Ecological speciation in darkness? Spatial niche partitioning in sibling subterranean spiders (Araneae: Linyphiidae: Troglohyphantes)

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Ecological speciation in darkness? Spatial niche partitioning in sibling subterranean spiders (Araneae: Linyphiidae: *Troglohyphantes*)

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Author contribution statement

EP, MI and SM performed fieldwork in the Pugnetto hypogean complex. EP sorted biological specimens collected in the subterranean sampling devices. SM and MI described the new species. NC measured specimens for the ecological analysis. SM analysed ecological data. SM and MI wrote the first draft of the paper. MA conducted the analyses of the molecular data and wrote the relative sections. PP supervised the preparation of morphological drawings, provided specimens stored in the museum collection and revised the species description. All authors contributed to the writing of the paper trough discussions and additions to the text.

Conflict of Interest statement

The authors declare no conflicts of interest.

Abstract

Speciation in subterranean habitats is commonly explained as the result of divergent selection in geographically isolated populations; conversely, the contribution of niche partitioning in driving subterranean species diversification has been rarely quantified. We integrated molecular and morphological data with a hypervolume analysis based on functional traits to study a potential case of parapatric speciation by means of niche differentiation in two sibling spiders inhabiting contiguous subterranean habitats within a small alpine hypogean site. *Troglohyphantes giachinoi* n. sp. and *T. bornensis* are diagnosed by small details of the genitalia, which are likely to be involved in a reproductive barrier. Molecular analysis recovered the two species as sister, and revealed a deep genetic divergence that may trace back to the Messinian (~6 My ago). The hypervolume analysis highlighted a marginal overlap in their ecological niches, coupled with morphological character displacement. Specifically, *T. giachinoi* n. sp. exhibits morphological traits suitable for thriving in the smaller pores of the superficial network of underground fissures (MSS), whereas *T. bornensis* shows a higher adaptation to the deep subterranean habitat. Our results suggest that different selective regimes within the subterranean environment, i.e. deep caves versus MSS, may either drive local speciation or facilitate contiguous distributions of independently subterranean adapted species.

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Although speciation is most often explained as the result of divergent selection in geographically isolated populations, alternative evolutionary scenarios have been documented by scientists. We describe how two sibling spiders may have originated via ecological differentiation in two contiguous and interconnected subterranean habitat with different size of void spaces. Our data demonstrates that habitat size may drive species differentiation within the subterranean realm, by minimizing intraspecific competition through niche partitioning, and provides further evidence of the importance of space heterogeneity as an evolutionary driver.

Introduction

While allopatric speciation — divergence with geographic isolation — is the most common process originating biological diversity (Coyne and Orr 2004), a body of evidence indicate that species divergence may occasionally occur with limited or even without geographical isolation — parapatric and sympatric speciation, respectively (Futuyma and Mayer 1980; Tregenza and Butlin 1999; Gavrilets 2003; Jiggins and Chris 2006; Bolnik and Fitzpatrick 2007). For instance, such speciation processes have been observed in fruit flies (Filchak *et al.* 2000), palm trees (Savolainen *et al.* 2006) and cichlid fish (Berluenga *et al.* 2006; Gavrilets *et al.* 2007). Parapatric and sympatric mode of speciation are often the result of ecologically-based divergent selection, i.e. ecological speciation (Mayr 1942, 1947). Three main ecological drivers of divergent selection leading to speciation have been put forward in literature (Rundle and Nosil 2005; Schluter and Conte 2009): sexual selection (Panhuis *et al.* 2001; Kirkpatrick and Ravigné 2002), species interaction (Schluter 2000), and environmental differentiation (Schluter 2000, 2001). The latter mechanism is probably the best documented and understood, and can occur when two populations occupy distinct ecological niches — here intended as the set of environmental conditions and/or functional traits maximizing the fitness of a certain species — and hence are subject to two different selective regimes, within the same habitat (Rundle and Nosil 2005).

Since the beginning of modern subterranean biology in the 1900's, researchers have recognized the subterranean domain as a well-suited system in which to test eco-evolutionary theories, especially those related to the processes of convergent (Jones et al. 1992; Wiens *et al.* 2003; Wilcox *et al.* 2004; Juan *et al.* 2010; Protas and Jeffery 2012; Gonzalez et al. 2018) and regressive evolution (Heuts 1953; Porter and Crandall 2003; Jeffery 2009; Rétaux and Casane 2013; Klaus *et al.* 2013). Also, subterranean habitats represent ideal model systems in which to study allopatric speciation. Most caves are indeed regarded as "islands" surrounded by inhospitable habitats (e.g., Culver 1970a, 1971, Snowman *et al.* 2010; Esposito *et al.* 2015; Fattorini *et al.* 2016), resulting in low gene flow among cave populations (e.g. Caccone 1985; Mammola *et al.* 2015b; Weckstein *et al.* 2016) and frequent speciation events connected to this geographic isolation (Barr and Holsinger 1985). This pattern is empirically confirmed by the documented higher proportion of subterranean endemic species relative to their surface counterparts (e.g., Sharratt *et al.* 2000; Christman *et al.* 2005; Cardoso 2012; Niemiller and Zigler 2013; Wynne *et al.* 2014). Nevertheless, mechanisms other than geographic isolation have been sometime invoked to explain the origin of subterranean organisms (Juan *et al.* 2010). Niche-based processes, for example, may play a key role in shaping subterranean species diversity (e.g., Culver 1970b; Fišer C. *et al.* 2012, 2015; Fišer Z. *et* *al.* 2015), whereas parapatric or even sympatric speciation modes may explain the origin of certain taxa, such as the *Astyanax* cave fishes from Mexico (Wilkens and Hüppop 1986; Strecker *et al.* 2012), the Tennessee cave salamanders (Niemiller *et al.* 2008; also discussed in Nosil 2008), the subterranean diving beetles from Australia (Cooper *et al.* 2002; Leys *et al.* 2003; Leys and Watts 2008) or the dysderid spiders from the Canary Islands (Arnedo *et al.* 2007).

In subterranean biology, the habitats of focus are not exclusively underground voids of wide dimensions (i.e. caves), but all the aphotic air- and water-filled underground spaces harbouring specialised subterranean species, even interstices with sizes that are not commensurable to the human scale. Among the latter, the so-called *Milieu Souterrain Superficiel* (MSS) is one of the most intensively studied (Mammola *et al.* 2016b). As a general definition, the MSS is the system of empty air-filled interstices within rocky debris that have accumulated for various morphogenetic reasons above the bedrock, offering suitable environmental conditions for the survival of subterranean species (Juberthie 1980, 1981; Uéno 1987; Culver and Pipan 2014; Mammola *et al.* 2016b). The topsoil layers, the MSS and the deep subterranean voids (caves and deep fissures) are often contiguous and intimately interconnected, generating a vertical gradient *"from soil to cave*" (Gers 1998). This physical interconnection directly implies exchanges of different types, such as nutrient circling, species migration and faunal commingling between the soil, cave and MSS habitats (Gers 1998, Giachino and Vailati 2010, Rendoš *et al.* 2012; Culver and Pipan 2014, Nitzu *et al.* 2014; Mammola *et al.* 2016b, 2017).

During a one-year ecological study in the hypogean complex of Pugnetto (Graian Alps, NW-Italy), we sampled invertebrates dwelling in the caves and in the associated superficial network of underground fissures (Mammola *et al.* 2017). The hypogean complex of Pugnetto consists of five natural caves and a well developed MSS, made of rocky fragments forming a network of fissures ranging from 0.1 to 10–20 cm, covered by mature beech forest soil. This underground system is an important hot-spot of subterranean biodiversity in Europe, hosting a diversified cave invertebrate fauna including numerous local endemics (Capra, 1924; Arcangeli 1940; Sturani 1942; Capra and Conci 1951; Casale 1980; Vailati 1988; Isaia and Pantini 2008; Mammola *et al.* 2015a, 2016a, 2017; Isaia *et al.* 2017). The caves of the Pugnetto complex host, among others, two spider of the genus *Troglohyphantes* (Araneae: Linyphiidae): *T. bornensis* Isaia & Pantini and *T. lucifer* Isaia, Mammola & Pantini (Isaia and Pantini 2008; Isaia *et al.* 2011, 2017). In the MSS, we collected individuals of a putative third species of *Troglohyphantes* (see Appendix I in Mammola *et al.* 2017). The examination of morphological details of the male palp as well as the female epigyne, revealed a close similarity to *T. bornensis.* In this paper, we combine a detailed morphological study with molecular and ecological analyses and confirm that the MSS-dwelling *Troglohyphantes* is a distinct species, sister to *T. bornensis.* We discuss the origin of these two sibling species in light of their niche differentiation and divergence time.

Material and Methods

Taxonomy

We stored specimens in 75% ethanol at the Museo Civico di Scienze Naturali "E. Caffi" (Bergamo, Italy), except a few specimens — labelled with the acronym "CI" — which we stored in Marco Isaia's collection at Department of Life Sciences and Systems Biology, University of Turin (Torino, Italy). We studied specimens using a Leica M80 stereoscopic binocular. Illustrations were made by Elena Pelizzoli, using a camera lucida. All measurements are in millimetres (mm). We referred to Isaia *et al.* (2017) for *Troglohyphantes* anatomical terms and to World Spider Catalog (2018) for current nomenclature. For the toponomastic and classification of the different sectors and sub-sectors of the Alps, we followed the standard partition of the alpine chain (SOIUSA; Marazzi 2005). Whenever applicable, we gave the speleological cadastral codes of the caves in squared brackets ['regional code' and 'number']. We used the following abbreviations in the text: ALE = anterior lateral eyes; AME = anterior median eyes; Cg = copulatory groove; E = Embolus; Fg = fertilization groove; LC = Lamella characteristica; MSS = *Milieu Souterrain Superficiel* (as defined in Mammola *et al.* 2016b); PC = Paracymbium; PLE = posterior lateral eyes; PME = posterior median eyes; Pp = Posterior plate; S = spermathecae; SA = Suprategular apophysis; SSD = Subterranean Sampling Device (López and Oromì 2010); Te = Tip of the embolus; TLL = total leg length; TmI = position of trichobothrium on metatarsus of first leg.

Analysis of morphological traits and niche overlap

From a morphologically oriented perspective, niche partitioning can be studied by analysing variations in morphological traits in multi-dimensional morphospace (Blonder 2017). We examined twenty-five female specimens for six characters related to body size, trophic specialisation and degree of subterranean adaptation (troglomorphism *sensu* Christiansen 2012). See Table 1 for the full list of morphological traits considered and their ecological and adaptive function. We calculated pairwise Pearson *r* correlations between the morphological variables to avoid autocorrelation in morphometric data, setting a threshold for collinearity at $r > |0.7|$ (Zuur *et al.* 2010). In turn, we used uncollinear variables to calculate the Hutchinsonian niche hypervolumes of the two species, as implemented in the *hypervolume* R package (Blonder 2015). To calculate the hypervolumes, we rescaled each variable by subtracting mean and dividing by standard deviation. We automated the choice of bandwidth for each variable through a Silverman estimator, using a threshold that included 100% of the total probability density (Blonder et al. 2014). To compare the morphospace of the two species, we calculated total volume dimension, intersection of both hypervolumes and the Sørensen–Dice niche overlap using the relevant functions of the *hypervolume* R package (Blonder 2015).

Molecular analysis

We added 11 new cytochrome *c* oxidase subunit 1 gene (COI) sequences to the data matrix of Isaia *et al.* (2017). The increased taxonomic sampling consisted of two individuals of the new species, four individuals of the putative sister species *Troglohyphantes bornensis*, one individual each of additional species belonging to the same species complex as the new species (*T. microcymbium*) and a species belonging to a *Troglohyphantes* species complex not sampled before (*T. sbordonii*; *Diurnus* species complex). We also included a few additional individuals of species already sampled in the former study (Supplementary Material Table S1). In all analyses we used the Canarian species *T. oromii* (Ribera & Blasco) as an outgroup.

Wet lab methods followed the protocols detailed in Mammola *et al.* (2015b). We edited and managed sequences using Geneious R10.2.3 (Kearse *et al.* 2012). We detected no evidence of indel mutations and hence alignment was trivial. The data matrix was subject to parsimony, maximum likelihood and Bayesian phylogenetic analyses. We conducted parsimony analysis with TNT v.1.1 (Goloboff *et al.* 2008) using 1,000 iterations of Wagner trees, followed by TBR branch swapping, and clade support assessed with 1,000 Jackknife resampling replicates for a removal probability of 36%. We assessed best partitioning schemes and substitution models simultaneously with PartitionFinder v.1.0.1 (Lanfear *et al.* 2012) under a Bayesian information criterion (BIC). Maximum likelihood (ML) analysis was conducted in RAxML v.8.11 (Stamatakis 2014), under the raxmlGUI v.1.5 (Silvestro and Michalak 2011) frontend. We inferred the best ML tree and bootstrap support, using the MRE convergence criteria to automatically determine the right number bootstrap replicates. We conducted Bayesian (BI) analysis in BEAST v.1.8 (Drummond *et al.* 2012). We defined partitions and models following results of Partitionfinder, i.e. a birth and death tree prior and relaxed uncorrelated lognormal. We estimated absolute divergence times by assigning a normal distributed on the substitution rate prior (ucdl.mean), truncated at 0, with a starting value of 0.0199, mean value 0.02, standard deviation 0.006 (95% probability interval 0.0136–0.0270) based on spider COI rates available in the literature (Bidegaray-Batista and Arnedo 2011). The tree obtained with RAxML was used as starting tree for the Bayesian analyses. We ran three independent chains of 10 million generation each, sampling every 1,000 generations. We monitored the chain convergence, the correct mixing (EES) and the number of generations to discard as burn-in with Tracer v.1.6 (Rambaut and Drummond 2013). We discarded the first 10% of trees in each run as burn-in.

We further used the BEAST ultrameric tree to identify coalescent groups (i.e. putative species) using the Generalized Mixed Yule-Coalescent method (Fujisawa and Barraclough 2013). We estimated a neighbor-joining tree based on the uncorrected genetic distances between all the specimens in MEGA7 (Kumar *et al.* 2016), and subsequently exported it into Geneious to estimate species delimitation parameters using the Species Delimitation plugin (Masters *et al.* 2011).

Results

Taxonomic account

Family **LINYPHIIDAE** Blackwall

Genus ***Troglohyphantes*** Joseph

Troglohyphantes giachinoi Isaia & Mammola, n. sp.

Type series

<u>Holotype.</u> Italy, Piemonte, Province of Torino, Mezzenile, Pugnetto: SSD in MSS 0.60 m deep, N 45° 16’ , E 5° 02’, 01.iv–01.v.2014, Isaia, Mammola & Piano leg. 1♂

<u>Paratypes.</u> Italy, Piemonte, Province of Torino, Mezzenile, Pugnetto: SSD in MSS 0.60 m deep, 4.vi.2012–1.ix.2012, Isaia, Mammola & Piano leg. 2♂♂, 1 juv.; SSD in MSS 0.60 m deep, 1.v.2014–1.vi.2014, Isaia, Mammola & Orlandini leg. 1♂; pitfall trap in deep leaf litter, 1.v.2014–1.vi.2014, Isaia, Mammola & Orlandini leg. 1♀; pitfall trap in deep leaf litter, 1.v.2014–1.vi.2014, Isaia, Mammola & Orlandini leg. 2♂♂ (CI); SSD in MSS 0.40 m, 01.iv–01.v.14, Mammola & Piano leg. 1 ♀; pitfall trap in deep leaf litter, 12ix.2013, Isaia, Mammola & Orlandini leg. 1♀; Italy, Piemonte, Province of Torino, Almese, Viù: Colle del Lys, 1200 m, 10.x.1972, Thaler leg. 4♀♀ 1♂1juv; Colle del Lys, 1300 m, 9.x.1973, Thaler leg. 1♀, 1juv.

Diagnosis

Males of *Troglohyphantes giachinoi* n. sp. are primarily distinguished from other species of *Troglohyphantes* by the shape of the *lamella characteristica* (Fig. 1a), better viewed in lateral view. The new species is close to *T. bornensis* (*Microcymbium* complex), from which it is distinguishable by the gun-like lamella chracteristica, with the horizontal branch short and the upper branch sub-triangular, connected at its base to the horizontal branch. The shape of cymbium, paracymbium, median apophysis and embolus are undistinguishable to that of *T. bornensis*.

Females are best diagnosed by the epigynum viewed ventrally, although differences with *T. bornensis* are very subtle (*cf.* Fig. 2b and 2d). The epigyne bears a subtriangular scape, rounded apically, approximately as wide as long. In comparison with *T. bornensis,* the base of the scape is narrower and the scape is longer. The posterior plate of the epigyne in ventral view is trapezoidal in *T. giachinoi* n. sp., and rectangular and wider in *T. bornensis*. Subtle additional diagnostic characters — better viewed ventrally on cleared epigyne — are found in the shape of the copulatory grooves, diverging medially towards the base of the scape. In comparison, in *T. bornensis* the copulatory grooves are bent backwards, towards the outer margin of the scape, recalling the shape of a heart. Compared to other congeneric species of the Western Italian Alps, male and female overall size and leg length are smaller. The only species of comparable size is *T. iulianae* Brignoli.

Description

Male holotype: overall size and leg length small. Prosoma 1.34 long, 0.93 wide, light-yellowish. Thoracic region slightly swollen, yellowish with grey shades. Cephalic region slightly elevated, interspersed with black bristles between eyes (Fig. 1c). Clypeus slightly indented under the eyes, then convex, 0.34 long. Eyes normally developed, with pigment and black margins. AME smallest. PLE very slightly bigger than PME, ALE slightly larger than PLE. ALE and PLE nearly contiguous (distance = 0.01). PLE–PME distance = 0.07, ALE–AME distance = 0.06, PME–PME distance = 0.06. Eye diameters: AME 0.03, PME 0.06, ALE 0.06, PLE 0.06. Sternum heart-shaped, yellowish with flimsy darkened anterior edges. Chelicerae 0.62 long, light brownish, with 18 lateral stridulatory ridges and armed with three anterior teeth. Legs uniformly light yellowish. Leg measurements as in Table 2. Abdomen 1.56 long, 0.93 wide, light-yellowish with dark setae. Palp (Fig. 1a) with cymbium faintly convex, subtriangular when seen from above, ending proximally in a single tooth-like apophysis, rounded at the proximal border (Fig. 1b). Posterior part of paracymbium identical to *T. bornensis*, sub-triangular, apical part gradually narrowed anteriorly.

Suprategular apophysis directed upwards, with a sharp end. Tip of the embolus spiky. Lamella characteristica gun-like shaped with the horizontal branch short. The upper branch sub-triangular, connected at its base to the horizontal branch. Spination: femur I with one dorsal and one prolateral spines; Femur II, III and IV with one dorsal spine. Patella I–IV with one dorsal and one retrolateral spines. Tibia I with one dorsal, one prolateral, and two retrolateral spines. Tibia II with one dorsal, and two retrolateral spines; tibia III and IV with one dorsal and one retrolateral spines. Metatarsus I–IV with no spine. Patella of the Palp with one curved spine. Position of TmI: 0.23. Trichobothrium on Mt IV absent.

Female (paratype from the same locality as holotype): overall size and leg length small when compared to other congeneric in the Western Italian Alps. Prosoma 0.87 long, 0.67 wide, slightly darker than in male. Cephalic region greyish. Carapace, ocular area, clypeus, and sternum similar to male in all features except cephalic bristles, being smaller. Clypeus 0.18 long, chelicerae 0.70 long. Anterior margin of the chelicerae armed with three teeth. PLE–PME distance = 0.02, ALE–AME distance = 0.03, PME–PME distance = 0.03, AME–AME distance = 0.00, ALE–PLE distance = 0.00. Eye diameters: AME 0.01, PME 0.02, ALE 0.02, PLE 0.03. Abdomen 1.35 long, 0.93 wide, greyish, with black hairs. Leg measurements as in Table 2. Epigyne strongly protruding. Epigynal plate excised, defining a U-shaped scape, with two small lateral incisions (Fig. 2a). Scape scarcely arched from a lateral view, half covering the inner part of the epigyne. Stretcher tongue-shaped almost straight, slightly bent upwards toward the scape, bearing a pitted knob at its end clearly visible from a ventral point of view. Posterior plate of the epigyne trapezoidal (ventral view). Internal genitalia as in Fig. 2b. Spination: Femur I with one dorsal and one prolateral spine; Femur II–IV with one dorsal spine. Patella I–IV with one dorsal spine. Tibia I–II with two dorsal, one prolateral and one retrolateral spines; tibia III with two dorsal spines; tibia IV with one dorsal and one prolateral spines. Metatarsus I–IV with one dorsal spine. Tibia of the palp with one dorsal and two prolateral spines. Tarsus of the palp with one dorsal, one prolateral and two retrolateral spines. Patella of the palp with one dorsal spine. Position of TmI: 0.23. Trichobothrium on Mt IV absent.

Etymology

The species is dedicated to our friend Pier Mauro Giachino, Piedmontese coleopterologist who drove our attention on the *Milieu Souterrain Superficiel* in which most of the type material here reported was collected.

Distribution and sampling notes

We regard the species as a steno-endemic element of the southern Graian Alps (NW Alps). We primarily collected specimens of *T. giachinoi* n. sp. in the MSS, using subterranean sampling devices installed at depths comprised between 0.40 and 0.80 m . We collected additional material in ordinary pitfall traps placed in deep beech forest leaf litter, i.e. at the interface soil/MSS (sampling details in Mammola et al. 2017). All the traps were installed within the area of the hypogean complex of the Pugnetto caves (Site of Communitary Importance IT 1110048 Grotte del Pugnetto), in the municipality of Mezzenile (about 40 km NW of Turin) at an altitude between 800 and 870 m asl. Further material was collected in 1972–1973 by Konrad Thaler, in an unspecified habitat within the Natural Park of Col del Lys, approximately 10 km South to Mezzenile.

***Troglohyphantes bornensis* Isaia & Pantini 2008**

Troglohyphantes bornensis Isaia & Pantini 2008: 428, f. 1-8 (♀♂)

Troglohyphantes bornensis Isaia *et al.* 2011: 124, f. 2.34A-C, 2.35 (♀♂)

Material examined. Italy, Piemonte, Province of Torino, Mezzenile, Pugnetto: [Pi 1501] Borna Maggiore di Pugnetto, pitfall trap, 1.vi.2012–1.vi.2013, Isaia & Piano leg. 7♀♀, 3♂♂ (CI).

Literature data

Italy, Piemonte, Province of Torino, Mezzenile, Pugnetto: [Pi 1501] Grotta del Pugnetto (=Borna Maggiore del Pugnetto) (Casale *et al.* 1997 sub *Troglohyphantes* sp.; Arnò and Lana 2005 sub *Troglohyphantes* sp.; Isaia and Pantini 2008; Isaia *et al.* 2010, 2011); [Pi 1502] Grotta inferiore del Pugnetto (=Tana del Lupo) (Isaia and Pantini 2008; Isaia *et al.* 2011); [Pi 1503] Grotta superiore del Pugnetto (=Creusa d'le Tane) (Isaia and Pantini 2008; Isaia *et al.* 2011); [Pi 1504] Tana della Volpe (Isaia and Pantini, 2008; Isaia *et al.* 2011)

Notes

We here provide new diagnostic drawings of the female for comparisons with *Troglohyphantes giachinoi* n. sp. (Fig. 2c,d).

Morphospace analysis

Measurements of the morphological traits considered for the multi-dimensional hypervolume analysis are summarized in Fig. 3. Pearson *r* correlations revealed a high degree of multicollinearity among some of the considered variables. Leg II–IV lengths were collinear with Leg I length (all pairwise $r > 0.9$), and thus we only used the latter variable in the analysis. Leg I was further collinear with the ratio between sternum length and width ($r = 0.7$), which we also excluded from the analysis. As a result, we used five morphological traits to estimate the hypervolume representing the morphological niche of the two species (Fig. 4). The overall 5-dimensional hypervolume of *T. bornensis* was almost two times bigger than that of *T. giachinoi* n. sp. (504.4 and 315.3, respectively), and the two geometrical figures only partially intersected with each other in the 5-dimensional space (Intersection = 46.9). There was a low niche overlap between the two species (Sørensen–Dice = 0.11). Specifically, the species displayed character displacement in most morphological traits considered (Fig. 4), *T. giachinoi* having shorter legs, shorter sternum and smaller chelicerae compared to *T. bornensis,* but larger eyes and chephalothorax height/length ratio (Fig. 3).

Molecular data

The new sequences obtained in the present study are available in GenBank® (MG836283–MG836291). The information of the complete set of sequences used for the molecular analyses is summarized in Supplementary Material Table S1. The 59 COI sequences available yielded 52 unique haplotypes. We obtained two most parsimonious trees of 1,333 steps. The best partition scheme was by codon position, and the preferred models TrN+I+G, HKY and TrN+G for the first, second and third positions, respectively. We recovered the maximum likelihood support from 400 replicates. The topologies mostly mirrored those obtained in Isaia *et al.* (2017) with a smaller data set. Overall, interspecific relationships were poorly supported, except for the Pesarini's species complexes (Pesarini 2001; Isaia *et al.* 2017) that were supported in some analyses and recovered in most. The only exception is the *Microcymbium* complex, represented by *Troglohyphantes microcymbium*, *T. lanai*, *T. bornensis* and the new species, that we never recovered as monophyletic. All analyses, on the other hand, recovered as expected, the sister species relationship of *T. bornensis* and the new species, albeit with partial support (Fig. 5).

The GMYC model provided a better fit than the single coalescent model ($p<0.001$) and circumscribed 26 coalescent groups (confidence interval 19–34). The coalescent groups mostly coincided with the morphologically-defined species, including the new species, which was delimited as a single coalescent group. Only exceptions were the species *T. vignai*, *T. nigraerosae* and *T. bolognai*, which were split into 5, 2 and 2 coalescent groups, respectively (Fig. 5). The intraspecific uncorrected genetic divergence between the two sample individuals of the new species, collected in the same site, was higher than that observed in the 5 specimens sequenced of *T. bornensis*, from three different sites (1.4% and 0.3%, respectively). The closest intraspecific distance between the new species and *T. bornensis* was 12.6%, while the average across all species was 10% (s.d.=0.02) (Supplementary Material Table S2).

The estimated divergence times derived from the COI gene tree were compatible with those reported in Mammola *et al.* (2015b) and suggested that the split between the haplotypes of the new species and *T. bornensis* traced back to the Messinian (6.1 My ago), although the confidence interval span most of the Pliocene and the late Miocene (2.3–12.6 My ago).

Discussion

Systematics

The new species can be diagnosed by both genitalic and somatic characters. Morphological differences are restricted to small details of the copulatory organs (male: Fig. 1; female: Fig. 2) and the overall size and leg length (Fig. 3). The molecular data based on the COI gene provide further evidence for their species status (Fig. 5). The uncorrected genetic divergence between *T. giachinoi* n. sp. and *T. bornensis* (~13%) is well-above the 7 to 7.6% threshold divergence recently proposed to identify *Troglohyphantes* species (Isaia *et al.* 2017). The GMYC method, a species delimitation approach based on single markers, further confirms that the two species constitute independent coalescent groups. Interestingly, three of the additional *Troglohyphantes* species analysed, consisted in more than one coalescent group, suggesting the existence of high population geographic structuring.

The attribution of *T. giachinoi* n. sp. to a *Troglohyphantes* species complex (*sensu* Pesarini 2001, Isaia *et al.* 2017) remains controversial. Due to its similarity with *T. bornensi*s, the new species may be assigned to the *Microcymbium* complex. However, as already advanced by Isaia et al. (2017), the monophyly of this species complex is not supported by genetic data. This result suggest that the characters used to define this complex may be either ancestral or have evolved independently. Alternatively, the apparent polyphyly of this complex may be the result of the lack of informative characters, since most of the deeper branches were weakly supported. Further analyses are needed to better define this species complex and to clarify the position of *T. bornesis* and *T. giachinoi* n. sp. within respect to the other species belonging to the genus.

Niche partitioning in subterranean habitats

We documented co-occurrence of three species of *Troglohyphantes* in the same hypogean complex (Pugnetto), namely *T. bornensis*, *T. giachinoi* n. sp., and *T. lucifer*. The coexistence of unrelated congeneric species has already been documented in few caves in Slovenia (Deeleman-Rehinold 1978) and in the Western and Central Italian Alps (Isaia and Pantini 2010; Isaia *et al.* 2011, 2017). Species co-existence in *Troglohyphantes* is mostly observed in phylogenetically distant species and often involves spatial segregation (e.g. occupation of different zones of the same cave; Deeleman-Rehinold 1978). Also in the case considered here, we found the three species to exploit different habitats. *Troglohyphantes lucifer* was associated to the cave entrance, *T. giachinoi* n.sp. was primarily associated to the MSS and *T. bornensis* occurred in the deep cave habitat. However, in disagreement with Deeleman-Reinhold (1978) observations on coexisting *Troglohyphantes* species, *T. bornensis* and *T. giachinoi* n. sp. are each other closest relatives.

In view of their close phylogenetic relationships and coexistence at the local scale in interconnected habitats, an ecological segregation should be expected to prevent or reduce competition between the two sibling species (Amarasekare 2003). In this regard, subterranean co-occurring species of *Niphargus* amphipods, for instance, have been shown to occupy non-overlapping regions of the morphospace (Fišer Z. *et al.* 2015). In spiders, documented mechanisms of niche partitioning between sibling and other closely related species include divergence in prey preference, phenology and habitat occupation (Poulson 1977, Novak *et al.* 2010, Mammola and Isaia 2014, 2017b).

In accordance with our expectations, the multi-dimensional hypervolume analysis confirmed that there is only marginal overlap in the morphological space of the two species, especially when considering traits related to body size. In a first step, differences in size may imply different target prey. Prey segregation could explain non-overlapping cheliceral size in the two species. Moreover, larger body and longer legs in *T. bornensis* are congruent with its preferential occurrence into the larger habitat spaces within the deep subterranean domain (Isaia and Pantini 2008). Appendage elongation is a well-documented troglomorphic character in cave-dwelling spiders (Mammola and Isaia 2017a), found in numerous species of *Troglohyphantes* (Deeleman-Reinhnold 1978) and in other genera and families (e.g., Gertsch 1992, Cokendolpher 2004, Miller 2005). Conversely, shorter legs and overall smaller body size of *T. giachinoi* n. sp. (Fig. 3) may reflect a specialisation for inhabiting smaller habitat pores, such as the air-filled spaces in MSS and deep leaf litter and soil strata (see discussion in Mammola and Isaia 2017a). In agreement with this interpretation, *T.*

giachinoi n. sp. is among the smaller species of alpine *Troglohyphantes*. A comparable size is found in *T. iulianae,* which has been collected both in MSS-like habitat (Pesarini 2001) and in caves (Isaia *et al.* 2011), but never found coexisting with any congeneric species in the same hypogean complex.

When comparing the two species, *T. bornensis* showed the most pronounced troglomorphic traits, namely a higher appendage elongation, flattening of the cephalothorax and eye regression (remarkable for the anterior eyes; Fig. 3). Once again, these results fit well with its preferential occurrence within the deep cave habitat. Both species exhibit complete depigmentation, which in *Troglohyphantes* is the first character undergoing selection during the process of subterranean adaptation (Deeleman-Reinhnold 1978). On the other hand, despite *T. bornensis* showing more pronounced eye reduction, none of the two species is eyeless, which may suggest either a relative recent adaptation to the subterranean medium or a complex pleiotropic effect of eye reduction with other adaptive traits (Réaux and Casane 2013).

The process of morphological niche differentiation here documented, offers new and potentially interesting arguments for feeding the recent debate about the role of habitat size as an important evolutionary force in subterranean habitats (see Culver and Pipan 2014, 2016); e.g., the extent to which the size of subterranean invertebrates is directly related/constrained by the pore volume of the habitat (Pipan and Culver 2017). Web-spinning spiders are particularly interesting models for testing hypotheses on the evolutionary role of the spatial habitat, as their preference for a certain pore size is not exclusively determined by the overall body size, but also by the need for a 3-dimensional space where to spin their webs (Mammola and Isaia 2017a). It is well documented that most cave-dwelling *Troglohyphantes*, including *T. bornensis,* wave the typical horizontal sheet-web of Linyphiidae (Deeleman-Reinhold 1978; Mammola and Isaia 2016) — the size of which is hardly commensurable to the size of MSS pores. Unfortunately, because *T. giachinoi* n. sp. has only been collected in pitfall traps, we lack any information about the shape and size of its web. Deeleman-Reinhold (1978) suggested that a pore size larger than 5 cm is necessary to *Troglohyphantes* spiders for spinning their web — in fact, in cave-dwelling species the size of the web ranges from 5 to 30 cm depending both on the species and individual size. Therefore, *T. giachinoi* n. sp. either inhabits exclusively larger (>5 cm in diameter) MSS voids or it spins smaller webs than its congeneric species. As an alternative explanation, it could have abandoned web-building behaviour and become a wandering species. Although web loss is unknown within the family Linyphiidae (see Benjamin and Zschokke 2004; Arnedo *et al.* 2009; Wang *et al.* 2015), it has been documented in orb-weaving families, for example in the spiny-leg clade of Hawaiian *Tetragnatha* spiders (Gillespie and Croom 1995).

With the data at hand, it is difficult to ascertain whether the two *Troglohyphantes* species adapted independently to the subterranean environment or, alternatively, the common ancestor was already a cave or deep soil dweller. Although time estimates should be taken with caution, given that we relied on a single gene and a universal substitution rate prior, our results suggest that the two species probably split during the late Miocene or Pliocene. This was a time of major climatic changes, including the deterioration of the previous subtropical conditions and the onset of the Mediterranean climate (Suc 1984; Shevenell *et al.* 2004; Jiménez-Moreno *et al.* 2010). These climatic changes could have prompted the colonization of the subterranean environment by fauna adapted to more humid and warmer climates, in search for stable conditions. We inferred similar time windows for the origin of other cave spider species in the region, for example *T. vignai* and *T. nigraerosae* and two species of the genus *Pimoa* (Araneae: Pimoidae) (Mammola *et al.* 2015b, 2016a).

It also remains unresolved whether the two species originated in parapatry, as a result of niche-based segregation in contiguous habitat (i.e. ecological speciation; Rundle and Nosil 2005) or, alternatively, if they originated in allopatry and independently colonized the two different underground habitats. On the other hand, our study highlights the importance of ecological factors in maintaining local diversity by minimizing direct interspecific competition through niche partitioning. The results here presented provide further support for the key role of competition in shaping morphology when competing species are present in the subterranean environments (Arnedo *et al.* 2007, Culver and Pipan 2016).

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SUPPLEMENTARY MATERIAL

Table S1. List of specimens sequenced in this study with voucher information and DNA code

Table S2. Species delimitation results.

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TABLES

Table 1. Morphological traits considered in the hypervolume analysis, with information on their adaptive meaning.

Trait	Description	Adaptive meaning
Leg length	Length of leg I-IV.	In subterranean spiders, leg dimension is often related with habitat — pore — size (Mammola *et al.* 2016bm Mammola and Isaia 2017a).
Sternum ratio	Ratio between sternum maximum length and width.	A *proxy* for body size. Overall body-size can be related to habitat (pore) size (Pipan and Culver 2017).
Cephalothorax height/length	Ratio between height and length of the cephalothorax. Height measured at the eye region, starting from the clypeus base to the top of the profile.	In *Troglohyphantes*, the relative height of the cephalothorax is a measure of subterranean adaptation — i.e., flattening of the cephalothorax profile at increasing troglomorphism (Deeleman-Reinhold 1978, Isaia and Pantini 2010)
Anterior eyes ratio	Sum of AME and ALE diameters, divided by the total length of eye region.	In spiders, eye regression is a well-documented adaptation to the hypogean medium (Mammola and Isaia 2017a). Moreover, according to Deeleman-Reinhold (1978), the anterior median eyes are often the first undergoing regression.
Posterior eyes ratio	Sum of PME and PLE diameters, divided by the total length of the eye region.	
Chelicerae	Height of the basal segment of chelicerae (paturon)	Shape and height of chelicerae give information on dietary requirements and possible trophic segregation.

Table 2. Leg measurements (mm) of *Troglohyphantes giachinoi* n. sp. (male holotype and female paratype). “NA” indicate missing articles.

	Femur	**Patella**	**Tibia**	**Metatarsus**	**Tarsus**	**Total**
			Male holotype			
Leg I	1.88	0.66	1.94	1.69	0.94	7.09
Leg II	1.59	0.66	1.69	1.56	0.97	6.47
Leg III	1.84	0.63	1.28	1.13	0.75	5.63
Leg IV	1.88	0.63	NA	NA	NA	NA
Pedipalp	0.5	0.13	0.16	-	0.3 (Cy)	1.09
			Female paratype			
Leg I	1.31	0.31	1.53	1.25	0.81	5.22
Leg II	1.28	0.59	1.34	1.19	0.75	5.16
Leg III	1.13	0.16	0.78	0-88	0.59	3.53
Leg IV	1.41	0.59	1.63	1.25	0.75	5.63
Pedipalp	0.66	0.06	0.53	-	0.44	1.69

FIGURE LEGEND

Figure 1. *Troglohyphantes giachinoi* n. sp. Holotype male. a) Retrolateral view of left male pedipalp; b) Embolus; c) Eyes and chelicerae, frontal view. Scales: a–b = 0.2 mm; c = 0.5 mm. Abbreviations: E = Embolus; LC = Lamella characteristica; PC = Paracymbium; SA = Suprategular apophysis; Te = Tip of the embolus. Illustration by Elena Pelizzoli.

Figure 2. a–b) *Troglohyphantes giachinoi* n. sp. Female paratype. c–d) *Troglohyphantes bornensis* Isaia & Pantini. Female from [Pi 1503] Borna Superiore di Pugnetto, Mezzenile (TO), Italy (1.xii.2006, Isaia M. *legit*). a,c) Epigyne, dorsal view. c,d) Epigyne, ventral view. Scales: a–d = 0.16 mm. Abbreviations: Cg = Copulatory groove; Fg = Fertilization groove; Pp = posterior plate; S = Spermatheca. Illustration by Elena Pelizzoli.

Figure 3. Boxplots showing the variation of morphological measurements between the individuals of *Troglohyphantes bornensis* and *T. giachinoi* n. sp. Morphological variables are explained in Table 1. Circles represent outlying values.

Figure 4. Estimated 5-dimensional hypervolume for *Troglohyphantes bornensis* and *T. giachinoi* n. sp. Variables have original units as in Table 1, but have been standardized for the analysis. For each inset, the coloured dots represent random points sampled from the inferred hypervolume (20,000 random points for each species are shown). Contour lines delimiting random points are drawn for visual presentation.

Figure 5. Chronogram obtained from the BEAST analysis. Circles on internal nodes denote support values as follow: upper left = Bayesian posterior probabilities (PP); upper right = maximum likelihood bootstraps (BS), bottom = parsimony jackknifing (PJ). Filled box = PP>95% or BS>75 or PJ>0.75. Grey boxes = clades recovered with support values below former thresholds. Empty sectors: clades not recovered. The tree was rooted using *Troglohyphantes oromii* (Ribera & Blasco). Pesarini's (2001) species complexes recovered as monophyletic in grey boxes. GMYC clusters are indicated with a white node on the more ancestral node. Bars denote 95% HPD confidence intervals. Species name in bold denotes troglomorphic species, otherwise troglophilic.

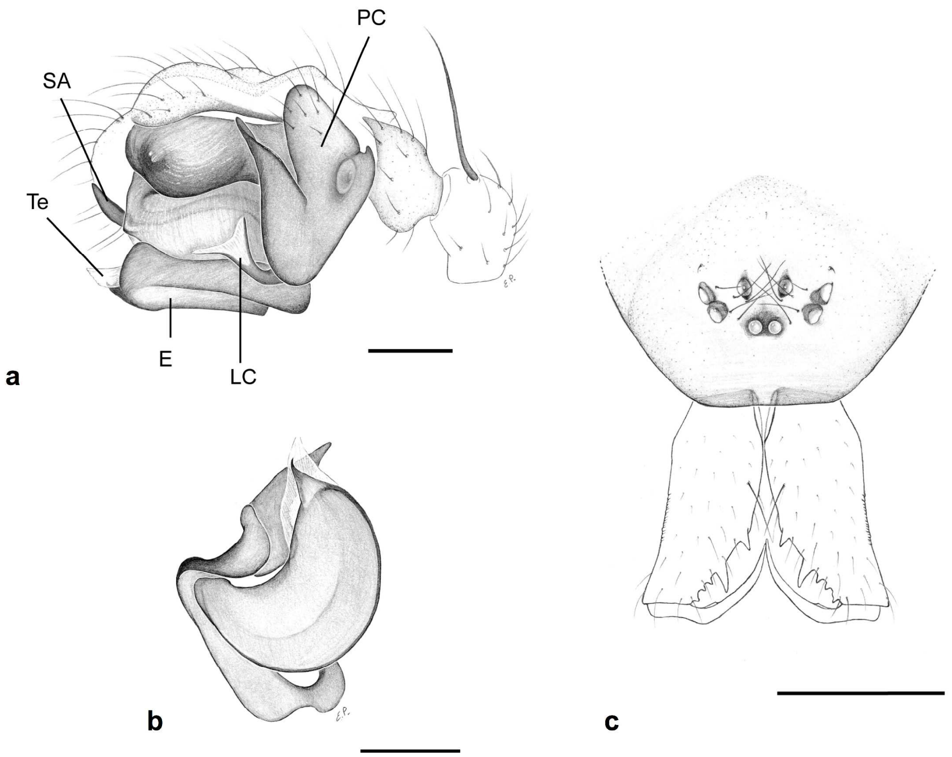


Figure 1

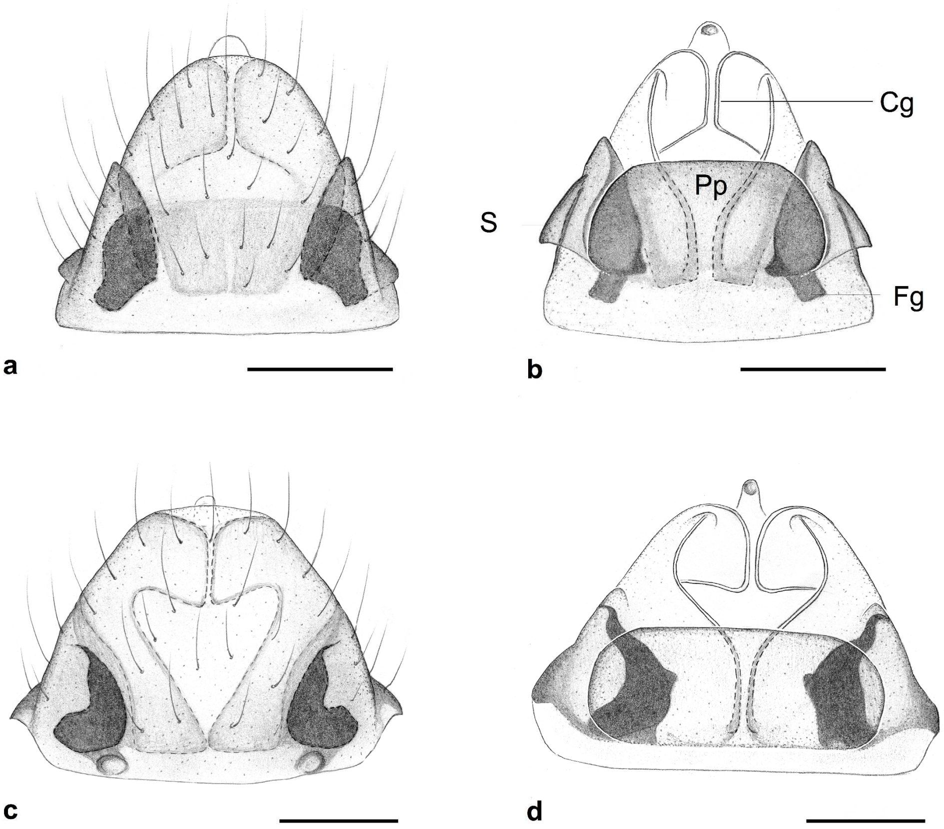


Figure 2

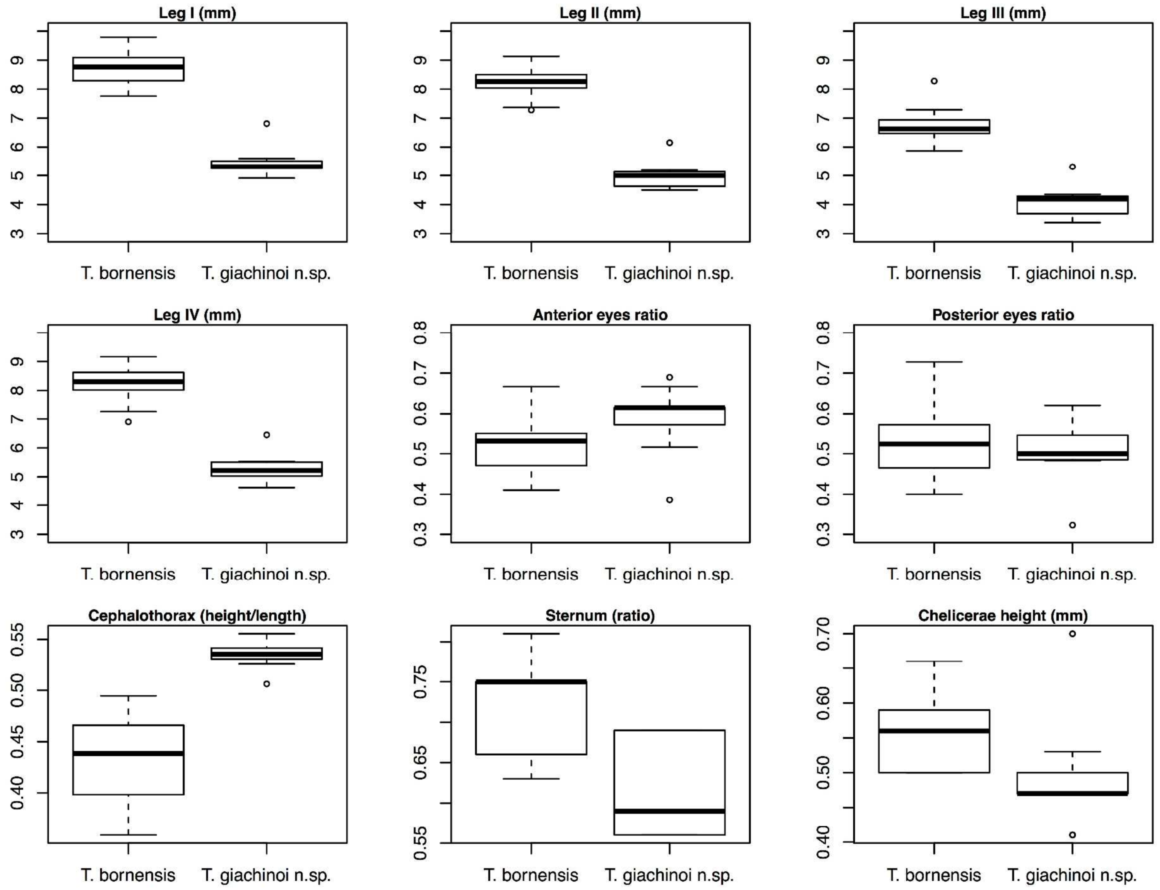

Leg I (mm)
Leg II (mm)
Leg III (mm)
3
4
5
6
7
8
9
T. bornensis
T. giachinoi n.sp.
Leg IV (mm)
Anterior eyes ratio
Posterior eyes ratio
0.3
0.4
0.5
0.6
0.7
0.8
Cephalothorax (height/length)
0.40
0.45
0.50
0.55
Sternum (ratio)
0.55
0.65
0.75
Chelicerae height (mm)
0.40
0.50
0.60
0.70

Figure 3

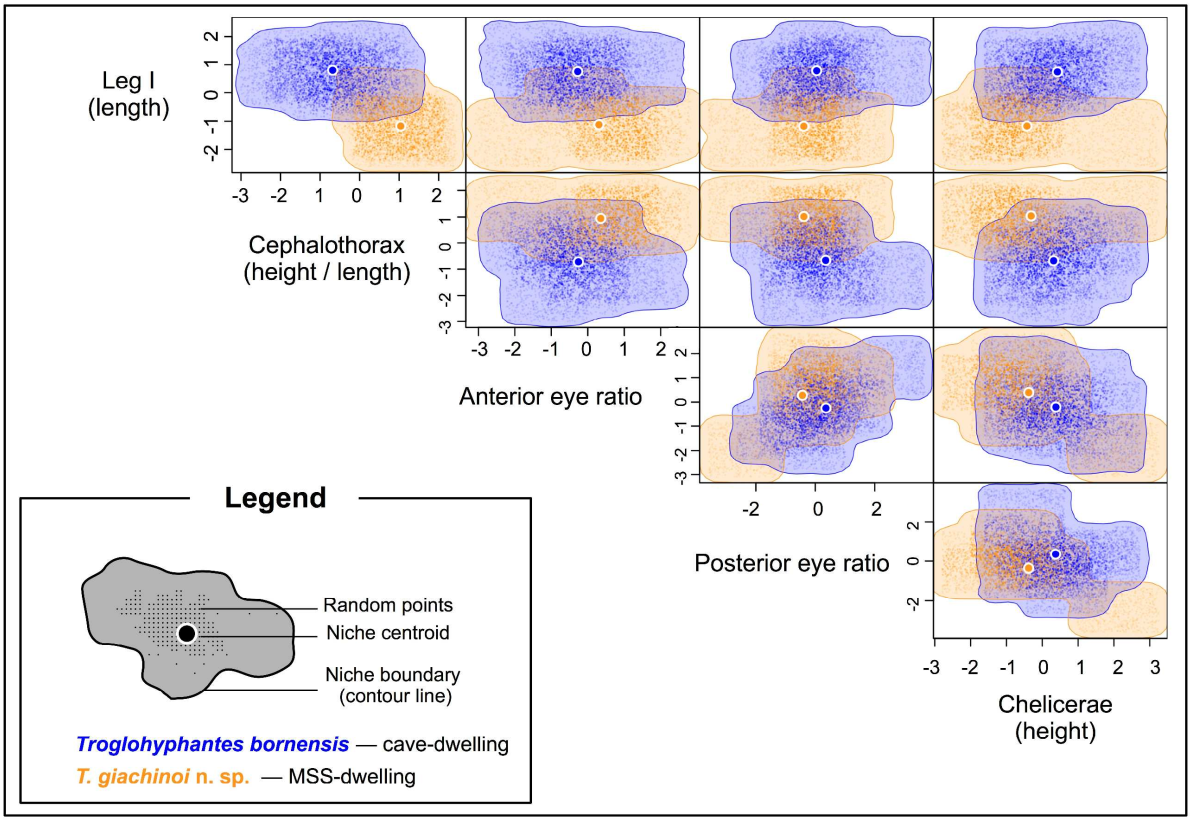

Leg I
(length)
Cephalothorax
(height / length)
Anterior eye ratio
Posterior eye ratio
Chelicerae
(height)
-3
-2
-1
0
1
2
3
Legend
Random points
Niche centroid
Niche boundary
(contour line)
Troglohyphantes bornensis — cave-dwelling
T. giachinoi n. sp. — MSS-dwelling

Figure 4

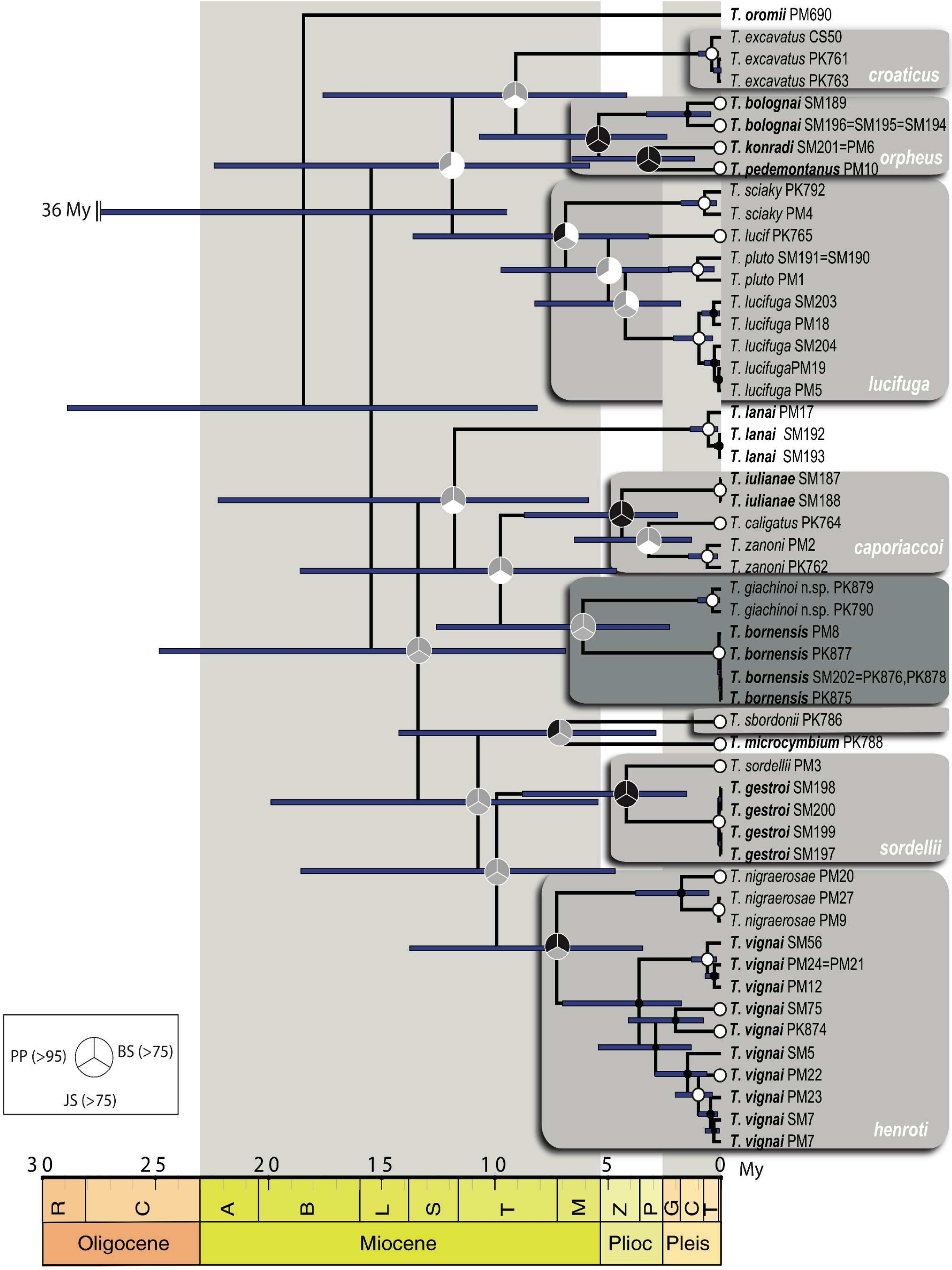

T. oromii PM690
T. excavatus CS50
T. excavatus PK761
T. excavatus PK763
croaticus
T. bolognai SM189
T. bolognai SM196=SM195=SM194
T. konradi SM201=PM6
T. pedemontanus PM10
orpheus
36 My
T. sciaky PK792
T. sciaky PM4
T. lucif PK765
T. pluto SM191=SM190
T. pluto PM1
T. lucifuga SM203
T. lucifuga PM18
T. lucifuga SM204
T. lucifugaPM19
T. lucifuga PM5
lucifuga
T. lanai PM17
T. lanai SM192
T. lanai SM193
T. iulianae SM187
T. iulianae SM188
T. caligatus PK764
T. zanoni PM2
T. zanoni PK762
caporiaccoi
T. giachinoi n.sp. PK879
T. giachinoi n.sp. PK790
T. bornensis PM8
T. bornensis PK877
T. bornensis SM202=PK876,PK878
T. bornensis PK875
T. sbordonii PK786
T. microcymbium PK788
T. sordellii PM3
T. gestroi SM198
T. gestroi SM200
T. gestroi SM199
T. gestroi SM197
sordellii
T. nigraerosae PM20
T. nigraerosae PM27
T. nigraerosae PM9
T. vignai SM56
T. vignai PM24=PM21
T. vignai PM12
T. vignai SM75
T. vignai PK874
T. vignai SM5
T. vignai PM22
T. vignai PM23
T. vignai SM7
T. vignai PM7
henroti
PP (>95)
BS (>75)
JS (>75)
30
25
20
15
10
5
0
My
R
C
A
B
L
S
T
M
Z
P
G
C
T
Oligocene
Miocene
Plioc
Pleis

Figure 5

APPENDIX

Table S1. List of specimens sequenced in this study with voucher information, DNA code and GenBanl

Genus	Species	DNA CODE	Genebank Code	Sex
Troglohyphantes	*nigraerosae*	PM27	KT832079	M
Troglohyphantes	*vignai*	SM5	KT832082	J
Troglohyphantes	*vignai*	SM7	KT832083	J
Troglohyphantes	*vignai*	SM56	KT832098	J
Troglohyphantes	*vignai*	SM75	KT832105	J
Troglohyphantes	*excavatus*	PK761	KX831559	Leg
Troglohyphantes	*excavatus*	PK763	KX831560	Leg
Troglohyphantes	*lucifer*	PK765	KX831561	L
Troglohyphantes	*zanoni*	PM2	KX831562	F
Troglohyphantes	*vignai*	PM21	KX831563	M
Troglohyphantes	*vignai*	PM24	KX831563	M
Troglohyphantes	*vignai*	PM23	KX831564	F
Troglohyphantes	*vignai*	PM22	KX831565	F
Troglohyphantes	*vignai*	PM12	KX831566	F
Troglohyphantes	*vignai*	PM7	KX831567	F
Troglohyphantes	*sordellii*	PM3	KX831568	M
Troglohyphantes	*sciakyi*	PM4	KX831569	F
Troglohyphantes	*pluto*	SM190	KX831570	M
Troglohyphantes	*pluto*	SM191	KX831570	F
Troglohyphantes	*pluto*	PM1	KX831571	F
Troglohyphantes	*pedemontanus*	PM10	KX831572	F
Troglohyphantes	*nigraerosae*	PM20	KX831573	M
Troglohyphantes	*nigraerosae*	PM9	KX831574	M
Troglohyphantes	*lucifuga*	SM204	KX831575	J
Troglohyphantes	*lucifuga*	SM203	KX831576	J
Troglohyphantes	*lucifuga*	PM19	KX831577	F
Troglohyphantes	*lucifuga*	PM18	KX831578	M
Troglohyphantes	*lucifuga*	PM5	KX831579	M
Troglohyphantes	*lanai*	SM193	KX831580	F
Troglohyphantes	*lanai*	SM192	KX831581	F
Troglohyphantes	*lanai*	PM17	KX831582	F
Troglohyphantes	*konradi*	PM6	KX831583	Leg
Troglohyphantes	*konradi*	SM201	KX831583	F
Troglohyphantes	*iulianae*	SM188	KX831584	J
Troglohyphantes	*iulianae*	SM187	KX831585	L
Troglohyphantes	*gestroi*	SM200	KX831586	J
Troglohyphantes	*gestroi*	SM199	KX831587	J
Troglohyphantes	*gestroi*	SM198	KX831588	J
Troglohyphantes	*gestroi*	SM197	KX831589	J
Troglohyphantes	*excavatus*	CS50	KX831590	Leg
Troglohyphantes	*bornensis*	PK876	KX831591	J

Troglohyphantes	*bornensis*	PK878	KX831591	J
Troglohyphantes	*bornensis*	SM202	KX831591	F
Troglohyphantes	*bolognai*	SM189	KX831592	F
Troglohyphantes	*bolognai*	SM194	KX831593	F
Troglohyphantes	*bolognai*	SM195	KX831593	F
Troglohyphantes	*bolognai*	SM196	KX831593	Leg
Troglohyphantes	*caligatus*	PK764	KX831594	Leg
Troglohyphantes	*zanoni*	PK762	KX831595	Leg
Troglohyphantes	*oromii*	CRBA000690	KX831596	F
Troglohyphantes	*sbordonii*	pk786	MG836283	M
Troglohyphantes	*microcymbium*	PK788	MG836284	F
Troglohyphantes	*giachinoi* n.sp.	pk789	MG836285	M
Troglohyphantes	*giachinoi* n.sp.	pk790	MG836286	F
Troglohyphantes	*sciakyi*	pk792	MG836287	F
Troglohyphantes	*vignai*	PK874	MG836288	F
Troglohyphantes	*bornensis*	PK875	MG836289	J
Troglohyphantes	*bornensis*	PK877	MG836290	J
Troglohyphantes	*bornensis*	PM8	MG836291	J

k® access code.

Cave/Locality	Cadastrial cave N°	x
Borna del Servais B	artificial	7.32763
Buco di Valenza	Pi 1009	7.17197
Grotta superiore delle Camoscere	Pi 250	7.65899
Prospetto di miniera di Boccetto	artificial	7.08510
Tana dell'Orso di Casteldelfino	Pi 1019	7.09835
Jobokova Luknja Belsko	-	NA
Betalov Spodmol	SI 859 (RKD)	NA
Grotta del Ghiaccio di Bosconero	Pi 1580	7.04508
Astino (in forest)	-	9.64157
Tana del diavolo	Pi 1591	7.12206
Tana del diavolo	Pi 1591	7.12206
Abisso Arrapanui	Pi 772	NA
Voragine della Ciuaiera	Pi 146	7.88660
Prospetto di miniera di Boccetto	artificial	7.08510
Grotta Superiore delle Camoscere	Pi 250	7.65899
Baite del Sedernello	-	9.76894
Rino Olmi	-	10.03200
Grotta del Caudano	Pi 121	7.78982
Grotta del Caudano	Pi 121	7.78982
Abisso Artesinera	Pi 197	7.78882
Pozzo del Rospo	Pi 3015	7.78882
Borna del Servais B	artificial	7.32763
Borna del Servais B	artificial	7.32763
Ca d'lom Salvej	Pi 2588	7.95800
Grotta la Custreta	Pi 1593	7.54550
Grotta della Soldanella	Ao 2072	7.19700
Buco della Bondaccia	Pi 2505	8.31183
Borna del Servais B	artificial	7.32763
Grotta delle Arenarie	Pi 2509	8.31447
Grotta delle Arenarie	Pi 2509	8.31447
Buco della Bondaccia	Pi 2505	8.31183
Sotterranei del forte (B) di Vernante, Opera 14 Tetto Filibert	artificial	7.52797
Sotterranei del forte (B) di Vernante, Opera 14 Tetto Filibert	artificial	7.52797
Grotta Rio dei Corvi	Pi 884	7.99283
Grotta Rio dei Corvi	Pi 884	7.99283
Bus del Frate	Lo 1	10.41891
Bus del Frate	Lo 1	10.41891
Bus del Frate	Lo 1	10.41891
Bus del Frate	Lo 1	10.41891
Zegnana jama	-	NA
Borna Maggiore di Pugnetto	Pi 1501	7.41360

Borna Minore di Pugnetto	Pi 1503	7.41033
Borna Minore di Pugnetto	Pi 1503	7.41033
Sgarbu du ventu	Li 619	7.93683
Tana di Bertrand	Li 144	7.86699
Tana di Bertrand	Li 144	7.86699
Tana di Bertrand	Li 144	7.86699
Grotta Tacchi	Lo 2029	9.21078
Grotta Ferrera	Lo 1502	9.37454
Cueva del Bucio	-	NA
Grotta Mainarie dal Puint	Fr 242	NA
Grotte Nala Ca'Maquela	Lo 1135	9.51709
MSS in Pugnetto hypogean complex	-	7.41360
MSS in Pugnetto hypogean complex	-	7.41360
Cima Verde, artificial shelter near the ridge	-	11.08100
Topalinda cave (Maissa 2)	Pi 1210	7.40520
Borna Maggiore di Pugnetto	Pi 1501	7.41360
Borna Minore di Pugnetto	Pi 1503	7.41033
Borna Minore di Pugnetto	Pi 1503	7.41033

y	Municipality	Province	Region
45.32259	Ala di Stura	TO	Piemonte
44.68180	Oncino	CN	Piemonte
44.21640	Chiusa Pesio	CN	Piemonte
44.95640	Bocetto	TO	Piemonte
44.55902	Casteldelfino	CN	Piemonte
NA	-	PO	Postojna
NA	Zagon	PO	Postojna
45.19017	Novalesa	TO	Piemonte
45.70614	Astino	BG	Lombardia
45.02634	Roreto Chisone	TO	Piemonte
45.02634	Roreto Chisone	TO	Piemonte
NA	Briga Alta	CN	Piemonte
44.19102	Garessio	CN	Piemonte
44.95640	Prali	TO	Piemonte
44.21640	Chiusa Pesio	CN	Piemonte
45.86104	Colzate	BG	Lombardia
45.91100	Castione della Presolana	BG	Lombardia
44.29332	Frabosa Sottana	CN	Piemonte
44.29332	Frabosa Sottana	CN	Piemonte
44.23432	Frabosa Sottana	CN	Piemonte
44.23432	San Giacomo di Roburent	CN	Piemonte
45.32259	Ala di Stura	TO	Piemonte
45.32259	Ala di Stura	TO	Piemonte
45.70660	Piedicavallo	TO	Piemonte
45.44632	Sparone	TO	Piemonte
45.75424	Aosta	AO	Val d'Aosta
45.71145	Borgosesia	VC	Piemonte
45.32259	Ala di Stura	TO	Piemonte
45.71196	Valduggia	VC	Piemonte
45.71196	Valduggia	VC	Piemonte
45.71145	Borgosesia	VC	Piemonte
44.25250	Vernante	CN	Piemonte
44.25250	Vernante	CN	Piemonte
44.30125	Lisio	CN	Piemonte
44.30125	Lisio	CN	Piemonte
45.49921	Prevalle	BS	Piemonte
45.49921	Prevalle	BS	Piemonte
45.49921	Prevalle	BS	Piemonte
45.49921	Prevalle	BS	Piemonte
NA	Orehek	PO	Postojna
45.27162	Mezzenile	TO	Piemonte

45.27076	Mezzenile	TO	Piemonte
45.27076	Mezzenile	TO	Piemonte
44.00206	Pieve di Teco	IM	Liguria
43.91566	Badalucco	IM	Piemonte
43.91566	Badalucco	IM	Piemonte
43.91566	Badalucco	IM	Piemonte
45.89043	Zelbio	CO	Lombardia
45.92933	Rongio	LC	Lombardia
NA	La Orotava	ES-TF	Santa Cruz de Tenerife
NA	Clauzetto	PN	Friuli-Venezia Giulia
45.80178	Sant'Ombrono Imagna	BG	Bergamo
45.27162	Mezzenile	TO	Piemonte
45.27162	Mezzenile	TO	Piemonte
46.02600	Cima Verde	BG	Lombardia
44.26160	Valdieri	CN	Piemonte
45.27162	Mezzenile	TO	Piemonte
45.27076	Mezzenile	TO	Piemonte
45.27076	Mezzenile	TO	Piemonte

Country	ollection_Dat	Legit
Italy	14 Oct 2009	Isaia, Paschetta
Italy	12 Nov 2014	Mammola, Isaia, Paschetta
Italy	26 Nov 2014	Isaia, Mammola
Italy	12 Sep 2014	Isaia, Mammola
Italy	21 Jul 2013	Mammola
Slovenia	24 May 2016	Isaia
Slovenia	23 May 2016	Isaia
Italy	18 Feb 2016	Isaia
Italy	06 Oct 2009	Pantini
Italy	11 Nov 2006	Isaia
Italy	11 Nov 2006	Isaia
Italy	13 Aug 2001	Lana
Italy	12 Oct 2008	Lana
Italy	21 Feb 2007	Isaia
Italy	21 Dec 2006	Isaia, Lana
Italy	18 Sep 2009	Schoenhofer
Italy	18 Sep 2009	Schoenhofer
Italy	08 May 2013	Isaia, Mammola, Paschetta, Piano, Dalle
Italy	08 May 2013	Isaia, Mammola, Paschetta, Piano, Dalle
Italy	10 Sep 2009	Lana
Italy	07 Jul 2007	Isaia, Lana
Italy	09 Sep 2007	Isaia, Elenia
Italy	14 Oct 2009	Isaia, Paschetta
Italy	24 Sep 2014	Mammola,Paschetta
Italy	07 Nov 2014	Isaia, Mammola
Italy	26 Oct 2008	Lana
Italy	26 Jan 2008	Isaia, Lana
Italy	14 Oct 2009	Isaia, Paschetta
Italy	14 Jun 2013	Isaia, Mammola
Italy	14 Jun 2013	Isaia, Mammola
Italy	26 Jan 2008	Isaia, Lana
Italy	14 Sep 2013	Isaia, Paschetta
Italy	27 Jan 2011	Isaia
Italy	26 Dec 2014	Isaia, Mammola
Italy	26 Dec 2014	Isaia, Mammola
Italy	29 Nov 2014	Isaia, Racchetti
Italy	29 Nov 2014	Isaia, Racchetti
Italy	29 Nov 2014	Isaia, Racchetti
Italy	29 Nov 2014	Isaia, Racchetti
Slovenia	04 Aug 2010	Gasparo
Italy	25 Nov 2016	Isaia, Mammola

Italy	04 Jun 2013	Isaia, Mammola
Italy	05 Dec 2012	Isaia
Italy	21 Dec 2014	Isaia, Mammola
Italy	27 Dec 2014	Isaia, Mammola
Italy	27 Dec 2014	Isaia, Mammola
Italy	27 Dec 2014	Isaia, Mammola
Italy	07 May 2016	Isaia, Mammola
Italy	08 May 2016	Isaia, Mammola
Spain (Canary Islands)	30 Apr 2004	Arnedo, Macías, De La Cruz
Italy	01 May 2014	Gasparo
Italy	10 Oct 2016	Santinelli
Italy	01 May 2014	Piano, Mammola, Isaia
Italy	01 May 2014	Piano, Mammola, Isaia
Italy	09 Sep 2015	Mazzoleni, Pantini
Italy	19 Nov 2016	Isaia, Mammola, Manenti, Santinelli, Barzaghi
Italy	25 Nov 2016	Isaia, Mammola
Italy	04 Jun 2013	Isaia, Mammola
Italy	05 Dec 2012	Isaia

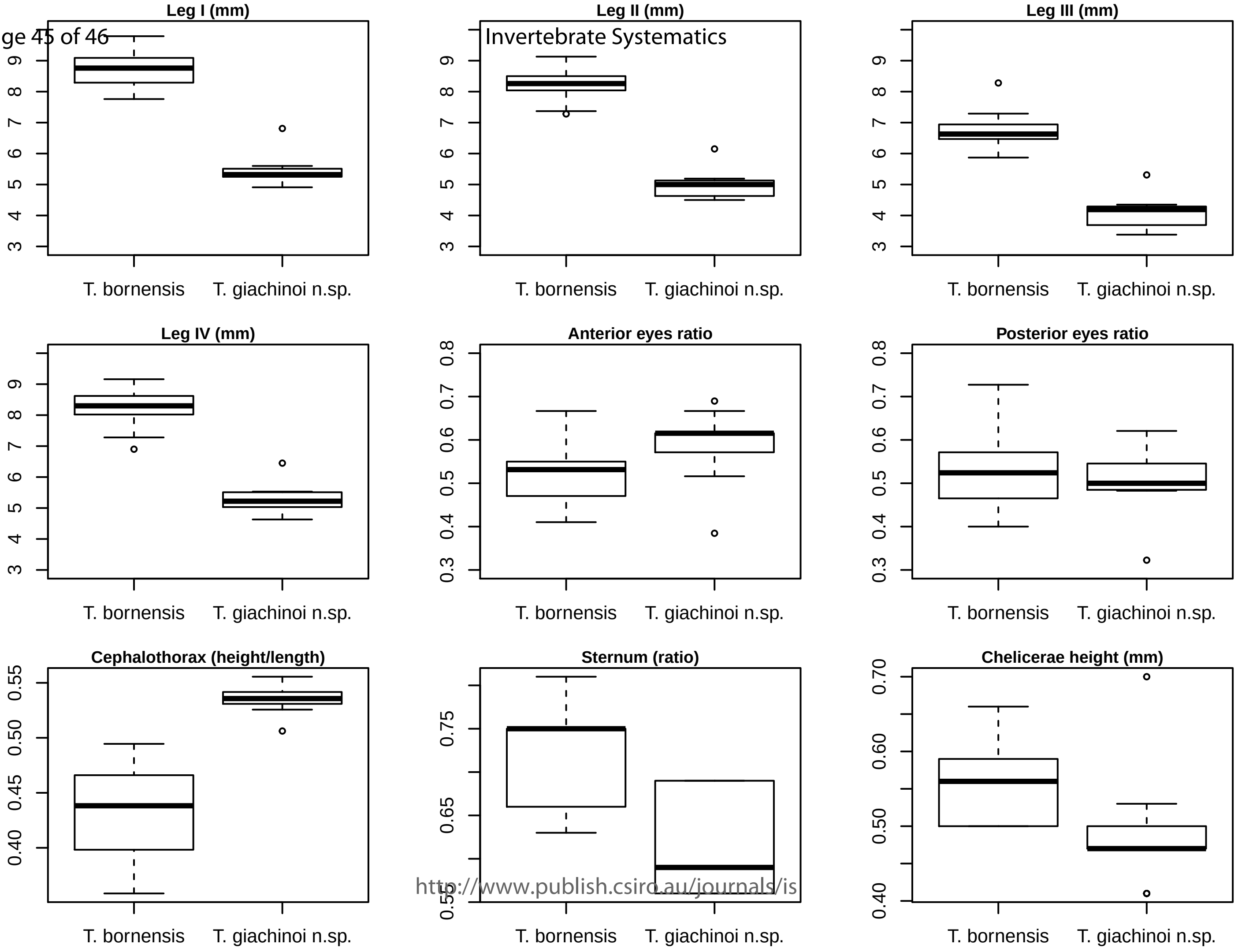
APPENDIX

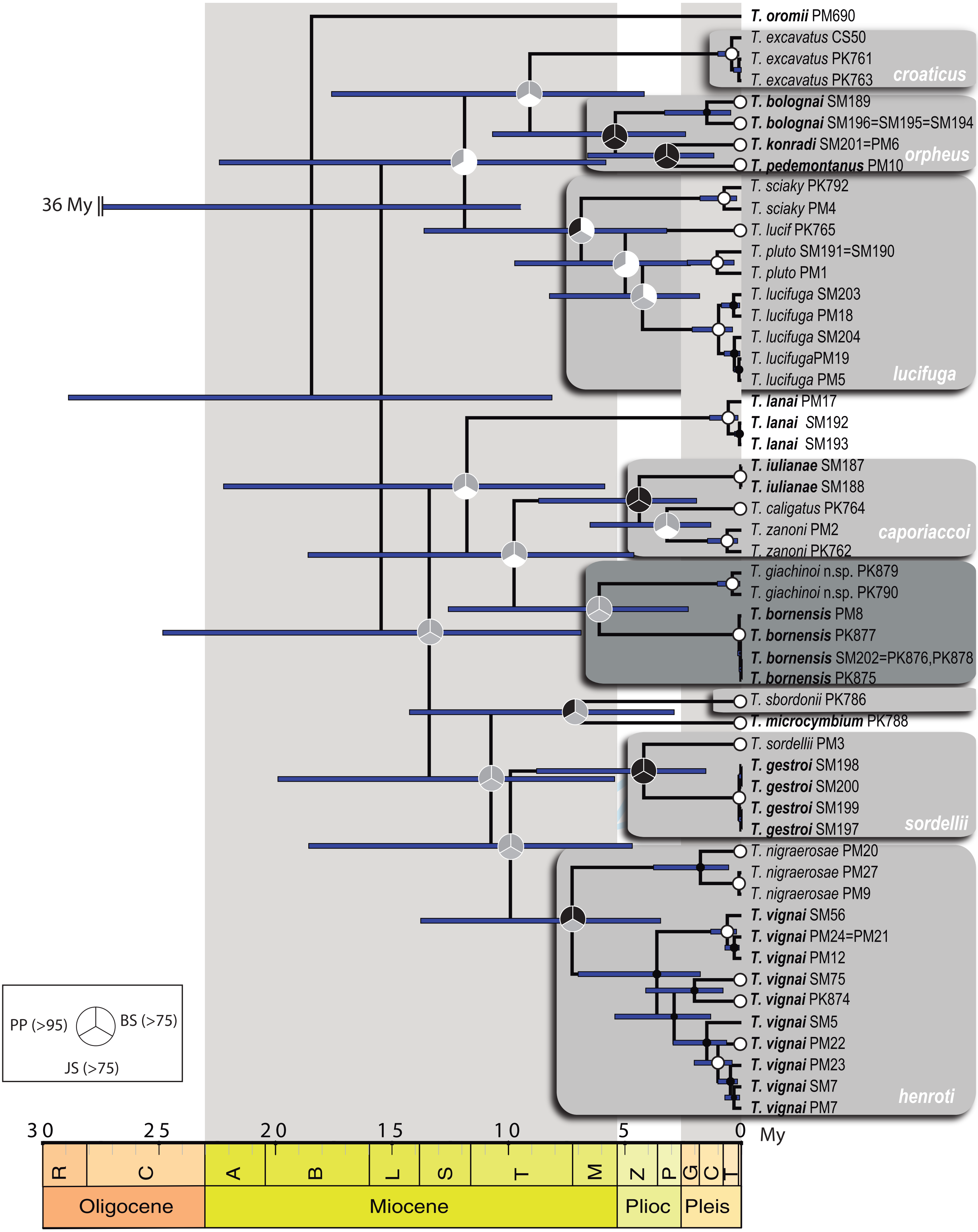
Table S2. Species Delimitation Results

Species	Closest Species	Monophyletic?	Intra Dist
20: vig	15: sord	yes	0.073
17: sbo	18: mic	yes	0.00E+00
18: mic	17: sbo	yes	0.00E+00
19: nigr	15: sord	yes	0.037
14: lana	15: sord	yes	0.011
5: exec	8: bolo	yes	0.009
12: gia	13: bor	yes	0.014
13: bor	12: gia	yes	0.003
8: bolo	7: konr	yes	0.021
9: cal	11: zan	yes	0.00E+00
1: sci	4: luc	yes	0.023
10: iuli	11: zan	yes	3.90E-05
11: zan	10: iuli	yes	0.023
15: sord	16: gest	yes	0.00E+00
16: gest	15: sord	yes	6.29E-04
2: plu	3: luci	yes	0.025
3: luci	4: luc	yes	0.02
4: luc	3: luci	yes	0.00E+00
6: pede	7: konr	yes	0.00E+00
7: konr	6: pede	yes	0.00E+00

Inter Dist - Closest	Intra/Inter	P ID(Strict)	P ID(Liberal)
0.145	0.5	0.73 (0.65, 0.82)	0.92 (0.86, 0.97)
0.144	0.00E+00	0.00E+00 (0.00E+00, 0.00E+00)	0.96 (0.83, 1.0)
0.144	0.00E+00	0.00E+00 (0.00E+00, 0.00E+00)	0.96 (0.83, 1.0)
0.142	0.26	0.61 (0.44, 0.79)	0.86 (0.71, 1.0)
0.136	0.08	0.74 (0.56, 0.91)	0.96 (0.82, 1.0)
0.13	0.07	0.75 (0.57, 0.92)	0.97 (0.83, 1.0)
0.126	0.11	0.54 (0.38, 0.69)	0.92 (0.76, 1.0)
0.126	0.02	0.92 (0.80, 1.0)	0.98 (0.87, 1.0)
0.101	0.2	0.73 (0.59, 0.87)	0.93 (0.82, 1.0)
0.1	0.00E+00	0.00E+00 (0.00E+00, 0.00E+00)	0.96 (0.83, 1.0)
0.094	0.24	0.47 (0.31, 0.62)	0.83 (0.68, 0.99)
0.093	4.10E-04	0.59 (0.44, 0.74)	0.98 (0.83, 1.0)
0.093	0.24	0.47 (0.31, 0.62)	0.83 (0.68, 0.98)
0.093	0.00E+00	0.00E+00 (0.00E+00, 0.00E+00)	0.96 (0.83, 1.0)
0.093	0.01	0.87 (0.72, 1.0)	0.98 (0.87, 1.0)
0.091	0.27	0.61 (0.43, 0.79)	0.85 (0.71, 1.00)
0.09	0.22	0.79 (0.66, 0.91)	0.95 (0.85, 1.0)
0.09	0.00E+00	0.00E+00 (0.00E+00, 0.00E+00)	0.96 (0.83, 1.0)
0.084	0.00E+00	0.00E+00 (0.00E+00, 0.00E+00)	0.96 (0.83, 1.0)
0.084	0.00E+00	0.59 (0.44, 0.74)	0.98 (0.83, 1.0)

Av(MRCA-tips)	P(Randomly Distinct)	Clade Support	Rosenberg's P(AB)
0.0464	1	NA	4.20E-04
0.00E+00	NA	NA	1
0.00E+00	NA	NA	1
0.0291	1	NA	4.20E-04
0.008	1	NA	4.20E-05
0.0066	0.98	NA	1.85E-03
0.0068	NA	0.01	NA
0.0022	NA	0.01	NA
0.0223	0.99	NA	0.01
0.00E+00	NA	NA	0.1
0.0114	0.92	NA	0.33
1.95E-05	NA	0.11	NA
0.0114	0.99	NA	0.11
0.00E+00	NA	NA	0.1
5.93E-04	NA	0.1	NA
0.0189	0.94	NA	0.01
0.0144	0.74	NA	0.01
0.00E+00	NA	NA	0.33
0.00E+00	NA	NA	0.33
0.00E+00	NA	NA	0.33


Leg I (mm)
Leg II (mm)
Leg III (mm)
Leg IV (mm)
Anterior eyes ratio
Posterior eyes ratio
Cephalothorax (height/length)
Sternum (ratio)
Chelicerae height (mm)
T. bornensis
T. giachinoi n.sp.
3
4
5
6
7
8
9
0.3
0.4
0.5
0.6
0.7
0.8
0.40
0.45
0.50
0.55
0.65
0.75
0.40
0.50
0.60
0.70


T. oromii PM690
T. excavatus CS50
T. excavatus PK761
T. excavatus PK763
croaticus
T. bolognai SM189
T. bolognai SM196=SM195=SM194
T. konradi SM201=PM6
T. pedemontanus PM10
orpheus
36 My
T. sciaky PK792
T. sciaky PM4
T. lucif PK765
T. pluto SM191=SM190
T. pluto PM1
T. lucifuga SM203
T. lucifuga PM18
T. lucifuga SM204
T. lucifugaPM19
T. lucifuga PM5
lucifuga
T. lanai PM17
T. lanai SM192
T. lanai SM193
T. iulianae SM187
T. iulianae SM188
T. caligatus PK764
T. zanoni PM2
T. zanoni PK762
caporiaccoi
T. giachinoi n.sp. PK879
T. giachinoi n.sp. PK790
T. bornensis PM8
T. bornensis PK877
T. bornensis SM202=PK876,PK878
T. bornensis PK875
T. sbordonii PK786
T. microcymbium PK788
T. sordellii PM3
T. gestroi SM198
T. gestroi SM200
T. gestroi SM199
T. gestroi SM197
sordellii
T. nigraerosae PM20
T. nigraerosae PM27
T. nigraerosae PM9
T. vignai SM56
T. vignai PM24=PM21
T. vignai PM12
T. vignai SM75
T. vignai PK874
T. vignai SM5
T. vignai PM22
T. vignai PM23
T. vignai SM7
T. vignai PM7
henroti
PP (>95)
BS (>75)
JS (>75)
30
25
20
15
10
5
0
My
R
C
A
B
L
S
T
M
Z
P
G
C
T
Oligocene
Miocene
Plioc
Pleis