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Molecular phylogeny reveals three new water mite species of the genus *Teutonia* Koenike, 1889, from Portugal (Acari, Hydrachnidia, Teutoniidae)

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Abstract

Water mites of the genus *Teutonia* Koenike, 1889 are often common in cold montane lakes and pools of shaded streams and include the fastest swimmers among the water mites. This study provides the first-time calibrated phylogeny of this genus in Europe. Fossil-calibrated modelling reveals that the most recent common ancestor of the European *Teutonia* spp. originated in the Oligocene. Phylogenetic analyses show the presence of at least five species-level lineages within European teutoniid mites, with the *T. corsicana*-clade as the earliest branching lineage. We infer the origin of *Teutonia corsicana* at 29.0 million years ago, near the Eocene–Oligocene boundary, indicating that this species or its most recent ancestor was already present on the Corsica-Sardinia block when it broke off from the Iberian Peninsula. The remaining *Teutonia* clades are relatively younger and began their divergence in the early Miocene (ca. 21 million years ago) and continued with individual species throughout the Miocene. Finally, as a result of an integrative taxonomic approach, three species, i.e., *T. lusitanica* **sp. nov**., *T. herminiana* **sp. nov**. and *T. condei* **sp. nov**. are described as new for science, and the presence of *T. cometes* is for the first time molecularly confirmed for Portugal.

Keywords: Water mites, DNA-barcoding, cytochrome c oxidase subunit I (COI), new species, systematics

Introduction

The family Teutoniidae Koenike, 1910 comprises two genera, the monotypic *Limnolegeria* Motaş, 1928, and the widely distributed *Teutonia* Koenike, 1889. The latter genus, recorded from the Holarctic, comprises two subgenera, the nominate subgenus and *Subteutonia* Habeeb, 1958 known only from Canada (Smit 2020). Currently, the genus *Teutonia* in Western Palaearctic encompasses three species, i.e., the widely distributed *T. cometes* (Koch, 1837), inhabiting cold montane lakes and pools of shaded streams, *T. subalpina* Thor, 1897, restricted to the far north of Europe (Di Sabatino *et al*. 2010) and *T. corsicana* Pešić & Smit, 2022, the latter originally described from Corsica (Pešić

& Smit 2022) and recently also reported from Sardinia (Pešić & Goldschmidt 2023). As emphasized by Di Sabatino *et al*. (2010) probably all species of the genus are characterized by weak sclerotization and consequently palps and legs frequently become distorted or folded, making the verification of the characters states difficult. It seems that some of the *Teutonia* species which are challenging to separate morphologically, are at least sympatric, even syntopic in some places as shown in this study.

Recently, as the results of recent DNA barcoding initiatives conducted within the framework of the project Biodiversity Genomics Europe (BGE, https://biodiversitygenomics.eu/), a COI dataset of specimens of the genus *Teutonia* collected in continental Portugal was formed. The existence of such a dataset provides the opportunity to reconstruct the diversification of the genus *Teutonia* in Europe based on a time-calibrated phylogeny. Moreover, as a result of an integrative taxonomic approach three species new for science were described, and presence of *T. cometes* is for the first time molecularly confirmed for Portugal.

Material and Methods

Water mites were collected by hand netting and immediately preserved in 96% ethanol for the purpose of the molecular analyses. The specimens used for the molecular study are listed in Table 1. After DNA extraction, the specimen vouchers were stored in 96% EtOH and morphologically examined. Some of these vouchers were dissected and slide mounted in Faure's medium, while the rest was transferred to Koenike's fluid.

Morphological nomenclature follows Gerecke *et al*. (2016). The genital acetabula in both sexes and the genital plate in the female were measured on both sides, therefore their dimensions are given as a range of values, rather than a single number. The holotype and paratypes of the new species are deposited in the Naturalis Biodiversity Center in Leiden (RMNH).

All measurements are given in µm. The following abbreviations are used: Ac-1 = first acetabulum; asl = above sea level; Cx-I = first coxae; dL = dorsal length; H = height; I-L-4-6 = fourth-sixth segments of first leg; L = length; Ma (mega-anum) = million years; MRCA = the most recent common ancestor; P-1-P-5 = palp segment 1-5; RMNH = Naturalis Biodiversity Center, Leiden; W = width.

Molecular and DNA barcode analyses

The molecular analysis was conducted at the University of Florence (Florence, Italy). DNA was extracted using a non-destructive protocol. For the methods used for cytochrome *c* oxidase subunit I (COI) gene amplification and sequencing see Pešić *et al*. (2024). Raw reads were demultiplexed using the Pacific Biosciences SMRT Link software. Consensus sequences were generated with the PacBio Amplicon Analysis (pbaa) tool. Primer trimming, translation and stop codon checking were performed using Geneious Prime 2024.0.1.

Consensus sequences were made available in the Barcode of Life Data Systems (BOLD). Relevant voucher information, photos, and recently generated DNA barcodes are publicly accessible through the Dataset - DS-BGEPL02 (BGE Biodiversity Genomics Europe: Portuguese water mites II - Molecular phylogeny of *Teutonia*) in BOLD.

In this study DNA was extracted from 26 specimens of the genus *Teutonia* from Portugal listed in Table 1. For all other species, COI sequence data were taken from Pešić and Smit (2022) and Pešić and Goldschmidt (2023) and downloaded from the respective sequence data archives. In total we used an COI dataset with 47 sequences of *Teutonia* spp. for phylogenetic analyses.

TABLE 1. Details on DNA barcoded specimens, including coordinates of sampling sites, the barcode index number (^{N} indicates a new BIN that contains only current sequences) and associated data obtained from BOLD. BOLD data presented here was last accessed on 10th October 2024.

Locality	Coordinates	Voucher Code	BOLD Acc Nos	BIN BOLD
Teutonia lusitanica **sp. nov.**				
Beja, Zambujeira do Mar	37.399° N, 8.723 ° W	BGE_00110_F09	BSNTN544-23	^{N}BOLD:AFP7254
Faro, Odeceixe (Covão da Serva)	37.374° N, 8.642 ° W	BGE_00110_F10	BSNTN545-23	
		BGE_00110_F11	BSNTN546-23	
		BGE_00110_F12	BSNTN547-23	
		BGE_00228_E12	BSNTN915-23	
Beja, Herdade do Vale de Águia	37.398° N, 8.680° W	BGE_00110_E08	BSNTN531-23	
		BGE_00110_E09	BSNTN532-23	
		BGE_00110_E10	BSNTN533-23	
Beja, Odemira, Ribeira de Seixe, Zambujeira do Mar	37.399° N, 8.723° W	BGE_00228_D04	BSNTN895-23	
		BGE_00228_F04	BSNTN919-23	
		BGE_00228_F05	BSNTN920-23	
Beja, Mértola, Moinho de Alferes 2	37.503° N, 7.687° W	BGE_00228_E07	BSNTN910-23	
Guarda, Seia, Casa do Loureiro	40.433° N, 7.701° W	BGE_00109_H09	BBIOP093-24	
Guarda, Seia, Rio Alva, Central hidroeléctrica de Ponte dos Jugais	40.385° N, 7.706° W	BGE_00228_B07	BSNTN874-23	
Teutonia herminiana **sp. nov.**				
Guarda, Manteigas, Casa do Cantoneiro	40.418° N, 7.603° W	BGE_00227_E07	BSNTN1005-23	^{N} BOLD:AFU4959
Guarda, Manteigas, Zezere, Covão da Ametade	40.328° N, 7.587° W	BGE_00227_G08	BSNTN1030-23	
		BGE_00108_A08	BBIOP103-24	
Guarda, Manteigas, Poço do Inferno	40.373° N, 7.516° W	BGE_00227_C11	BSNTN985-23	
Teutonia condei **sp. nov.**				
Guarda, Manteigas, Casa do Cantoneiro	40.418° N, 7.603° W	BGE_00227_E06	BSNTN1004-23	^{N}BOLD:AFW3141
		BGE_00227_E08	BSNTN1006-23	
		BGE_00108_H05	BBIOP184-24	
Guarda, Manteigas, Zezere, Covão da Ametade	40.328° N, 7.587° W	BGE_00227_G10	BSNTN1032-23	
		BGE_00108_A07	BBIOP102-24	
Teutonia cometes (Koch, 1837)				
Guarda, Seia, Rio Alva, Central hidroeléctrica de Ponte dos Jugais	40.385° N, 7.706° W	BGE_00228_B04	BSNTN871-23	BOLD:ACH7884
Rio Alva, Praia Fluvial de Sabugueiro	40.401° N, 7.640° W	BGE_00227_A04	BSNTN954-23	
		BGE_00227_B06	BSNTN968-23	

Sequence comparisons were performed using MUSCLE alignment (Edgar 2004). Intra and interspecific genetic distances were calculated based on the Kimura 2-parameter (K2P) model (Kimura 1980), using MEGA 11 software (Tamura *et al*. 2021). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Additionally, the sequence data were analyzed using the Assemble Species by Automatic Partitioning (ASAP) method (Puillandre *et al*. 2021). We used the online ASAP version (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings and K2P distance model.

Phylogenetic analysis

The 3-partitioned phylogeny was based on *COI* dataset with 47 sequences of *Teutonia* spp. Representative of *Limnolegeria longiseta* Motaş, 1928 was used as outgroup. Maximum likelihood phylogenetic analysis was performed using the online server IQ-TREE v1.6.12 (W-IQ-TREE) with automatic identification of the most appropriate evolutionary models (Chernomor et al. 2016) and ultrafast bootstrapping algorithm (UFBoot) with 5000 replicates (Hoang et al. 2017). Models of sequence evolution for each partition calculated through Model Finder (Kalyaanamoorthy *et al*. 2017) based on Bayesian Information Criterion (BIC) were as follows: 1st codon of *COI*: HKY + G; 2nd codon of *COI*: K2P + I; and 3rd codon of *COI*: F81 + I. The same models were used for the Bayesian Inference (BI) phylogenetic analysis, which was performed in MrBayes v3.2.7 (Ronquist *et al*. 2012) at the San Diego Supercomputer Center through the CIPRES Science Gateway (Miller *et al*. 2010). The following parameters were implemented: two runs with four Markov chains (three heated and one cold, temperature = 0.2), 5,000,000 generations, and tree sampling every 1000th generation, 10% of trees were discarded as burn-in and the majority rule consensus tree was calculated from the remaining trees. Convergence of the MCMC chains to a stationary distribution was checked visually based on the plotted posterior estimates using an MCMC trace analysis tool (Tracer v1.7; Rambaut *et al*. 2018) and all ESS values were >300.

Divergence time estimates

For time-calibrated COI phylogeny we used a dataset with 14 mite species. Besides five *Teutonia* species, representatives of Hydrachnoidea (*Hydrachna conjecta* Koenike, 1895 and *Hydrachna globosa* (De Geer, 1778)), Arrenuroidea (*Horreolanus orphanus* Mitchell, 1955 and *Mideopsis roztoczensis* Biesiadka & Kowalik, 1979), Hygrobatidae (*Hygrobates longipalpis* (Hermann, 1804), *Hygrobates cyrnusensis* Pešić & Smit, 2023 and *Hygrobates fluviatilis* (Strøm, 1768)) and Erythraeoidea (*Lasioerythraeus* sp. and *Smaris* sp.) were included as outgroups. Calculations were performed in BEAST v2.7.3 with a log-normal relaxed clock and Yule speciation process as priors (Bouckaert *et al*. 2019). We used external mean rate 0.0168 ± 0.0018 of COI dataset with log normal distribution (M=0.0168, S=0.056) calculated for insects by Papadopoulou *et al*. (2010). We included two fossil calibration points for MRCA of Arrenuroidea, Miocene (Cook 1957; Dabert *et al*. 2016) with an exponential distribution (mean (lambda) = 3.11, offset = 11.5) and Erythraeoidea, Cretaceous, Albian (Dunlop 2007; Dabert *et al*. 2016) with an exponential distribution (mean (lambda) = 8, offset = 115).

Two independent runs of 15,000,000 generations were processed, with sampling every 1000 generations. The resulting tree sets were combined using LogCombiner v2.7.3 with 10% burn-in. The final ESS values were checked using Tracer v1.7 (Rambaut *et al*. 2018) and each value was recorded as >300. A maximum clade credibility tree has been computed with TreeAnnotator v2.7.3.

Results

Phylogenetic analysis

The COI phylogenetic analyses revealed five species-level clades in the *Teutonia* genus, i.e. previously known species *Teutonia corsicana* and *Teutonia cometes* as well as three undescribed lineages corresponding to the taxa new for science, i.e. *Teutonia condei* **sp. nov**., *Teutonia lusitanica* **sp. nov**. and *Teutonia herminiana* **sp. nov**. All lineages were registered as highly-supported (1.00/ 95 – 1.00/100) both in the Bayesian and ML analyses. Topologies of the phylogenetic trees were generally similar, besides Turkish haplotype of *Teutonia cometes*, which was divided in separate lineage in the Bayesian analysis and was mixtured with other haplotypes in the ML analysis. *Teutonia corsicana* represents a basal clade to the other species of *Teutonia. Teutonia cometes* was revealed as a sister clade to *T. condei* **sp. nov**., and *T. lusitanica* **sp. nov**. and *T. herminiana* **sp. nov**. were recovered as sister clades, but with moderate supports.

TABLE 2. Estimates of average genetic distance (K2P) within and between clades examined species of the genus *Teutonia* sequence pairs in and between COI haplogroups are shown.

Clade	Intraspecific distances	Interspecific distances				
		(1)	(2)	(3)	(4)	(5)
(1) *T. corsicana*	0.0013					
(2) *T. cometes*	0.0189	0.190				
(3) *T. condei* **sp. nov**.	0.0009	0.151	0.173			
(4) *T. lusitanica* **sp. nov**.	0.0045	0.146	0.170	0.146		
(5) *T. herminiana* **sp. nov**.	0.0008	0.140	0.172	0.148	0.114	
(6) *Limnolegeria longiseta*	n/c	0.245	0.272	0.260	0.240	0.239

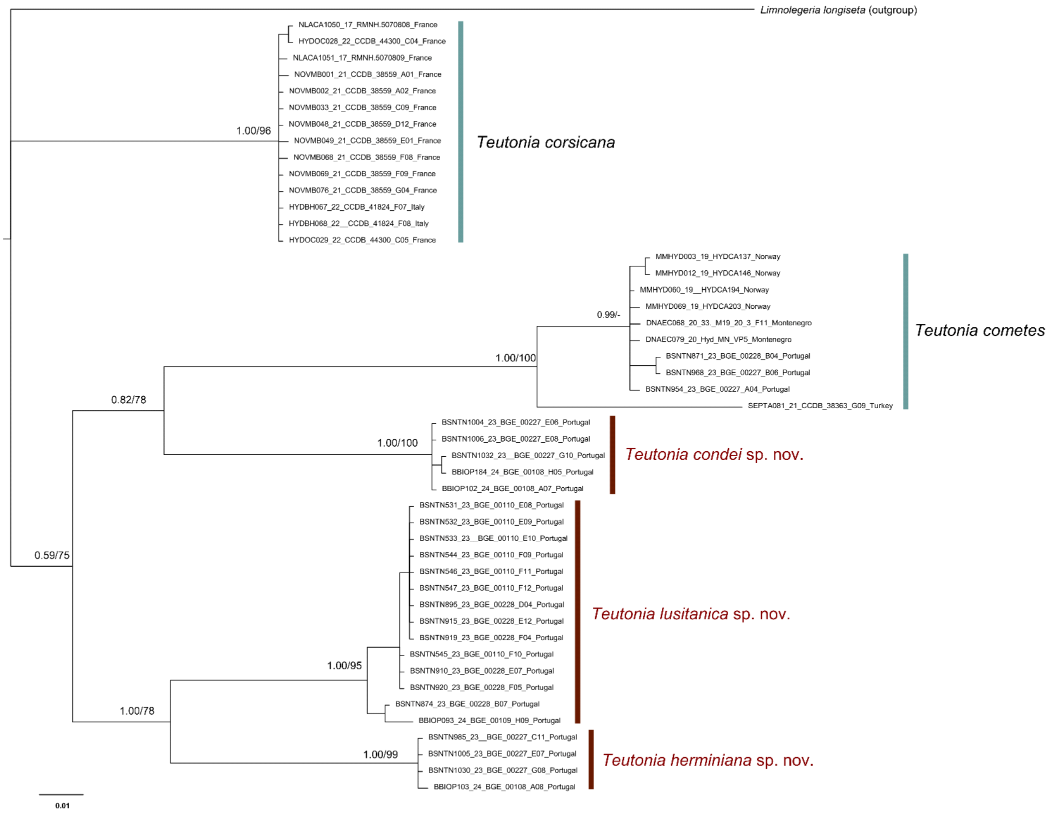

FIGURE 1. Bayesian phylogeny of the COI data set (three codons of COI) of the genus *Teutonia*. Scale bar indicates the branch lengths. Black numbers near nodes are Bayesian Posterior Probabilities (BPP)/ML ultrafast bootstrap support values (BS).

The mean genetic distance between COI sequence groups recovered in the ML analysis was ranged from 14.6% between *T. lusitanica* **sp. nov**. and *T. condei* **sp. nov**., to 19.0% between *T. cometes* and *T. corsicana* (Table 2). These genetic distances were higher than the barcoding gap found by the ASAP method (1% to 14%; Fig. 1A-B), which supports the species-status of the new species. On the other hand, the mean intraspecific distance within clades was very low, ranging from 0.08% K2P in *T. herminiana* **sp. nov**. to 1.89% K2P in *T. cometes*.

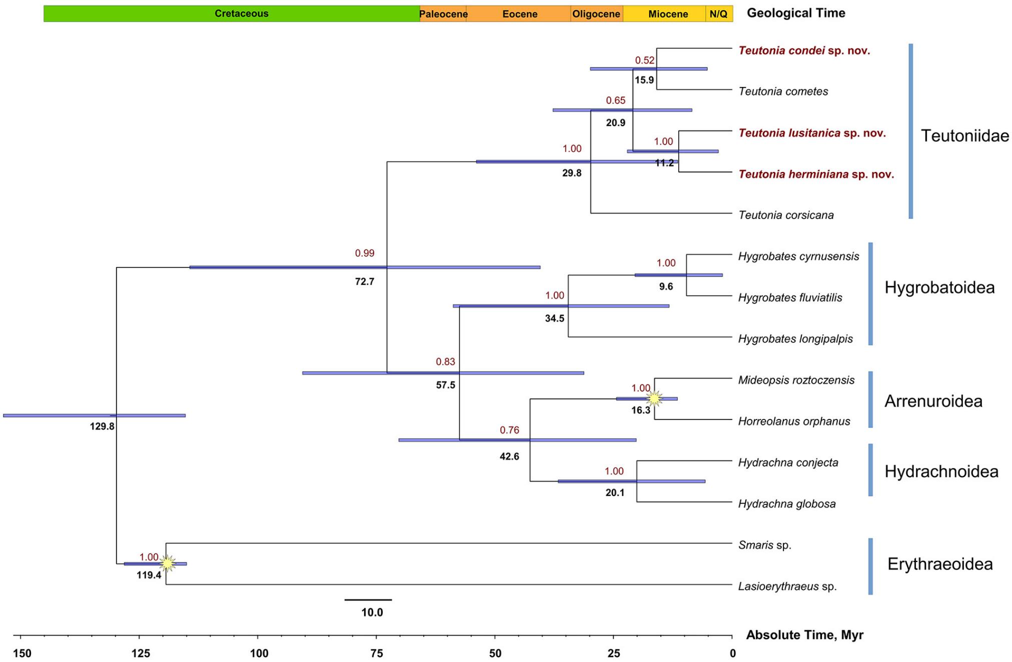

FIGURE 2. Time-calibrated phylogeny of *Teutonia* spp. based on the COI dataset reconstructed using external COI rate for insects (Suchard *et al.* 2018) and fossil records for Arrenuroidea (Cook, 1957) and Erythraeoidea (Dunlop, 2007; Dabert *et al*. 2016). Calibration points are marked by yellow stars. Red numbers near nodes are Bayesian posterior probability (BPP) values from BEAST v. 2.7.3. Black numbers near nodes are the mean node ages. Node bars are 95% HPD of divergence time. Stratigraphic chart according to the International Commission on Stratigraphy, 2022 (<https://stratigraphy.org/chart>).

Time-calibrated reconstruction of phylogeny

Time-calibrated COI phylogeny showed that the MRCA of *Teutonia* more likely originated in the Oligocene (mean age = 29.8 Ma, 95% highest posterior densities (HPD) = 11.4-53.9 Ma, BEAST BPP = 1.00). In that time the ancestors of *Teutonia corsicana* and the clade, including *Teutonia cometes*, *T. herminiana* **sp. nov**, *T. condei* **sp. nov**. and *T. lusitanica* **sp. nov**., were separated. The speciation processes in the latter group could start from the early Miocene 20.9 Ma (95% HPD = 8.4–37.8 Ma, BEAST BPP = 0.65) and continued during the late Miocene. The MRCA of *Teutonia cometes* and *T. condei* **sp. nov**. could originate around 15.9 Ma (95% HPD = 5.2–29.9 Ma, BEAST BPP = 0.52). In turn, *T. lusitanica* **sp. nov**. and *T. herminiana* **sp. nov**., most likely diverged from their MRCA approximately 11.2 Ma (95% HPD = 2.9–22.1 Ma, BEAST BPP = 1.00).

Systematics

Family Teutoniidae Koenike, 1910
Genus *Teutonia* Koenike, 1889

Teutonia cometes **(Koch, 1837)**

Fig. 3C

Material examined—**Guarda**, Seia, Rio Alva, Central hidroeléctrica de Ponte dos Jugais, river, 40.385° N, 7.706° W, 555 m asl., 28 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, 1♂ (sequenced); Seia, Rio Alva, Praia Fluvial de Sabugueiro, river, 40.401° N, 7.64° W, 1021 m asl., 24 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, 2♀ (sequenced).

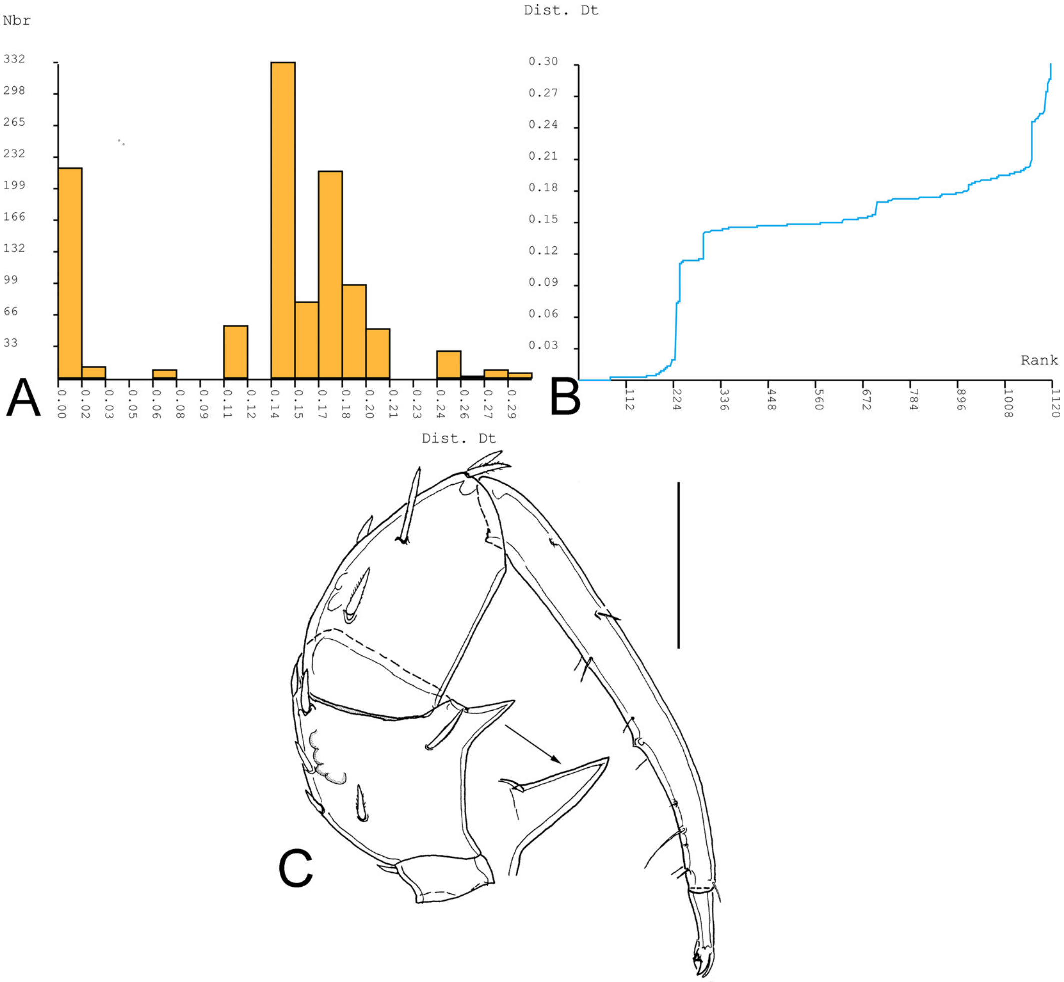

FIGURE 3. A-B Results of ASAP analysis for COI sequences. (A) Distribution of pairwise differences, (B) Ranked pairwise differences. (C) *Teutonia cometes*, ♀ (BGE_00227_B06): palp, medial view (inset: P-2 ventrodistal projection, 2x enlarged). Scale bars = 100 µm.

Remarks—The specimens from Portugal morphologically match the description of *Teutonia cometes*, a species reported as widely distributed in the Palaearctic (Di Sabatino *et al*. 2010). The sequenced specimens from Portugal cluster within BOLD:ACH7884, which, in addition to the specimens used in this study, includes specimens of *T. cometes* from Norway and Montenegro. There is an additional BIN for this species that includes currently a single specimen from Turkey (BOLD: AEN6005).

Distribution—Europe, from Norway to Portugal. In regard to the presence of four distinct lineages, the former records of *T. cometes* from Portugal by Lundblad (1956) require confirmation. Sympatric with *Teutonia lusitanica* **sp. nov.**

***Teutonia lusitanica* Pešić sp. nov.**

Figs. 4–5

Material examined—Holotype ♂ (sequenced; voucher id: BGE_00110_F09), Portugal, **Beja**, Odemira, Ribeira de Seixe, Zambujeira do Mar, river (Fig. 10C), 37.399° N, 8.723° W, 45 m asl., 23 May 2023, leg. Ekrem & Benitez-Bosco, dissected and slide mounted (RMNH). Paratypes: **Faro**, Aljezur, Ribeira de Seixe, Odeceixe (Covão da Serva), 37.374°N, 8.642° W, 100 m asl., 23 May 2023, leg. Ekrem & Benitez-Bosco, 1♂, 2♀ (sequenced), one ♀ (voucher id: BGE_00110_F11) dissected and slide mounted (RMNH); **Beja:** Odemira, Ribeira de Seixe, Herdade do Vale de Águia, river, 37.398° N, 8.680° W, 75 m asl., 23 May 2023, leg. Ekrem & Benitez-Bosco, 3♀ (sequenced; RMNH); Odemira, Ribeira de Seixe, Zambujeira do Mar, 37.399° N, 8.723° W, 45 m asl., 23 May 2023, leg. Ekrem & Benitez-Bosco, 1♂, 2♀ (sequenced; RMNH); Mértola, Moinho de Alferes 2, 37.503° N, 7.687° W, 19 May 2023, leg. Ferreira, Benitez-Bosco, Ekrem, Stur & Turaccio, 1♀ (sequenced; RMNH). **Guarda:** Seia, Casa do Loureiro, 40.433° N, 7.701° W, 415 m asl., 19 Jul. 2023 leg. Ferreira & Padilha, 1♀ (sequenced; RMNH); Seia, Rio Alva, Central hidroeléctrica de Ponte dos Jugais, river, 40.385° N, 7.706° W, 555 m asl., 28 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, 1♀ (sequenced; RMNH).

Diagnosis—*Morphological*: in both sexes P-2 with comparatively long, broad-based ventrodistal projection (L/W ratio 1.7-2.0), L ratio: distance from P-4 ventral tubercle to the end of the ventral margin of P-4/total length of P-4, 0.42-0.46. *Molecular*: this lineage differs from *T. corsicana* by 14.6%, from *T. cometes* by 17.0% K2P for COI.

Description. *General features*—Integument finely striated. Coxae medially touching, but not fused, with a few setae. Ac-1-2 rectangular, Ac-3 roundish. P-2 with an elongated and more or less a broad-based ventrodistal projection, P-3 with nearly straight, or slightly convex ventral margin, P-3 lateral seta long, shifted to medial surface, P-4 slender, bearing several fine hair-like setae, mainly in anteroventral part (Figs. 4B, 5B). Legs: claws with claw blade and ventral clawlets; swimming setae restricted to III-/IV-L-3-4, numbers: III-L-3, 2-3; III-L-4, 9-10; III-L-5, 10-11; IV-L-3, 4-5; IV-L-4, 9-11; IV-L-5, 13-15. *Male*—Glandularium on Cx-IV completely surrounded by coxal sclerotization. Genital flaps medially with a dense cover of fine setae, pregenital sclerite small, postgenital sclerite longer. *Female*—Glandularium on Cx-IV partly surrounded by coxal sclerite, medially in contact with the perigenital membrane. Genital flaps with a row of regularly arranged medial setae, pregenital sclerite large, crescent shaped, partly covered by genital flaps (Fig. 5C; but in some Koenike-fixed specimens pregenital sclerite not covered by genital flaps, as illustrated in Fig. 5D), gonopore all over its length flanked by Ac and genital flaps, postgenital sclerite shorter than pregenital sclerite.

Description. Male (holotype BGE_00110_F09; in parentheses some measurements of paratypes)—Idiosoma L 780 (828), W (700). Coxal field L: 454, W 677; Cx-III W 434. Genital field L/W 138/117, genital flaps L 131; pregenital sclerite W 37, postgenital sclerite W 125; L Ac-1-3: 38, 34, 31. Ejaculatory complex L 164.

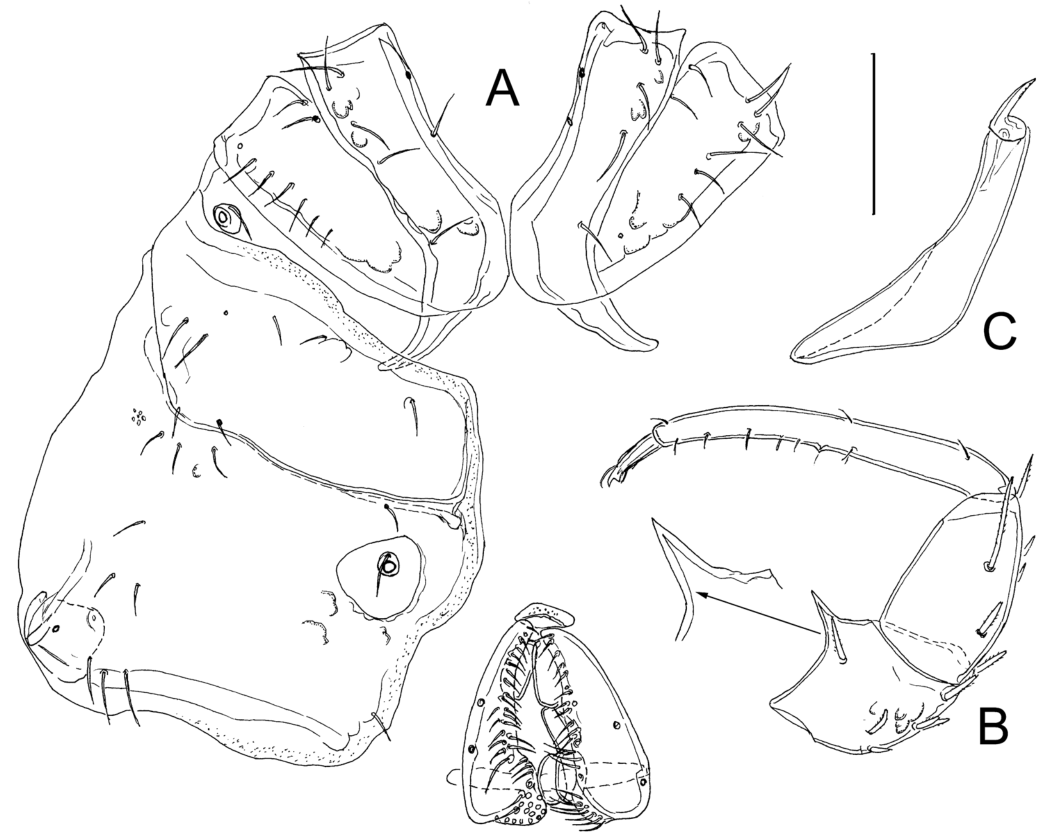

FIGURE 4. *Teutonia lusitanica* **sp. nov**., male holotype (BGE_00110_F09). A—coxal and genital field, partial view; B—palp, medial view (P-1 lacking) (inset: P-2 ventrodistal projection, 2x enlarged); C—chelicera. Scale bars = 100 µm.

Palp (in parentheses measurement of mounted palp of paratype BGE_00110_F05): total L 541, dL/H, dL/H ratio: P-1, 20/41, 0.5 (28/42, 0.67); P-2, 114/88, 1.3 (124/94, 1.32); P-3, 127/73, 1.72 (141/83, 1.7); P-4, 233/31, 7.56 (264/36, 7.35); P-5, 47/13, 3.75 (45/12, 3.7); L/W ratio P-2 ventrodistal projection 2.0 (1.9); L ratio P-2/P-4, 0.49 (0.47). Gnathosoma vL 139; chelicera L 231, basal segment L 188, claw L 42, L ratio chelicera basal segment/claw 4.8. Leg segments dL: I-L: 69, 104, 125, 173, 184, 210; longest I-L-5 ventrodistal seta L 73, L ventrodistal seta/I-L-5 ratio 0.35. dL IV-L: 161, 131, 163, 197, 209, 244.

Female (paratype, BGE_00110_F11; in parentheses some measurements of paratype BGE_00110_E08)—Idiosoma L (1220), W (1020). Coxal field: L 533, W 847; Cx-III W 553. Genital field L 230 (248), genital flaps L 182 (180), pregenital sclerite W 194 (191), postgenital sclerite W 128 (113); L Ac-1-3: 50, 47, 42 (53, 59, 43). Eggs maximum diameter (n= 3) 181-188.

Palp: total L 713, dL/H, dL/H ratio: P-1, 31/51, 0.6; P-2, 145/116, 1.26; P-3, 170/105, 1.62; P-4, 316/41, 7.77; P-5, 51/14, 3.62; L/W ratio P-2 ventrodistal projection 1.7; L ratio P-2/P-4, 0.46. Gnathosoma vL 178; chelicera L 295, basal segment L 244, claw L 54, L ratio chelicera basal segment/claw 4.5. Leg segments: dL I-L: 78, 134, 156, 212, 231, 256; longest I-L-5 ventrodistal seta L 94, L ventrodistal seta/I-L-5 ratio 0.37. dL IV-L-1-6: 156, 163, 218, 252, 275, 314.

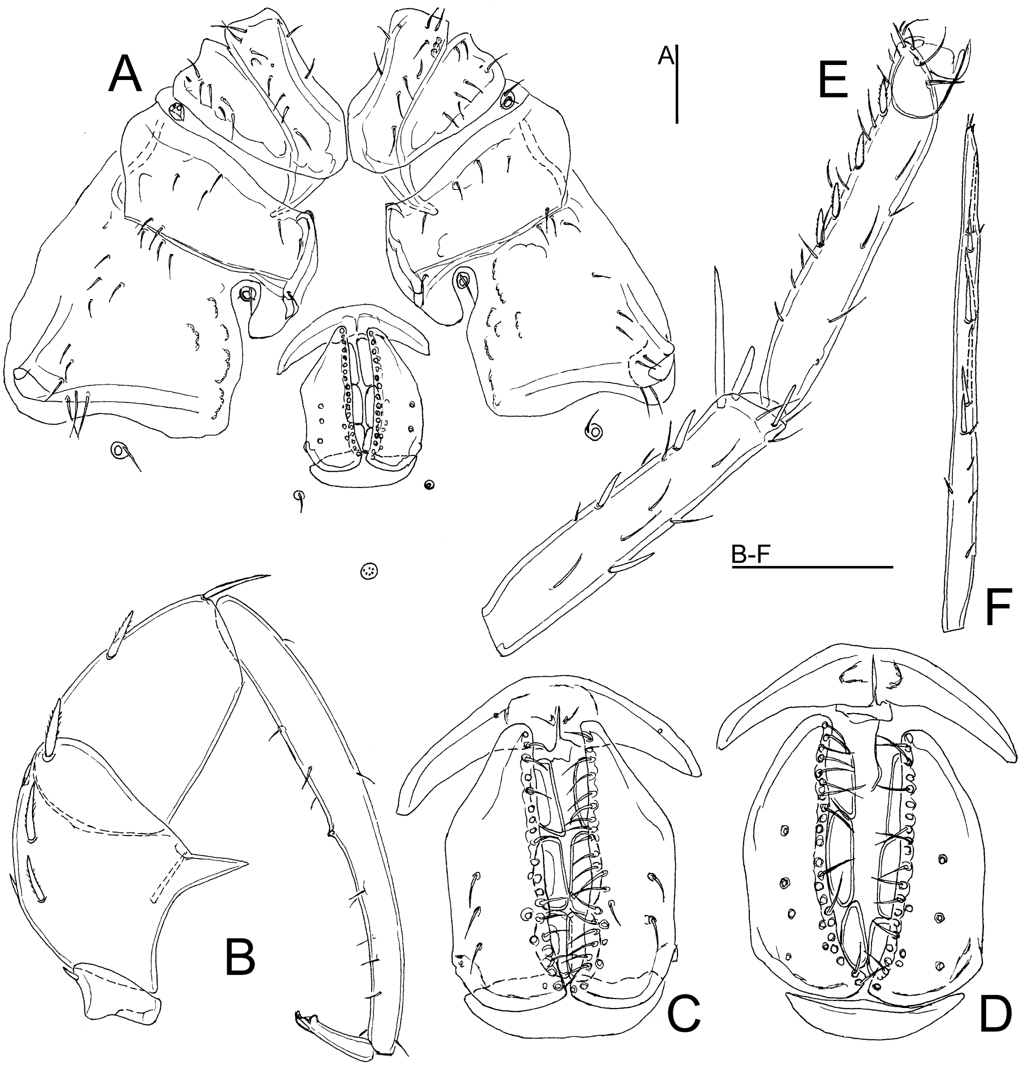

FIGURE 5. *Teutonia lusitanica* **sp. nov**., female (A-C, E-F paratype BGE_00110_F11; D paratype BGE_00110_E08). A—coxal and genital field; B—palp, lateral view; C-D—genital fields; E—I-leg-5 and -6; F—IV-leg-6. Scale bars = 100 µm.

Etymology—The name refers to the geographical provenance (from Latin *lusitanicus*, Lusitanian, Portuguese) from which the new species is currently only known, but where it seems to have a wide distribution.

DNA Barcoding data—The COI sequences of *T. lusitanica* **sp**. **nov**. form a unique cluster (BOLD:AFP7254), with the nearest neighbouring BIN being that of an unidentified *Limnesia* sp. (BOLD:AAE0971) from Canada. The genetic distance between these two clades in the BOLD was estimated at 11.86% *p*-distance.

Remarks—Currently, three species of the genus *Teutonia* are known from the Western Palaearctic, i.e., the widely distributed *T. cometes* (Koch, 1837), *T. subalpina* Thor, 1897 and *T. corsicana* Pešić & Smit 2022. The little-known *T. subalpina* can be distinguished in the female by the very long gonopore, anteriorly distinctly extending over the margin of the genital flaps (Di

Sabatino *et al.* 2010). From *T. corsicana,* the new species from Portugal in both sexes can be distinguished by the more elongated and consequently much slender (L/W ratio 1.5-1.7) ventrodistal projection of P-2. In *Teutonia cometes* the ventral projection of P-2 in both sexes is on a narrow base, and peg-like (L/W ratio 1.8 in specimen from Portugal). From the new species from Portugal, *T. cometes* can be separated by ventral tubercle on P-4 shifted more distally, reaching anterior third of the ventral margin of the segment (L ratio distance from ventral tubercle to the end of the ventral margin of P-4/total length of P-4 was 0.32-0.33 in *T. cometes vs*. 0.42-0.46 in *T. lusitanica*). The high genetic distance between the new species and *T. cometes* (COI 17.0% K2P) suggests a long independent history of these two species. For distinction from *Teutonia herminiana* **sp. nov** and *Teutonia condei* **sp. nov** see below.

Distribution—Portugal: Guarda, Beja and Faro districts.

Teutonia herminiana **Pešić sp. nov.**

Figs. 6–7

Material examined—Holotype ♂ (sequenced; voucher id: BGE_00108_A08), **Guarda**, Manteigas, Zezere, Covão da Ametade (Fig. 10A), 40.328° N, 7.587° W, 1431 m asl., 21 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, dissected and slide mounted (RMNH). Paratypes: 1♀ (sequenced; voucher id: BGE_00227_G08; juvenile specimen; RMNH), same data as the holotype, leg. Ferreira, Benitez-Bosco & Padilha; 1♂ (sequenced; voucher id: BGE_00227_E07, coxae and genital field partly damaged; RMNH), Manteigas, Casa do Cantoneiro, 40.418, 7.603° W, 1378 m asl., 24 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha; 1♀ (sequenced), Manteigas, Poço do Inferno, 40.373° N, 7.516° W, 1078 m asl., 21 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, dissected and slide mounted (voucher id: BGE_00227_C11; RMNH).

Diagnosis—*Morphological*: in both sexes P-2 with comparatively long, broad-based ventrodistal projection (L/W ratio 1.4-1.7); P-2 ventral seta placed posteriorly from the ventrodistal projection, ventral tubercle on P-4 located in the central third of the segment (L ratio distance from ventral tubercle to the end of the ventral margin of P-4/total length of P-4, 0.38-0.4). *Molecular*: this lineage differs from *T. lusitanica* **sp. nov**. by 11.4% K2P for COI.

Description. *General features*—Integument finely striated. Coxae with a few setae, in female first coxae slightly fused medially at its posterior end (as illustrate in Figure 4A; possibly this character state is age-dependent as a result of more or less developed development of secondary sclerotization). Ac-1-2 rectangular, Ac-3 roundish. P-2 with an elongated and more or less a broad-based ventrodistal projection, medial seta on P-2 placed proximally from the ventrodistal projection, P-3 with nearly straight, or slightly convex ventral margin, P-3 lateral seta long, shifted to medial surface, P-4 slender, bearing several fine hair-like setae, mainly in anteroventral part (Figs. 6D-E, 7B). Legs: claws with claw blade and ventral clawlets; swimming setae restricted to III-/IV-L-3-4, numbers: III-L-3, 3-5; III-L-4, 8-11; III-L-5, 8-12; IV-L-3, 3-5; IV-L-4, 10-11; IV-L-5, 12-16. *Male*—Glandularium on Cx-IV completely surrounded by coxal sclerotization (Figure 6A). Genital flaps medially with a dense cover of fine setae, pregenital sclerite small, postgenital sclerite longer. *Female*—Glandularium on Cx-IV partly surrounded by coxal sclerite, medially in contact with the perigenital membrane (Figure 7A). Genital flaps with a row of regularly arranged medial setae, pregenital sclerite large, crescent shaped, partly covered by genital flaps, gonopore all over its length flanked by Ac and genital flaps, postgenital sclerite shorter than pregenital sclerite.

Description. Male (holotype; BGE_00108_A08) — Idiosoma L 575, W 547. Coxal field L 412, W 506; Cx-III W 381. Genital field L/W 149/106, genital flaps L 98; pregenital sclerite W 45, postgenital sclerite W 192; maximum diameter Ac-1-3: 44, 51, 36.

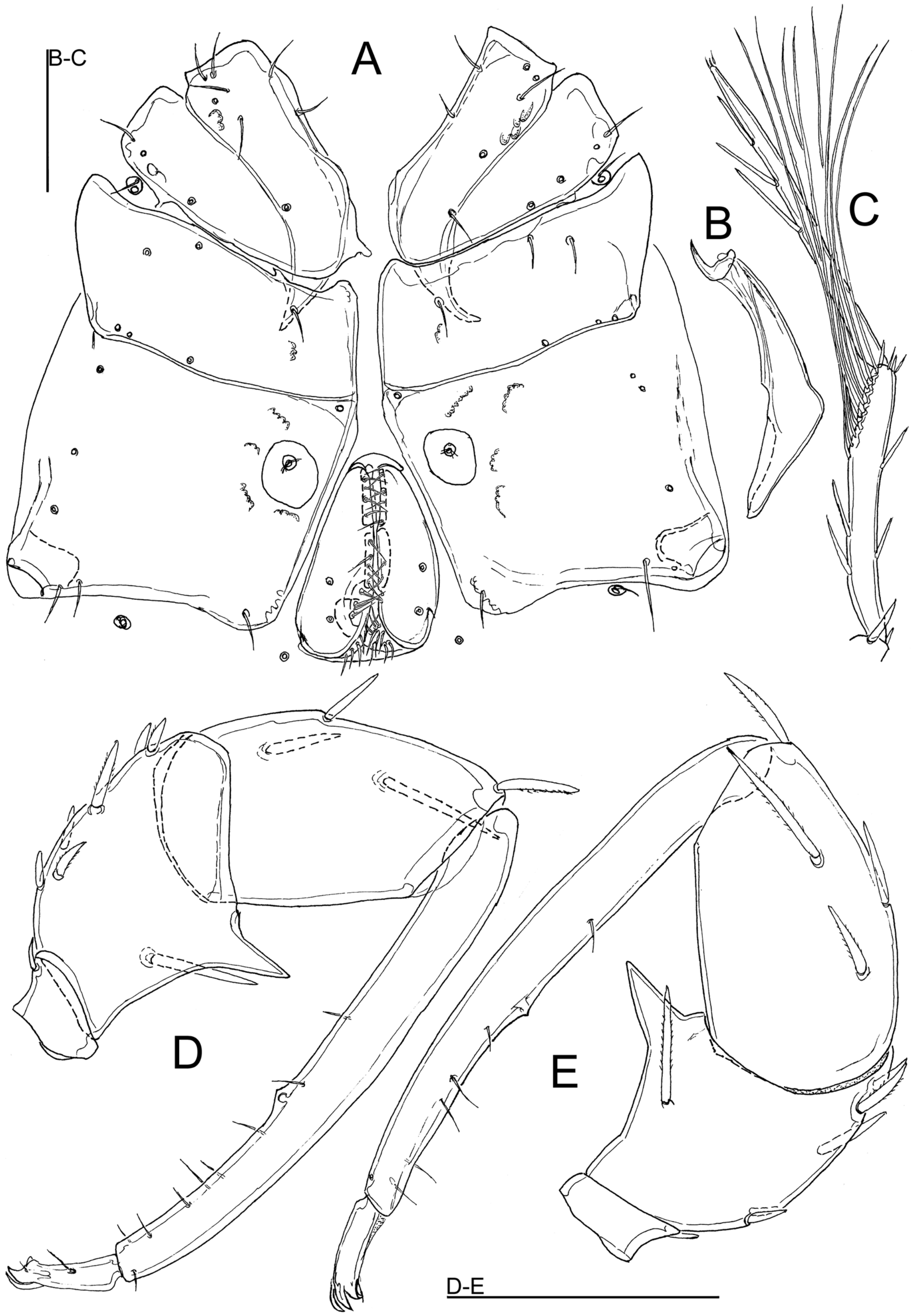

FIGURE 6. *Teutonia herminiana* **sp. nov.**, male holotype (BGE_00108_A08). A—coxal and genital field; B—chelicera; C—IV-L-5 and -6; D—palp, lateral view; E—palp, medial view. Scale bars = 100 µm.

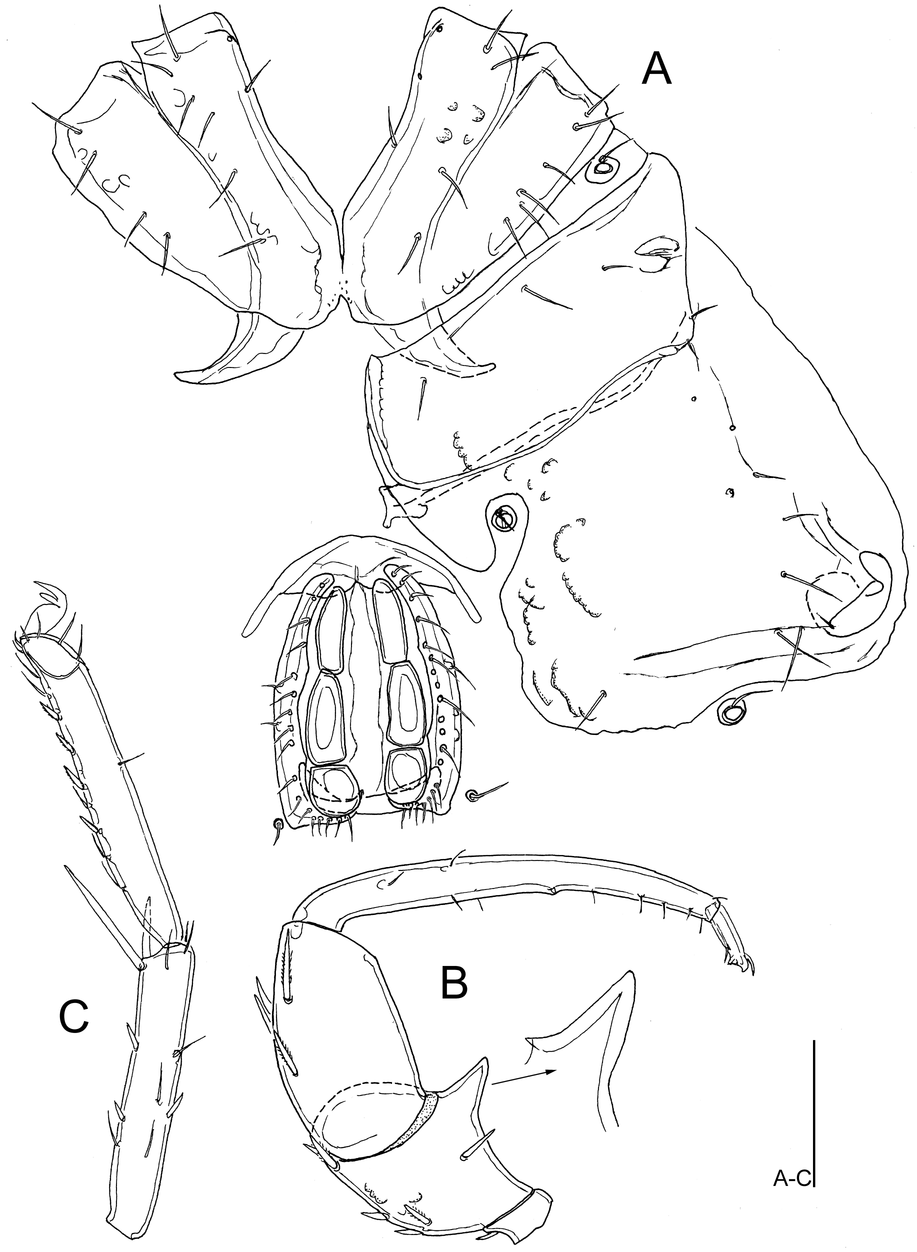

FIGURE 7. *Teutonia herminiana* **sp. nov.**, female paratype (BGE_00227_C11). A—coxal and genital field, partial view; B—palp, medial view (inset: P-2 ventrodistal projection, 2x enlarged); C—I-L-5 and -6. Scale bars = 100 µm.

Palp: total L 508, dL/H, dL/H ratio: P-1, 21/33, 0.64; P-2, 103/80, 1.29; P-3, 120/72, 1.67; P-4, 222/23, 9.48; P-5, 42/13, 3.4; L/W ratio P-2 ventrodistal projection 1.7; L ratio P-2/P-4, 0.46. Gnathosoma vL 144; chelicera L 203, basal segment L 172, claw L 34, L ratio chelicera basal segment/claw 5.0. Leg segments dL: I-L: 65, 94, 111, 150, 158, 188; longest I-L-5 ventrodistal seta L 75, L ventrodistal seta/I-L-5 ratio 0.4. dL IV-L: 120, 119, 156, 184, 193, 242.

Female (paratype; BGE_00227_C11)—Idiosoma L 925. Coxal field: L 515, W 783; Cx-III W 484. Genital flaps L 172, pregenital sclerite W 163; maximum diameter Ac-1-3: 59, 64, 38.

Palp: total L 678, dL/H, dL/H ratio: P-1, 27/42, 0.63; P-2, 139/103, 1.35; P-3, 165/89, 1.85; P-4, 292/35, 8.39; P-5, 55/14, 3.88; L/W ratio P-2 ventrodistal projection 1.4; L ratio P-2/P-4, 0.48. Gnathosoma vL 197; chelicera L 281.

Leg segments: dL I-L: 75, 119, 142, 198, 206, 243; longest I-L-5 ventrodistal seta L 94, L ventrodistal seta/I-L-5 ratio 0.39. dL IV-L: 150, 147, 197, 235, 238, 284.

Etymology—Named after the mountains (Mons Herminius, the Roman name for Serra da Estrela), where the new species was discovered.

Discussion—*Teutonia herminiana* **sp. nov**., resembles *T. lusitanica* **sp. nov**. in sharing an elongated and slender ventrodistal projection of P-2 (L/W ratio 1.4-1.7). From the latter species, *T. herminiana* **sp. nov**. can be easily distinguished by the position of the ventral seta on P-2, which is placed posterior to the ventrodistal projection (in *T. lusitanica* **sp. nov**. and other known species of the genus this seta is regularly placed laterally from the base of ventrodistal projection). In female *Teutonia herminiana* **sp. nov**, the coxae are medially slightly fused at their posterior end, while in *T. lusitanica* **sp. nov**. they are only touching but not fused.

Distribution—Portugal, known only from the three sites located in the Serra da Estrela Mountains, the highest elevation in mainland Portugal. Sympatric with *Teutonia condei* **sp. nov**.

***Teutonia condei* Pešić sp. nov.**

Figs. 8–9

Material examined—Holotype ♂ (sequenced; voucher ID: BGE_00227_G10), **Guarda**, Manteigas, Zezere, Covão da Ametade (Fig. 10A), 40.328° N, 7.587° W, 1431 m asl., 21 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, dissected and slide mounted. Paratypes: 1♂, 2♀ (sequenced), Manteigas, Casa do Cantoneiro, 40.418° N, 7.603° W, 1378 m asl., 24 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha, 1♀ (voucher id: BGE_00227_E06) dissected and slide mounted (RMNH).

Diagnosis—*Morphological*: in both sexes P-2 with moderately long, broad-based ventrodistal projection (L/W ratio 1.35-1.46), P-2 ventral seta placed laterally from the ventrodistal projection; ventral tubercle on P-4 located in the central third of the segment (L ratio distance from ventral tubercle to the end of the ventral margin of P-4/total length of P-4, 0.42-0.47). *Molecular*: this lineage differs from *T. corsicana* by 15.1% and from *T. cometes* by 17.3% K2P for COI.

Description. *General features*—Integument finely striated. Coxae medially touching, but not fused, with a few setae. Ac-1-2 rectangular, Ac-3 roundish. P-2 with a moderately slender and more or less a broad-based ventrodistal projection, P-2 ventral seta placed laterally from the ventrodistal projection, P-3 with nearly straight, or slightly convex ventral margin, P-3 lateral seta long, shifted to medial surface, P-4 slender, bearing several fine hair-like setae, mainly in anteroventral part (Figs. 8B-C). Legs: claws with claw blade and ventral clawlets; swimming setae restricted to III-/IV-L-3-4, numbers: III-L-3, 5; III-L-4, 8-10; III-L-5, 9-12; IV-L-3, 3-4; IV-L-4, 12; IV-L-5, 16-17.

Male—Glandularium on Cx-IV completely surrounded by coxal sclerotization. Genital field: genital flaps medially with a dense cover of fine setae, pregenital sclerite small, postgenital sclerite longer. *Female*—Glandularium on Cx-IV partly surrounded by coxal sclerite, medially in contact with the perigenital membrane. Genital flaps with a row of regularly arranged medial setae, pregenital sclerite very large, curved, crescent shaped, partly covered by genital flaps, gonopore all over its length flanked by Ac and genital flaps (Fig. 9A), postgenital sclerite shorter than pregenital sclerite.

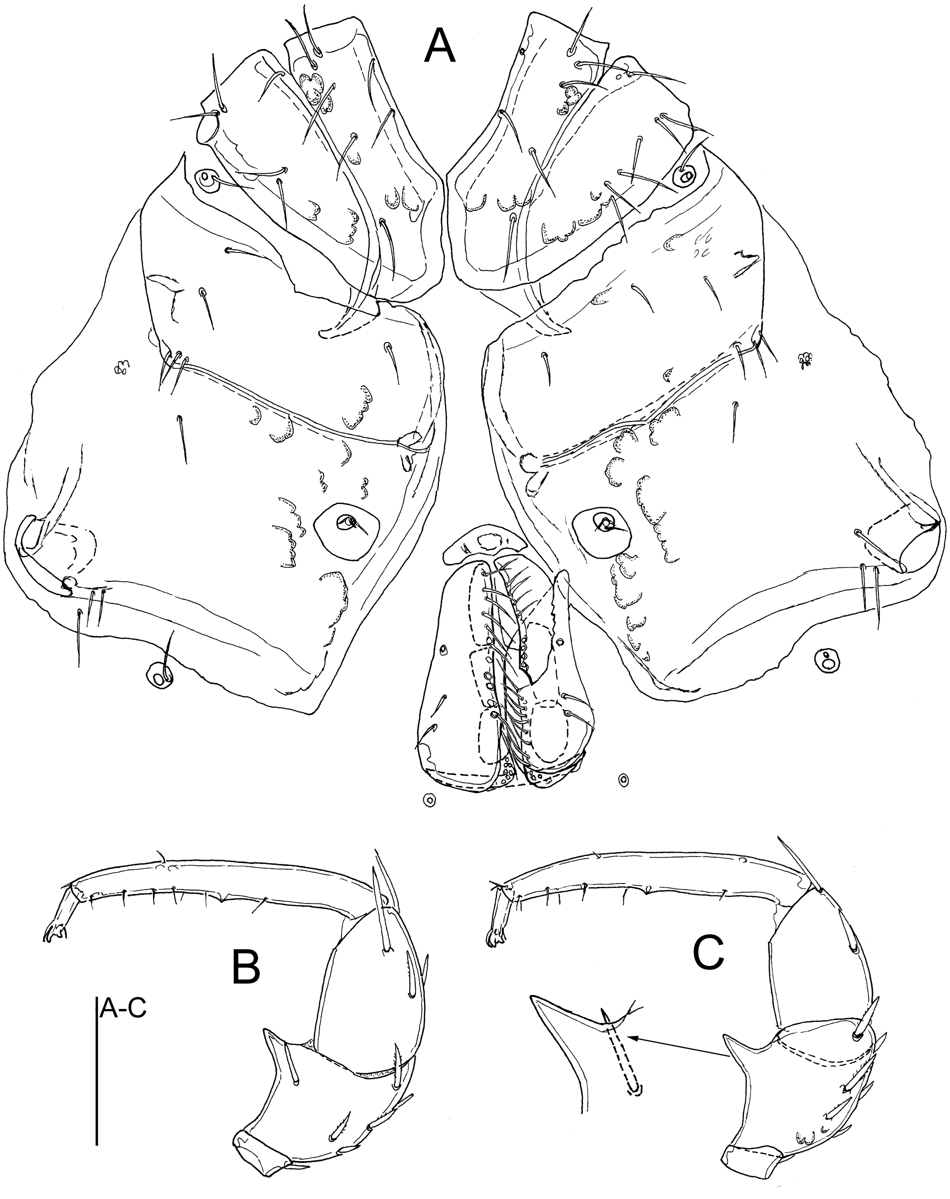

FIGURE 8. *Teutonia condei* **sp. nov.**, male holotype (BGE_00227_G10). A—coxal and genital field; B—palp, medial view; C—palp, lateral view (inset: P-2 ventrodistal projection, 2x enlarged). Scale bars = 100 µm.

Description. Male (holotype; BGE_00227_G10)—Idiosoma L 850, W 727. Coxal field L 467, W 631; Cx-III W 403. Genital field L/W 178/119, genital flaps L 156; pregenital sclerite W 57, postgenital sclerite W 107; maximum diameter Ac-1-3: 53. 56. 42. Ejaculatory complex L 219.

Palp: total L 505, dL/H, dL/H ratio: P-1, 24/36, 0.67; P-2, 109/79, 1.39; P-3, 114/69, 1.66; P-4, 217/30, 7.3; P-5, 41/14, 2.9; L/W ratio P-2 ventrodistal projection 1.46; L ratio P-2/P-4, 0.5. Gnathosoma vL 133; chelicera L 188, basal segment L 147, claw L 35, L ratio chelicera basal segment/claw 4.2. Leg segments: dL I-L-2-6: 98, 114, 169, 180, 211; longest I-L-5 ventrodistal seta L 63, L ventrodistal seta/I-L-5 ratio 0.3. dL IV-L: 122, 128, 163, 197, 218, 260.

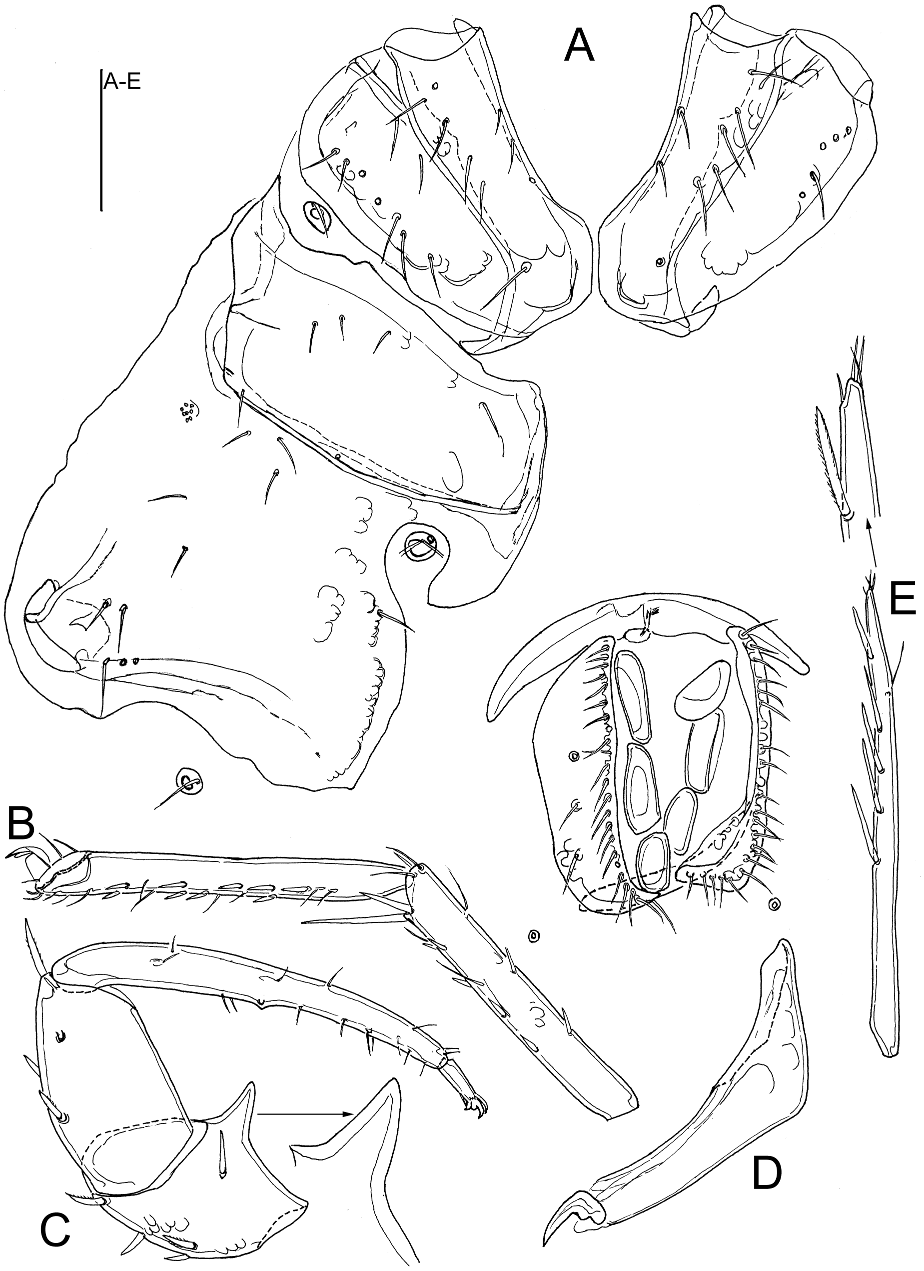

FIGURE 9. *Teutonia condei* **sp. nov.**, female holotype (BGE_00227_E06). A—coxal and genital field, partial view; B—I-L-5 and -6; C—palp, medial view (inset: P-2 ventrodistal projection, 2x enlarged); D—chelicera; E – IV-L-6 (inset: terminal part of segment, enlarged 2x). Scale bars = 100 µm.

Female (paratype; BGE_00227_E06)—Idiosoma L, W. Coxal field: L 550, W 918; Cx-III W 543. Genital flaps L 198, pregenital sclerite W 231; maximum diameter Ac-1-3: 69, 66, 47. Eggs maximum diameter (n=2) 184-194.

Palp: total L 683, dL/H, dL/H ratio: P-1, 34/48, 0.71; P-2, 142/109, 1.3; P-3, 162/91, 1.79; P-4, 291/37, 8.0; P-5, 54/15, 3.5; L/W ratio P-2 ventrodistal projection 1.35; L ratio P-2/P-4, 0.49. Gnathosoma vL 213; chelicera L 278, basal segment L 216, claw L 50, L ratio chelicera basal segment/claw 4.3. Leg segments: dL I-L: 81, 119, 144, 222, 240, 274. dL IV-L: 138, 153, 206, 263, 258, 334.

FIGURE 10. Photographs of selected sampling sites: A—Guarda, Covão da Ametade (type locality of *Teutonia herminiana* **sp. nov**. and *T. condei* **sp. nov**.). B—Guarda, Central hidroeléctrica de Ponte dos Jugais (sampling site of *T. lusitanica* **sp. nov**. and *T. cometes*). C—Beja, Zambujeira do Mar (type locality of *T. lusitanica* **sp. nov**.). Photos by E. Stur (A-B) and T. Ekrem (C).

Etymology—Named after Jose Conde (Serra da Estrela Interpretation Center, Municipality of Seia, Portugal) in appreciation of his dedication and outstanding contribution to studying and protecting biodiversity in Serra da Estrela region.

Discussion—Morphologically the new species from Portugal most closely resembles *T. corsicana*, a species known from Corsica and Sardinia. Both species share a broad-based and moderately slender ventrodistal projection on P-2. However, in comparison with the new species, P-2 ventrodistal projection in *T. corsicana* is somewhat broader (L/W ratio about 1.3). Further differences are found in IV-L-5 bearing only three ventral setae in *T. corsicana* (see Fig. 3G in Pešić & Smit 2022).

Phylogenetic analysis based on COI data placed *Teutonia condei* **sp. nov.** as a sister species of *T. cometes.* The latter species can easily be distinguished by the slender and peg-like ventral projection of P-2, and the ventral tubercle on P-4 shifted more distally (L ratio distance from ventral tubercle to the end of the ventral margin of P-4/total length of P-4 was 0.32-0.33 in *T. cometes vs*. 0.42-0.47 in *T. condei* **sp. nov**.). The high genetic distance between *T. cometes* and *T. condei* **sp. nov.** (COI 15.1% K2P), suggests a long independent history of the new species.

Distribution—Portugal; known only from two sites located in Serra da Estrela Mountains, the highest elevation in mainland Portugal. Sympatric with *T. herminiana* **sp. nov**.

Discussion

As emphasized in the introductory part, the morphological identification of the species of the genus *Teutonia* can be challenging because due to weak sclerotization of the coxae, genital field and appendages, the verification of these characters can be difficult. Despite the fact that many studies have highlighted the limitation of using a single gene barcode, its usefulness has been demonstrated in a number of recent studies on water mites. These studies have shown the utility of this approach in detecting previously overlooked lineages that would be difficult to be discovered based on morphological features alone (see e.g. Martin *et al*. 2017, 2023; Pešić *et al*. 2017, 2019, 2023).

Morphological stasis in *Teutonia* has resulted in three species of this genus being registered for the Western Palearctic so far, with the third species (*T. corsicana*) only recently described using an integrative approach that includes DNA barcoding (Pešić & Smit 2022). Most records of teutoniid mites in the water mite literature are attributed to *T. cometes*, a species often reported to be widespread in the Palaearctic (Di Sabatino *et al*. 2010). Another species, *T. subalpina* Thor, 1897, known from the far east of Europe and Siberia, is still poorly known and requires additional research to clarify its taxonomic status. Di Sabatino *et al*. (2010) argue that it is possible that this taxon is based on unsclerotized, newly hatched, deformed adults of *T. cometes*.

Currently all species of *Teutonia* in Europe are represented by a single BIN, with the exception of *Teutonia cometes* which is represented by two BINs, BOLD:ACH7884 which grouped from specimens from Montenegro, Norway and Portugal, and BOLD:AEN6005 which includes one specimen from Turkey. The *p*-distance between these two BINs was estimated at 6.67% indicating their historical isolation and phylogeographic break between European and Turkish populations of *T. cometes*.

Fossil-calibrated modeling reveals that the most recent common ancestor of European species of the genus *Teutonia* originated in the Oligocene. Phylogenetic and dating analyzes show the presence of at least five species-level clades within the genus *Teutonia* in Europe, with the *T. corsicana* clade as the earliest branching lineage. Our calibrated molecular clock data revealed that *T. corsicana*, a species endemic to Corsica and Sardinia, is quite old. We infer the origin of the *T. corsicana* clade at 29.0 Ma, near the Eocene-Oligocene boundary, indicating that this species or its most recent ancestor was already present on the Corsica-Sardinia Block (CSB) microplate when this microplate broke off from the Iberian Peninsula. This process has been dated to about 29 million years (Alvarez 1972) and was followed by the separation and counterclockwise (CCW) rotation of 23° to 53° rotation of the Corsica-Sardinia that stopped ~16 Ma (Speranza *et al*. 2002).

The remaining *Teutonia* clades are relatively younger and began their divergence in the early Miocene (ca. 21 Ma) and continued with individual species throughout the Miocene. The reconstruction of palaeogeological events that are possibly responsible for the hypothetical radiation events of these clades is almost impossible for now, primarily due to the fact that the distribution of these species is still not sufficiently known. For example, all four lineages (only exception is *T. corsicana* which is endemic to Corsica and Sardinia) are present in Portugal, three of them seem to be endemic to this country. Interestingly, two of these three species, *T. herminiana* **sp. nov**. and *T. condei* **sp. nov** are found only in the Serra da Estrela Mountain range, which indicates their possible limited distribution, but also emphasizes the high diversity of this genus on the Iberian Peninsula.

Several cases of high diversity of certain genera in Southern European Peninsulas have been described in other taxonomic groups, both in vertebrates and invertebrates (Weiss & Ferrand 2007). Specifically, the Iberian Peninsula has been shown to have been a glacial refugium with high internal complexity, that for many species, populations with a high degree of genetic structure have existed throughout the Pleistocene (Goméz & Lund, 2007). The Serra da Estrela Mountain range, and the Portuguese southwest coast are recognized areas of high endemism in the Iberian Peninsula and were found to be refugial areas identified by different case studies (e. g. Crochet *et al*. 2004; Gante *et al*. 2007; Corley & Ferreira 2021).

Our study revealed that speciation processes in European *Teutonia* spp. began much long before the beginning of the Pleistocene glaciations, which are often considered to be the main driver of the diversification of European biota (Hewitt 1996). Having in mind that we found three species in our study restricted to the Serra da Estrela Mountain range and the Portuguese southwest coast, it is likely that the present-day distribution of the European *Teutonia* spp. was primarily shaped by events in the Pleistocene. To address this interesting question, which is beyond the scope of this work, additional studies on the distribution and genetic structure of the European *Teutonia* populations are necessary.

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