	-	28S_1	Yes
PD-G 8431	CAS	Val di Ledro | Val Casalino	-	COI_1	28S_1	Yes
PD-G 8428	CAS	Val di Ledro | Val Casalino	16S_1	COI_3	28S_1	Yes
PD-G 8434	CAS	Val di Ledro | Val Casalino	16S_1	COI_1	28S_1	No
PD-G 9494	REC	Lessini hills | near Recoaro Mille	16S_3	COI_4	28S_1	Yes
PD-G 6728	BDD	Altopiano dei Sette Comuni | Bosco del Dosso	16S_1	COI_5	28S_1	Yes
PD-G 6741	BDD	Altopiano dei Sette Comuni | Bosco del Dosso	16S_1	COI_5	28S_1	Yes
PD-G 6982	BDD	Altopiano dei Sette Comuni | Bosco del Dosso	16S_1	COI_6	28S_1	Yes
PD-G 6986	BDD	Altopiano dei Sette Comuni | Bosco del Dosso	16S_1	COI_5	28S_1	Yes
PD-G 8731	CIS	M. Grappa | M. Cismon | NE slope	16S_4	COI_7	28S_2	Yes
PD-G 8928	CIS	M. Grappa | M. Cismon | NE slope	16S_4	COI_7	28S_2	Yes
PD-G 6708	MAR	Val di Lamen | Maragno	16S_4	COI_7	28S_2	Yes
PD-G 6702	MAR	Val di Lamen | Maragno	16S_4	COI_7	28S_2	Yes
PD-G 7245	MAR	Val di Lamen | Maragno	16S_4	COI_7	28S_2	Yes
PD-G 6703	MAR	Val di Lamen | Maragno	16S_4	COI_7	28S_2	No
PD-G 6163	STU	Dolomiti Bellunesi | Lago della Stua	16S_4	COI_7	28S_2	No
PD-G 7562	CAL	Val del Mis | California	16S_5	COI_8	28S_3	Yes
PD-G 7077	CAL	Val del Mis | California	16S_5	COI_8	28S_3	Yes
PD-G 7086	CAL	Val del Mis | California	16S_5	COI_8	28S_3	Yes
PD-G 7787	GUI	M. Cesen | Val Caldanè, near Guia	16S_6	COI_9	-	Yes
PD-G 7788	GUI	M. Cesen | Val Caldanè, near Guia	16S_7	COI_9	28S_3	Yes
PD-G 7809	GUI	M. Cesen | Val Caldanè, near Guia	16S_6	COI_9	28S_3	Yes
PD-G 7789	GUI	M. Cesen | Val Caldanè, near Guia	16S_6	-	-	Yes
PD-G 7791	GUI	M. Cesen | Val Caldanè, near Guia	16S_6	COI_9	28S_3	Yes
PD-G 7151	ROS	Ponte dei Ross	16S_5	-	28S_3	Yes
PD-G 7146	ROS	Ponte dei Ross	16S_5	COI_8	28S_3	No
PD-G 7161	ROS	Ponte dei Ross	16S_5	COI_8	28S_3	Yes
PD-G 6690	CPR	Cansiglio | Pich	-	COI_10	-	No
PD-G 7960	CPR	Cansiglio | Pian Rosada	16S_8	COI_11	28S_3	Yes
PD-G 7961	CPR	Cansiglio | Pian Rosada	16S_8	COI_10	28S_3	Yes
PD-G 7507	LES	Val Cellina | Bosco Lesis	16S_9	-	-	Yes
PD-G 7673	BRU	M. Valinis | Brusat	16S_9	COI_12	28S_4	Yes
PD-G 6790	BRU	M. Valinis | Brusat	16S_9	COI_12	28S_4	Yes
PD-G 8686	BRU	M. Valinis | Brusat	16S_9	COI_12	28S_4	Yes
PD-G 8694	BRU	M. Valinis | Brusat	16S_9	COI_13	-	Yes
PD-G 7983	BRU	M. Valinis | Brusat	16S_10	COI_14	28S_3	Yes
PD-G 7992	BRU	M. Valinis | Brusat	16S_10	COI_14	28S_3	Yes
PD-G 7902	BRU	M. Valinis | Brusat	-	COI_14	28S_3	Yes
PD-G 8764	ART	Rio Radina valley, near Arta Terme	16S_10	-	-	Yes
PD-G 8980	LUN	M. Sernio | Lunze	16S_10	-	-	No
PD-G 8736	SIM	M. San Simeone | Casera S. Simeone	16S_10	COI_15	28S_3	Yes
PD-G 8737	SIM	M. San Simeone | Casera S. Simeone	16S_10	-	28S_3	Yes
PD-G 8739	SIM	M. San Simeone | Casera S. Simeone	16S_10	COI_15	28S_3	Yes
PD-G 8649	BER	Le Bernadia | Monte Lédina | E slope	16S_9	COI_13	28S_5	Yes
PD-G 8660	BER	Le Bernadia | Monte Lédina | E slope	-	-	28S_5	No
PD-G 7924	CUM	Monte Cum | NW slope	16S_11	COI_16	286_6	Yes
PD-G 7925	CUM	Monte Cum | NW slope	-	COI_16	286_6	Yes
PD-G 7922	CUM	Monte Cum | NW slope	16S_11	COI_16	286_6	Yes
PD-G 9995	TRN	Trnovski gozd | Trnovo-Nemci	16S_12	COI_17	28S_7	Yes
PD-G 10128	TRN	Trnovski gozd | Trnovo-Nemci	-	COI_18	28S_7	Yes
PD-G 10017	RAV	Rodica | Kneža valley | Kneške Ravne	-	COI_19	28S_8	Yes
PD-G 10018	RAV	Rodica | Kneža valley | Kneške Ravne	-	COI_19	-	Yes
PD-G 9897	JAM	Jelovica | Jamnik	16S_13	COI_20	28S_8	Yes
PD-G 9793	KOM	Komen | Bezovec	16S_14	COI_21	28S_9	Yes
PD-G 9796	KOM	Komen | Bezovec	16S_14	COI_22	28S_9	Yes
PD-G 9971	VEL	Veliki vrh | Kos	16S_15	COI_23	-	Yes
PD-G 9972	VEL	Veliki vrh | Kos	16S_15	COI_23	28S_8	Yes
PD-G 9924	POL	Mala Polskava | Lobanškov kogel	16S_16	COI_24	28S_8	Yes
PD-G 9926	POL	Mala Polskava | Lobanškov kogel	16S_16	COI_25	-	Yes
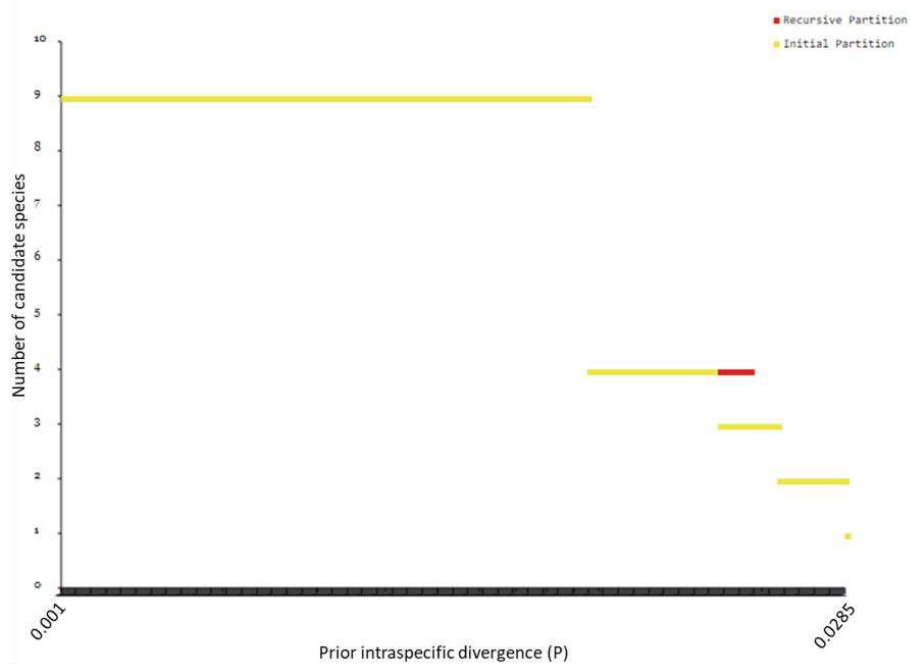
Figure S1: Number of candidate species suggested by the ABGD on 16S sequences before collapsing to 1 at P=0.0285.
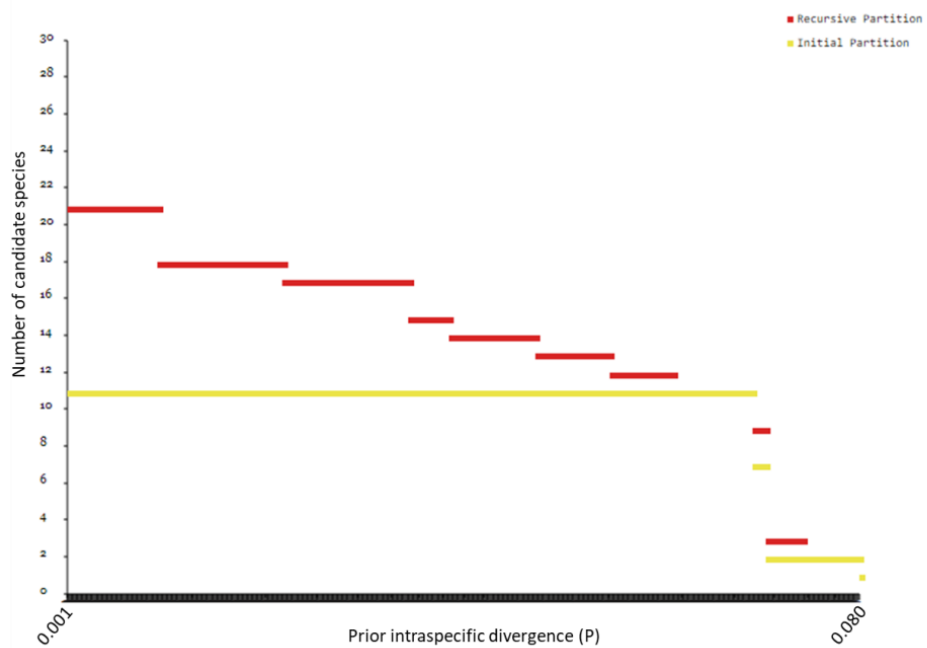

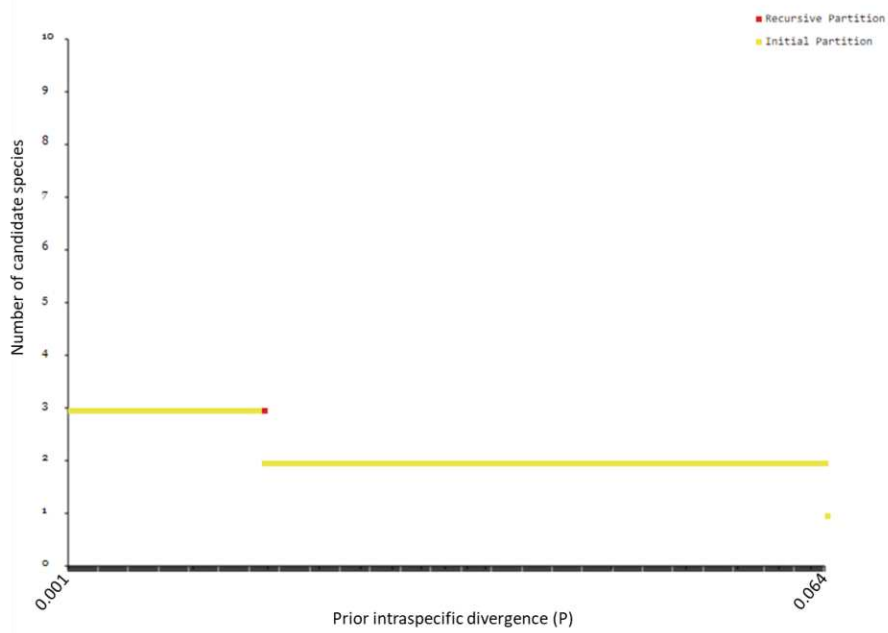Figure S2: Number of candidate species suggested by the ABGD on COI sequences before collapsing to 1 at P=0.0800.

Figure S3: Number of candidate species suggested by the ABGD on 28S sequences before collapsing to 1 at P=0.0640.

CONCLUSIVE REMARKS AND PERSPECTIVES

This thesis work provides new insights on some poorly known aspects of the diversity of centipedes and of soil-dwelling animals in general; however, knowledge on the investigated issues still remains fragmentary, as they were addressed here in the form of case studies.

The statistical models applied to estimate species richness in chapter I proved effective in 9 out of 10 investigated centipede communities. The application of these simple estimators in future studies involving abundance or incidence data of centipede communities would allow comparative studies on estimates from different habitats and biogeographic domains, thus providing useful insights for addressing other general aspects of community ecology such as niche partitioning.

In chapter II we successfully applied a protocol for integrative taxonomy, which includes multi-locus molecular-based and morphology-based species delimitation analyses, for the first time on strictly endogeic centipedes. Along with the increasing yet poor number of similar studies on epigeic centipedes (see Edgecombe & Giribet, 2019 for a review), this achievement will hopefully encourage the use of the integrative approach in the upcoming taxonomic studies on poorly vagile Chilopoda. On the other hand, future investigations may take advantage of the integration of new sources of inference, some of them already successfully employed for other soil invertebrates, e.g., chemotaxonomic characters (Wachter et al., 2015), changes in chromosome number and structure (Štundlová et al., 2019) and ecological niche differentiation (Bond & Stockman, 2008).

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