Phylogenetic analysis of European *Scutovertex* mites (Acari, Oribatida, Scutoverticidae) reveals paraphyly and cryptic diversity – a molecular genetic and morphological approach

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Abstract

The soil and moss dwelling oribatid mite family Scutoverticidae is considered to represent an assemblage of distantly related but morphologically similar genera. We used nucleotide sequences of one mitochondrial (COI) and two nuclear (28S rDNA, *ef-1α*) genes, and 79 morphological characters to elucidate the phylogenetic relationships among eleven nominal plus two undescribed European mite species of the family Scutoverticidae with a particular focus on the genus *Scutovertex*. Both molecular genetic and morphological data revealed a paraphyletic genus *Scutovertex*, with *S. pictus* probably representing a distinct genus, and *Provertex kuehnelti* was confirmed as member of the family Scutoverticidae. Molecular genetic data confirmed several recently described *Scutovertex* species and thus the high species diversity within this genus in Europe and suggest that *S. sculptus* represents a complex of several cryptic species exhibiting marked genetic, but hardly any morphological divergence.

Keywords

oribatid mites; *Scutovertex*; multidisciplinary approach; phylogeny; paraphylum; cryptic species

Introduction

In recent years, genetic studies have highlighted cryptic diversity in various groups of organisms, indicated by large genetic distances within traditionally recognized, sometimes even well-known, taxa (Edwards and Dimock, 1997; Hebert et al., 2004; Katongo et al., 2005; Kon et al., 2007; Mayer et al., 2007; Metzger et al., 2009). These cryptic species are so similar morphologically that they are almost or entirely indistinguishable based on morphological characters alone, albeit many cryptic species have been subsequently supported by subtle morphological differences found in post-hoc analyses of morphological data (Mathews et al., 2008; Padial and de la Riva, 2009). The potential for cryptic diversity seems particularly high in small-size and short generation time animals (Marzluff and Dial, 1991; Kon et al., 2007). Moreover, some biomes seem to home more cryptic species than others, and particularly in tropical and marine habitats it appears to be a widespread phenomenon (Baric and Sturmbauer, 1997; Wilcox et al., 1997; Bond and Sierwald, 2002; Hebert et al., 2004; Sáez and Lozano, 2005; Chan et al., 2007), whereas the number of

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cryptic species in temperate terrestrial biomes seems to be smaller (Schlick-Steiner et al., 2006; King et al., 2008; Murray et al,. 2008).

In mites, species identification is typically based on morphological character sets. There are many species which are morphologically very similar and thus hardly to distinguish. One example for such a group including morphologically very similar taxa is the oribatid mite family Scutoverticidae which is assigned to a subgroup of the Circumdehiscentiae ("Higher Oribatida") at the base of the Poronota. This subgroup shows wrinkled nymphs and adults which bear sacculi on the notogaster homologous to the so called octotaxic system consisting of four pairs of porose areas characterizing the Poronota (Grandjean, 1953; 1969).

Despite their systematic position the Scutoverticidae are often considered as a conglomeration of distantly related but morphologically similar genera. Bernini (1976) already pointed out the urgent need for a comprehensive and detailed revision of this family to check the membership of the different genera to this taxon, whereby *Scutovertex* as the eponymous genus should serve as reference. Meanwhile, several new taxa were described and Shtanchaeva and Netuzhilin (2003) published a revision of the Scutoverticidae, describing some new species and summing up the knowledge without any attempt to include additional characters to eliminate the taxonomic uncertainties. Up to now the systematic classification within the Scutoverticidae has been suffering from two major problems: the short, fragmentary and often inaccurate descriptions of species and genera, and the limited knowledge of the amount of intraspecific variation and the diversity of this mite family. These two factors led to the description of new taxa (some of them may represent synonyms) and caused some taxonomical confusion, e.g. in the genus *Provertex* (Krisper and Schuster, 2009).

To date, this family comprises eight genera with about 60 species worldwide (Subías, 2004; resp. 2008) whereof only one-third occurs in Europe. Most species can be found in the very south-western (Spain) or eastern (Russia) European part. They are adapted to extreme environmental conditions such as regular desiccation, inundation and temperature fluctuation because their preferred habitats are mosses, lichens or tussocks on sun exposed rocks and roofs (Krisper et al., 2002; Smrž, 2006), as well as saline soils, salt marshes or inundation meadows (Schuster, 1958; Weigmann, 2004). Due to their adaptation to extreme environmental conditions, scutoverticid mites play an important ecological role as pioneer species at the first steps of succession (Skubala, 1995). General information on life span, population size etc. is more or less lacking but most members of the family reproduce sexually and generation times vary from two to six months (Ermilov, 2008; personal observations). Recent data on the genetic diversity in two Austrian species revealed marked inter-specific differences suggestive of differences in population size and dispersal ability (Schäffer et al., in press).

Especially in the genus *Scutovertex* species diversity is very high. Problems to distinguish between the two most widespread species *S. minutus* and *S. sculptus* (e.g. mentioned by Weigmann, 2006) were solved recently by detailed re-descriptions of both species (Schäffer and Krisper, 2007; Pfingstl et al., 2008) and their taxonomic discreteness and different population structure was also confirmed by molecular genetic data (Schäffer et al., 2008). In Central, Northern and Western Europe eight *Scutovertex*-species are known (*S. alpinus*, *S. arenocolus*, *S. ianus*, *S. minutus*, *S. pictus*, *S. pileatus*, *S. pannonicus*, and *S. sculptus*), some of which have been described only recently (Schäffer et al., 2008; Pfingstl et al., 2009, in press, submitted), plus five additional species representing three different genera (*Lamellovertex caelatus*; *Exochocepheus hungaricus*; *Provertex delamarei*, *P. kuehnelti*, *P. mailloli*).

In the present study we attempt to i) evaluate the taxonomic status of eleven nominal and two undescribed European species of the family Scutoverticidae ii) elucidate the phylogenetic relationships between the *Scutovertex* species and iii) uncover a potential cryptic diversity within the genus *Scutovertex*. To achieve our aims, we used three molecular markers (one mitochondrial and two nuclear genes) which allow us to resolve both ancient and recent nodes in a phylogenetic tree. Additionally 79 well defined characters and character states were used to obtain a morphology-based phylogeny for comparative purpose.

Materials and Methods

Sample collection

This study includes eleven nominal plus two undescribed species of the family Scutoverticidae, collected from different localities in Central, Northern and Western Europe between 2005 and 2009. Based on Grandjean (1969) we chose *Unduloribates undulatus* (Unduloribatidae) and three specimens of *Cymbaeremaeus cymba* (Cymbaeremaeidae) as outgroup taxa because both families also belong to the subgroup of Circumdehiscentiae with wrinkled nymphs. Information on sampling localities is given in Table 1 and Figs. 1a-b. Specimens were extracted from mosses and lichens collected on sun exposed rocks and roofs or salt marshes with Berlese-Tullgren funnels. Individuals for morphological analyses were preserved in 70% ethanol, those for molecular genetic analyses in absolute ethanol.

Morphological data

79 morphological characters or character states, respectively, (see Figs. 1c-g) were recorded for five individuals per species. Character coding was based on unordered multistate characters (Appendix A+B).

Phylogenetic reconstruction was carried out by MP using PAUP* (search options: heuristic search; random addition of taxa; TBR branch swapping with 1,000 replicates), using one specimen per species, since there were no intraspecific differences. Statistical support was assessed by bootstrapping (1,000 pseudo-replicates).

Molecular genetic analyses

Total genomic DNA was extracted from single individuals applying the CTAB (hexadecyltriethylammonium bromide) method described in Schäffer et al. (in press) or the DNeasy Blood & Tissue Kit (Qiagen, Vienna, Austria).

Fragments of COI, *ef-1α* and 28S rDNA genes were amplified by polymerase chain reaction (PCR) using the following primers: COI_1fwd (5′-GNTCAACAAWTCATWAAG-3′) and COI_2rev (5′-TAAACTTCNGGYTGNCCAAAAAATCA-3′) for COI region1 (modified after Heethoff et al., 2007), Mite COI-2F and Mite COI-2R (Otto and Wilson, 2001) for COI region 2, D3A and D3B (Litvaitis et al., 1994) for the D3 fragment of the 28S rDNA, and 40.71F and 52.RC (Regier and Shultz, 1997) for *ef-1α*. Since last-mentioned primer pair did not work well in all specimens we designed new ones: EF-SyFwd (5′-GGACAAACTGAAGGHW GAGMG-3′) and EF-SyRev (5′-RKNGGTCKTGAGGGCGGTTCC-3′). Purification of PCR products, and sequencing reaction followed the protocol described in Schäffer et al. (2008). DNA fragments were purified with Sephadex[TM] G-50 (Amersham Biosciences) following the manufacturer's instruction and visualized on a 3130xl capillary sequencer (Applied Biosystems). Sequences are available from GenBank under the accession numbers listed in Table 1.

We sequenced 1,259 bp of the mitochondrial COI gene, 316-324 bp of the D3 region of the nuclear 28S rDNA and 504 bp of the nuclear *ef-1α* gene in 54 specimens (in the three *S. alpinus* individuals the fragment of the COI-region1 could not be amplified). Sequences were verified by comparisons with known oribatid sequences from GenBank and aligned by eye in MEGA 3.1 (Kumar et al., 2004). One 18-26 bp fragment of the 28S D3 region could not be aligned unambiguously and was excluded (also base position 153 from SpKal6) from the analyses. For the further phylogenetic analyses we always used all available sequences for each gene fragment.

In a first step, for testing the performance of the single genes, we constructed separate phylogenies for the three genes plus a phylogeny for the COI region2 (because of *S. alpinus*, see above) using Bayesian inference (BI) as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). For all Bayesian analyses genes (except 28S rDNA) were partitioned by codon position. Rate heterogeneity was set according to a gamma distribution with six rate categories (GTR model) for each data partition. Posterior probabilities were obtained from a Metropolis-coupled Markov chain Monte Carlo simulation (2 independent runs; 4 chains with 3 million generations each; chain temperature: 0.2; trees sampled every 100 generations), with parameters estimated from the data set. Depending on the data set we applied different burn-ins to allow likelihood values to reach stationarity, so that the average standard deviation of split frequencies was <0.01.

In a second step, the fragments of all three genes were combined for further analyses (length of concatenated data set = 2,059 bp). We analyzed two data sets, one with all available sequences, entitled full set (FS) and one without COI region1, entitled reduced set (RS). The RS data set served to get information on the phylogenetic placement of *S. alpinus*. Phylogenetic reconstructions by neighbor joining (NJ) and maximum parsimony (MP) were conducted in PAUP* (Swofford, 2002), maximum likelihood (ML) in RAxML-7.0.3-WIN (Stamatakis, 2006) and BI in MrBayes. For NJ of FS and RS, the best-fit substitution model selected by the hierarchical likelihood ratio test (hLRT) implemented in Modeltest 3.06 (Posada and Crandall, 1998) was GTR+I+G (parameters of FS/RS: base frequencies: A = 0.3057/0.3112, C = 0.1742/0.1749, G = 0.1604/0.1790, T = 0.3597/0.3349; R-matrix: A↔C = 0.6075/0.9541; A↔G = 10.6077/8.1492; A↔T = 1.0908/1.1455; C↔G = 1.3283/1.3145; C↔T = 12.0869/12.9849; G↔T = 1.0000; proportion of invariable sites: I = 0.6165/0.5742; gamma shape parameter: α = 0.6548/0.3888). Heuristic tree searches under MP criteria applied random addition of taxa and TBR branch swapping (1,000 replicates). Statistical support for the resulting NJ and MP topologies was assessed by bootstrapping (1,000 pseudo-replicates). To find the best-scoring ML tree we used the default algorithm with 40 distinct rate categories, the GTR+I+G substitution model and COI and *ef-1α* were partitioned by gene and by codon position. Nodes were supported by bootstrapping (500 replicates). Settings for Bayesian Inference were same as mentioned above and the partitioning of COI and *ef-1α* was the same as for ML analysis.

To assess whether the topologies obtained by the different tree building algorithms differed significantly, we performed Kishino-Hasegawa (KH; Kishino and Hasegawa, 1989) and Shimodaira-Hasegawa (SH) tests (Shimodaira and Hasegawa, 1999) in PAUP*. Alternative phylogenetic hypotheses were compared to a strict consensus topology of the NJ, MP, ML and BI trees also by means of KH and SH tests.

A Bayesian relative rates test according to the method describe by Wilcox et al. (2004) was conducted to test for significant differences in branch lengths and hence substitution rates at the COI gene, which is commonly used to estimated divergence times in arthropods (Brower, 1994; Juan et al., 1996; Quek et al., 2004), and also in oribatid mites (Salomone et al., 2002; Heethoff et al., 2007). The posterior probability distribution of branch lengths for

all branches was obtained by saving branch lengths for every 100th sampled tree (after burn-in) of the MrBayes run. For each tree the distance from the most recent common ancestor (MRCA) of the ingroup to each of the terminal taxa was calculated with Cadence v.1.0.1 (Wilcox et al., 2004; available at http://www.biosci.utexas.edu/antisense/). *Scutovertex alpinus* was excluded from this analysis because of the lacking COI region1 sequence. The distribution of branch lengths was plotted in the program SPSS ver. 16.0. Because of considerable variations in relative rates among the ingroup taxa (Fig. 2), we refrained from applying a molecular clock to estimate divergence times (Wilcox et al. 2004). Given a lack of possible calibration points we were not able use relaxed clock models on our data to reliably estimate divergence times either.

Results

Pairwise sequence divergence (uncorrected p-distance) between scutoverticid species ranged from 15 to 24 % in the COI gene, from 0 to 7.4 % in the D3 fragment of 28S rDNA, and from 0.6 to 9.6 % in the *ef-1α* gene. In the combined data set, pairwise differences ranged from 8 to 19 %.

Phylogenetic analyses based on Bayesian inference of the whole COI gene, COI region 2 and *ef-1α* (Figs. 3a, 3b and 3d) yielded similar results and revealed well resolved topologies with high statistical support for the monophyly of the family Scutoverticidae and the monophyly of each species except *S. sculptus*, whose specimens clustered in two well supported clades: one included individuals from Russia, Sweden, Germany and Austria ("sculptus1") and one comprised individuals from France, Ireland and Germany ("sculptus2"). By contrast, the 28S rDNA gene showed only a poorly resolved phylogeny (Fig. 3c). Only the monophyly of the genus *Scutovertex* and of the remaining genera was well supported. Single gene analyses resulted in different relative phylogenetic positions of the "sculptus1" and "sculptus2" clades.

All analyses with the combined data sets revealed highly consistent topologies (Figs. 4a-d). Only slight differences were observed with respect to the tree building algorithm used. MP of the FS/RS yielded 12/2 most parsimonious trees with a length of 3,519/1,916 steps (CI excluding uninformative characters = 0.3224/0.3421; RI = 0.7372/0.7576; RC = 0.2413/0.2633). An evaluation of the phylogenetic hypotheses obtained from NJ, MP, ML and BI by means of KH and SH tests revealed no significant differences between the alternative topologies except for the MP tree in the SH test (Table 2). Compared to the other three tree topologies the MP tree showed a slightly different branching order (branching order among *P. kuehnelti*, *L. caelatus*, *E. hungaricus* and *S. pictus*; placement of *S. pannonicus* and *S.* sp. 2 (specimens from Wangen/South Tyrol) albeit with low bootstrap support. A strict consensus tree of NJ, MP, ML and Bayesian Inference is shown in Figure 5a. *Scutovertex alpinus* was added manually based on its position in the analyses of the RS data (data not shown). Within the family Scutoverticidae two main clusters became evident, one with species of the genus *Scutovertex* and one with the members of the three other genera *P. kuehnelti*, *L. caelatus*, *E. hungaricus* plus *S. pictus*, rendering the genus *Scutovertex* paraphyletic. This result conforms to the morphology-based phylogeny (21 constant, 16 parsimony-uninformative and 42 parsimony-informative characters; 34 most parsimonious trees; tree length = 139; CI excluding uninformative characters = 0.7080; RI = 0.7027; RC = 0.5359), in which *S. pictus* clusters between *L. caelatus* and *E. hungaricus* with *P. kuehnelti* as sister taxon (Fig. 5b). The test enforcing a monophyletic genus *Scutovertex* in the molecular phylogeny with *S. pictus* representing the most ancestral split, resulted in a significantly worse fit to the data (KH-test: tree length difference = 64 steps, s.d. = 10.77344, *t* = 5.9405, *P* = <0.0001; SH-test: Δ-ln*L* = 130.98973, *P* = 0.000). With the exception of *S. sculptus*, all species within the genus *Scutovertex* were recovered, with high

statistical support, as monophyletic. The two "sculptus" clades resulted as sister taxa in all methods, except MP (Fig. 4b), which showed no resolution between *S. sculptus*, *S. ianus* and *S.* sp.1 specimens. *Scutovertex pileatus* and *S. alpinus* resulted as the most basal representatives of the genus *Scutovertex*. The phylogenetic relationship of the remaining species *S. minutus*, *S. pannonicus*, *S. arenocolus* and the undescribed species *S.* sp.2 with individuals from Wangen differs slightly depending on the method used. Unlike the molecular phylogeny, the morphological data (Fig. 5b) revealed, with good to high statistical support, the monophyly of all species but lacked resolution of the phylogenetic relationships among the *Scutovertex* species (with exception of *S. pictus* as already mentioned).

Discussion

Molecular genetic and morphological data revealed well resolved phylogenies, demonstrating the monophyly of all species, with the exception of *Scutovertex sculptus*. Despite only minor morphological differences to congeneric species, all recently described *Scutovertex* species included in this study appeared as genetically distinct. Thus, morphological differentiation in the studied European scutoverticid mites is accompanied by high degrees of genetic differentiation, which is not necessarily the case in other oribatid mite families (e.g., Avanzati et al., 1994).

The most important congruence between morphology and molecular genetic data concerned the phylogenetic reconstruction of the genus *Scutovertex* itself. Both trees revealed a paraphylum *Scutovertex*, supporting the morphology-based hypothesis of Sitnikova (1980) that *S. pictus* might not belong to the genus *Scutovertex*. Moreover, several morphological characteristics – e.g., the shape of the bothridium, the absence of the lenticulus and the type of respiratory organs in the legs - clearly separate *S. pictus* from all other members of the genus. Its definite position is still unclear because in the molecular tree it clusters, depending on the tree building algorithm used, with different members of the three other European scutoverticid genera. However, we hypothesize, that *S. pictus* does not belong to any other known genus of the family Scutoverticidae but rather constitutes a new genus pursuant to its distinct morphological characters mentioned above.

A further important finding concerns the phylogenetic placement of *P. kuehnelti*. In the molecular tree it was placed in a lineage together with *E. hungaricus*, *L. caelatus* and *S. pictus*, a grouping strongly supported by high bootstrap and posterior probability values. This result contradicts Woas' statement (2002) that the genus *Provertex* would belong to the Cymbaeremaeidae because of sharing some morphological characters. With regard to our investigations, his argument seems not to be substantive as there is no close relationship between *P. kuehnelti* and *Cymbaeremaeus cymba* in any of the phylogenetic trees. Even in our morphology-based phylogeny *P. kuehnelti* does not occupy the most ancestral branch within the Scutoverticidae, further rejecting a close affinity to *C. cymba*.

Despite well resolved phylogenies and congruencies between both data sets, unexpected results emerged from the molecular genetic data. The most obvious one was the high genetic divergence among samples classified as *S. sculptus*: morphologically indistinguishable individuals were separated into two well supported clades, "sculptus1" and "sculptus2" (Figs. 5a-b). This separation became evident in both the mitochondrial COI gene and the nuclear *ef-1α* gene, whereas the 28S rDNA lacked resolution at this divergence level. These two clades are allopatrically distributed with "sculptus1" in Eastern and "sculptus2" in Western Europe (Fig. 1a), pointing to an ancient geographic separation of these two clades. Moreover, despite their well supported genetic distinctness, two unidentified specimens from Hungary (*S.* sp.1) showed close morphological resemblance to *S. sculptus* - e.g., cuticle and cerotegument structure of notogaster, shape of notogastral setae. Given the congruence

among the different molecular markers, incomplete lineage sorting could be eliminated as possible cause for the patterns observed within *S. sculptus*. Instead, our findings are consistent with the possibility that *S. sculptus* actually represents a complex of cryptic species. Which one is representing the "real" *S. sculptus* can not be answered in this study since neither samples from the holo- or paratypes of this species (described by Michael, 1879) nor specimens from a location site in England (locus typicus) were available for our analyses.

There are several reasons why morphological characters might be not useful in discriminating species, but there appear to be two general and recurrent frames for cryptic species (Bickford et al., 2007): they are either differentiated by nonvisual mating signals (Byers and Struble, 1990; Henry, 1998; Feulner et al., 2006; Stuart et al., 2006) and/or appear to be under selection promoting morphological stasis (Vrijenhoek et al., 1994; Rothschild and Mancinelli, 2001; Lefébure et al., 2006; Finston et al., 2007). Regarding the first point, nonvisual mating signals could also be important in differentiating among the different *S. sculptus* lineages because within oribatid mites indirect sperm transfer occurs by means of spermatophores. For *S. sculptus*, the "completely dissociated transfer" after Proctor (1998) is applicable (Pfingstl, pers. observations), where males and females never meet, and chemical cues induce the uptake of spermatophores by the female. Moreover, since *S. sculptus* occurs in extreme environments such as saline soils, salt marshes and other very dry habitats, convergent evolution under harsh conditions in similar habitats likely produced similar morphologies in genetically distinct lineages (also see Vrijenhoek et al., 1994; Rothschild and Mancinelli, 2001; Lefébure et al., 2006; Finston et al., 2007). However, this raises the question 'what is really "extreme"'? Therefore we want to conform to Rothschild and Mancinelli (2001) who stated 'all physical factors are on a continuum, and extremes in the conditions that make it difficult for organisms to function are 'extreme" (p. 1093, lines 5-7). The main habitats of our investigated mite species are mosses and lichens on sun-exposed places. Considering that these habitats can both dry up and be flooded completely it is obvious that they are extreme for the specimens living in.

We emphasize that many European *Scutovertex* species are morphologically very similar and several species have been recognized only recently (Schäffer et al., 2008; Pfingstl et al., 2009; Weigmann, 2009). A good example is the new species *S. ianus* (Pfingstl et al., submitted) which exhibits morphological character states similar to either *S. minutus* or *S. sculptus*. Taking only a short look at *S. ianus* would certainly lead to wrong species identification. We note, that in the older literature species seem to have been mixed up, in particular *S. minutus* and *S. sculptus*, and clearly different morphological depictions have been referred to as one and the same species (Balogh, 1972; Giljarov and Krivolutsky, 1975; Pérez-Iñigo, 1993; Woas, 1998).

Furthermore, it should be noted that *S. minutus* is possibly not as common as it has been stated in literature. We received samples from many European countries but the "real" *S. minutus* could be identified hitherto only in Austria and in Germany (samples not included in this study). This suggests that the often-cited statement of the Palaearctic distribution of *S. minutus* (Subías, 2004, resp.2006; Weigmann, 2006) is not true. *Scutovertex sculptus* (or members of this cryptic species complex), on the other hand, seem to be very abundant throughout its Palaearctic distribution.

Conclusions

Molecular genetic and morphological data revealed a paraphyletic genus *Scutovertex*, with *S. pictus* likely representing a distinct genus, and confirmed the taxonomic placement of *Provertex kuehnelti* within the family Scutoverticidae. Furthermore, molecular genetic data

confirmed several recently described *Scutovertex* species and thus the high species diversity within this genus in Europe and suggest that *S. sculptus* is a complex of several cryptic species showing marked genetic, but little (if any) morphological divergence.

Acknowledgments

Appendix A: Morphological characters and character states

1. Prodorsal surface (P): smooth, no foveae (0); smooth, foveae (1); granular, no foveae, no wrinkles (2); granular, no foveae, wrinkles (3); granular, foveae, no wrinkles (4); granular, foveae, wrinkles (5).

2. Bothridium (bo): closed border, roundly shaped (0); closed border, longish shaped (1); open border, roundly shaped (2); open border, longish shaped (3).

3. Sensillus (bS) dimension: short, slim (0); short, thick (1); long, slim (2); long, thick (3).

4. Sensillus shape: spinose, broad, clavate and flattened (0); spinose, broad, clavate and spherical (1); spinose, slender, clavate and flattened (2); spinose, slender, clavate and spherical (3).

5. Lamella (cl): absent (0); short, collateral (1); short, convergent (2); long, collateral (3), long, convergent (4); broad, laterally overhanging (5).

6. Lamellar seta (*le*): short, slim, smooth (0); short, slim, spinose (1); short, thick, smooth (2); short, thick, spinose (3); long, slim, smooth (4); long, slim, spinose (5); long, thick, smooth (6); long, thick, spinose (7).

7. Interlamellar seta (*in*): absent (0); short, slim (1); short, thick (2); long, slim (3); long, thick (4).

8. Rostral seta (*ro*) dimension: short, thick (0); short, slim (1); long, thick (2); long, slim (3).

9. Rostral seta shape: smooth, spiniform (0); smooth, lanceolate (1); spinose, spiniform (2); spinose, lanceolate (3).

10. Exobothridial seta (*ex*): absent (0); short (1); long (2).

11. Rostrum (R): with one ridge (0); with two ridges (1); with two clear projections (2).

12. Lenticulus (Le): absent (0); lateral borders bend inward (1); oval (2); rectangular (3).

13. Translamella (TL): absent (0); narrow, straight (1); narrow, bent (2); broad, straight (3); broad, bent (4).

14. Cusps (cu): absent (0); small (1); large (2); broad, overhanging (3).

15. Prodorsal ridges (PR): absent (0); collateral, reaching TL (1); collateral, not reaching TL (2); converging, not fused, reaching TL (3); converging, not fused, not reaching TL (4); converging, fused, reaching TL (5); converging, fused, not reaching TL (6).

16. Notogastral surface (N): foveae, no blocs, no granules, no bars, not netlike (0); foveae, blocs, no granules, no bars, not netlike (1); foveae, blocs, granules, no bars, not netlike (2); foveae, no blocs, granules, no bars, not netlike (3); no foveae, no blocs, granules, no bars, not netlike (4); no foveae, no blocs, no granules, bars, not netlike (5); no foveae, no blocs, no granules, bars, netlike (6); no foveae, no blocs, granules, bars, not netlike (7); almost smooth (8).

17. Foveae on notogaster: absent (0); indistinct borders (1); distinct borders (2).

18. Lyrifissure *ia*: inconspicuous, not on a nodule (0); on a nodule (1).

19. Lyrifissure *im*: inconspicuous (0); very long (1).

20. Lyrifissure *ip*: inconspicuous (0); on a protuberance (1).

21. Pairs of notogastral setae: 10 (0); 12 (1); 13 (2); 14 (3); 15 (4).

22. Saccules (S) of the octotaxic system: absent (0); 1 pair (1); 2 pairs (2); 3 pairs (3); 4 pairs (4).

23.-37. Notogastral setae c_{1-3}, *da, dm, dp, la, lm, lp,* h_{1-3}, ps_{1-3}: absent (0); slim, not spinose (1); slim, spinose (2); broadened, not spinose (3); broadened, spinose (4); thick, not spinose (5); thick, spinose (6).

38. Lateral prodorsum (PL) surface: smooth (0); granular (1).

39. Tutorium (TU): absent (0); not V-shaped (1); V-shaped (2).

40. Pedotectum I (PtI): small, not triangular (0); small, triangular (1); large, not triangular (2); large, triangular (3).

41. Pedotectum II (PtII): small, Y-shaped (0); small, triangular (1); large, Y-shaped (2); large, triangular (3).

42. Dens tutorius: absent (0); small (1); large (2).

43. Subcapitulum (S) surface: smooth (0); granular (1).

44.-46. Subcapitular setae (*m, a, h*): smooth (0); spinose (1).

47. Rutellar teeth (RU): 2 (0); 3 (1); 4 (2).

48. Rib on mentum (MR): absent (0); slender, straight (1); slender, V-shaped (2); broad, straight (3); broad, V-shaped (4); massive, reaching anterior border (5).

49. Pedipalp (Pp) "corne double": absent (0); incomplete (1); complete (2).

50. Chaetome pedipalp (solenidion excluded): 0-2-1-3-9 (0); alternative (1).

51. Apophysis on palptarsus: absent (0); present (1).

52. Epimeral region (E) surface: smooth (0); granular (1).

53. Apodemata (Ap) III + IV: both absent (0); III present, IV absent (1); both present (2).

54. Epimeral setal formula: 3-1-2-2 (0); 3-1-3-2 (1); 3-1-3-3 (2); 3-1-3-4 (3); 3-1-3-1 (4).

55. Anogenital region (AG) surface: smooth (0); granular (1).

56. Genital valves (GV) shape: rounded, anteriorly broadened (0); rounded, posteriorly broadened (1); rectangular, anteriorly broadened (2); rectangular, posteriorly broadened (3).

57. Genital setal (g) formula: 5+5 (0); 6+6 (1); 9+9 (2); >9<12 (3); >12 (4).

58. Aggenital setal (ag) formula: setae absent (0); 1+1 (1); 2+2 (2).

59. Anal setal (an) formula: 2+2 (0); 3+3 (1).

60. Placement from g_1 to g_2: in a row (0); side by side (1).

61. Placement from g_3 to g_6: in a row (0); displaced laterally (1).

62. Placement of anal setae: medially on anal valve (0); next to inner border of anal valve (1).

63. Leg surface (LS): smooth (0); granular, no ridges (1); granular, ridges (2).

64. Respiratory organs (RO) in legs: absent (0); planar areae porosae (1); saccules (2); platytracheae (3), brachytracheae (4); tracheae (5).

65. Claw (C) number: monodactylous (0); bidactylous (2); tridactylous (3)

66. Claw shape: homodactylous (0); heterodactylous (1).

67. Dorsal setae on legs coupled with solenidia: lost in adult stage (1); present in all stages (2); lost in all stages (3).

68. Apophysis on tibia I: absent (0); small (1); large (2).

69. Lateral setae on legs: slender, smooth (0); slender, dentate (1); broadened, smooth (2); broadened, dentate (3); extremely broadened (4).

70. Position of the respiratory organs in leg I and II: femur (0); femur, tibia (1); femur, tibia, tarsus (2); femur, tarsus (3).

71. Position of the respiratory organs in leg III and IV: trochanter, femur (0); trochanter, femur, tibia (1), trochanter, femur, tibia, tarsus (2); trochanter, femur, tarsus (3); femur, tarsus (4)

72. Chaetome leg I: 1-3-3-4-16 (0); 1-4-2-4-18 (1); 1-4-3-4-18 (2); 1-4-3-4-19 (3); 1-5-3-4-18 (4).

73. Chaetome leg II: 1-4-2-4-15 (0); 1-4-3-4-15 (1); 1-3-3-5-14 (2); 1-5-3-4-15 (3).

74. Chaetome leg III: 2-2-1-3-15 (0); 2-3-1-3-15 (1); 2-2-2-3-14 (2); 2-4-1-3-15 (3).

75. Chaetome leg IV: 1-2-2-3-12 (0); alternative (1).

76. Solenidia leg I: 1-2-2 (0); 1-2-3 (1).

77. Solenidia leg II: 1-1-2 (0); 1-1-1 (1).

78. Solenidia leg III: 0-1-0 (0); 1-1-0 (1).

79. Solenidia leg IV: 0-1-0 (0); alternative (1).

Appendix B: Matrix for morphological characters and character states

|		1		2		3		4		5		6		7		8
		0		0		0		0		0		0		0		0
S. alpinus	22324	40200	11126	02101	04010	11010	11111	11123	20100	01310	01201	01101	00253	11230	02100	0010
S. arenocolus	22324	70200	11415	31100	03010	03010	33344	11123	20111	01410	01201	01101	10253	11230	02100	0010
S. ianus	22324	70200	11425	21100	03010	01010	11144	11123	20111	01310	01201	01101	10253	11230	02100	0010
S. minutus	22324	70200	11426	40100	13010	13310	33344	11123	20111	01410	01201	01101	10253	11230	02100	0010
S. pannonicus	22324	70200	11425	02100	03010	03010	33324	11123	20111	01010	01201	01101	10253	11230	02100	0010
S. pictus	32104	60200	00320	50000	00010	01010	11111	11103	20111	01400	11201	01100	01243	11231	13010	0010
S. pileatus	22323	00200	11324	70100	03010	33010	33331	11123	20111	01310	01201	01100	00253	11230	01000	0010
S. sculptus	22324	70200	11425	11100	03010	04010	66666	11123	20100	01410	01201	01101	10253	11230	02100	0010
L. caelatus	33304	70200	20120	60000	00101	11111	11111	10113	20100	01100	01201	01100	01240	01221	12110	0010
P. kuehnelti	22112	00220	00104	40000	30110	11111	11111	11101	20111	00010	01201	01100	01233	11131	12100	0010
E. hungaricus	22325	70220	00034	60001	01010	01010	11111	11113	20111	00010	01241	01100	01233	11241	12000	0010
C. cymba	30110	00101	00000	60000	20110	11111	11111	10103	30100	2300	11001	01100	01243	01202	20220	0110

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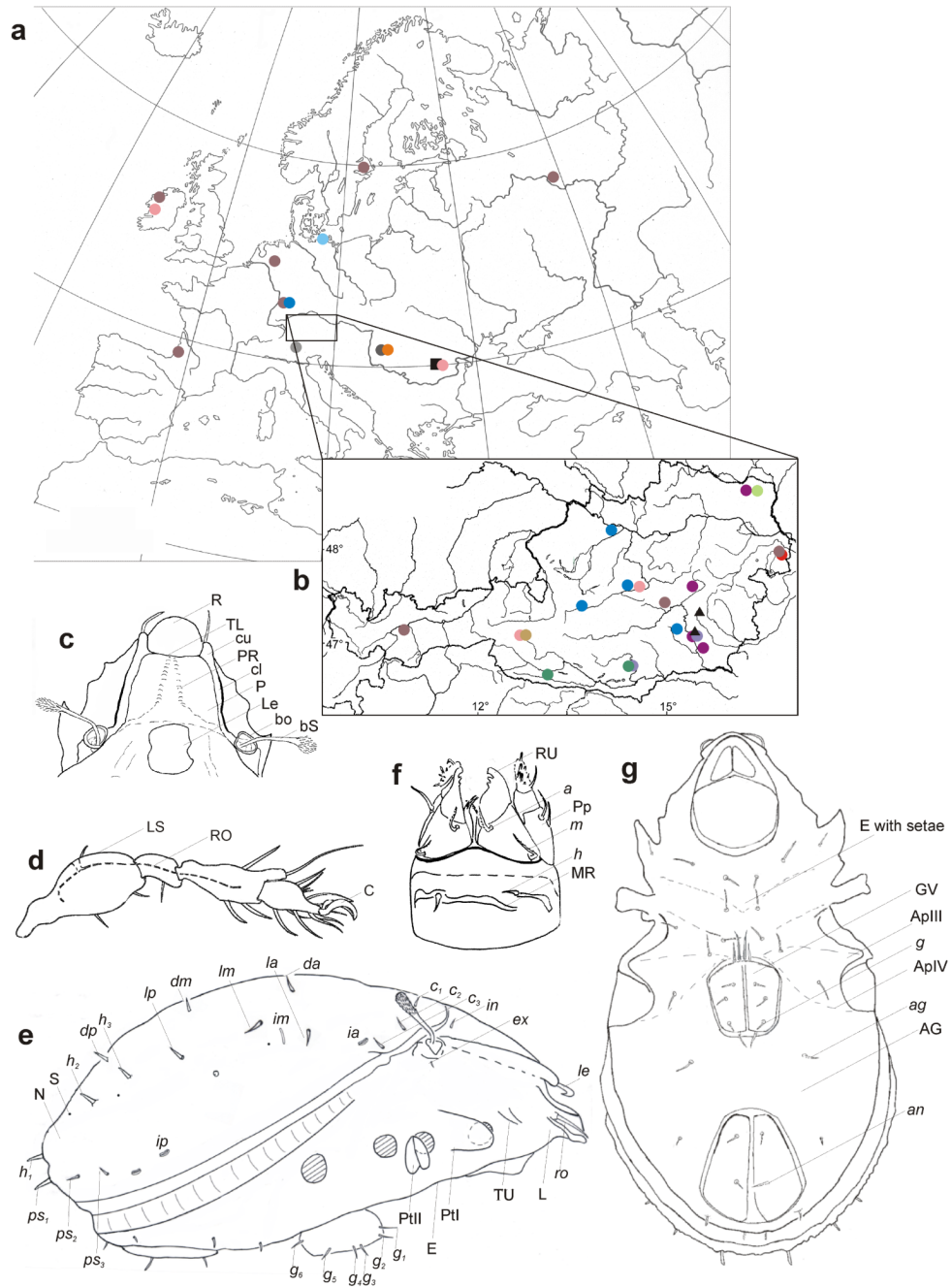
Fig. 1. (a-b) Sampling localities of the specimens used in this study: (a) Map of Europe. (b) Map of Austria.
Species are marked by different colors or symbols: ● = *Scutovertex alpinus*, ● = *S. arenocolus*, ● = *S. ianus*, ● = *S. minutus*, ● = *S. pannonicus*, ● = *S. pictus*, ● = *S. pileatus*, ● = *S.* sp.1, ● = *S.* sp.2, ● = *S. sculptus*, ● = *Exochocepheus hungaricus*, ● = *Lamellovertex caelatus*, ● = *Provertex kuehnelti*, ▲ = *Cymbaeremaeus cymba*, ▪ = *Unduloribates undulatus*. (c-g) Schematic representation of morphological characters investigated in this study: (c) prodorsum (dorsal view); (d) leg with setae (lateral view); (e) lateral body view; (f) subcapitulum (ventral view); (g) ventral body view. Abbreviations: *a*, anterior subcapitular seta; AG, anogenital region; *ag*, aggenital seta; *an*, anal seta; Ap,

apodem; bo, bothridium; bS, sensillus; C, claw; cl, lamella; cu, cusp; E, epimeral region; *ex*, exobothridial seta; GV, genital valve; *g*, genital seta; *h*, hysterostomatic seta; *in*, interlamellar seta; *ia, im, ip*, lyrifissures; *le*, lamellar seta; Le, lenticulus; LS, leg surface; *m*, median subcapitular seta; MR, rib on mentum; N, notogastral surface; P, prodorsal surface; PL, prodorsum lateral; Pp, pedipalp; PR, prodorsal ridges; PtI, PtII, pedotectum I/II; R, rostrum; RO, respiratory organ; *ro*, rostral seta; RU, rutellar teeth; S, saccule of the octotaxic system; TL, translamella; TU, tutorium; c_{1-3}, da, dm, dp, la, lm, lp, h_{1-3}, ps_{1-3}, notogastral setae.
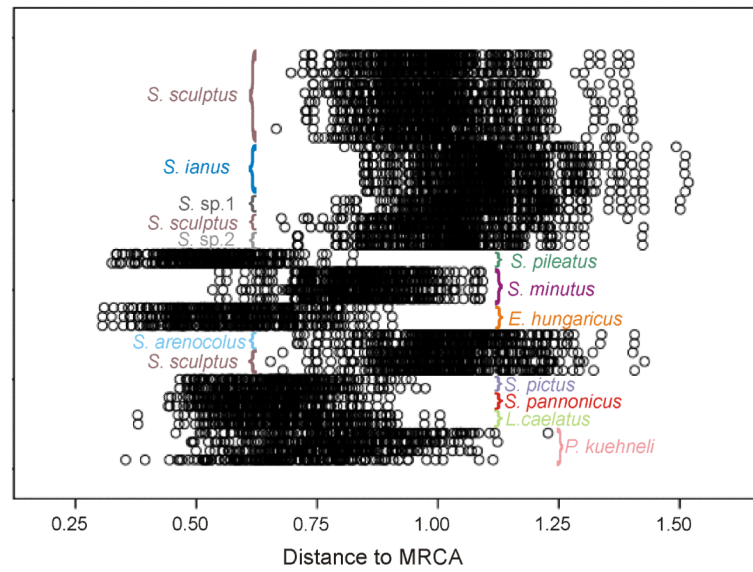
Fig. 2. Results of the Bayesian relative rates test.
The distribution of branch lengths from the most recent common ancestor (MRCA) to the terminal taxa (outgroup was excluded) is shown.
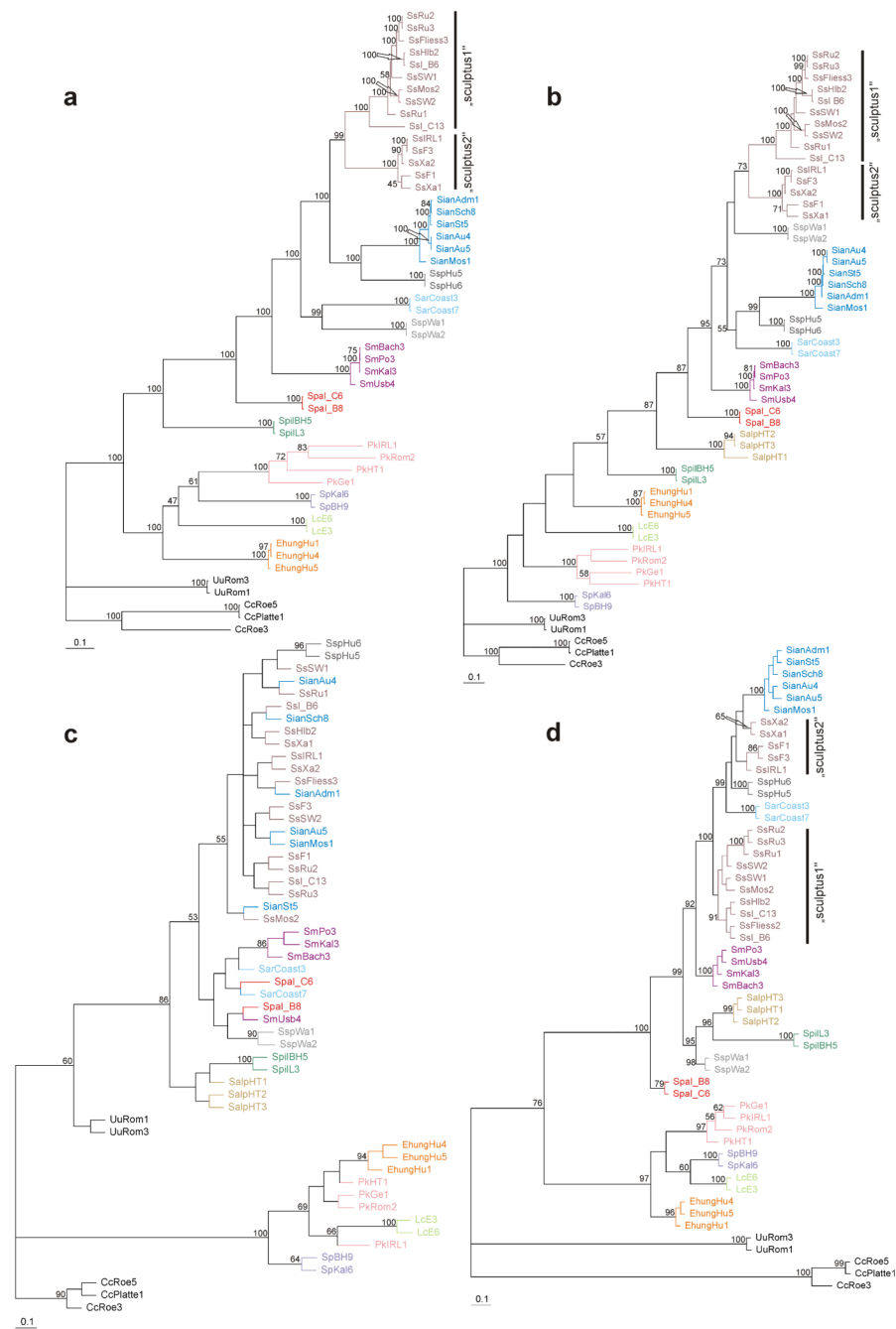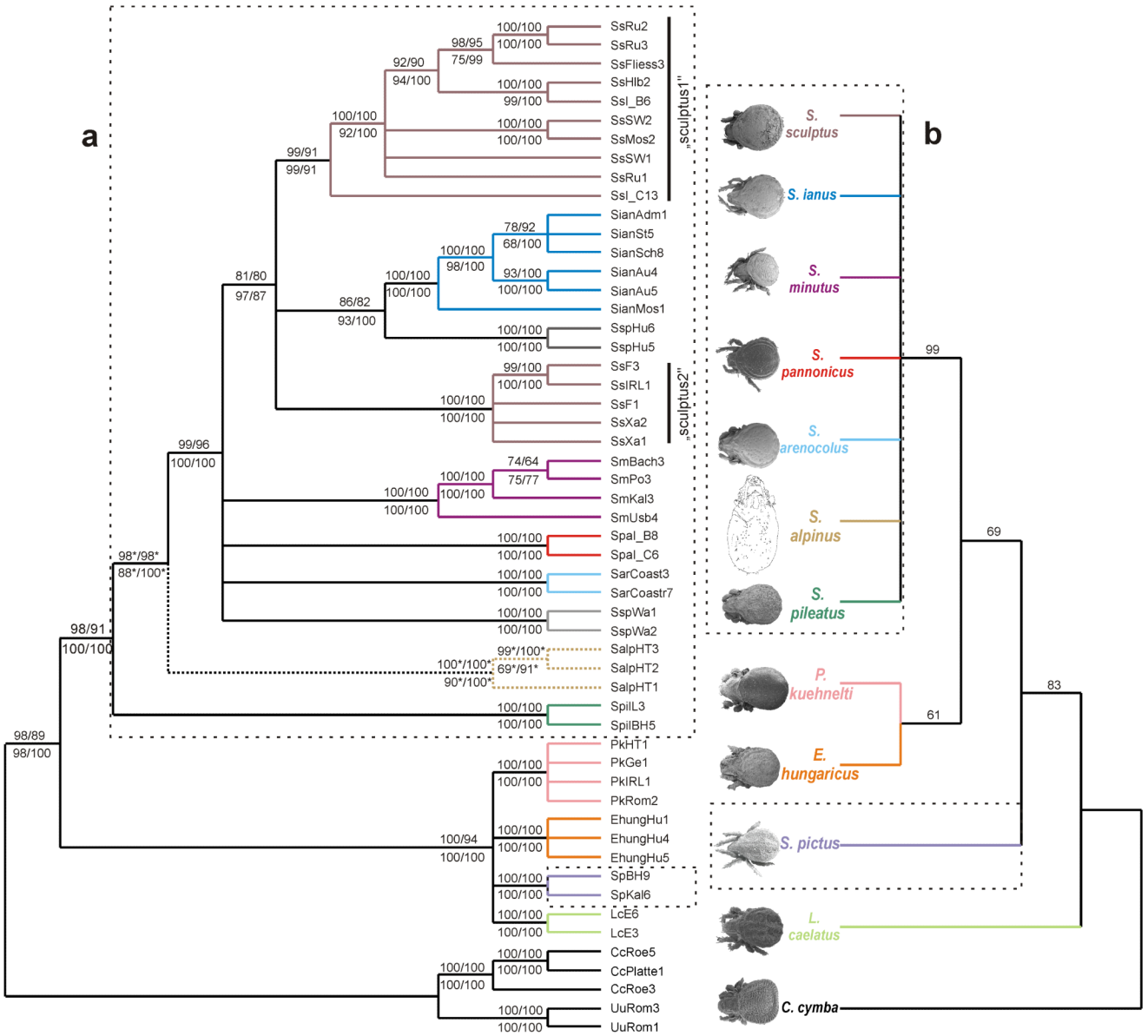
Fig. 3. Phylogeny of eleven nominal plus two undescribed European species of the family Scutoverticidae based on single gene analyses.

Bayesian inference (BI) tree of three studied genes: (a) mitochondrial COI; (b) COI region2; (c) nuclear 28S rDNA; (d) nuclear *ef-1α*. Only posterior probabilities >50 are shown. Colors are the same as in Fig. 1.
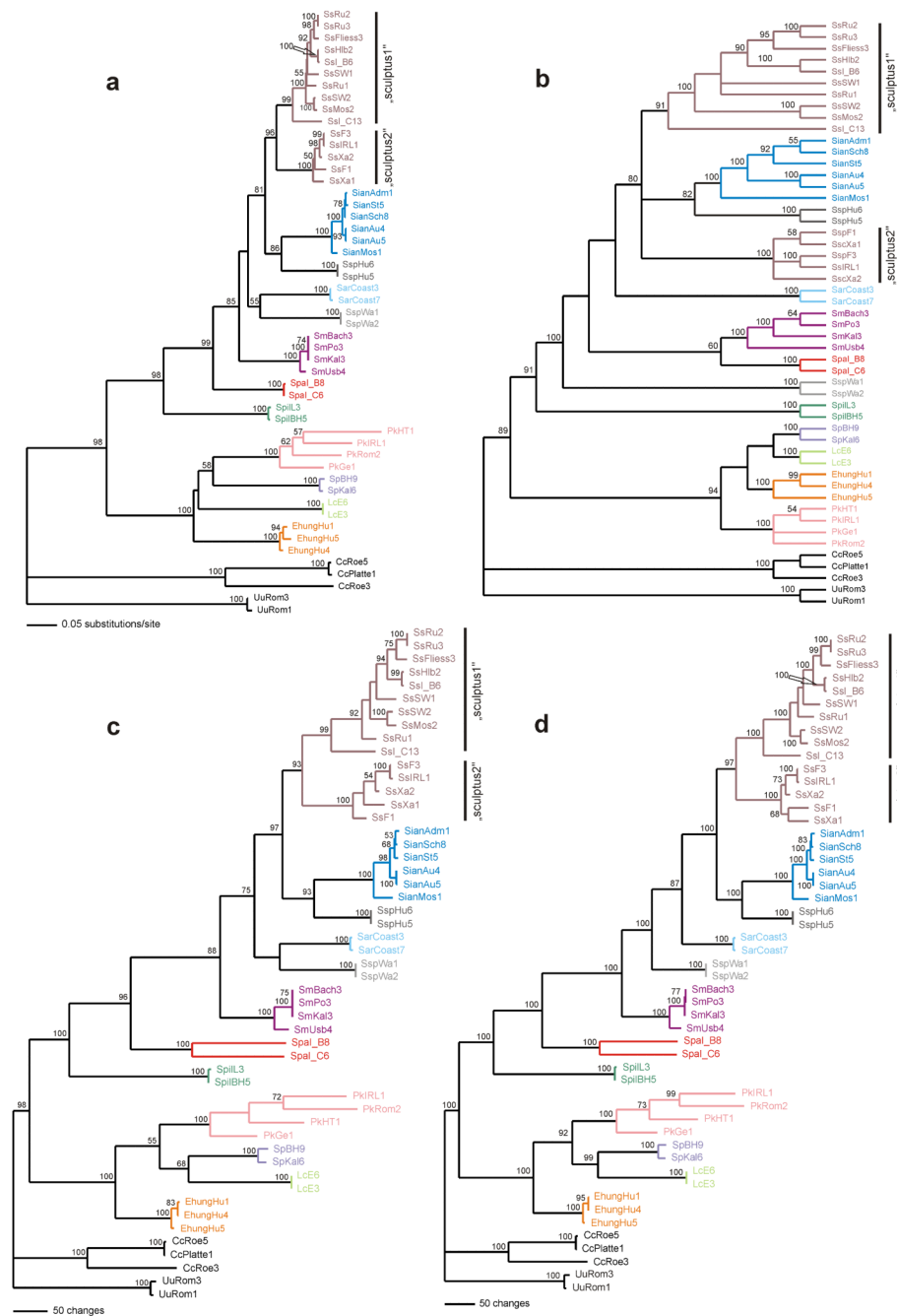
Fig. 4. Phylogeny of eleven nominal plus two undescribed European species of the family Scutoverticidae based on the concatenated data set of all available fragments of the COI, 28S rDNA and *ef-1α* genes.

(a) NJ tree using the GTR+I+G model; (b) strict consensus of 34 most parsimonious trees; (c) ML tree using the GTR+I+G; (d) Bayesian 50% majority rule consensus tree. Bootstrap values (for NJ, MP and ML), and posterior probabilities (for BI) are shown when >50. Colors are the same as in Fig. 1.

Fig. 5. Phylogeny of eleven nominal plus two undescribed European species of the family Scutoverticidae.
(a) Strict consensus of NJ, MP, ML and BI trees of the concatenated data set of all available fragments of the COI, 28S rDNA and *ef-1α* genes. Bootstrap values of NJ and MP are shown above the branches, bootstrap values for ML and posterior probabilities for BI below (only values >50 are shown). (b) Strict consensus tree of 34 most parsimonious trees based on 79 external morphological characters or character states. Bootstrap values >50 are shown. Stippled lines highlight investigated members of the genus *Scutovertex*. * = Support values from analyses of the RS data. Colors are the same as in Fig. 1.

Table 1

Specimens, sample ID, sampling location and GenBank accession numbers for the samples analyzed in this study.

| Species | Sample ID | GenBank Accession No. | | | Sampling locality |
		COI	28S	*ef-1α*	
Scutoverticidae					
Scutovertex					
S. alpinus	SalpHT1	GU208673[#]	GU208524	GU208619	Fuscherkarkopf/Großglockner/Carinthia - A
	SalpHT2	GU208674[#]	GU208525	GU208620	Fuscherkarkopf/Großglockner/Carinthia - A
	SalpHT3	GU208675[#]	GU208526	GU208621	Fuscherkarkopf /Großglockner/Carinthia - A
S. arenocolus	SarCoast3	GU208578	GU208527	GU208622	Darss-Zingst/Baltic Coast - D
	SarCoast7	GU208579	GU208528	GU208623	Darss-Zingst/Baltic Coast - D
S. ianus	SianSt5	GU208580	GU208529	GU208624	Stiwoll/Styria - A
	SianSch8	GU208581	GU208530	GU208625	Schladming/Styria - A
	SianAdm1	GU208582	GU208531	GU208626	Admont/Styria - A
	SianAu4	GU208583	GU208532	GU208627	Floodplain of Traun/Upper Astria - A
	SianAu5	GU208584	GU208533	GU208628	Floodplain of Traun/Upper Astria - A
	SianMos1	GU208585	GU208534	GU208629	Mosbach near Heidelberg - D
S. minutus	SmBach3	GQ890381[*]	GU208535	GU208630	Bachsdorf/Styria - A
	SmPo3	GQ890362[*]	GU208536	GU208631	Pogier/Styria - A
	SmKal3	GQ890373[*]	GU208537	GU208632	Graz/Styria - A
	SmUsb4	GQ890395[*]	GU208538	GU208633	Unterstinkenbrunn/Lower Austria - A
S. pannonicus	SpaI_B8	GQ890445[*]	GU208539	GU208634	Lake " Zicklacke"/Burgenland - A
	SpaI_C6	GQ890444[*]	GU208540	GU208635	Lake "Oberer Stinker"/Burgenland - A
S. pictus	SpKal6	GU208586	GU208541	GU208636	Graz/Styria - A
	SpBH9	GU208587	GU208542	GU208637	Castle Hochosterwitz/Carinthia - A
S. pileatus	SpilBH5	GU208588	GU208543	GU208638	Castle Hochosterwitz/Carinthia - A
	SpilL3	GU208589	GU208544	GU208639	Laas/Carinthia - A
S. species 1	SspHu5	GU208590	GU208545	GU208640	Fülöpháza/Kiskunság National Park - H
	SspHu6	GU208591	GU208546	GU208641	Fülöpháza/Kiskunság National Park - H
S. species 2	SspWa1	GU208592	GU208547	GU208642	Wangen am Ritten/South Tyrol - I
	SspWa2	GU208593	GU208548	GU208643	Wangen am Ritten/South Tyrol - I
S. sculptus	SsHlb2	GQ890440[*]	GU208549	GU208644	Häuslberg/Styria - A
	SsI_B6	GQ890427[*]	GU208550	GU208645	Lake "Oberer Stinker"/Burgenland - A
	SsI_C13	GQ890434[*]	GU208551	GU208646	Lake "Zicklacke"/Burgenland - A
	SsFliess3	GQ890441[*]	GU208552	GU208647	Fliess/Tyrol - A
	SsRu1	GU208594	GU208553	GU208648	Nizhniy Novgorod - RUS
	SsRu2	GU208595	GU208554	GU208649	Nizhniy Novgorod - RUS
	SsRu3	GU208596	GU208555	GU208650	Nizhniy Novgorod - RUS
	SsSW1	GU208597	GU208556	GU208651	Endeby near Uppsala - S

| Species | Sample ID | GenBank Accession No. | | | Sampling locality |
		COI	28S	*ef-1α*	
	SsSW2	GU208598	GU208557	GU208652	Endeby near Uppsala - S
	SsMos2	GU208599	GU208558	GU208653	Mosbach near Heidelberg - D
	SsIRL1	GU208600	GU208559	GU208654	Ceide-Fields - IRL
	SsF1	GU208601	GU208560	GU208655	Seignosse/Les Bourdaines - F
	SsF3	GU208602	GU208561	GU208656	Seignosse/Les Bourdaines - F
	SsXa1	GU208603	GU208562	GU208657	Xanten - D
	SsXa2	GU208604	GU208563	GU208658	Xanten - D
Lamellovertex					
L. caelatus	LcE3	GU208605	GU208564	GU208659	Ernstbrunn/Lower Austria - A
	LcE6	GU208606	GU208565	GU208660	Ernstbrunn/Lower Austria - A
Provertex					
P. kuehnelti	PkHT1	GU208607	GU208566	GU208661	F. Josef Höhe/Großglockner/Carinthia - A
	PkGe1	GU208608	GU208567	GU208662	Gesäuse/Styria - A
	PkIRL1	GU208609	GU208568	GU208663	Galway - IRL
	PkRom2	GU208610	GU208569	GU208664	Bra ov-Bucegi mountains - RO
Exochocepheus					
E. hungaricus	EhungHu1	GU208611	GU208570	GU208665	Fülöpháza/Kiskunság National Park - H
	EhungHu4	GU208612	GU208571	GU208666	Fülöpháza/Kiskunság National Park - H
	EhungHu5	GU208613	GU208572	GU208667	Fülöpháza/Kiskunság National Park - H
Unduloribatidae					
Unduloribates					
U. undulatus	UuRom1	GU208614	GU208573	GU208668	Bra ov-Bucegi mountains - RO
	UuRom3	GU208615	GU208574	GU208669	Bra ov-Bucegi mountains - RO
Cymbaeremaeidae					
Cymbaeremaeus					
C. cymba	CcRoe3	GU208616	GU208575	GU208670	Röthelstein/Styria - A
	CcRoe5	GU208617	GU208576	GU208671	Röthelstein/Styria - A
	CcPlatte1	GU208618	GU208577	GU208672	Graz/Styria - A

[#]Sequences from COI region2 fragment alone.

[*]Sequences not generated in the framework of this study were obtained from Schäffer et al. (in press).

Table 2

Comparison of alternative phylogenetic hypotheses.

Tree	tree length diff.	KH test			SH test		
		s.d. (diff)	t	P	$-\ln L$	Δ-$\ln L$	P
NJ	9	11.87557	0.7579	0.4486	16875.99005	19.70894	0.105
BI	best				16856.81429	0.53318	0.832
MP	20	13.48681	1.4829	0.1382	16886.10722	29.82611	0.026[*]
ML	5	7.68222	0.6509	0.5152	16856.28111	best	

Kishino-Hasegawa (KH; Kishino and Hasegawa, 1989) and Shimodaira-Hasegawa tests (SH; Shimodaira and Hasegawa, 1998) were used to assess whether the topologies of NJ, MP, BI and ML differed significantly.

[*]
$P<0.05$.