Invertebrate Systematics



A multi-layered approach uncovers overlooked taxonomic and physiological diversity in Alpine subterranean spiders (Araneae: Linyphiidae: Troglohyphantes)

Journal:	Invertebrate Systematics
Manuscript ID	IS21054.R1
Manuscript Type:	Research paper
Date Submitted by the Author:	16-Oct-2021
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Keyword:	Araneae, species delineation, biodiversity





337x506mm (300 x 300 DPI)

Recent studies on Italian Alpine cave-dwelling species of spiders covered taxonomy and faunistic, biogeography, and ecology. However, our knowledge about these spiders is far from being complete.

We used a combination of different approaches to explore diversity in a species with an apparent broader distribution compared to its congeners.

Our approach **proofed** proved effective in uncovering overlooked diversity and supporting the description of two new species for science, which distribution ranges show striking resemblances with other cave-dwelling spiders in the same region.

In this work we describe the new species and discuss their origin in light of biogeographical, taxonomical, physiological and ecological aspects.

1	A multi-layered approach uncovers overlooked taxonomic and physiological
2	diversity in Alpine subterranean spiders (Araneae: Linyphiidae: Troglohyphantes)
3	
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15	
16	ABSTRACT
17	The integration of multiple lines of evidence in the delimitation of taxa is becoming the gold
18	standard in modern taxonomy and systematics. Yet, multi-layered taxonomy is still incipient when
19	it comes to species description within mega-diverse group of organisms (e.g., arthropods),
20	especially those inhabiting secluded environments such as caves. This may represent a significant
21	shortcoming, because species in subterranean habitats generally show deep convergence in their
22	somatic traits, perplexing our ability to delimit species via traditional approaches. The genus
23	Troglohyphantes (Araneae: Linyphiidae) is presently known to include 129 species and 5
24	subspecies, which are primarily distributed in subterranean habitats of the European mountain

25 ranges. Recent studies on Italian Alpine species covered taxonomy and faunistics, biogeography, 26 and ecology. However, our knowledge about the actual species diversity of *Troglohyphantes* 27 spiders is far from being complete, and it is expected that significant levels of overlooked diversity 28 should exist within the genus. In this work, we combined morphological with target gene 29 molecular data, functional trait analysis, and thermal physiology to explore overlooked diversity in 30 Troglohyphantes vignai aut., a species of subterranean spider in the Western Italian Alps with an 31 apparent broader distribution compared to its congeners. Our approach proved effective in 32 uncovering overlooked diversity and further supporting the split of *T. vignai* aut. into three distinct 33 species, whose distribution ranges show striking resemblances with that of other subterranean 34 spiders. We here describe the new species and discuss their origin in light of their geographical 35 vicariance, niche convergence, thermal ecology and divergence time.

36

37 INTRODUCTION

The use of different sources of information in the delimitation of taxa has become the gold standard 38 39 in modern systematics. Traditional taxonomy (i.e. morphology based) is often challenged by the 40 existence of evolutionary lineages displaying little, if any, diagnostic anatomical features, resulting 41 in the classification of two or more distinct species as a single one (i.e. cryptic species, Bickford et 42 al. 2007). The term "integrative taxonomy" (Dayrat 2005) was coined to refer to the combination of 43 multiple sources of evidence to effectively establish species boundaries, avoiding the inherent 44 limitations of single disciplines to convey the inherent complexity of the speciation process, and 45 increase rigorousness in species delimitation (Schlick-Steiner et al. 2010). Moreover, uncovering 46 separately evolving lineages previously overlooked due to the lack of or the misinterpretation of 47 morphological diagnostic traits, provides fundamental information to devise more detailed and 48 efficient conservation strategies (Ryder 1986; Bickford et al. 2007).

49 A significant impulse to unveiling previously overlooked diversity came from the widespread adoption of DNA barcoding approaches for species identification and discovery (Hebert et al. 2003), and 50 51 their recent high-throughput sequencing derivatives (i.e. metabacording and metagenomics), in 52 biodiversity studies. Genomic analyses of individual specimens and bulk samples have led to the detection 53 of morphologically similar species in different habitats and biomes. Subsequently, more detailed analyses 54 often result in finding additional ecological, ethological, or phenological traits that covary with the 55 molecular results and thereby confirm the existence of complexes of several species that may not differ 56 from each other in their external morphology (Janzen et al. 2017; Fišer et al., 2018). According to Janzen 57 et al. (2017), "cryptic" species should be fairly common (10–20% of the traditional morphologically single 58 species may turn out to be two or more). For example, by combining morphological study and DNA 59 barcoding of museum specimens, Hebert et al. (2004) showed that what was known as a single species of 60 neotropical skipper comprised at least ten, largely sympatric, species.

61 The sheet-web spider genus *Troglohyphantes* Joseph 1882 (Arachnida: Araneae: 62 Linyphiidae) is presently known to include 129 species and five subspecies (World Spider Catalog 63 2021). The genus is primarily distributed through the European mountain ranges, from the Cantabric 64 Mountains, northern Iberian Peninsula, in the west to the Caucasus in the east. Four additional 65 species are found in northern Africa (Atlas) and two in the Canary Islands (World Spider Catalog 66 2021), but their congeneric status is open to question. In general, Troglohyphantes species are 67 short-range endemics (sensu Harvey 2002). Several species are restricted to a single or few 68 localities, generally subterranean habitats such as caves, mines, soil litter, rocky debris, and other 69 moist and shaded retreats (Isaia et al. 2017).

This genus has been intensively studied, with major focus on the Italian Alpine species.
Recent studies covered taxonomy and faunistics (Isaia and Pantini 2007, 2010; Isaia et al. 2010,
2011, 2017), biogeography (Mammola et al. 2015, 2018b), and ecology (Mammola and Isaia, 2016;

73 Mammola et al. 2018a, 2018b, 2019, 2020). However, even from a basic taxonomic standpoint, 74 knowledge of *Troglohyphantes* spiders is far from being exhaustive; given the rate of new species 75 description and the limited sampling of the subterranean environment, it is probable that more 76 species have yet to be described. A similar reasoning may apply to other species-rich groups of 77 cave-dwelling organisms, for which previous studies disclosed high levels of overlooked diversity, 78 including cavefish (Niemiller et al. 2012) and spiders (Hedin 2015) from North America, tailless 79 whip scorpions from the Caribbean (Esposito et al. 2015), or European crustaceans (Delić et al. 80 2017; Eme et al. 2018), among others.

In the present paper, we used a multi-layered approach to reveal overlooked diversity in *Troglohyphantes vignai* aut. In particular, we gathered evidence from a detailed morphological study coupled with molecular and ecological analyses to support the split of the relatively broadly distributed *T. vignai* aut. Into three distinct species. We here describe and illustrate the new species, summarise their phylogenetic affinities and diversification times, and provide insights into their ecology and natural history. Building on our multiple lines of evidence, we further discuss their origin in light of their geographical vicariance, niche convergence, and divergence time.

89 Material and methods

90 Morphological methods

91 We stored specimens in 70% ethanol in Marco Isaia's collection at the Department of Life Sciences

92 and Systems Biology, University of Turin (Torino, Italy), except for the type series and a few more

- 93 specimens—labelled with the acronym 'MCSNB'—stored at Museo Civico di Scienze Naturali
- 94 'E.Caffi', Bergamo, Italy. We studied specimens using a Leica M80 stereoscopic binocular
- 95 microscope. Illustrations were prepared by Elena Pelizzoli using a camera lucida. We referred to
- 96 Isaia et al. (2017) for *Troglohyphantes* anatomical terms and to the World Spider Catalog (2021)

97	for current nomenclature. All illustrated structures are from the right side. All morphological
98	measurements are in millimetres. Following Denis (1949), the position of the metatarsal
99	trichobothrium I (TmI) is expressed as the distance between the proximal end of the leg article and
100	the trichobothrial base divided by the total length of the leg article. For the toponymy and
101	classification of the different sectors and sub-sectors of the Alps, we followed the standard
102	partition of the Alpine chain (Marazzi 2005). Whenever applicable, we gave the speleological
103	cadastral codes of the caves in brackets ['regional code' and 'number'].
104	We used the following abbreviations in tables, text and figures: ALE, anterior lateral eyes;
105	AME, anterior median eyes; Cy, cymbium; E, Embolus; HB, horizontal branch of the lamella; P,
106	Paracymbium; PLE, posterior lateral eyes; PME, posterior median eyes; PL, Posterior lobes; PP,
107	posterior plate; RL, rounded lobe of the lamella; S, Scape; St, Stretcher; StA, Suprategular
108	apophysis; TLL, total leg length; TmI, position of trichobothrium on metatarsus of first leg; UB,
109	upper branch of the lamella.
110	
111	Molecular analyses

111 Molecular analyses

112 We compiled sequences for the cytochrome c oxidase subunit I (COI) and the Internal 113 Transcribed spacer 2 (ITS2) from previous studies (Mammola et al. 2015; Isaia et al. 2017; 114 Mammola et al. 2018a) available in Genbank. We generated additional sequences following the 115 laboratory protocols detailed in Mammola et al. (2015). We edited and managed sequences using 116 Geneious v. R10.2.6 (Kearse et al. 2012) . Since no indel mutations were inferred, COI was aligned 117 manually. We aligned ITS2 sequences using the online version of the program PRANK (Löytynoja 118 and Goldman 2010) available at the EBMBL website (https://www.ebi.ac.uk/goldman-srv/) with 119 default parameters.

120 We inferred the maximum likelihood tree of the COI data matrix (676 bp) with IQ-TREE v. 121 1.6.11 (Nguyen et al. 2015). We used IQ-TREE to first select the best-fit partitioning scheme and 122 corresponding evolutionary models (Kalyaanamoorthy et al. 2017), and then to infer the best 123 tree and estimate clade support by means of 1000 replicates of non-parametric bootstrapping. 124 Similarly, we conducted Bayesian (BI) analysis and divergence time estimation in BEAST v. 1.8.4 125 (Drummond et al. 2012) on the COI data matrix only, defining the partition scheme and models 126 selected by the BIC criterion in Partition Finder v2.1.1 (Lanfear et al. 2017). We specified a birth 127 and death tree prior and an unlinked relaxed uncorrelated lognormal clock for each gene. We 128 estimated absolute divergence times by defining a normal distribution on the substitution rate 129 prior (ucld.mean), truncated at 0, with a starting and mean value of 0.0125 and standard deviation 130 0.02, based on spider substitution rate estimates available in the literature for spiders (Bidegaray-131 Batista and Arnedo 2011). We ran three independent chains of 10 million generations each, 132 sampling every 1,000 generations. We monitored the chain convergence, the correct mixing, and 133 the number of generations to discard as burn-in (10%) with Tracer v. 1.7 (Rambaut et al. 2018). 134 We determined species boundaries using the multirate PTP (m-PTP) species delimitation 135 model (Kapli et al. 2017). This single marker method has been shown to outperform other 136 delimitation methods by providing more stable outputs without requiring the ultrametric 137 transformation of tree branches (Blair and Bryson 2017). We implemented the m-PTP model using 138 a Markov chain Monte Carlo approach, which allows estimates of support values on the 139 delimitations, on the COI matrix. The analyses were conducted on the best IQ-TREE. We ran 5 140 chains of 100 million generations each, removing the first 2 million as burn-in, and discarding all 141 branches with lengths smaller or equal to 0.0001. Additionally, we implemented the GMYC 142 delimitation method (Fujisawa and Barraclough 2013) to corroborate our results using an 143 ultrametric tree instead. We inferred the tree using the same settings as defined above for the

- 144 BEAST analysis but assuming a coalescent tree prior (constant population size), which has been
- 145 suggested to provide a more rigorous test of delimitation since the GMYC model assumes a single
- 146 species as the null option (Monaghan et al. 2009).
- 147 We determined the number of alleles in the ITS-2 matrix considering the gaps as
- 148 absence/presence data using the simple coding methods described by Simmons and Ochoterena
- 149 (2000) and implemented in the R package 'haplotypes' (Aktas 2015). We constructed the allele
- 150 network using the statistical parsimony method (Templeton et al. 1992; Clement et al. 2000), with
- a confidence limit of 90% also using 'haplotypes'.
- Uncorrected pairwise genetic distances were calculated in MEGA X (Kumar et al. 2018). The
 phylogenetic tree was edited for aesthetic purposes using FigTree
- 154 (http://tree.bio.ed.ac.uk/software/figtree/).
- 155
- 156 Analysis of morphological traits and niche overlap

157 We studied morphological differentiation among the three species by analysing variations 158 in morphological traits in a multi-dimensional morphospace (Blonder et al. 2014; 2018). For each 159 species, we measured 10 female specimens for different morphological traits: sternum length and 160 width, cephalothorax height (measured at the eye region, starting from the clypeus base to the 161 top of the profile), leg I and II length, and six more traits related to eye regression (diameter of 162 ALE, AME, PME, PLE, and the total length of the anterior and posterior eye lines). The functional 163 meaning of these traits is discussed in Mammola et al. (2018a, 2019, 2020). We acquired 164 morphological measurements for the three species and constructed their *n*-dimensional kernel 165 density hypervolumes following the approach described in Mammola et al. (2020). In short, we 166 applied a Gower dissimilarity measure to the complete trait matrix and analysed the resulting 167 distance matrix through Principal Coordinate Analysis (PCoA) in order to extract orthogonal

168 morphological axes for hypervolume construction. To obtain comparable data with that in 169 Mammola et al. (2020), we selected the first three PCoA to delineate the shape of the 170 hypervolumes (>75% variance explained). We constructed hypervolumes using a Gaussian kernel 171 density estimator and a cross-validation estimator to assess the optimal bandwidth value for each 172 axis (Blonder et al. 2018). We calculated niche differentiation using one overlap and one distance 173 metric (Mammola 2019), respectively the pairwise overall differentiation among kernel 174 hypervolumes (β total; Mammola and Cardoso 2020) and the distance between centroids. All 175 morphological measures used to construct the hypervolume are deposited in the World Spider 176 Trait database (Pekár et al., 2021a, 2021b). 177 178 Analysis of thermal tolerance 179 We performed experimental tolerance trials to evaluate the thermal breadth of each 180 species. Full details on experiment protocol and statistical analyses are presented in Mammola et 181 al. (2019) and all thermal tolerance estimates are available in the World Spider Trait database (Pekár et al., 2021a, 2021b). In short, we collected 85 al we specimens of the three lineages of 182 183 *Troglohyphantes vignai* aut. from subterranean sites across their range. In each cave, we collected 184 spiders by hand and placed them in individual Eppendorf Tubes[®] of 5 ml. We stored all vials in a 185 cool bag and transported it the same day to the laboratory at the University of Turin, where we 186 conducted the experiments. In the laboratory, we placed specimens in a IPP 30 Peltier Memmert 187 climatic chamber, each specimen in individual Petri dish, and acclimated them for one day at the temperature of the cave. Air humidity was kept at 100% during the acclimation and the 188 189 experiments, by placing two Petri dishes filled with water inside the climatic chamber. Owing to 190 the high resistance to starvation of subterranean spiders (Mammola and Isaia, 2017), specimens

191 were not fed throughout the experiments. For each sampled cave, we derived the internal mean

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192	annual temperature from continuous temperature measurements taken between 2012 and 2013
193	(Mammola et al. 2018b), or unpublished temperature records taken via the same methodology.
194	For two caves lacking field-collected temperature records, we downloaded annual temperature
195	series from the same period from the nearest thermo-hygro-pluviometric weather station. After
196	correcting the data with the standard environmental lapse rate (0.57°C/100 m; Rubel et al. 2017),
197	we calculated the mean annual temperature and used this value as a direct proxy of the cave
198	temperature (Badino 2010; Mammola et al., 2018b; Sánchez-Fernández et al. 2018).
199	The day after the sampling, we started the experiment, progressively raising the
200	temperature within the chamber with an increasing rate of 1°C/day. We evaluated the individual
201	critical temperature as the total paralysis, namely the temperature at which the individual was
202	immobile and no appendage movements were visible. For each species tested, we kept 10% of the
203	specimens as controls in stable climatic conditions. No mortality was recorded in controls during
204	the experiments.
205	We constructed survival curves with binomial generalized linear models (GLMs), modelling

206 the survival rate of each species as a function of ΔT , namely the difference between critical 207 temperature and the mean temperature of the cave where each species was collected. We 208 evaluated differences in ΔT between each species and between populations of each species via 209 analysis of variance (ANOVA).

- 210
- 211 **RESULTS**
- 212 Morphology

The comparative examination of male pedipalps of 38 specimens from eighteen different localities across the range of *Troglohyphantes vignai* aut. allowed a first preliminary identification of three morpho-species which formed the basis of comparison for the subsequent analyses (Fig. 216 1). We based the distinction of the three putative morpho-species on the overall shape of the 217 *lamella characteristica* of the male palps, which is considered the most important diagnostic 218 character in males of Troglohyphantes (Deeleman-Reinhold 1978, Isaia et al. 2017). Specimens 219 from the northernmost localities (Northern Cottian Alps) clustered in a geographically isolated 220 group which was provisionally named "T. achillis". Specimens from the remaining localities 221 clustered in two groups, the first ("T. vignai") comprised the type locality of T. vignai Brignoli 1971 and two nearby caves plus all localities in the southernmost sector of the range. The second ("T. 222 223 *delphinicus*") included all remaining localities scattered in the central part of the distribution 224 range. 225 In particular, when examining the A/B ratio (see Fig. 2) of the male palps from each group, we 226 detected a significant effect of on species delimitation based on ANOVA [F_(2.15)= 20.41, p< .001]. A 227 post hoc Tukey test showed that specimen assigned to T. delphinicus was significantly different 228 from both *T. achillis* and *T. vignai* at p < .05; conversely, the comparison between *achillis* and 229 vignal only approached statistical significance (p= .08). When examining females from the 230 corresponding localities, there was a significant effect of the epigyne ratio C/D (see Fig. 2) on 231 species delimitation based on ANOVA $[F_{(2,16)} = 4.11, p = .036]$. A post hoc Tukey test revealed that 232 values of *T. vignai* were significantly different from *T. achillis* at p < .05; conversely, the other 233 species comparisons were not significant.

234

235 Molecular analysis

We gathered 49 sequences, 676 bp long COI sequences — 4 new for the present study and 41,412 position long ITS2 aligned sequences — 5 new. The data matrices included sequences of *Troglohyphantes nigraerosae* Brignoli 1971 (3 of cox1, 1 of ITS2) following a former study that identified this species as the closest relative of *T. vignai* and the new species with high support

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240 (Mammola et al. 2020). Localities of sequenced material are listed in Table S1 available as 241 Supplementary Material to this paper. The sequences of the COI corresponded to 28 haplotypes 242 and the ITS2 to 11 alleles. None of the haplotypes or alleles were shared by more than one of the 243 morphologically delineated species. The Maximum likelihood and the time-aware Bayesian 244 inference analyses also agreed in recovering three main lineages corresponding to the 245 morphological delineations, and show similar relationships, but with contrasting levels of support. 246 The mPTP method confirmed the three lineages as potential candidate species the reinafter 247 indicated as T. vignai, T. delphinicus sp. nov. and T. achillis sp. nov.), although the split between T. 248 vignal and T. delphinicus sp. nov. was poorly supported. The GMYC method could not reject the 249 single species model over the multiple species (p= .62), which resulted in four clusters that 250 matched *T. vignai* and *T. achillis* sp. nov. but further split *T. delphinicus* sp. nov. into two different 251 coalescent clusters grouping haplotypes from the two neighbouring localities of Buco del Nebin 1 252 and Tana dell'Orso (PK1105 and SM02) versus the remaining haplotypes (see Tab. 1 and Fig. 3). 253 ITS allele were exclusive to each species. However, the ITS allele network suggested 254 contrasting population structures for each species. We did recover star-like relationships for the 255 ITS2 alleles of *T. vignai* and *T. achillis* sp. nov., while *T. delphinicus* sp. nov. included highly 256 divergent alleles, some of them being more similar to those of the other two species (Fig. 4). 257 Divergence time estimation analyses inferred a late Pliocene-early Pleistocene origin of the 258 three main lineages (Fig. 3). Coalescent times for *T. delphinicus* sp. nov. were older than for the 259 other two species, although confidence intervals were very wide and largely overlapped. 260 Accordingly, T. delphinicus sp. nov. showed the highest intraspecific genetic variability in both COI 261 and ITS-2 (5% and 1%, respectively) (see Tab. 1). Uncorrected genetic distances to T. nigraerosae 262 ranged from 14 to 15.4% and 1.2 to 1.3% in COI and ITS-2, respectively, and genetic divergence 263 between the three lineages ranged between 7.8% (T. delphinicus sp. nov. to T. achillis sp. nov.) to

264	9.1% (T. vignai to T. achillis	sp. nov.) and from 0.6% (T. vignai to T. achillis s	p. nov.) to 0.9%	(T
			· · ·	3		•

265 *delphinicus* sp. nov. to *T. achillis* sp. nov.) in COI and ITS-2, respectively.

266

267 Analysis of morphological traits

268	We measured 10 female specimens of each of the three species to estimate morphological
269	n-dimensional hypervolumes (Fig. 5). Troglohyphantes delphinicus sp. nov. had the most
270	voluminous hypervolume (0.42), followed by <i>T. vignai</i> (0.22), and <i>T. achillis</i> sp. nov. (0.11). The
271	overlap in the trait space of the three species was comparable with that of other Troglohyphantes
272	species adapted to the same microhabitat within Western Alpine caves (see Mammola et al.
273	2018a, 2020 for comparative data). Distance between centroids of the three species
274	morphospaces was below 0.2, and β diversity always above 0.3. Troglohyphantes vignai and T.
275	delphinicus sp. nov. showed the highest morphospace overlap, whereas T. achillis sp. nov. had the
276	most divergent morphospace (Fig. 5).
277	
278	Thermal tolerance
279	A total of 85 specimens were successfully tested in the thermal chamber trials (11

total of 85 specimens were successfully tested in the thermal chamber trials (.19 280 specimens of *T. delphinicus* sp. nov., from 1 cave; 23 specimens of *T. vignai* from 2 caves; 51 281 specimens of *T. achillis* sp. nov. from 4 caves). A general narrow thermal tolerance was observed 282 for the three species, with critical temperature values comparable to that of other high and 283 intermediate specialized subterranean Troglohyphantes species (see Mammola et al. 2019 for 284 comparative data). Specifically, we detected a significant effect of thermal tolerance on species 285 delimitation based on ANOVA [F_(2.67)= 15.16, p < .001] (Fig. 6). Accordingly, *Troglohyphantes* 286 achillis sp. nov. showed intermediate thermal tolerance (mean $\Delta T\pm s.d.: 4.82\pm 2.92; \Delta T$ range: 0-287 12), T. delphinicus sp. nov. was the least tolerant species, reaching 50% mortality at temperature

- values 1°C above its cave temperature (mean ΔT±s.d.: 0.67±0.77; ΔT range: 0–2) and *T. vignai*
- proved to be the species with the greatest thermal tolerance (mean $\Delta T \pm s.d.: 8.00 \pm 5.43$; ΔT range:
- 290 1–17). More specifically, a *post hoc* Tukey test revealed that all species were significantly different
- in their ΔT at p < .05. For each species, there were no significant differences between cave
- 292 populations with the exception of one population of *T. achillis* from Tuna dal Diau cave
- 293 (highlighted with an asterisk in Fig. 6), displaying a significantly higher thermal tolerance compared
- to the other populations of the same species based on ANOVA $[F_{(3,38)} = 9.41, p < .001]$.
- 295
- 296
- 297 Taxonomy
- 298 Family LINYPHIIDAE Blackwall 1859
- 299 Genus *Troglohyphantes* Joseph 1882
- 300 Troglohyphantes achillis Isaia and Mammola, sp. nov.
- 301 Figs. 7, 10a-b
- 302 Taxonomic references
- 303 Troglohyphantes vignai Pesarini 2001: 116 (S of T. rupicapra)
- 304 Troglohyphantes vignai Isaia et al. 2011b: 141, 2.68 (f)
- 305
- 306 Type series
- Holotype male. Italy, Piemonte, Province of Torino: Roure, Tana del Diavolo [1591 Pi] (Fig. 11a),
 7.122°E, 45.026°N, 1414 m, 19.IX.2007, Isaia M. leg. (MCSNB).
- 309 Paratypes. Italy, Piemonte, Province of Torino: Perrero, Grotta di Chiabrano or Tuna dal Diau [1621] Pi], 7.106°E 44.947°N, 1080 m, 23.X.2006, Isaia M. and Lana E. leg. 1m, 5f, 3 juv (MCSNB); same 310 311 locality, 4.XII.2006, Lana E. leg. 4f (MCSNB); same locality, 27.V.2019, Isaia M. and Nicolosi G. leg. 312 2f; Perrero, Prospetto di Miniera di Boccetto [Art. Pi], 7.062°E, 44.949°N, 1237 m, 21.II.2007, Isaia 313 M. leg. 1f, 1 juv.; Pramollo, Prospetto di Miniera di Tornini [Art. Pi], 7.198°E, 44.908°N, 990 m, 314 13.I.2015, Isaia M. and Mammola S. leg. 1m, 1f; same locality, 14.X.2010, Isaia M. leg. 2m, 1f, 3 juv.; 315 same locality, 27.V.2019, Isaia M. and Mammola S., leg; same locality, 14.VII.2017, Isaia M. and 316 Mammola S. leg. 2m,7f, 3juv; Roure, Tana del Diavolo [1591 Pi], Isaia M. and Paschetta M. leg. 2m; 317 same locality, 19.IX.2007, Isaia M. leg. 1f (MCSNB); same locality, 14.VII.2017, Isaia M. and 318 Mammola S. leg. 7m, 6f, 9juv.
- 319
- 320 Other material examined
- 321 Italy, Piemonte, Province of Torino: **Perrero**, Grotta di Chiabrano or Tuna dal Diau [1621 Pi], 322 19.IX.2007, Isaia M. leg. 1m, 1f; same locality, 6.XII.2007, Isaia M. leg. 1m, 1f; same locality,

19.I.2007, Isaia M. leg. 1m, 1f, 3 juv; Perrero, Prospetto di Miniera di Boccetto superiore [Art. Pi],
7.083°E, 44.958°N, 1310 m, 8.I.2019, Isaia M. and S. Mammola leg. 1m, 2f; Roure, Tana del Diavolo
[1591 Pi], 15.IV.1995, Lana E. leg. 1m; same locality, 12.XI.2006, Lana E. leg. 2m, 1f; same locality,
23.XI.2002, Lana E. leg. 2f, 1 juv.

328 Re-examined material

Former literature records (sub *T. vignai* or otherwise specified) are here revised and assigned to *T. achillis* sp. nov. on the basis of morphological examination. The identification of juveniles is based on the identification of adults occurring in the same locality. For details about material see the original publications.

333

327

334 Italy, Piemonte, Province of Torino: Perrero, Grotta di Chiabrano or Tuna dal Diau [1621 Pi]: Isaia 335 and Pantini 2010 ; Isaia et al. 2010; Isaia et al. 2011a; Isaia et al. 2011b ; Mammola et al. 2015 ; 336 Mammola et al. 2018b; Perrero, Prospetto di miniera di Boccetto [Art. Pi]: Isaia et al. 2010; Isaia et 337 al. 2011b ; Pramollo, Prospetto di miniera di Tornini [Art. Pi]: Mammola et al. 2015 ; Mammola and 338 Isaia, 2016; Mammola et al. 2018b; Roure, Balm Chanto [1575 Pi]: Pesarini, 2001; Isaia et al. 2011b; 339 Roure, Tana del Diavolo [1591 Pi]: Arnò and Lana, 2005 sub Troglohyphantes sp.; Isaia et al. 2010; 340 Isaia and Pantini 2010 Fig. 15-16; Isaia et al. 2011b (see note in re-examined material of T. 341 delphinicus about the misprint in Fig. 2.67); Mammola et al. 2015; Mammola et al. 2018b.

- 342
- 343 Diagnosis

344 Males of *Troglohyphantes achillis* sp. nov. are primarily distinguished from other species of

345 *Troglohyphantes* by the shape of the *lamella characteristica* (Figs. 1, 7a). The new species is close to

346 T. vignai and other species of the Henroti complex (sensu Pesarini 2001), from which it is

347 distinguishable by the short lower branch of the *lamella characteristica*. In particular, the A/B ratio

348 (Fig. 2a) is between 0.8 and 1.0. The identification of females in absence of males remains doubtful

- in most cases.
- 350
- 351 Description

352 *Male paratype* from type locality (Roure, Tana del Diavolo [1591 Pi], Isaia M. and Paschetta M. leg.

353 12.XI.2006): prosoma 1.07 long, 0.96 wide, yellowish. Thoracic region slightly swollen, yellowish

354 with grey shades. Cephalic region elevated, interspersed with black bristles between the eyes, with

355 few small black bristles around the eye region and continuing backwards in three longitudinal rows

356 converging at the thoracic furrow. One bristle just below AME. Carapace with darker margins.

Invertebrate Systematics

357 Clypeus slightly indented under the eyes, then convex, 0.34 long. Eyes normally developed, with 358 pigment and black margins. AME smallest. PME and PLE slightly bigger than ALE. ALE and PLE 359 contiguous. PLE-PME distance = 0.07, ALE-AME distance = 0.04, PME-PME distance = 0.9. Eye 360 diameters AME 0.04, PME 0.07, ALE 0.06, PLE 0.08. Sternum heart-shaped, yellowish with faintly 361 darkened anterior edges. Chelicerae light brownish, 0.51 long, with ca. 35 lateral stridulatory ridges 362 and armed with three anterior teeth. Legs yellowish, uniform in colour. Leg I: femur 2.59, patella 363 0.47, tibia 2.83, metatarsus 2.56, tarsus 1.45, TLL 9.90; leg II: femur 2.54, patella 0.41, tibia 2.70, 364 metatarsus 2.36, tarsus 1.36, TLL 9.37; leg III: femur 2.20, patella 0.31, tibia 2.08, metatarsus 2.03, 365 tarsus 1.02, TLL 7.64; leg IV: femur 2.76, patella 0.40, tibia 2.58, metatarsus 2.41, tarsus 1.22, TLL 366 9.37. Abdomen greyish with darkened distal pattern (Fig. 7d, 10b), 1.64 long, 0.97 wide. Palp: femur 367 0.73, patella 0.23 tibia 0.28 (see Tab. S2 for measurements of additional specimens). Male palp (Fig. 368 7a) with cymbium faintly convex, roughly triangular when seen from above, ending proximally with 369 a pointed tip. Posterior part of paracymbium subrectangular, apical part narrowed anteriorly. 370 Lamella characteristica similar to T. vignai, clearly visible in lateral view without extraction. Distal 371 part of the *lamella* gun-shaped with one superior horizontal branch and an inferior rounded lobe 372 directed anteriorly. One additional upper short branch is directed upwards, almost perpendicular to 373 the horizontal branch. Suprategular apophysis directed upwards, with a sharp end. Tip of the 374 embolus sharply pointed. A/B ratio between 0.8 and 1.0 (n= 7, mean±s.d.= 0.878±0.057; see Fig. 375 2a).

376 Spination: Femur I with one prolateral and one dorsal spine; Femur II–IV with one dorsal spine. 377 Patella I–IV with one dorsal spine. Tibia I with two dorsal, four ventral, two retrolateral and two 378 prolateral spines; Tibia II with two dorsal, three ventral, two retrolateral, and two prolateral spines; 379 tibia III with two ventral, two prolateral, two dorsal and three retrolateral spines; Tibia IV with three 380 ventral, three dorsal, two prolateral, and three retrolateral spines. Metatarsus I–IV with one dorsal 381 spine. Patella of the Palp with one long, curved spine. Position of TmI: 0.17. Trichobothrium on Mt382 IV absent.

383

384 Female paratype (Tornini, Prospetto di miniera [Art. Pi], Isaia M. and Mammola S. leg. 13.I.2015): 385 prosoma 1.38 long, 1.05 wide, slightly darker than male. Cephalic region light-brownish. Carapace, 386 ocular area, clypeus, and sternum are similar in all features to the analogous male body parts. 387 Sternum light brownish with dark margins. Anterior margin of the chelicerae armed with three 388 teeth. Clypeus 0.24 long, chelicerae 0.70 long. PLE–PME distance = 0.05, ALE–AME distance = 0.04, 389 PME–PME distance = 0.07, AME–AME distance = 0, ALE–PLE distance = 0. Eye diameters: AME 0.03, PME 0.07, ALE 0.06 and PLE 0.07. Abdomen 1.82 long, 1.15 wide, greyish with darkened distal 390 391 pattern (Fig. 7d). Leg I: femur 2.65, patella 0.44, tibia 2.70, metatarsus 2.44, tarsus 1.50, TLL 9.73; 392 leg II: femur 2.70, patella 0.46, tibia 2.53, metatarsus 2.34, tarsus 1.42, TLL 9.45; leg III: femur 2.18, 393 patella 0.43, tibia 2.07, metatarsus 1.88, tarsus 1.07, TLL 7.63; leg IV: femur 2.71, patella 0.33, tibia 394 2.30, metatarsus 2.32, tarsus 1.25, TLL 8.91. Female palp: femur 0.73, patella 0.20, tibia 0.43, tarsus 395 0.82, total palp length 2.18 (see Tab. S2 for measurements of additional specimens). Spination 396 approximately like in male. Position of TmI: 0.17. Trichobothrium on Mt IV absent. Epigyne (Fig. 7b, 397 c) protruding, with subtriangular scape, not much wider than long, covering less than half of the 398 inner part of the epigyne, C/D ratio between 1.7 and 1.9 (n= 4; mean±s.d.= 1.80±0.19; see Fig. 2b). 399 Lateral margins of the scape converging, posterior margin rounded. Stretcher tongue-shaped, 400 straight (Fig. 7c), emerging halfway in the profile in lateral view, bearing a pitted knob at its end, 401 visible in ventral view. Lateral lobes emerging at the posterior end of the epigyne. Fertilization ducts 402 sclerotized.

403

404 Etymology

- 405 The species epithet derives from the name of the first collector and our esteemed colleague Prof.
- 406 Achille Casale, renowned Italian zoologist and biospeleologist.
- 407
- 408 Distribution
- 409 The species is known from localities of a small inner sector of the Northern Cottian Alps (Chisone
- 410 and Germanasca Valleys) (Fig. 1). The distribution range of the species covers an area of
- 411 approximately 50 km².
- 412
- 413 Biospeleological notes

This species was first collected by Achille Casale in 1983, in a cave of Chisone Valley, Northern 414 415 Cottian Alps. The record was firstly published by Pesarini (2001), who mentioned that this specimen 416 was collected at the locality Balm Chanto [1575 Pi], a rock shelter renown for the findings of 417 archaeological remains in the early 1980s. As specified later (Arnò and Lana, 2005; Isaia et al. 2011b) 418 and recently confirmed by the original collector (A. Casale, pers. comm. 2019), the exact locality 419 where the collection took place was in fact the nearby cave Tana del Diavolo [1591 Pi] (Fig. 11a). On 420 the contrary, the rock shelter of Balm Chanto mentioned in the original publication is open and 421 exposed to natural light, thus unsuitable for the occurrence of this subterranean species. Specimens 422 were collected by Achille Casale among the floor debris and on the walls of the dark zone of the 423 cave. All later collectors report findings of the species in similar habitats of natural caves and mine 424 prospects of Chisone and Germanasca Valleys. Troglohyphantes achillis sp. nov. shares the type 425 locality with *T. lucifer* Isaia, Mammola and Pantini 2017.

- 426
- 427
- 428 *Troglohyphantes delphinicus* Isaia and Mammola, sp. nov.
- 429 Fig. 8, 10c-d

- 430 Taxonomic references
- 431 *Troglohyphantes vignai* Isaia et al. 2011b: 140, f. 2.67A (m, misprinted locality in figure caption).
- 432
- 433 Type series
- 434 Holotype male. Italy, Piemonte, Province of Cuneo: Becetto, Pertus del Drai [1017 Pi], 7.223°E,
- 435 44.610°N, 1930 m, 25.VIII.2001, leg. Lana E. (MCSNB)
- 436 Paratypes. Italy, Piemonte, Province of Cuneo: Becetto, Pertus dei Drai [1017 Pi], 16.VII.2015, Isaia
- 437 M. and Mammola S. leg. 2m, 2f, 1 juv.; same locality, 25.VIII.2001 leg. Lana E. 5f (MCSNB); same
- 438 locality, 5.IV.2019, Isaia M., Mammola S. leg., 1m, 4f; same locality, 14.VII.2021, Isaia M., Biggi E.
- 439 leg., 5f; **Casteldelfino,** Tana dell'Orso [1019 Pi], 7.098°E, 44.559°N, 2360 m, 19.I.2018, Isaia M. and
- 440 Mammola S. leg. 2m; Isasca, Pertus d'Ia Rocho (Pertus d'Ia Tundo) [1265 Pi], 7.362°E, 44.585 N,
- 1000 m, 26.IX.2010, Lana E. and Chesta M. leg. 1m, 4f; **Sampeyre,** Buco del Nebin 1 (Buco della
- 442 Pioggia) [1158 Pi], 44.543°N, 7.143°E, 2480 m, 14.I.2019, Mammola S. and Isaia M. leg. 1f;
- 443 **Sampeyre,** Buco del Nebin 2 (Buco della Crozza) [1159 Pi], 44.544°N, 7.142°E, 2440 m,
- 444 18.VIII.2006, Lana E. leg. 2m, 2f (MCSNB); Valdieri, Grotta Topalinda (Maissa 2) [1210 Pi], 7.405°E,
- 445 44.261°N, 1200 m, 19.XI.2016, Isaia M. and Mammola S. leg. 1f; same locality, 9.VII.2017, Isaia M.
- and Mammola S. leg. 1f; same locality, 11.VI-22.IX.2017 in pitfall traps Isaia M., Chiappetta N. and
 Mammola S. leg. 1m.
- 448
- 449

450 *Material examined*

Italy, Piemonte, Province of Cuneo: Casteldelfino, Tana dell'Orso [1019 Pi], 14.I.2019, Isaia M. and
Mammola S. leg. 2m; Sampeyre, Buco del Nebin 1 [1158 Pi], 19.XI.2008, Lana E. leg. 1f (MCSNB);
Manuel Lagitu 14 L 2010, Manura la Candidació M. lag. 1f Maldiari, Create Tanalinda [1210 Bi]

- same locality 14.I.2019, Mammola S and Isaia M. leg. 1f; Valdieri, Grotta Topalinda [1210 Pi],
- 454 30.IX.2017, Isaia M. leg. 1f.
- 455 456
- 457 *Re-examined material*
- 458 Former literature records (sub *T. vignai* or otherwise specified) are here revised and assigned to *T.*
- 459 *delphinicus* sp. nov. on the basis of morphological examination. The identification of juveniles is
- 460 based on the identification of adults occurring in the same locality. For details about material see 461 original publications.
- 462

463 Italy, Piemonte, Province of Cuneo: Casteldelfino, Tana dell'orso [1019 Pi]: Lana, 2000 sub

- 464 Troglohyphantes sp.; Casale et al. 2000 sub Troglohyphantes sp.; Arnò and Lana, 2005 sub
- 465 *Troglohyphantes* sp.; Isaia et al. 2010; Isaia et al. 2011b; Mammola et al. 2015; Mammola et al.
- 466 2018b. Sampeyre, Buco del Nebin 1 [1158 Pi]: Isaia et al. 2010; Isaia et al. 2011b; Sampeyre,
- 467 Pertus dal Drai [1017 Pi]: Casale et al. 2000 sub *Troglohyphantes* sp.; Lana et al. 2002 sub
- 468 *Troglohyphantes* sp.; Arnò and Lana, 2005 sub *Troglohyphantes* sp.; Isaia et al. 2010; Isaia et al.
- 469 2011b (the caption of Fig. 2.67 A: 140 contains a misprint: the illustration of the male palp do not
- 470 refer to tana del Diavolo [1591 Pi] but to Pertus del Drai [1017 Pi] and thus to *T. delphinicus* sp.
- 471 nov.). Isasca, Pertus d'la Tundo (or d'la Rocho) [1265 Pi]: Isaia et al. 2011b.
- 472
- 473 Diagnosis

474 Males of *Troglohyphantes delphinicus* sp. nov. are primarily distinguished from other species of 475 *Troglohyphantes* by the shape of the *lamella characteristica* (Fig. 1, 8a). The new species is close to 476 *T. vignai* and other species of the Henroti complex, from which it is distinguishable by the longer 477 upper branch of the *lamella characteristica*. In particular, the A/B ratio (Fig. 2a) is between 1.1 and 478 1.3, higher than in *T. vignai* as currently defined and in *T. achillis* sp. nov.

479 **The** identification of females in absence of males remains doubtful in most cases.

- 480
- 481 Description

482 Male paratype (Becetto, Pertus del Drai [1017 Pi] (Fig. 11b), Isaia M. and Mammola S. leg. 5.IV.2019): 483 prosoma 1.44 long, 1.21 wide, yellowish. Thoracic region slightly swollen, yellowish with grey 484 shades. Cephalic region elevated, interspersed with many black bristles between the eyes, with 485 several small black bristles forming the eye region and continuing backwards in three longitudinal 486 rows converging at the thoracic furrow. One bristle just below AME. Carapace with darker margins. 487 Clypeus slightly indented under the eyes, then convex, 0.40 long. Eyes normally developed, with 488 pigment and black margins. AME smallest. PLE and PME equal in size. ALE and PLE contiguous. PLE-489 PME distance = 0.10, ALE-AME distance = 0.14, PME-PME distance = 0.11. Eye diameters AME 0.02, 490 PME 0.04, ALE 0.05, PLE 0.05. Abdomen 1.83 long, 0.90 wide, pale greyish with some very faint spots 491 only visible on macrophotographs of alive specimens (Fig. 10c). Sternum heart-shaped, yellowish 492 with flimsy darkened anterior edges. Chelicerae light brownish, 0.55 long, with ca. 35 lateral 493 stridulatory ridges and armed with three anterior teeth. Legs yellowish, uniform in colour. Leg I: 494 femur 2.50, patella 0.70, tibia 2.54, metatarsus 2.14, tarsus 1.41, TLL 9.29; leg II: femur 2.52, patella 495 0.75, tibia 2.48, metatarsus 2.22, tarsus 1.29, TLL 9.26; leg III: femur 2.19, patella 0.38, tibia 2.30, 496 metatarsus 1.90, tarsus 1.10, TLL 7.87; leg IV: femur 2.29, patella 0.39, tibia 2.60, metatarsus 2.38, 497 tarsus 1.19, TLL 8.85. Palp: femur 0.59, patella 0.15, tibia 0.19 (see Tab. S2 for measurements of 498 additional specimens). Male palp (Fig. 8a) with cymbium faintly convex, roughly triangular when 499 seen from above, ending proximally with one stout apophysis. Posterior part of paracymbium 500 subtriangular, apical part narrowed anteriorly. Lamella characteristica similar to T. vignai and T. 501 achillis sp. nov., clearly visible in lateral view without extraction. Distal part of the lamella gun-502 shaped with one long horizontal branch and a small inferior, almost contiguous, rounded lobe. One 503 additional upper short branch is directed upwards, perpendicular to the horizontal branch. 504 Suprategular apophysis directed upwards, with a sharp end. Tip of the embolus sharply pointed. A/B 505 ratio between 1.1 and 1.3 (see Fig. 2a) (n= 4; mean±s.d.= 1.20±0.22).

506 Spination: femur I with one prolateral spine; all femurs with one dorsal spine. Patella I–IV with one 507 dorsal spine. Tibia I with three dorsal, three ventral, two retrolateral and two prolateral spines; Tibia 508 II with two dorsal, two ventral, two retrolateral, and two prolateral spines; tibia III with two 509 prolateral, two dorsal and two retrolateral spines; Tibia IV with two dorsal, two prolateral, and two 510 retrolateral spines. Metatarsus I–IV with one dorsal and one prolateral spine. Patella of the palp 511 with one long, curved spine. Position of TmI: 0.18. Trichobothrium on Mt IV absent.

512

513 Female (paratype from type locality, Isaia M. and Mammola S. leg. 16.VII.2015): prosoma 1.23 long, 514 1.15 wide, slightly darker than male. Cephalic region light-brownish. Carapace, ocular area, clypeus, 515 and sternum are similar in all features to the analogous male body parts. Light brownish sternum 516 with dark margins. Anterior margin of the chelicerae armed with three teeth. Clypeus 0.22 long, 517 chelicerae 0.60 long. PLE–PME distance = 0.06, ALE–AME distance = 0.04, PME–PME distance = 0.06, 518 AME-AME distance = 0, ALE-PLE distance = 0. Eye diameters: AME 0.05, PME, AME and PLE = 0.07. 519 Abdomen pale greyish with no pattern, cardiac mark slightly visible, 2.19 long, 1.84 wide. Leg I: 520 femur 2.45, patella 0.52, tibia 2.49, metatarsus 2.13, tarsus 1.49, TLL 9.08; leg II: femur 2.40, patella 521 0.51, tibia 2.42, metatarsus 1.97, tarsus 1.41, TLL 8.70; leg III: femur 2.04, patella 0.47, tibia 1.96, 522 metatarsus 1.67, tarsus 0.98, TLL 7.12; leg IV: femur 2.56, patella 0.42, tibia 2.47, metatarsus 2.15, 523 tarsus 1.26, TLL 8.86. Female palp: femur 0.69, patella 0.18, tibia 0.39, tarsus 0.81, total palp length 524 2.07 (see Tab. S2 for measurements of additional specimens). Spination: Femur I with one prolateral 525 spine; Femur I–IV with one dorsal spine. Patella I–IV with one dorsal spine. Tibia I with two dorsal, 526 two prolateral, five ventral and two retrolateral spines; Tibia II with two dorsal, three prolateral, 527 three ventral and two retrolateral spines; Tibia III with two dorsal, two prolateral and two 528 retrolateral spines. Tibia IV with two dorsal, two ventral and two retrolateral spines. Metatarsus I-529 IV with one dorsal and one prolateral spine. Tarsus of the palp with four dorsal, three retrolateral, 530 four ventral and one prolateral spines. Position of Tml: 0.18. Trichobothrium on Mt IV absent. 531 Epigyne (Fig. 8b, c) protruding, with subtriangular scape, not much wider than long, covering less 532 than half of the inner part of the epigyne, C/D ratio between 1.8 and 2.0 (n= 8, mean±s.d.= 533 1.96±0.16, see Fig. 2b), higher than *T. achillis* sp. nov. and lower than *T. vignai* (see Fig. 2b). Posterior 534 margin of the scape with almost parallel lateral margins, posterior profile less rounded than T. 535 achillis sp. nov. Stretcher tongue-shaped, straight, emerging halfway in the profile in lateral view 536 (Fig. 8c), bearing a pitted knob at its end, visible in ventral view. Lateral lobes emerging at the 537 posterior end of the epigyne. Fertilization ducts sclerotized.

538

539 Etymology

The species epithet is an adjective referring to the historical region of Dauphiné (Delfinato) of the French *ancien régime*, before the French revolution. The species shares the etymology with *Pimoa delphinica* Mammola, Hormiga and Isaia 2016 (family Pimoidae), a Western Alpine endemic species recently described from the same area (Mammola et al. 2016).

544

545 Distribution

546	The species is known in a few caves of Varaita Valley (Southern Cottian Alps) and an isolated
547	locality in Gesso Valley (Maritime Alps). The area covered by the minimum convex polygon
548	encompassing the known localities of this species is about 400 km ² , when excluding the isolated
549	population of Valle Gesso (Topalinda cave, Maritime Alps), the size of the range is 70 Km ² (see Fig.
550	1).
551	
552	Biospeleological notes
553	This species was firstly collected by Enrico Lana and Mike Chesta in two caves in Varaita valley in
554	summer 1999. The validity of the species was originally pointed out in 2006 by MI studying
555	specimens collected by Enrico Lana in Nebin 1 cave [Pi 1158], located a few meters under the top of
556	Mount Nebin (2510 m) on the ridge separating Maira and Varaita valleys (Southern Cottian Alps).
557	The availability of more material collected during the years by the authors and the support of
558	molecular analysis here presented have now confirmed the validity of this species.
559 560	
561	Troglohyphantes vignai Brignoli 1971
562	Fig. 9, 10e-f
563	Taxonomic references
565	Troglohyphantes runicapra Brignoli 1971: 170, 1. 52-58 (Diff)
566	Troglohyphantes rupicapra Brignoli 1979: 321. f. 13-14 (f)
567	Troglohyphantes rupicapra Pesarini 1988: 238, f. 3 (Dm)
568	Troglohyphantes vignai Isaia et al. 2011b: 140, f. 2.67B-C (f)
569	
570	
571	Material examined
572	Italy, Piemonte, Province of Cuneo: Chiusa Pesio , Grotta delle Camoscere Superiore [250 Pi],
573	/.600°E, 44.200°N, 1140 m, 29.XI.2007, Isaia M. leg. 1m; same locality, 26.XII.2014, Isaia M.,
374 575	iviammola S. leg. 1m, 21, Same locality, US.XII.2018, ISala IVI., Iviammola S. leg. 1m, 1f; Same
576	locality, 03.711.2010, Isala IVI. and IVIanimola 3. Ieg. 2111, Chissolo, Balina di Valenza [1009 Pl] (type locality Fig. 11c) 7.017°F 44.681°N 1440 m 13 XI 2014 Isala M. Mammola and Paschetta M.
577	leg. 2f, 2juv; same locality, 8.I.2019, Isaia M. and Mammola S. leg. 1m, 9f, 2juv; same locality,

578 13.VII.2021, Isaia M. and Biggi E. leg. 2f; **Frabosa Soprana**, Grotta Beppe Bessone [3303 Pi],

579 7.818°E, 44.208°N, 1419 m, 22.V.2015, Isaia M. and Mammola S. leg. 1m, 2f; Frabosa Sottana,

580 Tana del Bergamino [175 Pi], 7.767°E, 44.255°N, 1175 m, 21.VI:2021, Isaia M. and Tolve M. leg. 2f,

- 581 2m; Garessio, Pozzo Ciuaiera [146 Pi], 7.886°E, 44.191°N, 2099 m, 12.VI.1999, T. Pascutto leg. 1m;
- same locality, 18.VIII.2009, Lana E. leg. 1m, same locality, 12.X.2008, Lana E. leg. 3m, 4f; Ostana,
- 583 Pertui de l'Oustanetto [1251 Pi], 7.197°E, 44.714°N, 2180 m 8.VII.2001, Lana E. leg. 1f; **Crissolo**,
- 584 Pertugio Stopponetto [1047 Pi], 7.148°E, 44.682°N, 1815 m, 16.VII.2006, Lana E. leg. 1 juv;
- 585 Roccaforte Mondovì, F12 del Mongioie [3050 Pi], 7.781°E, 44.169°N, 2471 m, 27.VIII.2018, Isaia M.
 586 and Mammola S. leg. 3f.
- 587

591

588 Re-examined material

589 Former literature records are here revised and assigned to *T. vignai* on the basis of morphological 590 examination. For details about material see original publications.

592 Italy, Piemonte, Province of Cuneo: Briga Alta, Voragine di Scarasson [221 Pi]: Arnò and Lana, 2005 593 sub Troglohyphantes sp.; Isaia et al. 2010 sub T. rupicapra); Isaia et al. 2011b; Briga Alta, Pozzo 2-6 594 delle Carsene o Abisso Arrapa Nui [772 Pi]: Lana et al. 2002 sub T. cf. rupicapra; Arnò and Lana, 595 2005 sub Troglohyphantes sp.; Isaia et al. 2010, sub T. rupicapra; Isaia et al. 2011b; Briga Alta 596 Pozzo 1-5 delle Carsene o Abisso Rangipur [761 Pi]: Lana et al. 2002 sub T. cf. rupicapra; Arnò and 597 Lana, 2005 sub Troglohyphantes sp.; Isaia et al. 2010, sub T. rupicapra; Isaia and Pantini, 2010 Fig. 598 17-18; Isaia et al. 2011b (see previous note under T. delphinicus sp. nov. about the misprint in the 599 legend of Figure 2.67); Briga Alta, Abisso Vento [3500 Pi]: Isaia and Pantini, 2010; Isaia et al. 600 2011b; Briga Alta, Unknown cave near Colle del Pas: Mammola et al. 2015; Chiusa Pesio, Grotta 601 delle Camoscere [105 Pi]: Bologna and Vigna Taglianti, 1982 sub T. rupicapra; Casale, 1986 sub T. 602 rupicapra; Morisi in GSAM, 1987 sub T. rupicapra; Lana, 2001 sub T. rupicapra; Arnò and Lana, 603 2005 sub T. rupicapra; Isaia et al. 2011b; Chiusa Pesio, Grotta superiore delle Camoscere [250 Pi]: 604 Vigna Taglianti and Follis, 1968 sub Troglohyphantes prope ghidini; Morisi, 1971 sub T. rupicapra; 605 Casale and Longhetto, 1970 sub Troglohyphantes prope ghidini; Brignoli, 1971 sub T. rupicapra; 606 Casale, 1971 sub T. rupicapra; Brignoli, 1972 sub T. rupicapra; Brignoli, 1975 sub T. rupicapra; 607 Bologna and Vigna Taglianti, 1985 sub T. rupicapra; Casale, 1986 sub T. rupicapra; Pesarini, 1988 608 sub T. rupicapra; Lana, 2001 sub T. rupicapra; Arnò and Lana, 2005 sub T. rupicapra; Isaia et al. 609 2007; Isaia et al. 2011b; Mammola et al. 2015; Mammola et al. 2018b. Crissolo, Buco di Valenza o 610 Balma dell'inglese [1009 Pi]: Morisi, 1971; Brignoli, 1971; Casale, 1971; Brignoli, 1972; Bologna and 611 Vigna Taglianti, 1982; Arnò and Lana, 2005; Isaia et al. 2011b; Mammola et al. 2015. Crissolo, 612 Fortino a ovest della Balma di Rio Martino, Opera 372 Rocca di Granè [Art. Pi]: Arnò and Lana, 613 2005 sub Troglohyphantes sp.; Isaia et al. 2010; Isaia et al. 2011b; Garessio, Voragine della 614 Ciuaiera [146 Pi]: Isaia and Pantini, 2010; Isaia et al. 2011b; Frabosa Soprana, Grotta Beppe 615 Bessone [3303 Pi]: Isaia et al. 2017; Ostana, Pertui de l'Oustanetto [1251 Pi]: Lana et al. 2002 sub 616 T. cf. vignai; Isaia et al. 2011b; Pertugio di Stopponetto [1047 Pi].

- 617
- 618 New diagnosis
- 619 In light of the split of *T. vignai* aut. here proposed, males of *Troglohyphantes vignai* Brignoli 1971
- 620 are primarily distinguished from other species of *Troglohyphantes* by the shape of the *lamella*
- 621 *characteristica* (Fig. 9a). The species is distinguishable from *T. achillis* sp. nov. and *T. delphinicus* sp.

- 622 nov. by the shape of the lamella characteristica, with A/B ratio between 0.6 and 0.8 (n= 6,
- 623 mean±s.d.= 0.71±0.05), lower than in *T. delphinicus* and *T. achillis* sp. nov. (see Fig. 2a). The
- 624 identification of females in absence of males remains doubtful in most cases.
- 625
- 626
- 627 Distribution
- 628 On the base of this work, *Troglohyphantes vignai* shows a disjunct distribution in Cottian (Po
- 629 Valley, northern clade) and Ligurian Alps (from Pesio to Tanaro Valley, southern clade) (Fig. 1). The
- 630 area covered by the minimum convex polygon encompassing all known localities of this species is
- 631 about 600 km². When considering the two clades, the northern one covers a range of nearly 10
- 632 km² and the southern one of approximately 80 km², for a total disjunct range of approximately 90
- 633 km².

634 Biospeleological notes

- 635 Specimens of the southern clade (Val Pesio and Tanaro) exhibit higher degree of troglomorphism
- 636 (higher depigmentation, reduction of PLE and PME, lowering of the profile of cephalothorax; see
- 637 Figs. 15—18 in Isaia et al., 2010) in respect to other populations of the same species). Southern
- 638 localities also include the former type locality of *T. rupicapra* Brignoli 1971 (= *T. vignai* Brignoli
- 639 after Pesarini 2001) considered by Brignoli (1971) a different species on the basis of the higher
- 640 level of troglomorphism adaptation to subterranean life (i.e. troglomorphic traist; Christiansen
- 641 2012) and small differences in the shape of the epigyne.
- 642
- 643 **DISCUSSION**
- 644 Systematics and phylogeography

645	The new species are diagnosed by small details of the male copulatory organs (Figs. 2a, 7-
646	9). In particular the A/B ratio of the male <i>lamella characteristica</i> proved to be diagnostic in most
647	cases, with negligible overlapping among species (Fig. 2a). Our attempt to separate females
648	according to C/D ratio revealed a certain degree of overlap among individuals of different species
649	(Fig. 2b). The overall size, leg length and abdominal pattern also provide useful comparative
650	details for separating <i>T. achillis</i> from the other two species, being the biggest in most anatomical
651	features and the only one with pigment on the distal part of the abdomen; see measurements in
652	Tab. S2 and Figs. 7d, 10). However, due to the intrinsic variability of these traits, we did not
653	mention them in the species diagnosis.
654	The COI and ITS-2 gene trees corroborate our morphological delineations, in as much they
655	recovered monophyly of the haplotypes and alleles of each putative species (Fig. 3) and mPTP
656	supported the split of <i>T. achillis</i> sp. nov. from the other species. Additionally, the level of genetic
657	divergence in the COI between the three species (~8%, Tab. 1) was above the threshold divergence
658	proposed to identify Troglohyphantes species (Isaia et al. 2017; see also Leaché et al., 2021 for
659	threshold divergence in other taxa). However, GMYC failed to reject the alternative hypothesis of
660	a single species and mPTP provided low support for the split of <i>T. delphinicus</i> sp. nov. and <i>T. vignai</i>
661	(Fig. 3). The deeper coalescent of COI haplotypes and ITS2 alleles observed in <i>T. delphinicus</i> sp.
662	nov. compared to the other two species may be partly responsible for the inability of single
663	markers delimitation methods to delimit the three species. In this regard, the estimated time
664	frame of diversification is compatible with the involvement of the Quaternary glacial cycles in the
665	generation of the present-day patterns of genetic variability and geographic structuring of the
666	three species. It is worth mentioning that <i>T. delphinicus</i> sp. nov. not only showed the highest

667	levels of genetic variability but was the only lineage that did not show a star-like structure in the
668	ITS alleles (Fig. 4), indicating that this species may have not undergone population bottlenecks as
669	severe as those experienced by the other species. A similar pattern was recovered in the
670	troglophile endemic Alpine species Pimoa delphinica Mammola, Hormiga and Isaia 2016 sharing
671	the same small range of <i>T. delphinicus</i> (Mammola et al. 2015; Mammola et al. 2016), which may
672	point to the existence of an important glacial refugia in this area. Unlike Pimoa, however, we
673	found neither cases of co-occurrence of congeneric species, nor any indication of hybridization
674	(Mammola et al. 2016). This is probably due to the higher level of genetic isolation seen in species
675	with higher level of subterranean adaptation such as Troglohyphantes.
676	
677	Habitat and natural history
678	Despite them never being found in syntopy, <i>Troglohyphantes achillis</i> sp. nov., <i>T. delphinicus</i> sp.
679	nov. and <i>T. vignai</i> were found consistently in the same habitat, i.e. in the dark zone of natural
680	caves or artificial subterranean habitats such as mines and bunkers. In rare occasions, specimens
681	of T. delphinicus sp. nov. and T. vignai were also collected outside, in the vicinity of cave entrances
682	in deep litter or under big stones. Interestingly, similarities in habitat preference are also mirrored
683	in the life cycle of the three species. In all cases, males are rarely found in spring or early autumn
684	and almost disappear in winter, while females are present all year-long.
685	
686	Ecological adaptation and incipient speciation

- 687 The ability of these species to maintain permanent underground populations is reflected in their
- 688 level of adaptation to subterranean life (i.e. troglomorphic traits; Christiansen 2012). In this

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689 respect, cave-adapted traits are particularly remarkable in the populations at the southern margin 690 of the range of *Troglohyphantes vignai* as currently defined, which are more depigmented, show 691 higher degree of eye reduction (PLE and PME) and a more pronounced lowering of the 692 cephalothorax profile—a trait that has been noticed in high subterranean adapted species of 693 Troglohyphantes (Isaia and Pantini 2010; Isaia et al., 2017; Mammola et al., 2020). Accordingly, in 694 our previous work (Isaia and Pantini, 2010), we assumed these populations were in a stage of 695 incipient speciation. Such findings were successively re-interpreted in the light of the first 696 molecular study conducted on this species (Mammola et al., 2015) uncovering high genetic 697 structuring (Mammola et al., 2015) and pointing out climatic isolation as the main factor 698 determining, from one side, the presence of different lineages in *T. vignai* aut. —that we now 699 recognize as separate species— and, from the other, the higher development of troglomorphism 700 observed in the southern populations. This framework is also coherent with our current findings 701 and with the different levels of support recovered for the species level status of the three lineages. 702 In fact, the three species are well delimited by male palp morphology and physiological traits, but 703 only partially by female genitalia and the analysis of morphospaces. Similarly, we found 704 contrasting results in molecular data, with support from mPTP on the COI data matrix, although 705 only two delimitations (*T. vignai* and *T. achillis* sp. nov.) were well-supported. GMYC failed to 706 reject the null hypothesis of a single species and the ITS2 recovered *T. delphinicus* sp. nov. as non-707 monophyletic.

Such mixed levels of support recovered by our multi layered approach confirms the context of an incipient speciation for these three species, which is echoed in the framework of the unified species concept (de Queiroz, 2005). Such incongruences reflect the continuous nature of the speciation process, which is particularly remarkable in our model organism, showing low dispersal coupled with high microhabitat specificity. 713

714 Distribution patterns and thermal tolerance

715	Troglohyphantes achillis sp. nov. prefers the medium alpine montane belt, from 1000 up to
716	1400 m above sea level (mean = 1180 m; n = 4), and inhabits sites characterised by mean annual
717	temperatures from 6.1 to 8.9°C (mean=7.5 °C; range: 2,8; n=4). Troglohyphantes delphinicus sp.
718	nov. was mainly collected in natural caves at an altitude ranging from 1000 to 2480 m
719	(mean=1901 m; n=6) and inhabits sites with mean annual temperatures from 0 to 8°C (mean =
720	2.8°C; range: 8; n = 6). <i>Troglohyphantes vignai</i> shows a general preference for the medium alpine
721	montane belt, from 1140 up to 2471 m above sea level (mean = 1794 m; n = 8), and inhabits
722	subterranean sites with mean annual temperatures ranging from 7.3 to 12.1°C (mean=9.28°C;
723	range: 4.8; n=8).
724	Results of thermal tolerance tests (Fig. 6) align well with the general pattern observed in
725	Mammola et al. (2019) for <i>Troglohyphantes</i> , as well as that obtained with other subterranean
726	arthropods (e.g., Raschmanová et al. 2018; Pallarés et al. 2019, 2020a, 2020b; Colado et al. 2021;
727	Jones et al, 2021), which predicts that specialized subterranean species show low thermal
728	tolerances than related surface species. In <i>Troglohyphantes</i> , this narrow thermal niche breadth
729	often translates into restricted dispersal ability, as reflected in the small distribution ranges. This is
730	particularly evident in the three species here considered, with <i>T. delphinicus</i> sp. nov. and <i>T. achillis</i>
731	sp. nov. being poorly tolerant and short ranged, followed by <i>T. vignai</i> , exhibiting higher tolerance
732	and a greater range. Interestingly, results on the thermal tolerance of Western Alpine specie of
733	Troglohyphantes reported in Mammola et al. (2019), revealed a high variability in this
734	physiological trait in <i>T. vignai</i> aut., which now appear to be fully coherent with the delimitation of

735 the three species proposed here. Results on thermal tolerance support the hypothesis that the 736 present-day pattern of isolated distributions in the three species is mostly explained by the 737 combination of low dispersal ability and low thermal tolerance (Mammola et al. 2019), and 738 historical biogeographic events (see Mammola et al. 2015). 739 It is worth noting that thermal tolerance tested on a single population of *T. delphinicus* sp. 740 nov. may be poorly representative for the species, which has actually been found in a number of 741 sites characterised by different altitudes and temperatures. This apparent contradiction could 742 provide and additional explanation for the highest genetic variability found in this species, which 743 would be the result of local adaptation to particular temperatures coupled with the inability of 744 disperse to localities with different temperatures. 0.5 745

746 CONCLUSIONS

747

748 Using a multi-layered set of morphological, genetic, physiological and ecological evidence, we 749 revealed the existence of three distinct species in a lineage of spiders characterized by a high level 750 of adaptation to the subterranean environment. As previously observed for other subterranean 751 organisms in the Alps, the process prompting diversity is most likely related to pulses of 752 population contraction and expansion during the Quaternary cycles. We found evidence for 753 differences in thermal tolerance between the species, which most likely had an impact on their 754 dispersal ability and ultimately on their ability to expand their distribution ranges and retain 755 genetic variability. The same approach can be extended to other subterranean taxa showing 756 similar distributions in the Alps (e.g., Doderotrechus and Duvalius beetles, Ischyropsalis 757 harvestmen) or in other similar biogeographical settings to broaden our knowledge on the 758 processes that shaped subterranean biodiversity.

759

760

761 **ACKNOWLEDGEMENTS**

- This work is dedicated in memory of our estemeed colleague and extraordinary natural scientist
- 763 Prof. Augusto Vigna-Taglianti, who recently passed away. Special thanks go to Elena Pelizzoli and
- 764 Paolo Pantini for the illustrations and to John Dejanaz and Nicolò Chiappetta for measuring the
- 765 specimens. The authors would like to thank all people mentioned in the Material sections for
- 766 fieldwork assistance. Specimen were photographed by Emanuele Biggi, alive in the designated
- type locality during a dedicated field expedition led by MI on July 13 and 14, 2021.
- 768

769 **DECLARATION OF FUNDINGS**

- 770 Funding was provided by the Agency for Management of University and Research Grants of
- 771 Catalonia (2017SGR83) to MA.

772 CONFLICT OF INTEREST

The authors declare no conflicts of interest.

774 DATA AND CODE AVAILABILITY

- 775 Data and R script to reproduce the ecological analyses is available in GitHub
- 776 (https://github.com/StefanoMammola/Troglohyphantes_vignai_overlooked_diversity.git).
- All morphological and physiological traits have been deposited in the Spider Trait Database (Pekár
 et al., 2021a, 2021b) (upon acceptance).
- 779 Specimens analyses in the present study with geographic and sequence information; cox1
- accession #: GeneBank accession code; cox1 haplo: cox1 haplotypes arbitrarily named with one of
- the sequences; GMYC: GMYC clusters; ITS2 Accession #: GeneBank accession code, ITS2 alleles:
- arbitrarily named after one of the sequences; Locality #: locality number as seen in Fig. 4.
- 783

784 SUPPLEMENTARY MATERIALS

Table S1. List of specimens used for molecular analysis with voucher information, DNA code and
 GenBank[®] access code. Sequences new for this study are marked in bold.

787 Table S2. Measurements of additional specimens (in mm).

788

789 AUTHOR'S CONTRIBUTION

- 790 MI and SM conceived the idea, designed methodology and performed fieldwork. MI described the
- new species, assembled figures and led the writing of the manuscript. SM analysed ecological
- 792 data, wrote the relative sections and helped MI in the species description. MA conducted the
- analyses of the molecular data and wrote the relate sections. All authors contributed to the
- 794 writing of the paper trough discussions and additions to the text.

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1088	TABLE CAPTIONS
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1090	Table 1. Uncorrected genetic distances in COI (white cells) and ITS-2 (grey cells). Intraspecific
1091	distances in italics. Standard error estimates are shown above the diagonal and were obtained by
1092	a bootstrap procedure.
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1096	FIGURE CAPTIONS
1097	
1098	Figure 1. Distribution of <i>Troglohyphantes achillis</i> sp. nov., <i>T. delphinicus</i> sp. nov. and <i>T. vignai</i> and
1099	intraspecific variability in <i>lamella significativa</i> of males. For a number of occurrences, including
1100	type localities, we report the outlines of the corresponding <i>lamella significativa</i> of males. To make
1101	comparison clearer, lamella significativa outlines are in the same orientation. Samples used for
1102	molecular analysis are highlighted with double points (see Tab. S1).
1103	
1104	Figure 2. Genitalic features in <i>Troglohyphantes achillis</i> sp. nov., <i>T. delphinicus</i> sp. nov. and <i>T.</i>
1105	vignal. (a) Ratio between the length of the upper branch (A) and height (B) of the lamella
1106	characteristica in males. (b) Ratio between width (C) and height (D) of the epigyne scape in
110/	remaies.
1108	Figure 2. Bayesian concensus chronogram informed from the COI convenses with time axis (in
1109	million years. My) and goological time scale. Pars denote 95% highest posterior density (HPD)
1110	confidence intervals. Circles on internal nodes denote support values as follows: right half –
1111	Bayesian posterior probabilities (PP): left half = maximum likelihood bootstraps (BS). Black colour =
1112	PP>0.95 or BS >75 grey colour - clades recovered with support values below former thresholds
1113	Triangles on nodes indicate clusters recovered in species delineation analyses (m-PTP) Black
1115	colour = delimitation support>0.95, grev colour = delimitation >0.95. The tree was rooted using
1116	Troglohyphantes nigrgerosge Brignoli, Clades coloured after morphological delineations. For DNA
1117	coding see Table S1 (column C).
1118	
1119	Figure 4. Statistical parsimony ITS-2 allele network. <i>Troglohyphantes vignai</i> (blue), <i>T. delphinicus</i>
1120	sp. nov. (yellow) and <i>T. achillis</i> sp. nov. (purple). <i>T. nigraerosae</i> (grey) is the outgroup. Numbers on
1121	pies indicate different alleles. The size of each circle is proportional to the number of sampled

1122	individuals with each allele. Numbers in specimen labels correspond to localities reported in Table
1123	S1 (column L).
1124	
1125	Figure 5. Pairplot showing the functional morphospace of the three species of <i>Troglohyphantes</i> .
1126	For each plot, 20,000 random points sampled from the estimated 3-dimensional kernel density
1127	hypervolumes are shown. Note that original morphological traits have been converted by applying
1128	Gower distance and a Principal Coordinate Analyses before hypervolume estimation. Arrows and
1129	overlapping ovals in the bottom-left scheme are, respectively, niche centroid distances and niche
1130	differentiation values (measured as eta -total; Mammola and Cardoso 2020) among the three
1131	species.
1132	
1133	Figure 6. Thermal tolerance of the three species of <i>Troglohyphantes</i> , estimated using the
1134	experimental protocol detailed in Mammola et al. (2019). Thermal tolerance is here expressed as
1135	ΔT , namely the difference between the critical temperature and the mean temperature of the
1136	cave of each species. (a) Survival rate as a function of ΔT . Survival curves represent best fits to the
1137	data, according to binomial generalized linear models. (b) Differences in ΔT between cave
1138	populations.
1139	
1140	Figure 7. Troglohyphantes achillis sp. nov. (Tana del Diavolo [1591 Pi], Roreto Chisone, Italy,
1141	19.09.2007 leg. Isaia M.). Right pedipalp of the male (a), epigyne in dorsal view (b), epigyne in
1142	lateral view (c) and abdomen (d). Abbreviations: Cy, cymbium; HB, horizontal branch of the
1143	lamella; PL, Posterior lobes; PP: posterior plate; RL, rounded lobe of the lamella; S, Scape; St,
1144	Stretcher; StA, Suprategular apophysis.
1145	
1146	Figure 8. Troglohyphantes delphinicus (Pertus del Drai [1017 Pi], Sampeyre, Italy, 25.08.2001 leg.
1147	Lana E.). Right pedipalp of the male (a), epigyne in dorsal view (b) and epigyne in lateral view (c).
1148	Abbreviations: Cv. cymbium: HB. horizontal branch of the lamella: PL. Posterior lobes: PP:
1149	posterior plate: RL, rounded lobe of the lamella: S. Scape: St. Stretcher: StA. Suprategular
1150	apophysis.
1151	
1152	Figure 9. Troglohyphantes vignai (Buco di Valenza [1009 Pi], Oncino, Italy, 8.01.2019 leg. Isaia M.
1153	and Mammola S) Right pedinaln of the male (a) enjoyne in dorsal view (b) and enjoyne in lateral
1154	view (c) Abbreviations: Cy cymbium: HB horizontal branch of the lamella: PL Posterior lobes: PP:
1155	nosterior plate: RL rounded lobe of the lamella: S. Scape: St. Stretcher: StA. Suprategular
1156	anonhysis
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1158	Figure 10 Alive specimen of Troglobyphante achillis sp. nov. (a, b) T. delphinicus sp. nov. (c, d) and
1150	<i>T vianai</i> Brignoli (e. f) Photos of alive specimen taken in their natural habitat on July 13 –14, 2021
1160	hy Emanuele Biggi
1161	by Enditacle Diggi.
1162	Figure 11 Type localities of Tradabynhante achillis sp. pov. T. delphinicus sp. Nov. and T. vianai
1163	Brignoli: Tana del Diavolo [Di 1591] (a) Dertus del Drai [Di 1017] (b) and Balma di Valenza [Di 1009]
1164	(c) respectively. Photos by Marco Isaja (a, c) and Emanuele Riggi (b)
1165	(c), respectively. Photos by Marco Isala (a, c) and Emandele biggi (b).
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1160	Cover photo - Tradlohyphantes achillis p.sp. photographed by Emanuele Diggi (July 12, 2021)
1109	cover photo – mogionyphunites uchinis n.sp. photographied by chianuele biggi (July 13, 2021)

1	A multi-layered approach uncovers overlooked taxonomic and physiological
2	diversity in Alpine subterranean spiders (Araneae: Linyphiidae: Troglohyphantes)
3	
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15	
16	ABSTRACT
17	The integration of multiple lines of evidence in the delimitation of taxa is becoming the goldmine
18	standard in modern taxonomy and systematics. Yet, multi-layered taxonomy is still incipient when
19	it comes to species description within mega-diverse group of organisms (e.g., arthropods),
20	especially those inhabiting secluded environments such as caves. This may represent a significant
21	shortcoming, because species inhabitingin subterranean habitatssuch habitats generally show
22	deep convergence in their somatic traits, perplexing our ability to delimit species via traditional
23	approaches. The genus Troglohyphantes (Araneae: Linyphiidae) is presently known to include 129
24	species and five-5 _subspecies, which are primarily distributed in <u>subterranean habitats</u> of the the

25 European mountain ranges. Recent studies on Italian Alpine species covered taxonomy and 26 faunistics, biogeography, and ecology. However, our knowledge about the actual species diversity 27 of Troglohyphantes spiders is far from being complete., and it is expected that significant levels of 28 overlooked diversity should exist within the genus. In this work, we combined morphological with 29 target gene molecular data, functional trait analysis, and thermal physiology to explore cryptic 30 overlooked diversity in *Troglohyphantes vignai* aut., a species of subterranean spider in the 31 Western Italian Alps with an apparent broader distribution compared to its congeners. Our 32 approach proofed proved effective in uncovering overlooked diversity and further supporting the 33 split of *T. vignai* aut. into three distinct species, which whose distribution ranges show striking 34 resemblances with that of other cave-dwellingsubterranean spiders. We here describe the new 35 species and discuss their origin in light of their geographical vicariance, niche convergence, 36 thermal ecology and divergence time.

37

38 INTRODUCTION

39 The use of different sources of information in the in taxa delimitation of taxa has become the gold 40 standard in modern systematics. Traditional taxonomy (i.e. morphology based) is often challenged 41 by the existence of evolutionary lineages displaying little, if any, diagnostic anatomical features, 42 resulting in the classification of two or more distinct species as a single one (i.e. cryptic species, Bickford et al. 2007). The term "integrative taxonomy" (Dayrat 2005) was coined to refer to the 43 44 combination of multiple sources of evidence to effectively establish species boundaries, avoiding 45 the inherent limitations of single disciplines to convey the inherent complexity of the speciation 46 process, and increase rigorousness in species delimitation (Schlick-Steiner et al. 2010). Interest in 47 integrative species delimitation has gained momentum in the light of biodiversity loss and species 48 conservation, given that separately evolving lineages are meant to be the only reasonable

49 conservation unit (Ryder 1986; Bickford et al. 2007; De Queiroz 2007). Moreover, uncovering 50 separately evolving lineages previously overlooked due to the lack of or the misinterpretation of 51 morphological diagnostic traits, provides fundamental information to devise more detailed and 52 efficient conservation strategies (Ryder 1986; Bickford et al. 2007).

53 A significant impulse to unveiling previously overlooked diversity came from the widespread 54 adoption of DNA barcoding approaches (Hebert et al. 2003) for species identification and discovery (Hebert 55 et al. 2003), and their recent high-throughput sequencing derivatives (i.e. metabacording and 56 metagenomics), τ in biodiversity studies. Genomic analyses of individual specimens and bulk samples have 57 led to the detection of morphologically similar species in different habitats and biomes. Subsequently, more 58 detailed analyses often result in finding additional ecological, ethological, or phenological traits that covary 59 with the molecular results and thereby confirm the existence of complexes of several species that may not 60 differ from each other in their external morphology (Janzen et al. 2017; Fišer et al., 2018). According to 61 Janzen et al. (2017), "cryptic" species should be fairly common (10–20% of the traditional morphologically 62 single species may turn out to be two or more). For example, by combining morphological study and DNA 63 barcoding of museum specimens, Hebert et al. (2004) showed that what was known as a single species of 64 neotropical skipper comprised at least ten, largely sympatric, species.

The sheet-web spider genus *Troglohyphantes* Joseph <u>1882</u> (Family Arachnida: Araneae: Linyphiidae) is presently known to include 129 species and five subspecies (World Spider Catalog 2021). The genus is primarily distributed through the European mountain ranges, from the Cantabric Mountains, northern Iberian Peninsula, in the west to the Caucasus in the east. Four additional species are found in northern Africa (Atlas) and two in the Canary Islands (World Spider Catalog 2021), but their congeneric status is open to question. In general, *Troglohyphantes* species are rare and show narrow distributionsshort-range endemics (sensu Harvey 2002). Several species are restricted to a single or few localities, generally inhabiting subterranean habitats such as caves,
 mines, soil litter, rocky debris, and other moist and shaded retreats (Isaia et al. 2017).

74 This genus has been intensively studied, with major focus on the Italian Alpine species. 75 Recent studies covered taxonomy and faunistics (Isaia and Pantini 2007, 2010; Isaia et al. 2010, 76 2011, 2017), biogeography (Mammola et al. 2015, 2018b), and ecology (Mammola and Isaia, 2016; 77 Mammola et al. 2018a, 2018b, 2019, 2020). However, even from a merely-basic taxonomic 78 standpoint, knowledge of *Troglohyphantes* spiders is far from being exhaustive; and given the rate 79 of new species description and the limited sampling of the subterranean environment, it is 80 probable that more species have yet to be described. A similar reasoning may apply to other 81 species-rich groups of cave-dwelling organisms, for which previous studies disclosed high levels of 82 overlooked diversity, including cavefish (Niemiller et al. 2012) and spiders (Hedin 2015) from 83 North America, tailless whip scorpions from the Caribbean (Esposito et al. 2015), or European 84 crustaceans (Delić et al. 2017; Eme et al. 2018), among others.

85 In the present paper, we used a multi-layered approach to reveal overlooked diversity in 86 Troglohyphantes vignai aut. In particular, we gathered evidence from a detailed morphological 87 study coupled with molecular and ecological analyses to support the split of the relatively broadly 88 distributed *T. vignai* aut. into three distinct species. We here describe and illustrate the new 89 species, summarise their phylogenetic affinities and diversification timelinetimes, and provide 90 insights into their ecology and natural history. Building on our multiple lines of evidence, we 91 further discuss their origin in light of their geographical vicariance, niche convergence, and 92 divergence time.

93

94 Material and methods

95 Morphological methods

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96	We stored specimens in 70% ethanol at in the Marco Isaia's collection at the Department of Life
97	Sciences and Systems Biology, University of Turin (Torino, Italy), except for <u>the type series and a</u>
98	few more specimens—labelled with the acronym 'MCSNB'—stored at Museo Civico di Scienze
99	Naturali 'E.Caffi', Bergamo, Italy. We studied specimens using a Leica M80 stereoscopic binocular
100	microscope. Illustrations were prepared by Elena Pelizzoli using a camera lucida. We referred to
101	Isaia et al. (2017) for Troglohyphantes anatomical terms and to the World Spider Catalog (2021)
102	for current nomenclature. <u>All illustrated structures are from the right sideRight structures are</u>
103	illustrated. All morphological measurements are in millimetres. Following Denis (1949), the
104	position of the metatarsal trichobothrium I (TmI) is expressed <u>as</u> the distance between the
105	proximal end of the leg article and the trichobothrial base divided by the total length of the leg
106	article. For the toponymy and classification of the different sectors and sub-sectors of the Alps, we
107	followed the standard partition of the Alpine chain (Marazzi 2005). Whenever applicable, we gave
108	the speleological cadastral codes of the caves in brackets ['regional code' and 'number'].
109	We used the following abbreviations in tables, text and figures: ALE, anterior lateral eyes;
110	AME, anterior median eyes; Cy, cymbium; E, Embolus; HB, horizontal branch of the lamella; P,
111	Paracymbium; PLE, posterior lateral eyes; PME, posterior median eyes; PL, Posterior lobes; PP,
112	posterior plate; RL, rounded lobe of the lamella; S, Scape; St, Stretcher; StA, Suprategular
113	apophysis; TLL, total leg length; TmI, position of trichobothrium on metatarsus of first leg; UB \div
114	upper branch of the lamella.
115	

116 *Molecular analyses*

We compiled sequences for the cytochrome *c* oxidase subunit I (COI) and the Internal
Transcribed spacer 2 (ITS2) from previous studies (Mammola et al. 2015; Isaia et al. 2017;
Mammola et al. 2018a) available in Genbank. We generated additional sequences following the

laboratory protocols detailed in Mammola et al. (2015). We edited and managed sequences using
Geneious v. R10.2.6 (Kearse et al. 2012)a...)a. Since no indel mutations were inferred, COI was
aligned manually. We aligned ITS2 sequences using the online version of the program PRANK
(Löytynoja and Goldman 2010) available at the EBMBL website (https://www.ebi.ac.uk/goldmansrv/) with default parameters.
We inferred the maximum likelihood tree of the COI data matrix (676 bp) with IQ-TREE v.
1.6.11 (Nguyen et al. 2015)a. We used IQ-TREE to first select the best-fit partitioning scheme and

127 corresponding evolutionary models (Kalyaanamoorthy et al. 2017), and then to infer the best 128 tree and estimate clade support by means of 1000 replicates of non-parametric bootstrapping. 129 Similarly, We we conducted Bayesian (BI) analysis and divergence time estimation in BEAST v. 130 1.8.4 (Drummond et al. 2012) on the COI data matrix only, defining the partition scheme and 131 models selected by the BIC criterion in Partition Finder v2.1.1 (Lanfear et al. 2017). We specified a 132 birth and death tree prior and an unlinked relaxed uncorrelated lognormal clock for each gene. We 133 estimated absolute divergence times by defining a normal distribution on the substitution rate 134 prior (ucld.mean), truncated at 0, with a starting and mean value of 0.0125 and standard deviation 135 0.02, based on spider substitution rate estimates available in the literature for spiders (Bidegaray-136 Batista and Arnedo 2011). We ran three independent chains of 10 million generations each, 137 sampling every 1,000 generations. We monitored the chain convergence, the correct mixing, and the number of generations to discard as burn-in (10%) with Tracer v. 1.7 (Rambaut et al. 2018). 138 139 We determined species boundaries using the multirate PTP (m-PTP) species delimitation 140 model (Kapli et al. 2017). This single marker method has been shown to outperform other

141 delimitation methods by providing more stable outputs without requiring the ultrametric

transformation of tree branches (Blair and Bryson 2017). We implemented the m-PTP model using

143 a Markov chain Monte Carlo approach, which allows estimates of support values on the

144	delimitations, on the COI matrix. The analyses were conducted on the best IQ-TREE. We ran 5
145	chains of 100 million generations each, removing the first 2 million as burn-in, and discarding all
146	branches with lengths smaller or equal to 0.0001. Additionally, we implemented the GMYC
147	delimitation method (Fujisawa and Barraclough 2013) to corroborate our results using an
148	ultrametric tree instead. We inferred the tree using the same settings as defined above for the
149	BEAST analysis but assuming a coalescent tree prior (constant population size), which has been
150	suggested to provide a more rigorous test of delimitation since the GMYC model assumes a single
151	species as the null option (Monaghan et al. 2009).
152	We determined the number of alleles in the ITS-2 matrix considering the gaps as
153	absence/presence data using the simple coding methods described by Simmons and Ochoterena
154	(2000) and implemented in the R package 'haplotypes' (Aktas 2015). We constructed the allele
155	network using the statistical parsimony method (Templeton et al. 1992; Clement et al. 2000), with
156	a confidence limit of 90% also using 'haplotypes'.
157	Uncorrected pairwise genetic distances were calculated in MEGA X (Kumar et al. 2018). The
158	phylogenetic tree was edited for aesthetic purposes using FigTree
159	(http://tree.bio.ed.ac.uk/software/figtree/).
160	
161	Analysis of morphological traits and niche overlap
162	We studied niche partitioningmorphological differentiation among the three species by
163	analysing variations in morphological traits in a multi-dimensional morphospace (Blonder et al.
164	2014; 2018). For each species, we measured 10 female specimens for different morphological
165	traits: sternum length and width, cephalothorax height (measured at the eye region, starting from
166	the clypeus base to the top of the profile), leg I and II length, and six more traits related to eye

regression (diameter of ALE, AME, PME, PLE, and the total length of the anterior and posterior eye

168 lines). The functional meaning of these traits is discussed in Mammola et al. (2018a, 2019, 2020). 169 We acquired morphological measurements for the three species and constructed their n-170 dimensional kernel density hypervolumes following the approach described in Mammola et al. 171 (2020). In short, we applied a Gower dissimilarity measure to the complete trait matrix and 172 analysed the resulting distance matrix through Principal Coordinate Analysis (PCoA) in order to 173 extract orthogonal morphological axes for hypervolume construction. To obtain comparable data 174 with that in Mammola et al. (2020), we selected the first three PCoA to delineate the shape of the 175 hypervolumes (>75% variance explained). We constructed hypervolumes using a Gaussian kernel 176 density estimator and a cross-validation estimator to assess the optimal bandwidth value for each axis (Blonder et al. 2018). We calculated niche differentiation using one overlap and one distance 177 178 metric (Mammola 2019), respectively the pairwise overall differentiation among kernel 179 hypervolumes (β total; Mammola and Cardoso 2020) and the distance between centroids. All 180 morphological measures used to construct the hypervolume are deposited in the World Spider 181 Trait database (Pekár et al., 2021a, 2021b). 182 183 Analysis of thermal tolerance 184 We performed experimental tolerance trials to evaluate the thermal breadth of each 185 species. Full details on experiment protocol and statistical analyses are presented in Mammola et

186 al. (2019).-) and all thermal tolerance estimates are available in the World Spider Trait database

187 (Pekár et al., 2021a, 2021b). In short, we collected 85 alive specimens of the three lineages of

188 *Troglohyphantes vignai* aut. from subterranean sites across their range. In each cave, we collected

- 189 spiders by hand and placed them in individual Eppendorf Tubes[®] of 5 ml. We stored all vials in a
- 190 cool bag and transported it the same day to the laboratory at the University of Turin (Italy), where
- 191 we conducted the experiments. In the laboratory, we placed specimens in a IPP 30 Peltier

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192 Memmert climatic chamber, each specimen in individual Petri dish, and acclimated them for one 193 day at the temperature of the cave. Air humidity was kept at 100% during the acclimation and 194 during the experiments, by placing two Petri dishes filled with water inside the climatic chamber. 195 Owing to the high resistance to starvation of subterranean spiders (Mammola and Isaia, 2017), 196 specimens were not fed throughout the experiments. For each sampled cave, we derived the 197 internal mean annual temperature from continuous temperature measurements taken between 198 2012 and 2013 (Mammola et al. 2018b), or unpublished temperature records taken via the same 199 methodology. For two caves lacking field-collected temperature records, we downloaded annual 200 temperature series from the same period from the nearest thermo-hygro-pluviometric weather 201 station. After correcting the data with the standard environmental lapse rate (0.57°C/100 m; Rubel 202 et al. 2017), we calculated the mean annual temperature and used this value as a direct proxy of 203 the cave temperature (Badino 2010; Mammola et al., 2018b; Sánchez-Fernández et al. 2018). 204 The day after the sampling, we started the experiment, progressively raising the 205 temperature within the chamber with an increasing rate of 1°C/day. We evaluated the individual 206 critical temperature as the total paralysis, namely the temperature at which the individual was 207 immobile and no appendage movements were visible. For each species tested, we kept 10% of the 208 specimens as controls in stable climatic conditions. No mortality was recorded in controls during 209 the experiments.

210 We constructed survival curves with binomial generalized linear models (GLMs), modelling 211 the survival rate of each species as a function of ΔT , namely the difference between critical 212 temperature and the mean temperature of the cave where each species was collected. We 213 evaluated differences in ΔT between each species and between populations of each species via 214 analysis of variance (ANOVA).

215

216 **RESULTS**

217 <u>Morphology</u>

218	The comparative examination of male pedipalps of 38 specimens from eighteen different
219	localities across the range of Troglohyphantes vignai aut. allowed a first preliminary identification
220	of three morpho-species which formed the basis of comparison for the subsequent analyses (Fig.
221	1). We based the distinction of the three putative morpho-species on the overall shape of the
222	lamella characteristica of the male palps, which is considered the most important diagnostic
223	<u>character in males of Troglohyphantes (Deeleman-Reinhold, 1978, Isaia et al., 2017). Specimens</u>
224	from the northernmost localities (Northern Cottian Alps) clustered in a geographically isolated
225	group which was provisionally named "T. T. achillis". Specimens from the remaining localities
226	clustered in two groups, the first ("T. T. vignai") comprised the type locality of T. vignai Brignoli
227	1971 and two nearby caves plus all localities in the southernmost sector of the range. The second
228	("T. Tdelphinicus") included all remaining localities scattered in the central part of the distribution
229	range.
230	In particular, when examining the A/B ratio (see Fig. 2) of the male palps from each group, we
231	detected a significant effect of on species delimitation based on ANOVA [F _(2,15) = 20.41, p< .001]. A
232	post hoc Tukey test showed that specimen assigned to T. T. delphinicus sp. nov. was significantly
233	different from both T. Tachillis sp. nov. and T. Tvignai at p < .05; conversely, the comparison
234	between T. achillis sp. nov. and T. vignai only approached statistical significance (p= .08). When
235	examining females from the corresponding localities, there was a significant effect of the epigyne
236	ratio C/D (see Fig. 2) on species delimitation based on ANOVA [F _(2,16) = 4.11, p= .036]. A post hoc
237	Tukey test revealed that 7. values of 7vignai wasere significantly different from 7. 7. achillis sp.
238	<u>nov. at $p < 0.05$; conversely, the other species comparisons were not significant.</u>
239	

240

Molecular analysis

241 We gathered 49 sequences, of 676 bp, long COI sequences — 4 new for the present 242 study—, and 41, of 412 position long ITS2 aligned sequences—sequences aligned positions, 5 new, 243 for the COI and ITS2, respectively,. The data matrices included including sequences of 244 *Troglohyphantes nigraerosae* Brignoli <u>1971 that were used as outgroup</u> (3 of cox1, 1 of ITS2).) 245 following a former studiesy that identified this species as the closest relative of *T. vignai* and the 246 new species with high support (Mammola et al. 2020). Localities of sequenced material are listed 247 in Table S1 available as Supplementary Material to this paper. The sequences of the COI 248 corresponded to 28 haplotypes and the ITS2 to 11 alleles. None of the haplotypes or alleles were 249 shared by more than one of the morphologically delineated species. The Maximum likelihood and 250 the time-aware Bayesian inference analyses also agreed in recovering three main lineages 251 corresponding to the -morphological delineations, and show similar relationships, but with 252 contrasting levels of support. The mPTP method confirmed the three lineages as potential 253 candidate species (hereinafter indicated as T. vignai, T. delphinicus sp. nov. and T. achillis sp. nov.), 254 although the split between T. vignai and T. delphinicus sp. nov. was poorly supported. The GMYC 255 method could not reject the single species model over the multiple species (p=0.62), which 256 resulted in four clusters that matched *T. vignai* and *T. achillis* sp. nov. but further splitted *T.* 257 *delphinicus* sp. nov. into two different coalescent clusters grouping haplotypes from the two 258 neighbouring localities of Buco del Nebin 1 and Tana dell'Orso (PK1105 and SM02) versus the 259 remaining haplotypes (see Tab. 1 and Fig. <u>13</u>). 260 The ITS allele network also recovered non overlapping relationships for the three 261 morphologically delineated species. ITS allele were exclusive to each species. However, the ITS 262 allele network suggested contrasting population structures for each species. The allele network

263 suggested contrasting population structures for each species. We did recover star-like

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relationships for the ITS2 alleles of *T. vignai* and *T. achillis* sp. nov., while *T. delphinicus* sp. nov.
 included highly divergent alleles (Fig. 2)., some of them being more similar to those of the other
 two species (Fig. 4).

267 Divergence time estimation analyses inferred a late Pliocene-early Pleistocene origin of 268 the three main lineages (Fig. 13). Coalescent times for *T. delphinicus* sp. nov. were older than for 269 the other two species, although confidence intervals were very wide and largely overlapped. 270 Accordingly, T. delphinicus sp. nov. showed the highest intraspecific genetic variability in both COI 271 and ITS-2 (5% and 1%, respectively) (see Table Tab. 1). Uncorrected genetic distances to T. 272 nigraerosae ranged from 14 to 15.4% and 1.2 to 1.3% in COI and ITS-2, respectively, and genetic 273 divergence between the three lineages ranged between 7.8% (T. delphinicus sp. nov. to T. achillis 274 sp. nov.-) to 9.1% (T. vignai to T. achillis sp. nov.-) and from 0.6% (T. vignai to T. achillis sp. nov.-) 275 to 0.9% (T. delphinicus sp. nov. to T. achillis sp. nov.-) in COI and ITS-2, respectively.

276

277 Analysis of morphological traits and niche overlap

278 We measured 10 female specimens of each of the three species to estimate morphological 279 *n*-dimensional hypervolumes (Fig. <u>35</u>). *Troglohyphantes- delphinicus* sp. nov. had the most 280 voluminous hypervolume (0.42), followed by T. vignai (0.22), and T. achillis sp. nov. (0.11). The 281 overlap in the trait space of the three species was comparable with that of other *Troglohyphantes* 282 species adapted to the same microhabitat within Western Alpine caves (see Mammola et al. 283 2018a, 2020 for comparative data). Distance between centroids of the three species niches 284 morphospaces was below 0.2, and β diversity always above 0.3. Troglohyphantes vignai and T. 285 *delphinicus* sp. nov. showed the highest <u>nichemorphospace</u> overlap, whereas *T. achillis* sp. nov. 286 had the most divergent niche morphospace (Fig. <u>35</u>).

287

288 Thermal tolerance

289	A total of 85 specimens were successfully tested in the thermal chamber trials (11
290	specimens of <i>T. delphinicus</i> sp. nov., from 1 cave; 23 specimens of <i>T. vignai</i> from 2 caves; 51
291	specimens of <i>T. achillis</i> sp. nov. from 4 caves). A general narrow thermal tolerance was observed
292	for the three species, with critical temperature values comparable to that of other high and
293	intermediate specialized subterranean Troglohyphantes species (see Mammola et al. 2019 for
294	comparative data). Specifically, we detected a significant effect of thermal tolerance on species
295	delimitation based on ANOVA [F _(2,67) = 15.16, p < .001] (Fig. 6). Accordingly, We found the three
296	species to be significantly different in their thermal tolerance (Fig. 4). Troglohyphantes achillis sp.
297	<u>nov showed intermediate thermal tolerance (mean $\Delta T \pm s.d.: 4.82 \pm 2.92; \Delta T range: 0–12),$</u>
298	<u>Troglohyphantes. delphinicus sp. nov. was the least tolerant species, reaching 50% mortality at</u>
299	temperature values 1°C above its cave temperature (mean $\Delta T \pm s.d.: 0.67 \pm 0.77$; ΔT range: 0–2) and
300	<u><i>T. vignai</i> proved to be the species with the greattest thermal tolerance (mean $\Delta T \pm s.d.: 8.00 \pm 5.43$;</u>
301	<u>ΔT range: 1–17). More specifically, a <i>post hoc</i> Tukey test revealed that all species were significantly</u>
302	different in their ΔT at p < .05. For each species, there were no significant differences between
303	cave populations with the exception of one population of <i>T. achillis</i> from Tuna dal Diau cave
304	(highlighted with an asterisk in Fig. 6), displaying a significantly higher thermal tolerance compared
305	to the other populations of the same species based on ANOVA $[F_{(3,38)} = 9.41, p < .001]$.
306	Troglohyphantes delphinicus sp. nov. was the least tolerant species, reaching 50% mortality at
307	temperature values 1°C above its cave temperature (mean ΔT±s.d.: 0.67±0.77; ΔT range: 0–2).
308	Troglohyphantes achillis sp. nov. showed intermediate thermal tolerance (mean $\Delta T \pm s.d.$:
309	4.82±2.92; ΔT range: 0–12), with the exception of one population diverging significantly from the
310	other ones in its thermal tolerance which we excluded from the analysis (highlighted with an

- 311 asterisk in Fig. 4). The species with the greatest thermal tolerance was *T. vignai* (mean ΔT±s.d.:
- 3 2 8.00±5.43; ∆T range: 1–17).
- 313
- 314
- 315 Taxonomy
- 3 6 Family LINYPHIIDAE Blackwall <u>1859</u>
- 317 Genus *Troglohyphantes* Joseph <u>1882</u>h
- 318 *Troglohyphantes achillis* Isaia and Mammola, sp. nov.
- 319 Fig<u>s</u>. <u>57</u>, <u>8a10a</u>, <u>8b-b</u>
- 320 Taxonomic references
- 321 Troglohyphantes vignai Pesarini 2001: 116 (S of T. rupicapra)
- 322 Troglohyphantes vignai Isaia et al. 2011b: 141, 2.68 (f)
- 323 324
- 325 Type series
- 326 Holotype male. Italy, Piemonte, Province of Torino: Roure, Tana del Diavolo [1591 Pi] (Fig. 9a11a),
 327 7.122°E, 45.026°N, 1414 m, 19.IX.2007, Isaia M. leg. (MCSNB).
- 328 Paratypes. Italy, Piemonte, Province of Torino: Perrero, Grotta di Chiabrano or Tuna dal Diau [1621 329 Pi], 7.106°E 44.947°N, 1080 m, 23.X.2006, Isaia M. and Lana E. leg. 1m, 5f, 3 juv (MCSNB); same 330 locality, 4.XII.2006, Lana E. leg. 4f (MCSNB); same locality, 27.V.2019, Isaia M. and Nicolosi G. leg. 331 2f; Perrero, Prospetto di Miniera di Boccetto [Art. Pi], 7.062°E, 44.949°N, 1237 m, 21.II.2007, Isaia 332 M. leg. 1f, 1 juv.; Pramollo, Prospetto di Miniera di Tornini [Art. Pi], 7.198°E, 44.908°N, 990 m, 333 13.I.2015, Isaia M. and Mammola S. leg. 1m, 1f; same locality, 14.X.2010, Isaia M. leg. 2m, 1f, 3 juv.; 334 same locality, 27.V.2019, Isaia M. and Mammola S., leg; same locality, 14.VII.2017, Isaia M. and 335 Mammola S. leg. 2m,7f, 3juv; Roure, Tana del Diavolo [1591 Pi], Isaia M. and Paschetta M. leg. 2m; 336 same locality, 19.IX.2007, Isaia M. leg. 1f (MCSNB); same locality, 14.VII.2017, Isaia M. and 337 Mammola S. leg. 7m, 6f, 9juv.
- 338

339 Other material examined

Italy, Piemonte, Province of Torino: Perrero, Grotta di Chiabrano or Tuna dal Diau [1621 Pi],
19.IX.2007, Isaia M. leg. 1m, 1f; same locality, 6.XII.2007, Isaia M. leg. 1m, 1f; same locality,
19.I.2007, Isaia M. leg. 1m, 1f, 3 juv; Perrero, Prospetto di Miniera di Boccetto superiore [Art. Pi],
7.083°E, 44.958°N, 1310 m, 8.I.2019, Isaia M. and S. Mammola leg. 1m, 2f; Roure, Tana del Diavolo
[1591 Pi], 15.IV.1995, Lana E. leg. 1m; same locality, 12.XI.2006, Lana E. leg. 2m, 1f; same locality,
23.XI.2002, Lana E. leg. 2f, 1 juv.

346

347 *Re-examined material*

- 348 Former literature records (sub *T. vignai* or otherwise specified) are here revised and assigned to *T.*
- 349 *achillis* sp. nov. on the basis of morphological examination. The identification of juveniles is based

350 on the identification of adults occurring in the same locality. For details about material see the 351 original publications.

352

353 Italy, Piemonte, Province of Torino: Perrero, Grotta di Chiabrano or Tuna dal Diau [1621 Pi]: Isaia 354 and Pantini 2010 ; Isaia et al. 2010; Isaia et al. 2011a; Isaia et al. 2011b ; Mammola et al. 2015 ; 355 Mammola et al. 2018 2018b; Perrero, Prospetto di miniera di Boccetto [Art. Pi]: Isaia et al. 2010; 356 Isaia et al. 2011b ; Pramollo, Prospetto di miniera di Tornini [Art. Pi]: Mammola et al. 2015 ; 357 Mammola and Isaia, 2016; Mammola et al. 2018-2018b; Roure, Balm Chanto [1575 Pi]: Pesarini, 358 2001; Isaia et al. 2011b; Roure, Tana del Diavolo [1591 Pi]: Arnò and Lana, 2005 sub Troglohyphantes 359 sp.; Isaia et al. 2010; Isaia and Pantini 2010 Fig. 15-16; Isaia et al. 2011b (see note in re-examined 360 material of *T. delphinicus* about the misprint in Fig. 2.67); Mammola et al. 2015-; Mammola et al. 361 2018<mark>b</mark>.

- 362
- 363 Diagnosis
- 364 Males of *Troglohyphantes achillis* sp. nov. are primarily distinguished from other species of
- 365 Troglohyphantes by the shape of the lamella characteristica (Figs. <u>1, 5a7a, 10a, 11</u>). <u>In particular</u>,
- 366 <u>the A/B ratio (Fig. 2a) is between 0.8 and 1.0.</u> The new species is close to *T. vignai* and other species
- 367 of the Henroti complex (sensu Pesarini 2001), from which it is distinguishable by the short lower
- 368 branch of the *lamella characteristica*. In particular, the A/B ratio (Fig. 2a) is between 0.8 and 1.0. In
- 369 particular, the A/B ratio (Fig. 10a) is between 0.8 and 1.0 (n= 7; mean±s.d.= 0. 88±0.06), smaller
- 370 than *T. delphinicus* sp. nov. and greater than *T. vignai*.
- 371 Females are diagnosed by the epigynum in frontal view (Fig. 5b), The identification of females in
- 372 <u>absence of males remains doubtful in most cases.</u>
- 373
- 374 Females are diagnosed by the epigynum in frontal view (Fig. 5b), with a C/D ratio (Fig. 10b) between
- 375 1.7 and 1.9 (n= 4; mean±s.d.= 1.80±0.19), smaller than both *T. delphinicus* sp. nov. and *T. vignai*.
- 376
- 377 Description
- 378 *Male paratype* from type locality (Roure, Tana del Diavolo [1591 Pi], Isaia M. and Paschetta M. leg.
- 379 12.XI.2006): prosoma 1.07 long, 0.96 wide, yellowish. Thoracic region slightly swollen, yellowish

380 with grey shades. Cephalic region elevated, interspersed with black bristles between the eyes, with 381 few small black bristles around the eye region and continuing backwards in three longitudinal rows 382 converging at the thoracic furrow. One bristle just below AME. Carapace with darker margins. 383 Clypeus slightly indented under the eyes, then convex, 0.34 long. Eyes normally developed, with 384 pigment and black margins. AME smallest. PME and PLE slightly bigger than ALE. ALE and PLE 385 contiguous. PLE–PME distance = 0.07, ALE–AME distance = 0.04, PME–PME distance = 0.9. Eye 386 diameters AME 0.04, PME 0.07, ALE 0.06, PLE 0.08. Sternum heart-shaped, yellowish with faintly 387 darkened anterior edges. Chelicerae light brownish, 0.51 long, with ca. 35 lateral stridulatory ridges 388 and armed with three anterior teeth. Legs yellowish, uniform in colour. Leg I: femur 2.59, patella 389 0.47, tibia 2.83, metatarsus 2.56, tarsus 1.45, TLL 9.90; leg II: femur 2.54, patella 0.41, tibia 2.70, 390 metatarsus 2.36, tarsus 1.36, TLL 9.37; leg III: femur 2.20, patella 0.31, tibia 2.08, metatarsus 2.03, 391 tarsus 1.02, TLL 7.64; leg IV: femur 2.76, patella 0.40, tibia 2.58, metatarsus 2.41, tarsus 1.22, TLL 392 9.37. Abdomen greyish with darkened distal pattern (Fig. 7d, 10b), 1.64 long, 0.97 wide. Palp: femur 393 0.73, patella 0.23 tibia 0.28 (see Tab. S2 for measurements of additional specimens). Male palp (Fig. 394 7a) with Cymbium cymbium faintly convex, roughly triangular when seen from above, ending 395 proximally with a pointed tip. Posterior part of paracymbium subrectangular, apical part narrowed 396 anteriorly. Lamella characteristica similar to T. vignai, clearly visible in lateral view without 397 extraction. Distal part of the lamella gun-shaped with one superior horizontal branch and an inferior 398 rounded lobe directed anteriorly. One additional upper short branch is directed upwards, almost 399 perpendicular to the horizontal branch. Suprategular apophysis directed upwards, with a sharp end. 400 Tip of the embolus spikysharply pointed. A/B ratio between 0.8 and 1.0 (n= 7, mean±s.d.= 401 0.878±0.057; see Fig. 10a2a).

402 Spination: Femur I with one prolateral and one dorsal spine; Femur II–IV with one dorsal spine.
403 Patella I–IV with one dorsal spine. Tibia I with two dorsal, four ventral, two retrolateral and two

404 prolateral spines; Tibia II with two dorsal, three ventral, two retrolateral, and two prolateral spines;
405 tibia III with two ventral, two prolateral, two dorsal and three retrolateral spines; Tibia IV with three
406 ventral, three dorsal, two prolateral, and three retrolateral spines. Metatarsus I–IV with one dorsal
407 spine. Patella of the Palp with one long, curved spine. Position of Tml: 0.17. Trichobothrium on Mt
408 IV absent.

409

410 Female paratype (Tornini, Prospetto di miniera [Art. Pi], Isaia M. and Mammola S. leg. 13.I.2015): 411 prosoma 1.38 long, 1.05 wide, slightly darker than male. Cephalic region light-brownish. Carapace, 412 ocular area, clypeus, and sternum are similar in all features to the analogous male body parts. 413 Sternum light brownish with dark margins. Anterior margin of the chelicerae armed with three 414 teeth. Clypeus 0.24 long, chelicerae 0.70 long. PLE–PME distance = 0.05, ALE–AME distance = 0.04, 415 PME–PME distance = 0.07, AME–AME distance = 0, ALE–PLE distance = 0. Eye diameters: AME 0.03, 416 PME 0.07, ALE 0.06 and PLE 0.07. Abdomen 1.82 long, 1.15 wide, greyish with darkened distal 417 pattern (Fig. 7d), 1.82 long, 1.15 wide. Leg I: femur 2.65, patella 0.44, tibia 2.70, metatarsus 2.44, 418 tarsus 1.50, TLL 9.73; leg II: femur 2.70, patella 0.46, tibia 2.53, metatarsus 2.34, tarsus 1.42, TLL 419 9.45; leg III: femur 2.18, patella 0.43, tibia 2.07, metatarsus 1.88, tarsus 1.07, TLL 7.63; leg IV: femur 420 2.71, patella 0.33, tibia 2.30, metatarsus 2.32, tarsus 1.25, TLL 8.91. Female palp: femur 0.73, patella 421 0.20, tibia 0.43, tarsus 0.82, total palp length 2.18 (see Tab. S2 for measurements of additional 422 specimens). Spination approximately like in male. Position of TmI: 0.17. Trichobothrium on Mt IV 423 absent. Epigyne (Fig. 7b, c) protruding, with subtriangular scape, not much wider than long, covering 424 less than half of the inner part of the epigyne, C/D ratio between 1.7 and 1.9 (n= 4; mean±s.d.= 425 1.80±0.19; (see Fig. 10b2b). Lateral margins of the scape converging, posterior margin rounded. 426 Stretcher tongue-shaped, straight (Fig. 7c), emerging halfway in the profile in lateral view, bearing 427 a pitted knob at its end, visible in ventral view. Lateral lobes emerging at the posterior end of the
428 epigyne. Fertilization ducts sclerotized.

429

430 Etymology

The species epithet derives from the name of the first collector and our estimated esteemed
colleague Prof. Achille Casale, renowned Italian zoologist and biospeleologist.

433

434 Distribution

The species is known from a few localities of a small inner sector of the Northern Cottian Alps
(Chisone and Germanasca Valleys) (Fig. 11). The distribution range of the species covers an area of
approximately 50 km².

438

439 Biospeleological notes

440 This species was first collected by Achille Casale in 1983, in a wild cave of Chisone Valley, Northern 441 Cottian Alps. The record was firstly published by Pesarini (2001), who mentioned that this specimen 442 was collected at the locality Balm Chanto [1575 Pi], an rock shelter renown for the findings of 443 archaeological remains in the early 1980s. As specified later (Arnò and Lana, 2005; Isaia et al. 2011b) 444 and recently confirmed by the original collector (A. Casale, pers. comm. 2019), the exact locality 445 where the collection took place was in fact the nearby wild cave Tana del Diavolo [1591 Pi] (Fig. 446 9a11a). On the contrary, the rock shelter of Balm Chanto mentioned in the original publication is 447 open and exposed to natural light, thus unsuitable for the occurrence of this cave-448 dwellingsubterranean species. Specimens were in fact collected by Achille Casale among the floor 449 debris and on the walls of the dark zone of the wild cave. All later collectors report about findings 450 of the species in similar habitats of natural caves and mine prospects of Chisone and Germanasca

- 451 Valleys. Troglohyphantes- achillis sp. nov. shares the type locality with T. lucifer Isaia, Mammola and
- 452 Pantini <u>2017</u>, recently described by the authors (Isaia et al. 2017).

453

- 454
- 455 *Troglohyphantes delphinicus* Isaia and Mammola, sp. nov.
- 456 Fig. <u>68</u>, <u>8c10c</u>, <u>8d-d</u>
- 457 *Taxonomic references*
- 458 *Troglohyphantes vignai* Isaia et al. 2011b: 140, f. 2.67A (m, misprinted locality in figure caption).
- 459
- 460 Type series
- 461 Holotype male. Italy, Piemonte, Province of Cuneo: Becetto, Pertus del Drai [1017 Pi], 7.223°E,
- 462 44.610°N, 1930 m, 25.VIII.2001, leg. Lana E. (MCSNB)
- 463 Paratypes. Italy, Piemonte, Province of Cuneo: Becetto, Pertus dei Drai [1017 Pi], 16.VII.2015, Isaia
- 464 M. and Mammola S. leg. 2m, 2f, 1 juv.; same locality, 25.VIII.2001 leg. Lana E. 5f (MCSNB); same
- 465 locality, 5.IV.2019, Isaia M., Mammola S. leg., 1m, 4f; same locality, 14.VII.2021, Isaia M., Biggi E.
- 466 leg., 5f; **Casteldelfino**, Tana dell'Orso [1019 Pi], 7.098°E, 44.559°N, 2360 m, 19.I.2018, Isaia M. and
- 467 Mammola S. leg. 2m; Isasca, Pertus d'la Rocho (Pertus d'la Tundo) [1265 Pi], 7.362°E, 44.585 N,
- 1000 m, 26.IX.2010, Lana E. and Chesta M. leg. 1m, 4f; **Sampeyre,** Buco del Nebin 1 (Buco della
- 469 Pioggia) [1158 Pi], 44.543°N, 7.143°E, 2480 m, 14.I.2019, Mammola S. and Isaia M. leg. 1f;
- 470 **Sampeyre,** Buco del Nebin 2 (Buco della Crozza) [1159 Pi], 44.544°N, 7.142°E, 2440 m,
- 471 18.VIII.2006, Lana E. leg. 2m, 2f (MCSNB); Valdieri, Grotta Topalinda (Maissa 2) [1210 Pi], 7.405°E,
- 472 44.261°N, 1200 m, 19.XI.2016, Isaia M. and Mammola S. leg. 1f; same locality, 9.VII.2017, Isaia M.
 473 and Mammola S. leg. 1f; same locality, 11.VI-22.IX.2017 in pitfall traps Isaia M., Chiappetta N. and
 474 Maxwella G. leg. 1g;
- 474 Mammola S. leg. 1m.
- 475 476
- 477 Material examined
- Italy, Piemonte, Province of Cuneo: Casteldelfino, Tana dell'Orso [1019 Pi], 14.I.2019, Isaia M. and
 Mammola S. leg. 2m; Sampeyre, Buco del Nebin 1 [1158 Pi], 19.XI.2008, Lana E. leg. 1f (MCSNB);
 same locality 14.I.2019, Mammola S and Isaia M. leg. 1f; Valdieri, Grotta Topalinda [1210 Pi],
- 481 30.IX.2017, Isaia M. leg. 1f.
- 482
- 483
- 484 *Re-examined material*
- 485 Former literature records (sub *T. vignai* or otherwise specified) are here revised and assigned to *T.* 486 *delphinicus* sp. nov. on the basis of morphological examination. The identification of juveniles is
- 487 based on the identification of adults occurring in the same locality. For details about material see
- 488 original publications.
- 489

490 Italy, Piemonte, Province of Cuneo: Casteldelfino, Tana dell'orso [1019 Pi]: Lana, 2000 sub
 491 *Troglohyphantes* sp.; Casale et al. 2000 sub *Troglohyphantes* sp.; Arnò and Lana, 2005 sub

- 492 *Troglohyphantes* sp.; Isaia et al. 2010; Isaia et al. 2011b; Mammola et al. 2015; Mammola et al.
- 493 2018b. Sampeyre, Buco del Nebin 1 [1158 Pi]: Isaia et al. 2010; Isaia et al. 2011b; Sampeyre,

- 494 Pertus dal Drai [1017 Pi]: Casale et al. 2000 sub *Troglohyphantes* sp.; Lana et al. 2002 sub
- 495 *Troglohyphantes* sp.; Arnò and Lana, 2005 sub *Troglohyphantes* sp.; Isaia et al. 2010; Isaia et al.
- 496 2011b (the caption of Fig. 2.67 A: 140 contains a misprint: the illustration of the male palp do not
- 497 refer to tana del Diavolo [1591 Pi] but to Pertus del Drai [1017 Pi] and thus to *T. delphinicus* sp.
- 498 nov.). Isasca, Pertus d'la Tundo (or d'la Rocho) [1265 Pi]: Isaia et al. 2011b.
- 499
- 500 Diagnosis
- 501 Males of *Troglohyphantes delphinicus* sp. nov. are primarily distinguished from other species of
- 502 Troglohyphantes by the shape of the lamella characteristica (Fig. <u>1, 68a, 10a, 11</u>). The new species
- is close to *T. vignai* and other species of the Henroti complex, from which it is distinguishable by the
- 504 longer upper branch of the *lamella characteristica*. In particular, the A/B ratio (Fig. 10a2a) is
- 505 between 1.1 and 1.3 (n= 6, mean±s.d.= 1.14±0.24), significantly higher than in in T. vignai as
- 506 <u>currently defined</u> and <u>in</u> *T. achillis* sp. nov.
- 507 <u>The identification of females in absence of males remains doubtful in most cases.</u>
- 508 Females are best diagnosed by the epigynum in frontal view (Fig. 6b), showing a C/D ratio between
- 509 1.8 and 2.0 (n= 8, mean±S.d.= 1.96±0.16), higher than *T. achillis* sp. nov. and lower than *T. vignai*
- 510 (see Fig. 10b)
- 511
- 512 Description

Male paratype (Becetto, Pertus del Drai [1017 Pi] (Fig. 9b11b), Isaia M. and Mammola S. leg. 5.IV.2019): prosoma 1.44 long, 1.21 wide, yellowish. Thoracic region slightly swollen, yellowish with grey shades. Cephalic region elevated, interspersed with many black bristles between the eyes, with several small black bristles forming the eye region and continuing backwards in three longitudinal rows converging at the thoracic furrow. One bristle just below AME. Carapace with darker margins. Clypeus slightly indented under the eyes, then convex, 0.40 long. Eyes normally developed, with pigment and black margins. AME smallest. PLE and PME equal in size. ALE and PLE contiguous. PLE–

52	20	PME distance = 0.10, ALE–AME distance = 0.14, PME–PME distance = 0.11. Eye diameters AME 0.02,
52	21	PME 0.04, ALE 0.05, PLE 0.05. Abdomen 1.83 long, 0.90 wide, pale greyish with no distinct pattern
52	22	but some very faint spots only are visible on macrophotographs of aalive specimens (Fig. 10c).
52	23	Sternum heart-shaped, yellowish with flimsy darkened anterior edges. Chelicerae light brownish,
52	24	0.55 long, with ca. 35 lateral stridulatory ridges and armed with three anterior teeth. Legs yellowish,
52	25	uniform in colour. Leg I: femur 2.50, patella 0.70, tibia 2.54, metatarsus 2.14, tarsus 1.41, TLL 9.29;
52	26	leg II: femur 2.52, patella 0.75, tibia 2.48, metatarsus 2.22, tarsus 1.29, TLL 9.26; leg III: femur 2.19,
52	27	patella 0.38, tibia 2.30, metatarsus 1.90, tarsus 1.10, TLL 7.87; leg IV: femur 2.29, patella 0.39, tibia
52	28	2.60, metatarsus 2.38, tarsus 1.19, TLL 8.85. Abdomen greyish with a very faint pattern at the
52	29	bottom, 1.83 long, 0.90 wide. Palp: femur 0.59, patella 0.15, tibia 0.19 (see Tab. S2 for
53	0	measurements of additional specimens). Male palp (Fig. 8a) with Cymbium cymbium faintly convex,
53	1	roughly triangular when seen from above, ending proximally with one stout apophysis. Posterior
53	2	part of paracymbium subtriangular, apical part narrowed anteriorly. Lamella characteristica similar
53	3	to <i>T. vignai</i> and <i>T. achillis</i> sp. nov., clearly visible in lateral view without extraction. Distal part of the
53	4	lamella gun-shaped with one long horizontal branch and a small inferior, almost contiguous,
53	5	rounded lobe. One additional upper short branch is directed upwards, perpendicular to the
53	6	horizontal branch. Suprategular apophysis directed upwards, with a sharp end. Tip of the embolus
53	57	spikysharply pointed. A/B ratio between 1.1 and 1.3 (see Fig. 10a2a) (n= 4; mean±s.d.= 1.20±0.22).
53	8	Spination: femur I with one prolateral spine; all femurs with one dorsal spine. Patella I-IV with one
53	9	dorsal spine. Tibia I with three dorsal, three ventral, two retrolateral and two prolateral spines; Tibia
54	0	II with two dorsal, two ventral, two retrolateral, and two prolateral spines; tibia III with two
54	1	prolateral, two dorsal and two retrolateral spines; Tibia IV with two dorsal, two prolateral, and two
54	2	retrolateral spines. Metatarsus I-IV with one dorsal and one prolateral spine. Patella of the palp
54	3	with one long, curved spine. Position of TmI: 0.18. Trichobothrium on Mt IV absent.

544

545 Female (paratype from type locality, Isaia M. and Mammola S. leg. 16.VII.2015): prosoma 1.23 long, 546 1.15 wide, slightly darker than male. Cephalic region light-brownish. Carapace, ocular area, clypeus, 547 and sternum are similar in all features to the analogous male body parts. Light brownish sternum 548 with dark margins. Anterior margin of the chelicerae armed with three teeth. Clypeus 0.22 long, 549 chelicerae 0.60 long. PLE-PME distance = 0.06, ALE-AME distance = 0.04, PME-PME distance = 0.06, 550 AME–AME distance = 0, ALE–PLE distance = 0. Eye diameters: AME 0.05, PME, AME and PLE = 0.07. 551 Abdomen pale greyish with no pattern, cardiac mark slightly visible, 2.19 long, 1.84 wide. Leg I: 552 femur 2.45, patella 0.52, tibia 2.49, metatarsus 2.13, tarsus 1.49, TLL 9.08; leg II: femur 2.40, patella 553 0.51, tibia 2.42, metatarsus 1.97, tarsus 1.41, TLL 8.70; leg III: femur 2.04, patella 0.47, tibia 1.96, 554 metatarsus 1.67, tarsus 0.98, TLL 7.12; leg IV: femur 2.56, patella 0.42, tibia 2.47, metatarsus 2.15, 555 tarsus 1.26, TLL 8.86. Female palp: femur 0.69, patella 0.18, tibia 0.39, tarsus 0.81, total palp length 556 2.07 (see Tab. S2 for measurements of additional specimens). Spination: Femur I with one prolateral 557 spine; Femur I–IV with one dorsal spine. Patella I–IV with one dorsal spine. Tibia I with two dorsal, 558 two prolateral, five ventral and two retrolateral spines; Tibia II with two dorsal, three prolateral, 559 three ventral and two retrolateral spines; Tibia III with two dorsal, two prolateral and two 560 retrolateral spines. Tibia IV with two dorsal, two ventral and two retrolateral spines. Metatarsus I-561 IV with one dorsal and one prolateral spine. Tarsus of the palp with four dorsal, three retrolateral, four ventral and one prolateral spines. Position of TmI: 0.18. Trichobothrium on Mt IV absent. 562 563 Epigyne (Fig. 8b, c) protruding, with subtriangular scape, not much wider than long, covering less 564 than half of the inner part of the epigyne, C/D ratio between 1.8 and 2.0 (n= 8, mean±S.d.= 565 1.96±0.16, (see Fig. 10b2b), . Females epygine are best diagnosed by the epigynum in frontal view 566 (Fig. 6b), showing a C/D ratio between 1.8 and 2.0 (n= 8, mean±s.d.= 1.96±0.16), higher than T. 567 achillis sp. nov. and lower than T. vignai (see Fig. 10b2b).

Posterior margin of the scape with almost parallel lateral margins, posterior profile less rounded than *T. achillis* sp. nov. Stretcher tongue-shaped, straight, emerging halfway in the profile in lateral view (Fig. 8c), bearing a pitted knob at its end, visible in ventral view. Lateral lobes emerging at the posterior end of the epigyne. Fertilization ducts sclerotized.

- 572
- 573 Etymology

The species epithet is an adjective referring to the historical region of Dauphiné (Delfinato) of the French *ancien régime*, before the French revolution. The species shares the etymology with *Pimoa delphinica* Mammola, Hormiga and Isaia 2016 (family Pimoidae), an a Western Alpine endemic species recently described from the same area (Mammola et al. 2016a).

578

579 Distribution

580 The species is known in a few caves of Varaita Valley (Southern Cottian Alps) and an isolated 581 locality in Gesso Valley (Maritime Alps). The area covered by the minimum convex polygon 582 encompassing the known localities of this species is about 400 km², when excluding the isolated 583 population dwelling in Topalinda cave (Alpi Marittime)of Valle Gesso (Topalinda cave, Maritime 584 Alps), the size of the range is 70 Km² (see Fig. 14).

585

586 Biospeleological notes

This species was firstly collected by Enrico Lana and Mike Chesta in two wild-caves in Varaita valley in summer 1999. The validity of the species was originally pointed out in 2006 by MI studying specimens collected by Enrico Lana in Nebin 1 cave [Pi 1158], located a few meters under the top of Mount Nebin (2510 m) on the ridge separating Maira and Varaita valleys (Southern Cottian Alps).

- 591 The availability of more material collected during the years by the authors and the support of
- 592 molecular analysis here presented have now confirmed the validity of this species.
- 593
- 594
- 595 Troglohyphantes vignai Brignoli 1971
- 596 Fig. 7<u>9</u>, 8e<u>10e</u>, 8f-f
- 597 Taxonomic references
- 598 *Troglohyphantes vignai* Brignoli 1971: 170, f. 52-58 (Dmf)
- 599 Troglohyphantes rupicapra Brignoli 1971: 172, f. 59-60 (Df)
- 600 Troglohyphantes rupicapra Brignoli 1979: 321, f. 13-14 (f)
- 601 *Troglohyphantes rupicapra* Pesarini 1988: 238, f. 3 (Dm)
- 602 Troglohyphantes vignai Isaia et al. 2011b: 140, f. 2.67B-C (f)
- 603
- 604
- 605 Material examined
- 606 Italy, Piemonte, Province of Cuneo: Chiusa Pesio, Grotta delle Camoscere Superiore [250 Pi],
- 607 7.600°E, 44.200°N, 1140 m, 29.XI.2007, Isaia M. leg. 1m; same locality, 26.XII.2014, Isaia M.,
- Mammola S. leg. 1m, 2f, same locality, 03.XII.2018, Isaia M., Mammola S. leg. 1m, 1f; same
- locality, 03.XII.2018, Isaia M. and Mammola S. leg. 2m; Crissolo, Balma di Valenza [1009 Pi] (type
- 610 locality, Fig. 9c11c), 7.017°E, 44.681°N, 1440 m, 13.XI.2014, Isaia M., Mammola and Paschetta M.
- 611 leg. 2f, 2juv; same locality, 8.I.2019, Isaia M. and Mammola S. leg. 1m, 9f, 2juv; same locality,
- 612 13.VII.2021, Isaia M. and Biggi E. leg. 2f; Frabosa Soprana, Grotta Beppe Bessone [3303 Pi],
- 613 7.818°E, 44.208°N, 1419 m, 22.V.2015, Isaia M. and Mammola S. leg. 1m, 2f; Frabosa Sottana,
- 614 Tana del Bergamino [175 Pi], 7.767°E, 44.255°N, 1175 m, 21.VI:2021, Isaia M. and Tolve M. leg. 2f,
- 615 2m; Garessio, Pozzo Ciuaiera [146 Pi], 7.886°E, 44.191°N, 2099 m, 12.VI.1999, T. Pascutto leg. 1m;
- 616 same locality, 18.VIII.2009, Lana E. leg. 1m, same locality, 12.X.2008, Lana E. leg. 3m, 4f; Ostana,
- 617 Pertui de l'Oustanetto [1251 Pi], 7.197°E, 44.714°N, 2180 m 8.VII.2001, Lana E. leg. 1f; **Crissolo**,
- 618 Pertugio Stopponetto [1047 Pi], 7.148°E, 44.682°N, 1815 m, 16.VII.2006, Lana E. leg. 1 juv;
- 619 **Roccaforte Mondovi**, F12 del Mongioie [3050 Pi], 7.781°E, 44.169°N, 2471 m, 27.VIII.2018, Isaia M.
- 620 and Mammola S. leg. 3f.
- 621

622 *Re-examined material*

Former literature records are here revised and assigned to *T. vignai* on the basis of morphological
examination. For details about material see original publications.

625

626 Italy, Piemonte, Province of Cuneo: Briga Alta, Voragine di Scarasson [221 Pi]: Arnò and Lana, 2005

- 627 sub *Troglohyphantes* sp.; Isaia et al. 2010 sub *T. rupicapra*); Isaia et al. 2011b; **Briga Alta,** Pozzo 2-6
- delle Carsene o Abisso Arrapa Nui [772 Pi]: Lana et al. 2002 sub *T*. cf. *rupicapra*; Arnò and Lana,
- 629 2005 sub Troglohyphantes sp.; Isaia et al. 2010, sub T. rupicapra; Isaia et al. 2011b; Briga Alta
- 630 Pozzo 1-5 delle Carsene o Abisso Rangipur [761 Pi]: Lana et al. 2002 sub T. cf. rupicapra; Arnò and
- 631 Lana, 2005 sub *Troglohyphantes* sp.; Isaia et al. 2010, sub *T. rupicapra*; Isaia and Pantini, 2010 Fig.
- 632 17-18; Isaia et al. 2011b (see previous note under *T. delphinicus* sp. nov. about the misprint in the
- 633 legend of Figure 2.67); Briga Alta, Abisso Vento [3500 Pi]: Isaia and Pantini, 2010; Isaia et al.
- 634 2011b; Briga Alta, Unknown cave near Colle del Pas: Mammola et al. 2015; Chiusa Pesio, Grotta

635 delle Camoscere [105 Pi]: Bologna and Vigna Taglianti, 1982 sub T. rupicapra; Casale, 1986 sub T. 636 rupicapra; Morisi in GSAM, 1987 sub T. rupicapra; Lana, 2001 sub T. rupicapra; Arnò and Lana, 637 2005 sub T. rupicapra; Isaia et al. 2011b; Chiusa Pesio, Grotta superiore delle Camoscere [250 Pi]: 638 Vigna Taglianti and Follis, 1968 sub *Troglohyphantes* prope *qhidini*; Morisi, 1971 sub *T. rupicapra*; 639 Casale and Longhetto, 1970 sub *Troglohyphantes* prope *qhidini*; Brignoli, 1971 sub *T. rupicapra*; 640 Casale, 1971 sub T. rupicapra; Brignoli, 1972 sub T. rupicapra; Brignoli, 1975 sub T. rupicapra; 641 Bologna and Vigna Taglianti, 1985 sub T. rupicapra; Casale, 1986 sub T. rupicapra; Pesarini, 1988 642 sub T. rupicapra; Lana, 2001 sub T. rupicapra; Arnò and Lana, 2005 sub T. rupicapra; Isaia et al. 643 2007; Isaia et al. 2011b; Mammola et al. 2015; Mammola et al. 2018b. Crissolo, Buco di Valenza o 644 Balma dell'inglese [1009 Pi]: Morisi, 1971; Brignoli, 1971; Casale, 1971; Brignoli, 1972; Bologna and 645 Vigna Taglianti, 1982; Arnò and Lana, 2005; Isaia et al. 2011b; Mammola et al. 2015. Crissolo, 646 Fortino a ovest della Balma di Rio Martino, Opera 372 Rocca di Granè [Art. Pi]: Arnò and Lana, 647 2005 sub Troglohyphantes sp.; Isaia et al. 2010; Isaia et al. 2011b; Garessio, Voragine della 648 Ciuaiera [146 Pi]: Isaia and Pantini, 2010; Isaia et al. 2011b; Frabosa Soprana, Grotta Beppe 649 Bessone [3303 Pi]: Isaia et al. 2017; Ostana, Pertui de l'Oustanetto [1251 Pi]: Lana et al. 2002 sub 650 T. cf. vignai; Isaia et al. 2011b; Pertugio di Stopponetto [1047 Pi].

- 651
- 652 *New diagnosis*

653	In light of the split of <i>T. vignai</i> aut. here proposed, males of <i>Troglohyphantes vignai</i> Brignoli <u>1971</u>
654	are primarily distinguished from other species of Troglohyphantes by the shape of the lamella
655	characteristica (Fig. 79a). The species is distinguishable from <i>T. achillis</i> sp. nov. and <i>T. delphinicus</i>
656	sp. nov. by the shape of the <i>lamella characteristica</i> , with A/B ratio between 0.6 and 0.8_ (n= 6,
657	mean±s.d.= 0.71±0.05)(n= 6, mean±s.d.= 0.71±0.05), significantly lower than in T. delphinicus and
658	T. achillis sp. nov. (see Fig. 10a2a). The identification of females in absence of males remains
659	doubtful in most cases.
660	Females are best diagnosed by the epigynum in frontal view (Fig. 10b), showing a C/D ratio between

- $661 \qquad 1.8 \text{ and } 2.5 \text{ (n= 6, mean \pm s.d. = 2.20 \pm 0.30; Fig. 10b), higher than T. achillis sp. nov. and T. vignai.}$
- 662
- 663 Distribution
- 664 On the base of this work, *Troglohyphantes vignai* shows a disjunct distribution in Cottian (Po
- 665 Valley, northern clade) and Ligurian Alps (from Pesio to Tanaro Valley, southern clade) (Fig. 14).
- 666 The area covered by the minimum convex polygon encompassing all known localities of this

species is about 600 km². When considering the two clades, the northern one covers a range of
nearly 10 km² and the southern one of approximately 80 km², for a total disjunct range of
approximately 90 km².

670 Biospeleological notes

Specimens of the southern clade (Val Pesio and Tanaro) exhibit higher degree of troglomorphism in respect to other populations of the same species (higher depigmentation, reduction of PLE and PME, lowering of the profile of cephalothorax; see Figs. 15-—18 in Isaia et al., 2010) in respect to other populations of the same species). Southern localities also include the former type locality of *T. rupicapra* Brignoli 1971 (= *T. vignai* Brignoli after Pesarini 2001) considered by Brignoli (1971) a different species on the basis of the higher level of troglomorphism adaptation to subterranean life (i.e. troglomorphic traist; Christiansen 2012) and small differences in the shape of the epigyne.

Still

678

679 **DISCUSSION**

680 *Systematics and phylogeography*

The new species can be diagnosed by both genitalic and somatic characters. Morphological
differences are restricted to small details of the copulatory organs (Fig. 5-8, 10, 11). The overall
size, leg length and abdominal pattern also provide useful details for identifying the species (see
measurements in Tab. S2 and Fig. 8). Owing to their similarity to *T. vignai, the two new species here described are assigned to the Henroti complex (sensu Deeleman Rehinold, 1978; Isaia et al.*2017).

687

688	The new species are diagnosed by small details of the male copulatory organs (Figs. 2a, 7-
689	9). In particular the A/B ratio of the male lamella characteristica proved to be diagnostic in most
690	cases, with negligible overlapping among species (see Fig. 2a). Our attempt to separate females
691	according to C/D ratio has partly failed, with revealed a certain degree of overlap among
692	individuals of different species (Fig. 2b). The overall size, leg length and abdominal pattern also
693	provide useful comparative details for separating T. achillis from the other two species, being the
694	biggest in most anatomical features and the only one with pigment on the distal part of the
695	abdomen; see measurements in Tab. S2 and Figs. 7d, and 10). However, due to the intrinsic
696	variability of these traits, we did not listmentioned them in the species diagnosis.
697	The COI and ITS-2 gene trees corroborate our morphological delineations, in as much they
698	recovered monophyly of the haplotypes and alleles of each sputative species (Fig. 23) and mPTP
699	supported the split of <i>T. achillis</i> sp. nov. from the other species. Additionally, the level of genetic
700	divergence in the COI between the three species (~8%, <u>Tab. 1</u>) was above the threshold divergence
701	proposed to identify Troglohyphantes species (Isaia et al. 2017; but see also Leaché et al., 2021 for
702	threshold divergence in other taxa). However, GMYC failed to reject the alternative hypothesis of
703	a single species and mPTP provided low support for the split of <i>T. delphinicus</i> sp. nov. and <i>T. vignai</i>
704	(Fig. <u>13</u>). The deeper coalescent of COI haplotypes and ITS2 alleles revealed observed - in <i>T.</i>
705	delphinicus sp. nov. compared to the other two species may be partly responsible for the inability
706	of single markers delimitation methods to delimit the three species. In this regard, the estimated
707	time frame of diversification is compatible with the involvement of the Quaternary glacial cycles in
708	the generation of the present-day patterns of genetic variability and geographic structuring of the
709	three species. It is worth mentioning that <i>T. delphinicus</i> sp. nov. not only showed the highest
1	

710 levels of genetic variability but was the only lineage that did not show a star-like structure in the 711 ITS alleles (Fig. 24), indicating that this species may have not undergone population bottlenecks as 712 severe as those experienced by the other species. A similar pattern was recovered in the 713 troglophile endemic Alpine species *Pimoa delphinica* Mammola, Hormiga and Isaia 2016 sharing 714 the same small range of *T. delphinicus* (Mammola et al. 2015; Mammola et al. 2016), which may 715 point to the existence of an important glacial refugia in this area. Unlike Pimoa, however, in this 716 area we found neither cases of co-occurrence of congeneric species, nor any indication of 717 hybridization (Mammola et al. 2016). This is probably due to the higher level of genetic isolation 718 seen in species with higher level of subterranean adaptation such as *Troglohyphantes*. 719

720 Ecology and Habitat and natural history

721 Despite we neverthem never being found them in syntopy, *Troglohyphantes achillis* sp. nov., *T*. 722 delphinicus sp. nov. and T. vignai were found consistently in the same habitat, i.e. in the dark zone 723 of natural caves or artificial subterranean habitats such as mines and bunkers. In a very fewrare 724 occasions, specimens of T. delphinicus sp. nov. and T. vignai were also collected outside, in the 725 vicinity of cave entrances in deep litter or under big stones (only T. delphinicus sp. nov. was 726 collected in pitfall trap in deep litter of a beech forest of Valdieri, Province of Cuneo). Interestingly, 727 such similarities in habitat preference is are also mirrored in the life cycle of the three species. In 728 all cases, males are rarely found in spring or early autumn and almost disappear in winter, while 729 females are present all year long.

730

731 <u>Ecological adaptation and incipient speciation</u>

Invertebrate Systematics

7	32	The ability of the three newly defined species-these species -to maintain permanent underground
7	33	populations is reflected in their level of adaptation to subterranean life (i.e. troglomorphic
7	34	traitssm; Christiansen 2012). In this respect, cave-adapted traits are particularly remarkable in the
7	35	populations at the southern margin of the range <u>of (Troglohyphantes</u> , vignai as currently
7	86	defined)in southern populations of <i>T. vignai</i>, which are more less depigmented, show higher
7	37	degree of eye reduction (PLE and PME) and a more pronounced lowering of the cephalothorax
7	38	profile—a trait that has been noticed in high subterranean adapted species of <i>Troglohyphantes</i>
7	<u>89</u>	(Isaia and Pantini 2010; Isaia et al., 2017; Mammola et al., 2020). Accordingly, in our previous work
74	40	(Isaia and Pantini, 2010), we assumed for these populations were in a stage of incipient speciation
74	11	an incipient phenomenon of speciation for these populations,. Such findings which were
74	12	successively re-interpreted in the light of the firsta molecular study- conducted on this species
74	13	(Mammola et al., 2015)_uncovering high genetic structuring (Mammola et al., 2015) and where we
74	14	pointed) and pointing pointing out climatic isolation as the main factor determining-, from one
74	15	side, the presence of different lineages in <i>T. vignai</i> autin <i>T. vignai aut.</i> — that we now recognize as
74	16	separate species—- and, from the other, the higher development of troglomorphism observed in
74	17	the southern populations. This frame <u>work</u> is also coherent with our current findings and with the
74	18	different levels of support recovered for the species level status off the three lineages. In fact, the
74	19	three species are well delimited by male palp morphology and physiological traits, but only
7	50	partially by female genitalia and the analysis of morphospaces. Similarly, we found contrasting
7	51	results in molecular data, with support from mPTP on the COI data matrix, although only two
7	52	delimitations (<i>T. vignai</i> and <i>T. achillis</i> n.sp.nov.) were well-supported. GMYC failed to reject the
7	53	null hypothesis of a single species and the ITS2 recovered <i>T. delphinicus</i> sp. nov. as non-
7	54	monophyletic.
Such mixed levels of support recovered by our multi layered approach confirms the context of an
incipient speciation for these three species, which is echoed in the framework of the unified
species concept (de Queiroz, 2005). Such incongruences reflect the continuous nature of the
speciation process, which is particularly remarkable in our model organism, showing low dispersal
coupled with high microhabitat specificity.

- 760
- 761

762 Distribution patterns and thermal tolerance

763	Troglohyphantes achillis sp. nov. prefers the medium alpine montane belt, from 1000 up to
764	1400 m above sea level (mean = 1180 m; n = 4), and inhabits sites characterised by mean annual
765	temperature <u>s</u> values ranging from 6.1 to 8.9°C (mean=7.5 °C; range: 2,8; n=4). <i>Troglohyphantes</i>
766	delphinicus sp. nov. was mainly collected in natural caves at an altitude ranging from 1000 to 2480
767	m (mean=1901 m; n=6) and inhabits sites characterised withby mean annual temperature-values
768	ranging from 0 to 8°C (mean = 2.8°C; range: 8; n = 6). <i>Troglohyphantes vignai</i> shows a general
769	preference for the medium alpine montane belt, from 1140 up to 2471 m above sea level (mean =
770	1794 m; n = 8), and inhabits subterranean sites characterised by with mean annual temperature
771	values ranging ranging from 7.3 to 12.1°C (mean=9.28°C; range: 4.8; n=8).
772	Results of thermal tolerance tests (Fig. 46) align well with the general pattern observed in
773	Mammola et al. (2019) for Troglohyphantes, as well as that obtained with other subterranean
774	arthropods (e.g., <u>Raschmanová et al. 2018;</u> Pallarés et al. 2019, 2020a, 2020b; Colado et al. 2021 <u>;</u>
775	Jones et al, 2021), which predicts that specialized subterranean species show low thermal
776	tolerances <u>than related surface species.</u> In <i>Troglohyphantes</i> , this narrow thermal niche breadth

777	often translates into restricted dispersal ability, as reflected in the small distribution ranges. This is
778	particularly evident in the three species here considered, with T. delphinicus sp. nov. and T. achillis
779	sp. nov. being poorly tolerant and short ranged, followed by <i>T. vignai</i> , exhibiting higher tolerance
780	and a greater range. Interestingly, results on the thermal tolerance of Western Alpine specie of
781	Troglohyphantes reported in Mammola et al. (2019), revealed a high variability in this
782	physiological trait in <i>T. vignai</i> aut., which now appear to be fully coherent with new-the
783	delimitation of the three species here proposed <u>here</u> . Results on thermal tolerance support the
784	hypothesis that the present-day pattern of isolated distributions in the three species is mostly
785	explained by the combination of low dispersal ability and low thermal tolerance (Mammola et al.
786	2019), and historical biogeographic events (see Mammola et al. 2015).
787	It is worth noting that Thermal thermal tolerance tested on a single population of T.
788	delphinicus sp. nov. may be poorly representative for the species, which has actually been found
789	in a number of sites characterised by different altitudes and temperatures. This apparent
790	contradiction could provide and additional explanation for the highest genetic variability found in
791	this species, which would be the result of local adaptation to particular temperatures coupled with
792	the inability of disperse to localities with different temperatures.
 793	

794 CONCLUSIONS

795

796 Using a multi-layered set of morphological, genetic, <u>physiological</u> and ecological evidence, we 797 revealed the existence of three distinct species in a lineage of spiders characterized by a high level 798 of adaptation to the subterranean environment. As previously observed for other subterranean 799 organisms in the Alps, the process prompting diversity is most likely related to pulses of 800 population contraction and expansion during the Quaternary cycles. We found evidence for 801 differences in thermal tolerance between the species, which most likely had an impact on their 802 dispersal ability and ultimately on their ability to expand their distribution ranges and retain 803 genetic variability. The same approach can be extended to other subterranean taxa showing 804 similar distributions in the Alps (e.g., Doderotrechus and Duvalius beetles, Ischyropsalis 805 harvestmen) or in other similar biogeographical settings to to shed broaden our knowledge on the 806 processes that shaped subterranean biodiversity. 807 808 809 ACKNOWLEDGEMENTS

8 10 This work is dedicated in memory of our esteinateed colleague and extraordinary natural scientist
8 11 Prof. Augusto Vigna-Taglianti, who recently passed away. Special thanks go to Elena Pelizzoli and
8 12 Paolo Pantini for the illustrations and to John Dejanaz and Nicolò Chiappetta for measuring the
8 13 specimens. The authors would like to thank all people mentioned in the Material sections for
8 14 fieldwork assistance. Spider pictures are by Emaneule Biggi, sSpecimen were photographed by
8 15 Emanuele Biggi, alive in the designated type locality during a dedicated field expedition led by MI
8 16 on July 13 and 14, 2021.

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8 8 DECLARATION OF FUNDINGS

819 Funding was provided by the Agency for Management of University and Research Grants of820 Catalonia (2017SGR83) to MA.

821 CONFLICT OF INTEREST

822 The authors declare no conflicts of interest.

823 DATA AND CODE AVAILABILITY

Data and R script to generatereproduce the ecological analyses is available in GitHub
 (https://github.com/StefanoMammola/Troglohyphantes vignai overlooked diversity.git).

All morphological and physiological traits have been deposited in the Spider Trait Database (Pekár et al., 2021<u>a</u>, <u>2021b</u>) (upon acceptance).

Specimens analyses in the present study with geographic and sequence information; cox1
accession #: GeneBank accession code; cox1 haplo: cox1 haplotypes arbitrarily named with one of
the sequences; GMYC: GMYC clusters; ITS2 Accession #: GeneBank accession code, ITS2 alleles:

arbitrarily named after one of the sequences; Locality #: locality number as seen in Fig. 24.

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833 SUPPLEMENTARY MATERIALS

Table S1. List of specimens used for molecular analysis with voucher information, DNA code and
 GenBank[®] access code. Sequences new for this study are marked in bold.

- 836 <u>Table S2. Measurements of additional specimens (in mm).</u>
- 837

838 AUTHOR'S CONTRIBUTION

839 MI and SM conceived the idea, designed methodology and performed fieldwork. MI described the

840 new species, assembled figures and <u>led the writingete</u> the first draft of the manuscript. SM

analysed ecological data, wrote the relative sections and helped MI in the species description. MA

conducted the analyses of the molecular data and wrote the related sections. All authors
 contributed to the writing of the paper trough discussions and additions to the text.

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1154 comparison clearer, lamella significativa outlines are in the same orientation. Samples used for

1155 molecular analysis are highlighted with double points (see Tab. S1).

1156	
1157	Figure 2. Genitalic features in <i>Troglohyphantes achillis</i> sp. nov., <i>T. delphinicus</i> sp. nov. and <i>T.</i>
1158	vignai. (a) Ratio between the length of the upper branch (A) and height (B) of the lamella
1159	characteristica in males (b) Batio between width (C) and height (D) of the enigyne scape in
1160	females
1161	
1162	Figure 13 Revesian consensus chronogram inforred from the COI sequences with time axis (in
1162	million years. Mu) and goological time scale. Pars denote QEV highest posterior density (HDD)
1164	confidence intervals. Circles on internal nodes denote support values as follows: right half –
1104	Confidence intervals. Circles on internal nodes denote support values as follows. Fight fiam –
1105	Bayesian posterior probabilities (PP), left nan – maximum incennood bootstraps (BS). Black colour –
1100	PP>0.95 of BS >75, grey colour = clades recovered with support values below former thresholds.
110/	I riangles on nodes indicate clusters recovered in species delineation analyses (m-PTP). Black
1108	colour = delimitation support>0.95, grey colour = delimitation >0.95. The tree was rooted using
1109	Trogionyphantes higraerosae Brignoli. Clades coloured after morphological delineations. For DNA
1170	coding see Table S1 (column C).
11/2	Figure <u>24</u> . Statistical parsimony ITS-2 allele network. <i>Troglohyphantes vignai</i> (blue), <i>T. delphinicus</i>
1173	sp. nov. (yellow) and <i>T. achillis</i> sp. nov. (purple). <i>T. nigraerosae</i> (grey) is the outgroup. Numbers on
1174	pies indicate different alleles. The size of each circle is proportional to the number of sampled
1175	individuals with each allele. Numbers in specimen labels correspond to localities reported in Table
1176	S1 (column L).
1177	
1178	
1179	Figure <u>35</u> . Pairplot showing the functional morphospace of the three species of <i>Troglohyphantes</i> .
1180	For each plot, 20,000 random points sampled from the estimated 3-dimensional kernel density
1181	hypervolumes are shown. Note that original morphological traits have been converted by applying
1182	Gower distance and a Principal Coordinate Analyses before hypervolume estimation. Arrows and
1183	overlapping ovals in the bottom-left scheme are, respectively, niche centroid distances and niche
1184	differentiation values (measured as eta -total; Mammola and Cardoso 2020) among the three
1185	species.
1186	
1187	Figure 4 <u>6</u> . Thermal tolerance of the three species of <i>Troglohyphantes</i> , estimated using the
1188	experimental protocol detailed in Mammola et al. (2019). Thermal tolerance is here expressed as
1189	ΔT , namely the difference between the critical temperature and the mean temperature of the
1190	cave of each species. (a) Survival rate as a function of ΔT . Survival curves represent best fits to the
1191	data, according to binomial generalized linear models. (b) Differences in ΔT between cave
1192	populations. There was a significant effect of thermal tolerance on species delimitation based on
1193	ANOVA [F _(2.67) = 15.16, p< .001]. A post hoc Tukey test revealed that all species were significantly
1194	different in their AT at p< .05. For each species, there were no significant differences between
1195	cave populations with the exception of <i>T. achillis</i> sp. nov. population from Tuna dal Diau cave (*),
1196	displaying a significantly higher thermal tolerance compared to the other <i>T. achillis</i> sp. nov.
1197	populations based on ANOVA [$F_{(3.38)}$ = 9.41, p< .001]. Note that this population was excluded in
1198	subsequent analyses.
1199	
1200	Figure 57 . <i>Troalohyphantes achillis</i> sp. nov. (Tana del Diavolo [1591 Pi]. Roreto Chisone, Italy
1201	19.09.2007 leg. Isaja M.). Right pedipalp of the male (a), epigyne in dorsal view (b), and epigyne in
1202	lateral view (c) and abdomen (d). Abbreviations: Cy. cymbium: HB. horizontal branch of the
r -	

- lamella; PL, Posterior lobes; PP: posterior plate; RL, rounded lobe of the lamella; S, Scape; St,
 Stretcher; StA, Suprategular apophysis.
- Figure <u>68</u>. *Troglohyphantes delphinicus* ([Pertus del Drai [1017 Pi], Sampeyre, Italy, 25.08.2001 leg.
 Lana E.). Right pedipalp of the male (a), epigyne in dorsal view (b) and epigyne in lateral view (c).
 Abbreviations: Cy, cymbium; HB, horizontal branch of the lamella; PL, Posterior lobes; PP:
 posterior plate; RL, rounded lobe of the lamella; S, Scape; St, Stretcher; StA, Suprategular
- 1210 apophysis.
- 1211

1221

1225

Figure 79. *Troglohyphantes vignai* (Buco di Valenza [1009 Pi], Oncino, Italy, 8.01.2019 leg. Isaia M.
and Mammola S.). Right pedipalp of the male (a), epigyne in dorsal view (b) and epigyne in lateral
view (c). Abbreviations: Cy, cymbium; HB, horizontal branch of the lamella; PL, Posterior lobes; PP:
posterior plate; RL, rounded lobe of the lamella; S, Scape; St, Stretcher; StA, Suprategular
apophysis.

- Figure 810. Alive specimen of *Troglohyphante achillis* sp. nov. (a, b), *T. delphinicus* sp. nov. (c, d)
 and *T. vignai* Brignoli (e, f). Photos of alive specimen taken in their natural habitat on July 13 –14,
 2021 by Emanuele Biggi.
- Figure 911. Type localities of *Troglohyphante achillis* sp. nov.-, *T. delphinicus* sp. Nov. and *T. vignai* Brignoli: -Tana del Diavolo [Pi 1591] (a), Pertus del Drai [Pi 1017], (b) and Balma di Valenza [Pi 1009] (c), respectively. <u>Photos by Marco Isaia (a, c) and Emanuele Biggi (b).</u>
- 1226 Figure 10. Diagnostic features in Troglohyphantes achillis sp. nov., T. delphinicus sp. nov. and T. 1227 vignai. (a) Ratio between the length of the upper branch (A) and height (B) of the lamella 1228 characteristica in males. There was a significant effect of the ratio A/B on species delimitation 1229 based on ANOVA [F_(2.15)= 20.41, p< .001]. A post hoc Tukey test showed that T. delphinicus sp. nov. 1230 was significantly different from both *T. achillis* sp. nov. and *T. vignai* at p< 0.05; conversely, the 1231 comparison between T. achillis sp. nov. and T. vignai only approached statistical significance (p= 1232 .09). (b) Ratio between width (C) and height (D) of the epigyne scape in females. There was a 1233 significant effect of the ratio C/D on species delimitation based on ANOVA [$F_{(2.16)}$ = 4.11, p= .036]. A 1284 post hoc Tukey test revealed that T. vignai was significantly different from T. achillis sp. nov. at p< 1235 0.05; conversely, the other species comparisons were not significant. 1236
- Figure 11. Distribution of *Troglohyphantes achillis* sp. nov., *T. delphinicus* sp. nov. and *T. vignai* and
 intraspecific variability in *lamella significativa* of males. For a number of occurrences, including
 type localities, we report the outlines of the corresponding *lamella significativa* of males. To make
 comparison clearer, lamella significativa outlines are in the same orientation. Samples used for
 molecular analysis are highlighted with double points (see Tab. S1).
- 1242
- 1243
- Cover photo *Troglohyphantes achillis* n.sp. phoptopgraphehrd by Emanuele Biggi (July 13, 2021)
- 1244

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http://www.publish.csiro.au/journals/is

FIG 03





11My

17	Μv		







FIG_04







http://www.publish.csiro.au/journals/is

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Figure 11. Type localities of Troglohyphante achillis sp. nov., T. delphinicus sp. nov. and T. vignai Brignoli: Tana del Diavolo [Pi 1591] (a), Pertus del Drai [Pi 1017], (b) and Balma di Valenza [Pi 1009] (c), respectively. Photos by Marco Isaia (a, c) and Emanuele Biggi (b).

132x178mm (150 x 150 DPI)

TABLES

Table 1

	T. nigraerosae		T. delphinicus		T. achillis		T. vignai	
			sp. nov.		sp. nov.			
T. nigraerosae	0.04	0.010	0.012	0.005	0.012	0.006	0.013	0.0065
T. delphinicus sp. nov.	0.154	0.013	0.050	0.010	0.009	0.003	0.009	0.004
<i>T. achillis</i> sp. nov.	0.139	0.012	0.078	0.009	0.010	0.000	0.010	0.004
T. vignai	0.145	0.013	0.080	0.010	0.091	0.006	0.030	0.000

Supplementary material

Table S1

Genus	Species	DNA CODE (FIG1)	COI
Troglohyphantes	achillis	PM12	KX831566
Troglohyphantes	achillis	PM21	KX831563
Troglohyphantes	achillis	PM24	KX831563
Troglohyphantes	achillis	SM03	KT832081
Troglohyphantes	achillis	SM177	KT832113
Troglohyphantes	achillis	SM178	KT832114
Troglohyphantes	achillis	SM28	KT832090
Troglohyphantes	achillis	SM29	KT832091
Troglohyphantes	achillis	SM30	KT832092
Troglohyphantes	achillis	SM31	KT832093
Troglohyphantes	achillis	SM54	KT832096
Troglohyphantes	achillis	SM55	KT832097
Troglohyphantes	achillis	SM56	KT832098
Troglohyphantes	achillis	SM57	KT832099
Troglohyphantes	achillis	SM58	KT832100
Troglohyphantes	achillis	SM71	KT832101
Troglohyphantes	achillis	SM72	KT832102
Troglohyphantes	achillis	SM73	KT832103
Troglohyphantes	achillis	SM74	KT832104
Troglohyphantes	achillis	SM77	KT832107
Troglohyphantes	achillis	SM78	KT832108
Troglohyphantes	achillis	SM79 🔍 🗙	KT832109
Troglohyphantes	achillis	SM80	KT832110
Troglohyphantes	achillis	SM81	KT832111
Troglohyphantes	achillis	SM82	КТ832112
Troglohyphantes	delphinicus	PK1105	Tsp_pk1105
Troglohyphantes	delphinicus	PK1116	Tro.cf.vignai_pk1116_coi
Troglohyphantes	delphinicus	PK1117	Tro.cf.vignai_pk1117_coi
Troglohyphantes	delphinicus	PK1118	Tro.cf.vignai_pk1118_coi
Troglohyphantes	delphinicus	PK874	MG836288
Troglohyphantes	delphinicus	SM02	KT832080
Troglohyphantes	delphinicus	SM75	KT832105
Troglohyphantes	delphinicus	SM76	KT832106
Troglohyphantes	nigraerosae	PM09	KX831574
Troglohyphantes	nigraerosae	PM20	KX831573
Troglohyphantes	nigraerosae	PM27	KT832079
Troglohyphantes	vignai	PM07	KX831567
Troglohyphantes	vignai	PM22	KX831565
Troglohyphantes	vignai	PM23	KX831564
Troglohyphantes	vignai	SM05	KT832082
Troglohyphantes	vignai	SM07	KT832083
Troglohyphantes	vignai	SM09	KT832084
Troglohyphantes	vignai	SM23	KT832085

Troglohyphantes vignai		SM24	KT832086
Troglohyphantes	vignai	SM25	KT832087
Troglohyphantes	vignai	SM26	KT832088
Troglohyphantes	vignai	SM27	KT832089
Troglohyphantes	vignai	SM33	KT832094
Troglohyphantes vignai		SM34	KT832095

Haplotypes	ITS-2	Alleles	males	females	legs
PM12				1	
PM21			1		
PM21			1		
SM03	KT832236	I			
SM177	KT832268	II			
SM58	KT832269	П			
SM03	KT832245	П			
SM03	KT832246	П			
SM03	KT832247	I			
SM31	KT832248				
SM54	KT832251	II			
SM54	KT832252	П			
SM56	KT832253	П			1
SM56	KT832254				
SM58	KT832255	II			
SM54	KT832256	II			
SM54	KT832257	II			
SM73	KT832258	II			
SM74	KT832259	П			
SM77	KT832262	IV			
SM56	KT832263				
SM58	KT832264				
SM58	KT832265				
SM58	KT832266	II			
SM58	KT832267				
PK1105	Tsp_pk1105	V			
PK1116	Tro.cf.vignai_pk1116_coi	VI	X	1	
PK1117	Tro.cf.vignai_pk1117_coi	VI	5	1	
PK1118	Tro.cf.vignai_pk1118_coi	VI		1	
PK874	tvigpk874	VII			1
SM02	KT832235	VIII			
SM02	KT832260	VIII			1
SM02	KT832261	VIII			
PM09			1		
PM20			1		
PM27	KT832234	XI	1		
PM07				1	
PM22				1	
PM23				1	
SM05	KT832237	Х			1
SM07	KT832238	Х			1
SM09	KT832239	IX			
SM23	KT832240	Х			

SM24	KT832241	Х		
SM24	KT832242	Х		
SM26	KT832243	Х		
SM26	KT832244	Х		
SM24	KT832249	Х		
SM24	KT832250	Х		

Range of distribution	Cave (FIG2)	Cave/Locality
Italy (Western Alps)	6	Prospetto di miniera di Boccetto
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	7	Tuna Du Diau
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	7	Tuna Du Diau
Italy (Western Alps)	7	Tuna Du Diau
Italy (Western Alps)	7	Tuna Du Diau
Italy (Western Alps)	7	Tuna Du Diau
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	6	Miniera di Bocetto
Italy (Western Alps)	6	Miniera di Bocetto
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	9	Miniera di Tornini
Italy (Western Alps)	6	Miniera di Bocetto
Italy (Western Alps)	6	Miniera di Bocetto
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	5	Tana del Diavolo
Italy (Western Alps)	33	Grotta del Nebin
Italy (Western Alps)	35	Pertus del Drai
Italy (Western Alps)	35	Pertus del Drai
Italy (Western Alps)	34	Pertus d'la Tundo
Italy (Western Alps)	36	Topalinda cave (Maissa 2)
Italy (Western Alps)	16	Tana dell' Orso di Casteldelfino -
Italy (Western Alps)	16	Tana dell' Orso di Casteldelfino
Italy (Western Alps)	16	Tana dell' Orso di Casteldelfino -
Italy (Graian Alps)	32	Borna del Servais B
Italy (Graian Alps)	32	Borna del Servais B
Italy (Graian Alps)	32	Borna del Servais B
Italy (Western Alps)	25	Grotta Superiore delle Camoscere
Italy (Western Alps)	31	Voragine della Ciuaiera
Italy (Western Alps)	30	Abisso Arrapanui
Italy (Western Alps)	14	Buco di Valenza
Italy (Western Alps)	25	Grotta superiore delle Camoscere
Italy (Western Alps)	27	Grotta senza nome presso colle del Pas
Italy (Western Alps)	25	Grotta superiore delle Camoscere -

Italy (Western Alps)	25	Grotta superiore delle Camoscere -
Italy (Western Alps)	25	Grotta superiore delle Camoscere -
Italy (Western Alps)	14	Buco di Valenza
Italy (Western Alps)	14	Buco di Valenza
Italy (Western Alps)	25	Grotta superiore delle Camoscere -
Italy (Western Alps)	25	Grotta superiore delle Camoscere -

Cadastrial cave N°	x	Y	Municipality	Prov	Reg	Country
artificial	349049	4980178	Prali	то	Piemonte	Italy
Pi 1591	352000	4987000	Roreto Chisone TO		Piemonte	Italy
Pi 1591	352000	4987000	Roreto Chisone	то	Piemonte	Italy
n.c.	350453	4978979	Chiabrano	CN	Piemonte	Italy
artificial	357817	4974337	Tornini	то	Piemonte	Italy
Pi 1593	352140	4987790	Roreto Chisone TO		Piemonte	Italy
n.c.	350453	4978979	Chiabrano	то	Piemonte	Italy
n.c.	350453	4978979	Chiabrano TO		Piemonte	Italy
n.c.	350453	4978979	Chiabrano	то	Piemonte	Italy
n.c.	350453	4978979	Chiabrano TO		Piemonte	Italy
artificial	357817	4974337	Tornini TO		Piemonte	Italy
artificial	357817	4974337	Tornini	то	Piemonte	Italy
artificial	7.0851	44.9564	Bocetto	то	Piemonte	Italy
artificial	349049	4980178	Bocetto	то	Piemonte	Italy
Pi 1594	352140	4987790	Roreto Chisone	то	Piemonte	Italy
artificial	357817	4974337	Tornini	то	Piemonte	Italy
artificial	357817	4974337	Tornini	то	Piemonte	Italy
artificial	357817	4974337	Tornini	то	Piemonte	Italy
artificial	357817	4974337	Tornini	то	Piemonte	Italy
artificial	349049	4980178	Bocetto	то	Piemonte	Italy
artificial	349049	4980178	Bocetto	то	Piemonte	Italy
Pi 1595	352140	4987790	Roreto Chisone	то	Piemonte	Italy
Pi 1596	352140	4987790	Roreto Chisone	то	Piemonte	Italy
Pi 1597	352140	4987790	Roreto Chisone	то	Piemonte	Italy
Pi 1598	352140	4987790	Roreto Chisone	то	Piemonte	Italy
Pi 1158	NA	NA	Sampeyre CN		Piemonte	Italy
Pi 1017	NA	NA	Becetto CN		Piemonte	Italy
Pi 1017	NA	NA	Becetto	CN	Piemonte	Italy
Pi 1265	NA	NA	Isasca	CN	Piemonte	Italy
Pi 1210	NA	NA	Valdieri	CN	Piemonte	Italy
Pi 1020	349058	4935922	Casteldelfino	CN	Piemonte	Italy
Pi 1019	7.09835	44.55902	Casteldelfino CN		Piemonte	Italy
Pi 1021	349058	4935922	Casteldelfino CN		Piemonte	Italy
artificial	369016	5020346	Ala di Stura TO		Piemonte	Italy
artificial	7.32763	45.32259	Ala di Stura	то	Piemonte	Italy
artificial	369016	5020346	Ala di Stura	то	Piemonte	Italy
Pi 250	392960	4896980	Chiusa Pesio	CN Piemonte		Italy
Pi 146	7.8866	44.19102	Garessio	CN	Piemonte	Italy
Pi 772	390892	4892856	Briga Alta	CN	Piemonte	Italy
Pi 1009	355106	4949418	Oncino	CN	Piemonte	Italy
Pi 250	392662	4896874	Chiusa Pesio	CN	Piemonte	Italy
n.c.	NA	NA		CN	Piemonte	Italy
Pi 251	392662	4896874	Chiusa Pesio	CN	Piemonte	Italy

Pi 252	392662	4896874	Chiusa Pesio	CN	Piemonte	Italy
Pi 253	392662	4896874	Chiusa Pesio	CN	Piemonte	Italy
Pi 1010	355107	4949419	Oncino	CN	Piemonte	Italy
Pi 1011	355108	4949420	Oncino	CN	Piemonte	Italy
Pi 255	392662	4896874	Chiusa Pesio	CN	Piemonte	Italy
Pi 256	392662	4896874	Chiusa Pesio	CN	Piemonte	Italy

DATE	Leg	
2/21/2007	Isaia	
11/11/2006	Isaia	
11/11/2006	Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
9/12/2014	Mammola, Isaia	
7/21/2013	Mammola	
9/12/2014	Isaia, Mammola	
9/12/2014	Mammola, Isaia	$\mathbf{O}_{\mathbf{A}}$
4/5/2019	Isaia, Mammola	
4/5/2019	Isaia, Mammola	N
9/26/2010	Lana, Chesta	
11/19/2016	Isaia, Mammola, Manenti, Santinelli, Barzaghi	
7/21/2013	Mammola	
7/21/2013	Mammola	
7/21/2013	Mammola	
10/14/2009	Isaia, Paschetta	
9/9/2007	Isaia, Lazzaro	
10/14/2009	Isaia, Paschetta	
12/21/2006	Isaia, Lana	
10/12/2008	Lana	
8/13/2001		
11/12/2014	Mammola, Isaia, Paschetta	
11/26/2014	Isaia, Mammola	
8/20/2014	Badino	
11/26/2014	Mammola, Isaia	

11/26/2014	Mammola, Isaia
11/26/2014	Mammola, Isaia
11/13/2015	Mammola,Isaia,Paschetta
11/13/2016	Mammola,Isaia,Paschetta
11/26/2014	Mammola, Isaia
11/26/2014	Mammola, Isaia

Table 2.

		T. achillis]	T. delphinicus sp.]	T. vianai	
		sp. nov.			nov.				gritt
		males females			males females			males	females
		(n=5)	(n=4)		(n=3)	(n=5)		(n=2)	(n=7)
Femur I	Mean	2,63	2,47		2,26	2,41		2,30	2,42
	SD	0,19	0,13		0,10	0,05		0,13	0,10
Tibia I	Mean	2,81	2,52		2,47	2,50		2,53	2,42
	SD	0,20	0,07		0,09	0,09		0,01	0,23
Metatarsus I	Mean	2,62	2,29		2,20	2,20		2,23	2,17
	SD	0,20	0,09		0,08	0,05		0,06	0,06
Tarsus I	Mean	1,42	1,36		1,42	1,26		1,37	1,29
	SD	0,07	0,06		0,02	0,15		0,06	0,09
Femur II	Mean	2,63	2,42		2,22	2,38		2,35	2,33
	SD	0,17	0,09		0,09	0,09		0,01	0,11
Tibia II	Mean	2,78	2,45		2,30	2,38		2,44	2,37
	SD	0,20	0,02		0,19	0,07		0,05	0,13
Metatarsus II	Mean	2,58	2,16		2,13	2,11		2,27	2,10
	SD	0,18	0,06		0,08	0,06		0,03	0,17
Tarsus II	Mean	1,37	1,24		1,35	1,29		1,32	1,22
	SD	0,06	0,04		0,05	0,09		0,09	0,10
Posterior eye row	Mean	0,42	0,38		0,39	0,45		0,46	0,44
	SD	0,04	0,01		0,02	0,05		0,01	0,03
Anterior eye row	Mean	0,40	0,35		0,35	0,38		0,41	0,38
	SD	0,04	0,02		0,01	0,01		0,01	0,02
Anterior median									
eyes (diameter)	Mean	0,05	0,04		0,03	0,04		0,05	0,05
	SD	0,02	0,01		0,01	0,01		0,01	0,01
Anterior lateral						X			
eyes (diameter)	Mean	0,07	0,07		0,05	0,06		0,06	0,07
	SD	0,01	0,01		0,01	0,01		0,00	0,00
Posterior median									
eyes (diameter)	Mean	0,06	0,07		0,05	0,06		0,07	0,07
	SD	0,00	0,01		0,00	0,00		0,00	0,01
Posterior lateral									
eyes (diameter)	Mean	0,07	0,06		0,07	0,06		0,07	0,07
	SD	0,01	0,01		0,01	0,01		0,01	0,01
Width of									
cephalothorax	Mean	1,15	1,07		1,08	1,11		1,19	1,17
	SD	0,06	0,03		0,02	0,05		0,04	0,06
Height of the									
cephalothorax	Mean	0,62	0,53		0,52	0,54		0,57	0,51
	SD	0,10	0,02		0,03	0,06		0,01	0,05
Sternum length	Mean	0,87	0,81		0,78	0,82		0,84	0,87
	SD	0,03	0,03		0,04	0,05		0,01	0,05
Sternum width	Mean	0,79	0,76	0,68	0,79	0,81	0,79		
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	SD	0,07	0,03	0,02	0,03	0,04	0,08		