Systematic & Applied Acarology 24(9): 1631–1652 (2019) ISSN 1362-1971 (print)
http://doi.org/10.11158/saa.24.9.5 ISSN 2056-6069 (online)

Article http://zoobank.org/urn:lsid:zoobank.org:pub:AF2E7D7B-99B7-4914-9CF2-B8ECD78DCA88

Supplementary descriptions and DNA barcodes of two rarely encountered *Trisetacus* species (Eriophyoidea, Phytoptidae) associated with Tertiary relict conifers from the Mediterranean region

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Abstract

New records and supplementary morphological descriptions of two rarely encountered *Trisetacus* species from Pinaceae, *T. abietis* Postner 1968 and *T. cedri* (Nalepa 1920), are reported. *Trisetacus abietis* was found in Abkhazia under the needle epidermis of *Abies nordmanniana* (Steven) Spach, a conifer endemic to the mountainous Asian coast of the Black Sea. *Trisetacus cedri* was found in buds of introduced *Cedrus deodara* (Roxb. ex D. Don) G. Don in Abkhazia and South Africa. It is the only member of *Trisetacus* known from *Cedrus* spp. For the first time we provide sequences of two genes (COI and D1–D2 28S) of *T. abietis* (MN022221, MN025333) and *T. cedri* (MN022222, MN022223, MN025334, MN025335), along with microphotographs of the damage caused by these mites on their coniferous hosts. Sequences of D1–D2 28S of *T. cedri* from Abkhazian and South African populations are identical; COI sequences from different populations differ by only one synonymous substitution in a codon for asparagine. Females of *T. abietis* have long asymmetrical 8/7-rayed empodia, whereas males have shorter symmetrical 6/6-rayed empodia and shorter solenidia ω I. Similar sexual dimorphism in tarsal appendages was previously reported in *Novophytoptus*, representing an endoparasitic lineage of phytoptids on monocots. In *T. cedri*, a "long form" and a "short form" of both males and females were detected, suggesting a complex life cycle in this species. The evolution of *Trisetacus* is discussed within the broader context of the molecular phylogenies of Pinaceae and Eriophyoidea, including estimations of divergence times.

Key words: gymnosperm pest, endoparasite, bud mite, Mediterranean, introduced conifer, COI, 28S

Introduction

Trisetacus Keifer (Phytoptidae, Nalepellinae) is a large genus of eriophyoid mites comprising about 60 species associated with the coniferous families Pinaceae and Cupressaceae. Most *Trisetacus* spp. have been recorded from the Holarctic, predominantly from North America and Europe, whereas a few species have been reported from elsewhere (e.g. *T. calvus* Navia & Flechtmann 2000 described from introduced *Cupressus sempervirens* L. in Brazil). Some of the members of *Trisetacus* are considered to be economically important pests because they are capable of causing various types of damage to ornamental conifers (Lindquist & Amrine 1996; Castagnoli *et al*. 2010). Along with widely distributed and frequently recorded species (e.g. *T. piceae* (Roivainen 1951) and *T. pini* (Nalepa 1887) in Europe), the genus *Trisetacus* includes rarely encountered species, many of which are associated with locally endemic host plants that are difficult to collect (Smith 1984a,b). Typically, *Trisetacus* live in natural shelters provided by their hosts (buds, seeds, needle sheaths, and bud scales), but some species are possible vagrants (Lewandowski *et al.* 2014 and papers cited). Three European species, *T. pini* (Nalepa 1887), *T. floricolus* (Trotter & Cecconi 1902) and *T. abietis* Postner 1968, are remarkable in their unusual relationship with hosts: *T. pini* causes the formation of bark galls on twigs of *Pinus* spp., *T. floricolus* attacks strobili of *Abies alba* Mill., and *T. abietis* lives under the needle epidermis of *A. alba*, causing subepidermal tissue necrosis (Postner 1968, Shevchenko *et al*. 1993, Hellrigl 2003).

Most of the *Trisetacus* species were discovered during the 20th century, and their descriptions are usually short and incomplete, with inadequate drawings that impede taxonomic studies. In summer 2018, we obtained fresh plant material from Abkhazia and South Africa, which provided us with two *Trisetacus* species: *T. abietis* Postner 1968 inside needles of *Abies nordmanniana* (Steven) Spach and *T. cedri* (Nalepa 1920) in buds of introduced *Cedrus deodara* (Roxb. ex D. Don) G. Don. These findings are the only records for both species since they were poorly described about 50 and 100 years ago, respectively. In this paper we give supplementary descriptions, provide COI and D1D2 28S sequences, and discuss morphological peculiarities of *T. abietis* and *T. cedri*. We also discuss the evolution of *Trisetacus* within the broader context of molecular phylogenies of Pinaceae and Eriophyoidea, including estimations of divergence times.

Material and Methods

Morphology

Live mites were collected from plants using a fine minuten pin and a dissecting microscope. The mites were mounted in a modified Berlese medium with Iodine (Amrine & Manson 1996) and cleared on a heating block at 90° C for 3 hours. For the investigation based on confocal laser scanning microscopy (CLSM), live mites were mounted in Hoyers's medium without Iodine to avoid loss of autofluorescent signal (Kirejtshuk *et al.* 2015). Slide-mounted specimens were examined with differential interference contrast (DIC) and phase contrast (PC) light microscopy (LM). In this study we used two microscopes: a Leica DM750 (equipped with a Hi-plan 100×/1.25 oil Ph3 objective) and a Leica DM5000 (with a HCX PL Fluotar 100×/1.30 oil DIC objective) compound microscope. Each microscope was equipped with a digital camera, ToupCam UCMOS09000KPB and Leica DFC320, respectively. Images and specimens were analyzed and measured using ToupTek ToupView and Leica DFC320 Imaging Software. In the supplementary descriptions of the two *Trisetacus* species, measurement ranges are given. All measurements are given in micrometers (μm) and are lengths except when mentioned otherwise. Classification, terminology of the genital anatomy, and terminology of external morphology follow Amrine *et al*. (2003), Chetverikov (2014),

and Lindquist (1996), respectively. Drawings were made with the aid of a video projector, using the setup detailed by Chetverikov (2016). The scientific names of host plants are given according to The Plant List (2013). Institutional acronyms follow Zhang (2018).

Confocal microscopy

In all females of *Trisetacus abietis* examined under conventional LM, internal genitalia were not seen, possibly because they were too thin and translucent. Therefore, we applied a previously described protocol for the investigation of internal genitalia of eriophyoids under confocal laser scanning microscopy (Chetverikov 2012, 2014). CLSM acquisition was carried out using a Spectral confocal & multiphoton system Leica TCS SP2 with objective 63x N.A. 1.4–0.60 Oil lBL HCX PL APO at an excitation wavelength of 405 nm (blue laser) at 12% intensity. Acquisition resolution was 1024x1024 pixels, level of gain 600–800, and the zoom range 1.5–2.0 x. Stacks of 12 to 26 optical slices were recorded digitally from each of the six studied females of *T. abietis*. These stacks were processed using Amira®5.3.2 software. The CLSM images, which were recorded using the "Snapshot" command in Amira®, were generated through Volume Rendering via a combination of the Voltex and Orthoslice modules of Amira®, with different transparency adjustments.

Comparative material

Holotype female of *Trisetacus neoabietis* Smith 1984a from *Abies amabilis* (Douglas ex Loudon) Douglas ex Forbes, mites were rinsed from foliage near growing tips, CANADA: British Columbia, East Sooke Park, Becher Bay, 11 July 1979, coll. I. Smith, slide M790308T; a female of *T. neoabietis* from *Picea sitchensis* (Bong.) (presumed accidental host association resulting from chance dispersal of mites from nearby specimens of *Abies* (Smith 1984, p. 176)), CANADA: British Columbia, Ucluelet, Pacific Rim National Park, mixed woods along beach, 16–18 July 1979, coll. I. Smith, slide M790317b; one female and one male of *T. neoabietis* from needle mesophyll of *Abies balsamea* (L.) Mill., USA, California, Lake Co. (specimens may have come from Clearlake, but the slide had no precise location, just Lake Co.; the trees were transplanted from Maine, L.L. Bean Company, Headquarters in Freeport, Maine), slide #2, 19 December 1991, coll. T. A. Stasny.

DNA extraction, PCR and sequencing

About 100 live mites of each *Trisetacus* species were placed in Eppendorf tubes filled with 96% ethanol. The ethanol-preserved material is kept in a freezer (–25°C) at the Zoological Institute of the Russian Academy of Science (ZIN RAS) in Saint-Petersburg. DNA was extracted from 2–3 mite specimens using Chelex® 100 Resin Bio Rad following the protocol detailed by Chetverikov *et al*. (2019). D1–D2 domains of the 28S rDNA gene and cytochrome oxidase subunit I (COI) gene fragment were amplified (Table 1). The D1–D2 28S fragment was amplified in accordance with the protocol of Chetverikov *et al*. (2019).

The COI fragment was amplified in a two-step PCR with the primers Cox1_16F 5′–TGANTW TTTTCHACWAAYCAYAA–3′ and Cox1_1384Rm 5′–TCDGARTAKCGDCGDGGTAT–3′ at the first step (PCR-1), and primers Cox1_25Fm 5′–TCHACHAATCAYAARGATAT–3′ and Cox1_1282Rm 5′–CCRTTNARNCCTAAAAARTGYTG–3′ at the second step (PCR-2), all primers modified from Klimov *et al.* (2018). The PCR-1 was carried out in 20 µl total reaction volume containing 2.5 µl of ScreenMix (#PK041B, Evrogen), 15.5 µl of distilled water, 0.6 of each primer (10µM) and 0.8 µl of DNA template. Thermocycling profiles for PCR 1 & 2 were used as specified by Klimov *et al*. (2018, Supplement).

After amplification, 4.5 µl of each reaction product was mixed with 0.5 µl of SYBR Green I (Lumiprobe) and analyzed by electrophoresis on a 1% agarose gel to assess the product size and concentration. PCR products were sequenced in both directions using the HCO2198 (Folmer *et al*.

1994)/Cox1_1282Rm primers for COI and D1D2fw1m/D1D2rev4E (Chetverikov *et al*. 2019) primers for D1–D2 28S. Sequences were obtained using BigDye Terminator v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA) and a 3500xl Genetic Analyzer (Applied Biosystems). Trace files were checked and edited using GeneStudioTM Professional 2.2.0.0. (*www.genestudio.com*). COI sequences were translated into amino acids; the absence of stop codons was checked with Mega 7 (Kumar *et al.* 2016).

TABLE 1. Collection data and GenBank accession numbers of COI and D1–D2 28S sequences of two *Trisetacus* species from Pinaceae.

Mite species	Host plant	Collecting data	GB accession number
Trisetacus abietis Postner 1968	*Abies nordmanniana* (Steven) Spach, inside needles	ABKHAZIA: mountain Ah-Ag, 280 m a.s.l., 43°28′38N″, 40°12′17E″, coll. G. Yu. Konechnaya, 6 July 2018	MN022221 (COI, 1203 bp, 401 aa)
			MN025333 (D1–D2 28S, 1032 bp)
Trisetacus cedri (Nalepa 1920)	*Cedrus deodara* (Roxb. ex D. Don) G. Don, in buds	ABKHAZIA: Tzandripsh, City Park, coll. G. Yu. Konechnaya, 08 July 2018,	MN022222 (COI, 1152 bp, 383 aa)
			MN025334 (D1–D2 28S, 1020 bp)
		SOUTH AFRICA: NW Province, 10 km WSW of Buffelspoort Dam, Barlett Farm, 25°50′00.7″S 27°23′53.4″E, coll. S. Neser, 10 October 2018	MN022223 (COI, 1175 bp, 391 aa)
			MN025335 (D1–D2 28S, 1020 bp)

Results

Trisetacus abietis Postner 1968—Figs 1–5.

Trisetacus abietis Postner 1968:106, fig. 1–4; Smith 1984b:1204, no fig.

Supplementary description of *T. abietis* (Abkhazian population). FEMALE (n=12). Body vermiform, whitish, 302–364, 52–59 wide at the level of setae *c2*. **Prodorsal shield** subrhomboid, 29–34, 36–40 wide; frontal lobe absent. Prodorsal shield ornamentation variable, usually including median line, paired admedian lines and one pair of submedian lines. Median lines straight, present in posterior ¾ or prodorsal shield, sometimes forked anteriorly. Admedian lines typically with three anteromedial branches, which look like separate short lines in some specimens. Submedian I commonly entire and slightly sinuous, but may be fragmented into 2–3 short lines (Fig. 2 E,F). Lateral field of prodorsal shield with numerous microgranules. Prodorsal shield setae: *vi* 10–14, directed up and anteriad; *sc* 39–47, 14–17 apart, directed up and anterolaterad; distance between tubercles of *vi* and *sc* 9–12. **Gnathosoma** directed obliquely down and forward; palps 19–22; chelicerae 15–17, outer infracapitular stylets 10–13, oral stylet (n=3) angled, 7–9. Gnathosomal setae: seta *v* not discernible; pedipalp genual seta *d* non-bifurcate, 7–10; pedipalp coxal seta *ep* 3–4. Suboral plate (formed by fused ventral palpcoxae) subcordate, smooth.

Leg I 29–33, tarsus 6–7, *u'* 2–5, *ft'* 11–15, *ft''* 23–28, *ω* 9–10 without knob; empodium 10–12, asymmetrical, 7/8-rayed, all rays except terminal pair with one subray each; tibia 6–8, *l'* 4–5; *φ* 8–10; genu 5–6, *l''* 21–26; femur 10–11, *bv* 9–13. **Leg II** 27–32, tarsus 6–7, *u'* 2–4, *ft'* 5–8, *ft''* 25–30, *ω* 9–10 without knob; empodium 10–12, similar to empodium I; tibia 5–7; genu 5–6, *l''* 10–16; femur 10–12, *bv* 8–12. **Coxal plates** with longitudinal ridges; coxal setae *1b* 20–27, 9–11 apart; *1a* 28–33, 7–10 apart; *2a* 50–62, 20–23 apart. Prosternal apodeme indistinct; cuticle between tubercles of coxal setae *1a* and *1b* with tiny microtubercles; 2–4 incomplete coxigenital annuli before epigynium.

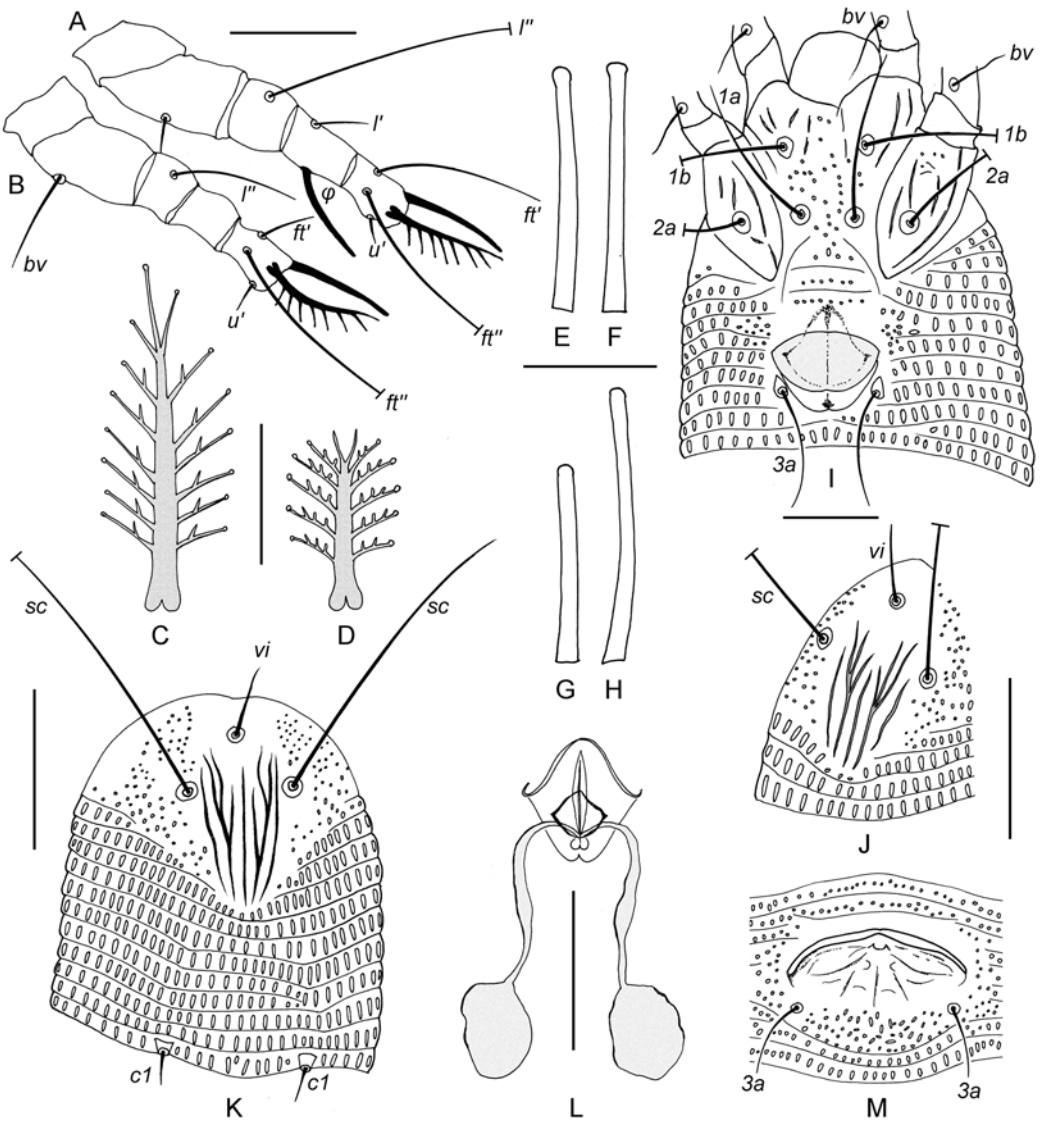

FIGURE 1. Drawings of *Trisetacus abietis* Postner 1968**.** A—leg I; B – leg II; C—female empodium I; D—male empodium I; E—female tarsal solenidion I; F—female tarsal solenidion II; G—male tarsal solenidion I; H—male tarsal solenidion II; I—coxigenital area; J—male prodorsal shield; K—female prodorsal shield; L—female internal genitalia; M—male external genitalia. Scale bar: A,B,I,L = 10 μm; C,D,E,F,G,H = 5 μm; J,K,L,M = 20 μm.

External genitalia. Genital coverflap rounded, smooth, with tiny medial indentation, 7–10 long, 14–17 wide; setae *3a* 14–18, 13–16 apart; pregenital plate (*sensu* Flechtmann *et al*. 2015) absent. **Internal genitalia (n=4).** Spermathecae ovoid, 10–13 long, 8–11 wide; spermathecal tubes recurved, 17–22 long, 2–3 wide, with notable widening in medial part; thorn-shaped spermathecal process (*sensu* Duarte *et al*. 2016) typical for many members of Eriophyidae absent; longitudinal bridge 8–11, post-spermathecal part of longitudinal bridge indistinct and rudimentary; anterior genital apodeme bell-shaped, distinct, clearly seen in all studied females; oblique apodeme (*sensu* Chetverikov *et al.* 2015) absent.

Opisthosoma dorsally with 92–102 annuli, ventrally with 91–104 annuli between posterior margin of coxae II and caudal lobes; dorsal and ventral annuli bear distinct oval microtubercles; microtubercles on ventral telosomal annuli elongate. Setal lengths: *c1* 4–5, *c2* 33–38, *d* 20–26, *e* 22–24, *f* 22–26; *h1* 7–9; *h2* 60–70; 8–9 annuli from rear shield margin to *c1*; 10–12 annuli from rear shield margin to *c2*; 11–13 annuli between *c2*–*d*; 19–22 annuli between *d* and *e*; 47–52 annuli between *e* and *f*; 4–5 annuli between *f* and *h2*.

MALE (n=4)

In comparison to females, males are slightly smaller (290–312) and narrower (49–54), with fewer opisthosomal annuli (89–94 dorsal annuli and 81–86 ventral annuli), shorter tarsal solenidia ω I (6–8 vs 9–10), similarly shaped ornamentation of prodorsal shield but with less distinct branches of admedian lines. Empodia symmetrical, 6/6-rayed, all rays except terminal pair with 2–3 subrays each; rays of the terminal pair short, 2–3 times shorter than in females. **External genitalia** 10–12, 21–23 wide; setae *3a* 11–14, 18–21 apart. Post-genital region (situated between tubercles of *3a*, delimited anteriorly by genital opening and posteriorly by an arch-shaped microtubeculate semi-annulus) with three diverging ridges and numerous, irregularly distributed elongate microtubercles (Fig. 2I); eugenital setae absent.

Material examined

Numerous females and males in slides #E5000, #E5001, #5002, and #E5003, collected from inside needles of *Abies nordmanniana* (Steven) Spach (all stages numerous) in ABKHAZIA: mountain Ah-Ag, 280 m a.s.l., 43°28′38N″, 40°12′17E″, coll. G. Yu. Konechnaya, 6 July 2018; material has been deposited in Acarological collection of ZIN RAS, Saint-Petersburg, Russia.

GenBank data

MN022221 (COI), MN025333 (28S).

Remarks

A blast search for COI showed highest similarity with sequences KY922367.1 (*Trisetacus pini*, 99% cover, 81.99% identity) and KY922366.1 (*Trisetacus piceae*, 100% cover, 80.55% identity). Blast search for D1–D2 28S showed highest values for sequences of the same two *Trisetacus* species: KY921990.1 (*T. piceae*, 99% cover, 93.30% identity) and KY921991.1 (*T. pini*, 99% cover, 92.35% identity).

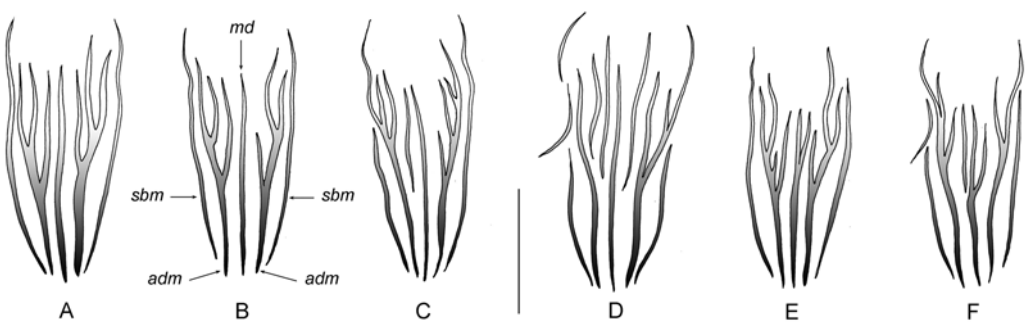

FIGURE 2. Variation of prodorsal shield ornamentation in six females of *Trisetacus abietis.* A, B—most common in studied population symmetrical ornamentation; C,D,E,F—aberrant forms of prodorsal shield ornamentation. Scale bar = 10 μm. *Note*: in all studied specimens the lines of prodorsal shield are notably more distinct (dark under PC LM) in their basal part than in anterior part (much lighter under PC LM). Notations: *md* —median line, *adm*—admedian line, *sbm*—submedian line.

Host plants and relation to host

Until now, *T. abietis* has been recorded from two *Abies* species: *Abies alba* Mill. and *A. nordmanniana* (Steven) Spach. *Abies alba* is a widespread conifer species growing in Central Europe including the Alps, Pyrenees, Carpathians, Balkans, Italy and Corsica, with the southernmost and northernmost extents respectively lying in Calarabia (Southern Italy) and the lowlands of Poland around 51°N (Farjon & Filer 2013). *Abies nordmanniana* is distributed in the mountains surrounding the Asian side of the Black Sea (in Turkey, Georgia, Abkhazia and Southern Russia). *Trisetacus abietis* are needle endoparasites that live under the needle epidermis, form large colonies consisting of hundreds of individuals, feed on needle mesophyll, and cause necrosis of leaf tissues. Damaged needles are brown and susceptible to falling (Fig. 6).

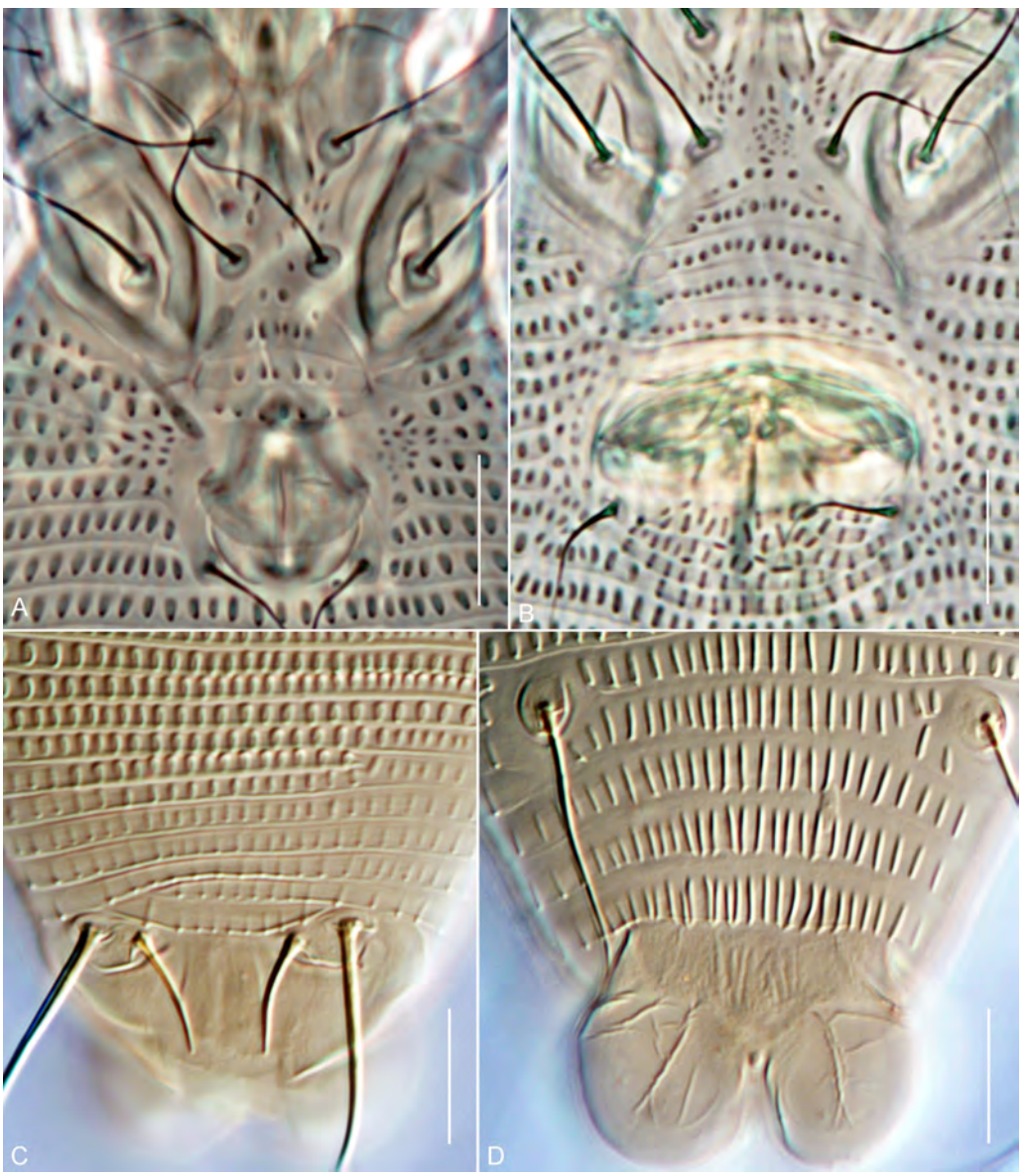

FIGURE 3. PC LM (A,B) and DIC LM (C,D) images of coxigenital area and caudal part of opisthosoma of *Trisetacus abietis* A—female coxigenital area, B—male coxigenital area, C—dorsal view of telosoma and anal lobes, D—ventral view of telosoma and anal lobes. Scale bar: A = 15 µm; B,C,D = 10 µm.

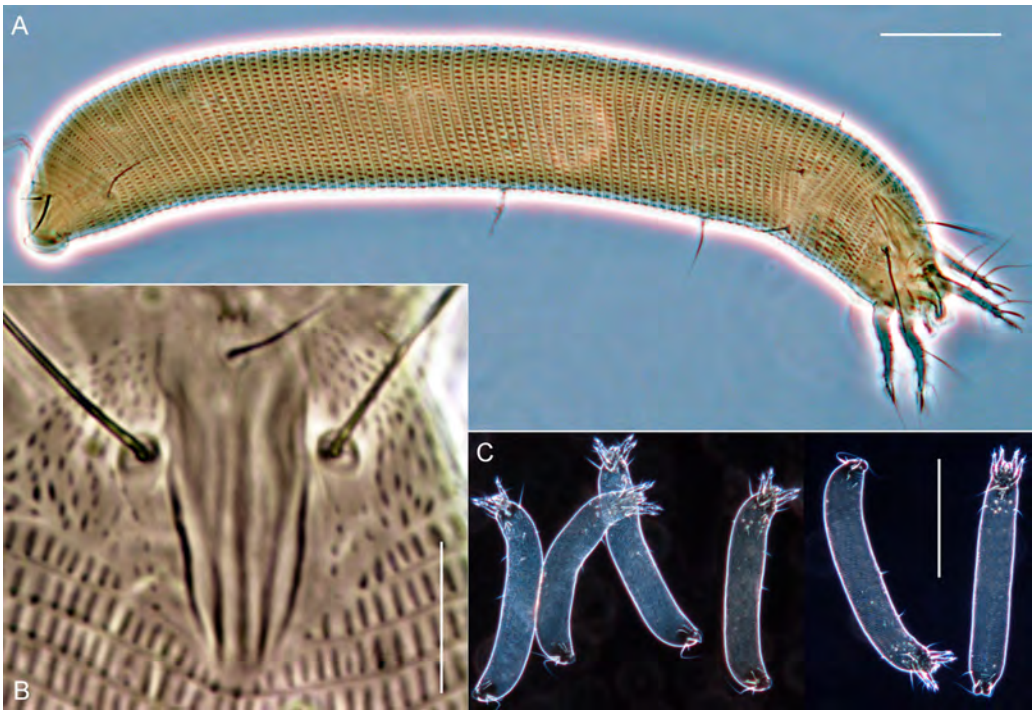

FIGURE 4. PC LM microphotographs of *Trisetacus abietis.* A—whole female in semilateral view, B—prodorsal shield, C—a group of mites in a slide (x20, pseudo dark-field). Scale bar: A = 40 µm, B = 10 µm, C = 150 µm.

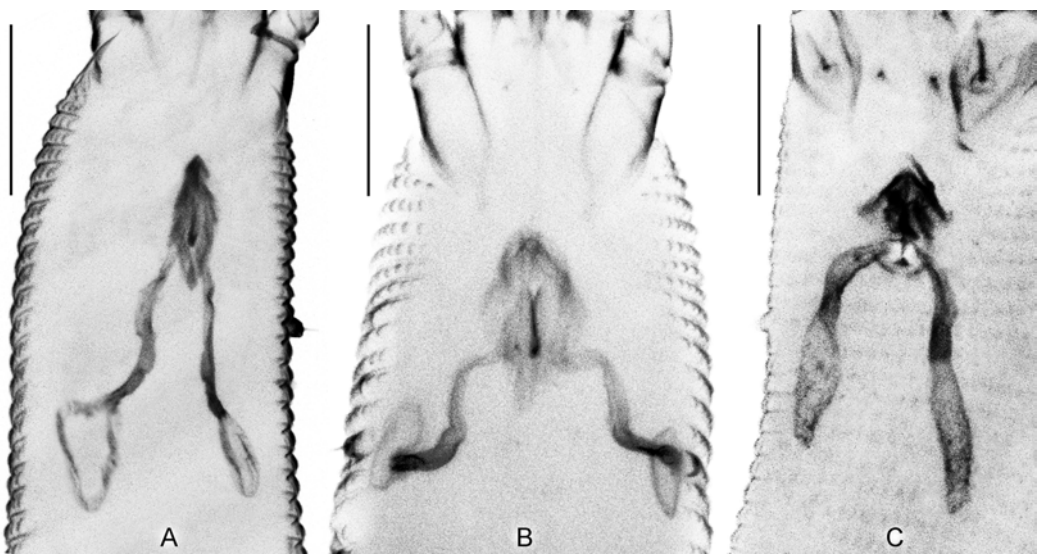

FIGURE 5. Internal genitalia in three females of *Trisetacus abietis* **n. sp.** (inverted black and white CLSM images). Scale bar = 20 µm. *Note*: spermathecae are deformed in Fig. 5 B &C; the shape of the left spermatheca in Fig. 5A is considered closest to the intact spermatheca.

Distribution

Trisetacus abietis was first recorded about 50 years ago in Germany inside needles of *A. alba* (Postner 1968). Since then, no records of this species have been published. In this paper we report

on a new finding of this species from Abkhazia (inside needles of *A. nordmanniana*). The distribution area of *T. abieties* may cover wider territories if this mite species is capable of infesting various *Abies* species native to the Mediterranean region.

Type material

Postner (1968) reported that the type material of *T. abietis* was deposited in "Sammlung des Instituts für angewandte Zoologie". This institute, which was part of the Ludwig-Maximilians-Universität München (LMU, University of Munich), has since been renamed as Biocenter LMU. The type material is now reported to be lost (Dr. Stefan Friedrich, Zoologische Staatssammlung München, ZSM, personal communication, 15 February 2019).

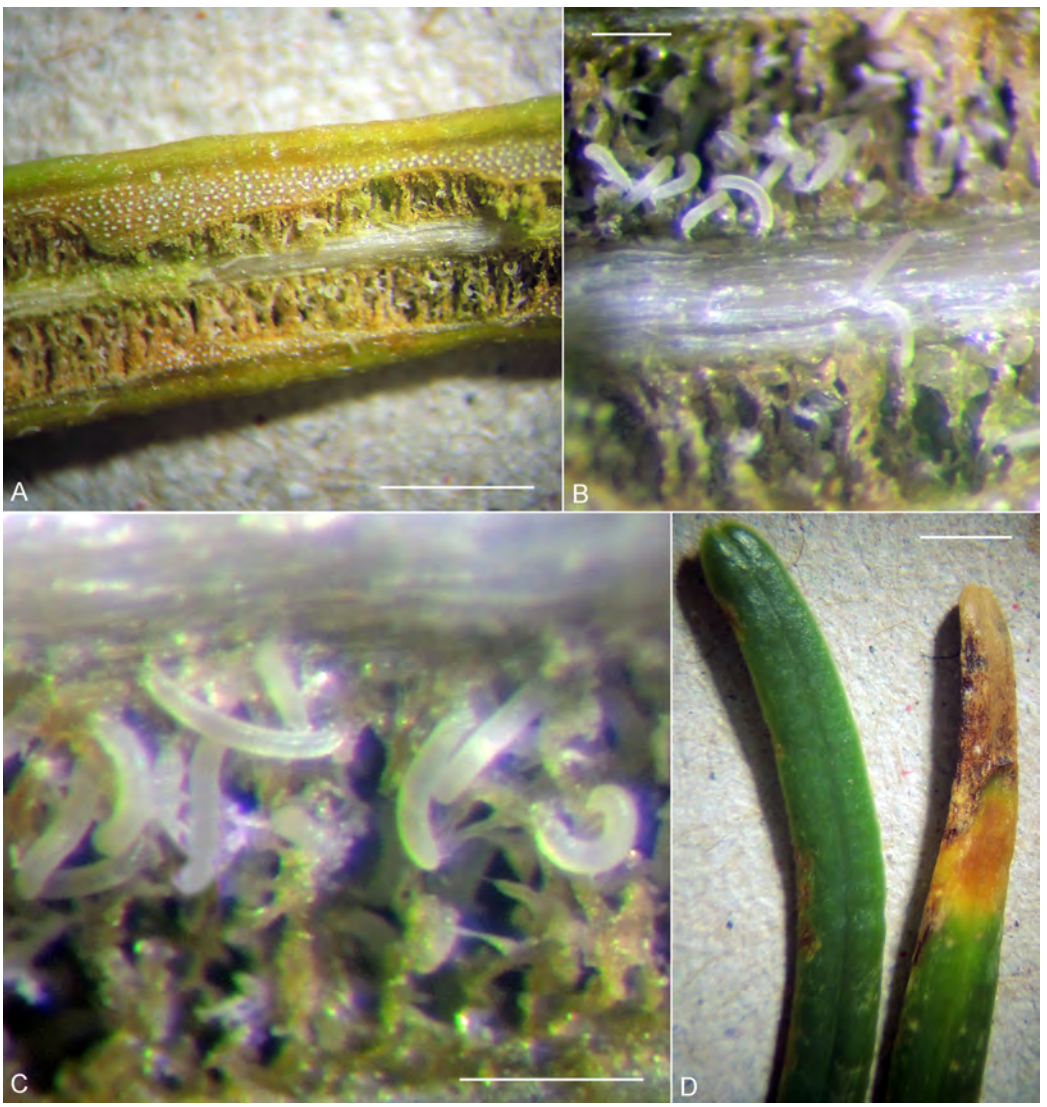

FIGURE 6. Damages on *Abies nordmanniana* caused by *Trisetacus abietis* (microphotographs). A—necrotizing brownish needle mesophyll inhabited by mites (ventral epidermis removed); B, C—mite colony within a needle; D—infested (on the right) and non-infested (on the left) needles. Scale bar: A = 1 mm; B, C = 300 μm; D = 2 mm.

FIGURE 7. World findings of *Trisetacus neoabietis* (1,2,3), *T. cedri* (4,5,6), and *T. abietis* (8,7). Map created with SimpleMappr (Shorthouse 2010) based on data from Nalepa (1920), Postner (1968), Smith (1984a,b), and original data; Southern hemisphere cropped. Notations: 1—*T. neoabietis* from *Abies amabilis* (Vancouver Island, Canada), 2—*T. neoabietis* from *A. balsamea* (Cape Breton Island, Canada), 3—*T. neoabietis* from *A. balsamea* (California, USA), 4—*T. cedri* from aboriginal *Cedrus atlantica* (Algeria), 5 and 6—*T. cedri* from introduced *C. deodara* (South Africa and Abkhazia), 7—*T. abietis* from *A. nordmanniana* (Abkhazia), 8—*T. abietis* from *A. alba* (Germany), 9—natural distribution area of *C. deodara* in India and Pakistan (based on data from The Gymnosperm database, *www.conifers.org*).

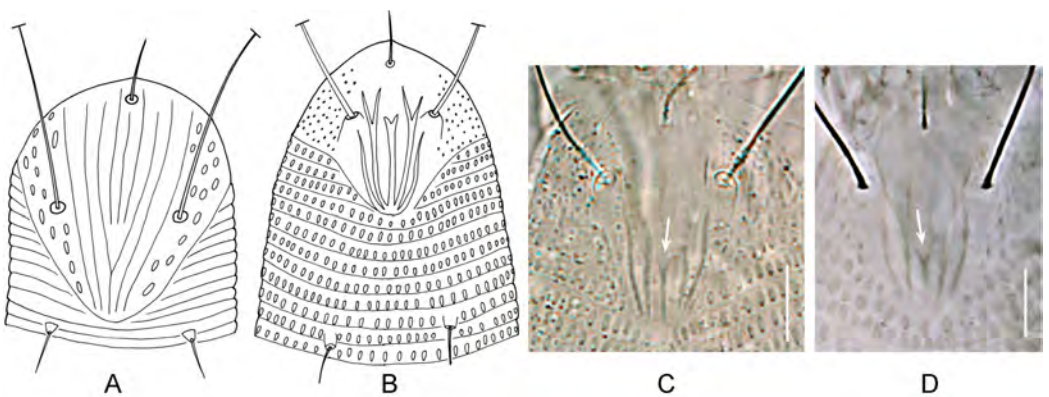

FIGURE 8. Prodorsal shields of two *Trisetacus* species from *Abies*. A—*T. abietis* (redrawn from Postner 1968); B—*T. neoabietis* (redrawn from Smith 1984a); C, D—PC LM images of prodorsal shields of two females of *T. neoabietis* used as comparative material (see for details in the section "Comparative material") from Canada (Fig. 8C, slide M790308T, courtesy of W. Knee and F. Beaulieu) and USA (Fig. 8D). *Note*: arrows in Fig. 8C and Fig. 8D indicate bifurcated median line. Scale bar: C,D = 8 μm.

Remarks

Trisetacus abietis is morphologically very close to the North American species *T. neoabietis* Smith 1984a. It is hard to find distinct characters discriminating these two species. Although data from the original descriptions suggests major differences between them in the ornamentation of the prodorsal shield (Fig. 8) and the number of empodial rays, careful examination shows that Postner's drawings and measurements of *T. abietis* are highly inadequate and do not provide a reliable basis

for comparison. Smith (1984a,b) believed that *T. abietis* and *T. neoabietis* differ in "degree of separation of the coxal plates" and number of empodial rays. Our data on *T. abietis* from Abkhazia indicate that these two species cannot be separated based on these characters. The North American specimens of *T. neoabietis* (see above in the section "Comparative material") were in poor condition; in most of them the ornamentation of the prodorsal shield was obscure. However, in some of them the median line of the prodorsal shield is short and forked anteriorly (Fig. 8 C, D arrows), whereas this line is notably longer and not forked in *T. abietis* from Abkhazia. It is not clear if these differences represent true morphological markers delimiting two species distributed in different continents (Europe and North America). *Trisetacus abietis* and *T. neoabietis* are associated with different *Abies* species which belong to ancient lineages of *Abies* that have been isolated phylogenetically and geographically since the Miocene or before (Semerikova *et al.* 2018). The taxonomic status and origin of *T. abietis* and *T. neoabietis* should be clarified by a further search for endoparasitic *Trisetacus* mites on conifers in the Holarctic, the redescription of *T. neoabietis,* and the application of species delimitation methods to molecular sequences of *Trisetacus*.

***Trisetacus cedri* (Nalepa 1920)—Fig. 9,10,11,12, Table 2.**
Eriophyes pini cedri Nalepa 1920:81; 1929:71

Supplementary description of *T. cedri.* Material was examined from buds of the same introduced conifer species (*Cedrus deodara*) from two remote localities (Abkhazia and South Africa; Fig. 7, green circles). In both localities, two morphotypes of females and males of *T. cedri* (herein called "long form", LF, and "short form", SF) were found. Below we give a description of these morphotypes based on the material from Abkhazia. Mites from the South African population were morphologically identical to the mites from Abkhazia, and therefore only barcode data for the sample from Africa is given.

"LONG FORM" FEMALE (n=12, Abkhazian population). Body vermiform, pale creamy, 370–450, 71–75 wide at the level of setae *c2*. **Prodorsal shield** subcordate, 30–33, 50–54 wide; frontal lobe absent. Prodorsal shield ornamentation weak. Median line very short, projects from posterior margin of prodorsal shield. Admedian lines thin, usually fragmented, forming a horseshoe-like figure. Submedian I arc-shaped, present only on the posterior half of the prodorsal shield. Short ridges and sparse microgranulations in lateral field of prodorsal shield. Group of 4–8 microtubercles present behind each tubercle of *sc*. Tiny longitudinal ridges or cuticular wrinkles present in central field of prodorsal shield. Prodorsal shield setae: *vi* 5–9, directed up and anteriad; *sc* 36–42, 28–32 apart, directed up and anterolaterad; distance between tubercles of *vi* and *sc* 16–18. **Gnathosoma** directed obliquely down and forward; palps 26–30; chelicerae 16–18, outer infracapitular stylets 12–14, oral stylet (n=3) angled, 6–9. Gnathosomal setae: seta *v* about 1; pedipalp genual seta *d* non-bifurcate, 8–10; pedipalp coxal seta *ep* 2–3. Suboral plate (formed by fused ventral palpcoxae) subcordate, smooth.

Leg I 28–33, tarsus 5–6, *u'* 2–3, *ft'* 14–18, *ft''* 18–24, *ω* 8–10 with tiny spherical knob; empodium 10–11, asymmetrical, usually 8/9-rayed, rarely 8/8 or 9/9, all rays except terminal pair with 2–3 subrays each; tibia 5–6, *l'* 4–6; *φ* 6–8; genu 4–5, *l''* 28–34; femur 9–11, *bv* 7–10. **Leg II** 25–30, tarsus 5–6, *u'* 2–3, *ft'* 9–12, *ft''* 17–25, *ω* 7–8 with tiny spherical knob; empodium 9–11, similar to empodium I; tibia 4–5; genu 3–4, *l''* 7–9; femur 8–10, *bv* 7–9. **Coxal plates** with longitudinal ridges; coxal setae *1b* 25–32, 16–18 apart; *1a* 50–65, 15–18 apart; *2a* 60–70, 35–43 apart. Prosternal apodeme indistinct; cuticle between tubercles of coxal setae *1a* with 4–5 tiny microtubercles; 2–3 incomplete coxigenital annuli before epigynium. **External genitalia.** Genital coverflap subtriangular, distally rounded, smooth, 13–17 long, 21–25 wide; setae *3a* 19–24, 15–18 apart; pregenital plate (*sensu* Flechtmann *et al*. 2015) absent. **Internal genitalia (n=5).** Spermathecae

large, ovoid, 12–15 long, 10–11 wide; spermathecal tubes recurved, 24–30 long, 2–4 wide, presence of median widening not apparent because in all studied specimens, spermathecal tubes were twisted and involute; thorn-shaped spermathecal process (*sensu* Duarte *et al.* 2016), typical for many members of Eriophyidae, absent; longitudinal bridge 9–10; anterior genital apodeme trapezoidal, distinct; oblique apodeme (*sensu* Chetverikov *et al.* 2015) absent; additional apodemes forming rhomboid figure and strengthening longitudinal bridge clearly seen under genital cuticle.

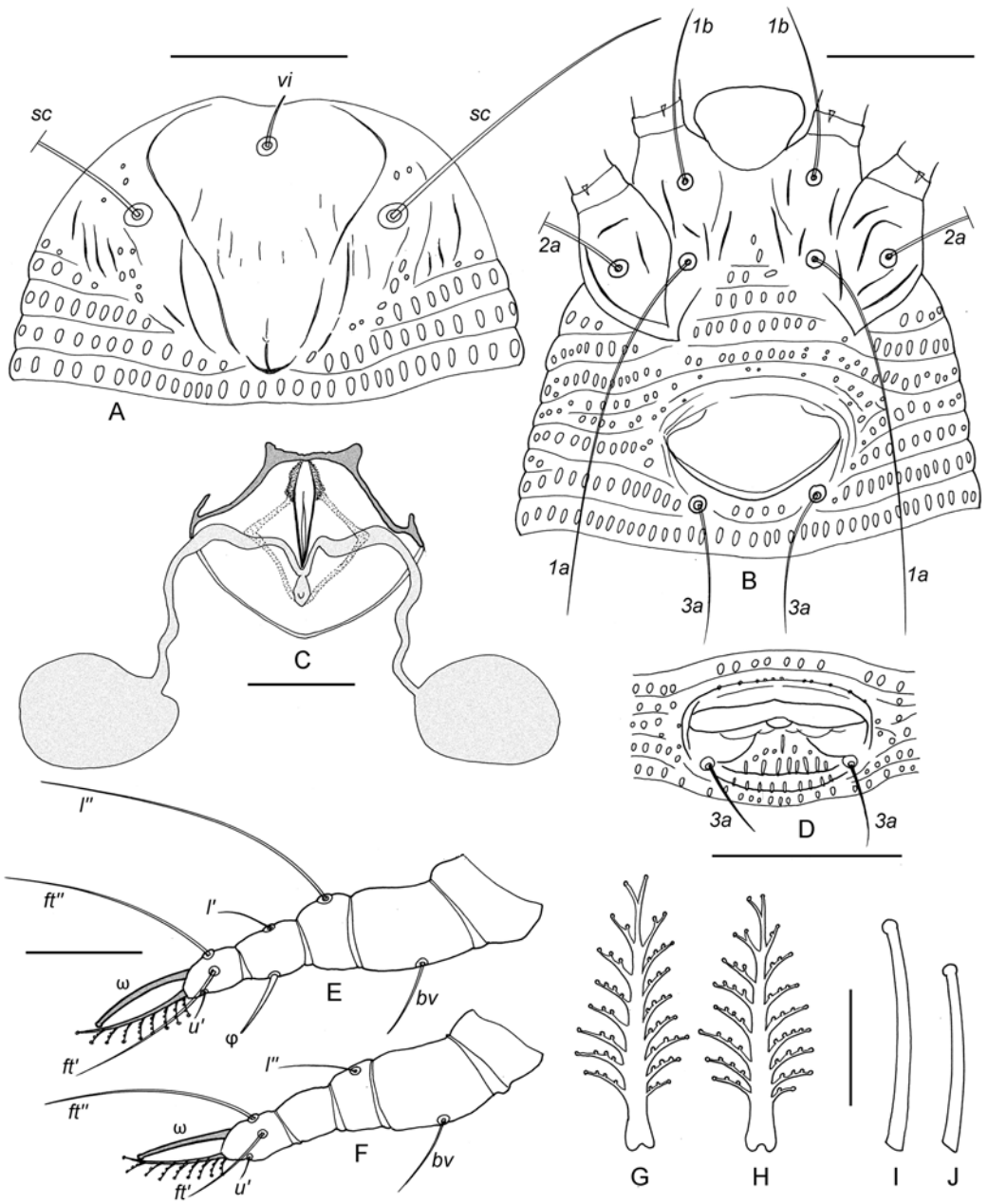

FIGURE 9. Drawings of long form female (A–C, E–J) and male (D) *Trisetacus cedri* (Nalepa 1920). A—prodorsal shield, B—coxigenital area, C—female internal genitalia, D—male external genitalia, E—leg I, F—leg II, G—empodium I, H—empodium II, I—tarsal solenidion I, J—tarsal solenidion II. Scale bar: A, B = 20 μm; C = 10 μm; D = 25 μm; E–J = 10 μm.

Opisthosoma dorsally with 79–87 annuli, ventrally with 70–81 annuli between posterior margin of coxae II and caudal lobes; dorsal and ventral annuli bear distinct oval microtubercles; microtubercles on ventral telosomal annuli elongate. Setal lengths: *c1* 8–10, *c2* 53–68, *d* 22–29, *e* 21–26, *f* 39–46; *h1* 12–15; *h2* 82–101; 8–9 annuli from rear shield margin to *c1*; 9–11 annuli from rear shield margin to *c2*; 10–12 annuli between *c2–d*; 17–19 annuli between *d* and *e*; 30–34 annuli between *e* and *f*; 4–5 annuli between *f* and *h2*.

"LONG FORM" MALE (n=10)

Body 305–330, 72–79 wide. Leg I 27–33, leg II 26–30, empodia 7/8-rayed in all studied specimens. Opisthosoma with 60–68 dorsal and 61–69 ventral annuli. Genital area 10–12 long, 23–25 wide; setae *3a* 10–13, 18–20 apart. Post-genital region (situated between tubercles of *3a*, delimited anteriorly by genital opening and posteriorly by an arch-shaped microtuberculate semi-annulus at the level of tubercles of *3a*) with irregularly distributed microtubercles; eugenital setae absent.

"SHORT FORM" FEMALES (n=8) & MALES (n=9)

In comparison to long form adults, the short form (SF) females and males are notably smaller, with fewer empodial rays and opisthosomal annuli, and similarly shaped ornamentation of the prodorsal shield (Table 2). Morphologically, SF adults from Abkhazian population fit the measurements from the brief original description of *T. cedri* from Algeria given by Nalepa (1920). However, Abkhazian SF are slightly longer and notably wider, which may be explained by differences in the slide mounting methodology. Nalepa (1920) also reported that he rarely observed notably larger males (210 long, 54 wide), which we consider putative members of the LF, in the Algerian population.

TABLE 2. Morphological differences between long and short form adults of *Trisetacus cedri* from Abkhazia (original data, ranges are given) and Algeria (data from Nalepa 1920).

Characters	Abkhazian population				Algerian population (Nalepa 1920)	
	Long form		Short form		Putative short form	
	Females (n=12)	Males (n=10)	Females (n=8)	Males (n=9)	Female (n=unknown)	Male (n=unknown)
Length of body	370–450	303–330	278–317	204–249	180	170
Width of body	71–75	72–79	62–67	59–65	37	47
Number of empodial rays	8/9, rarely 9/9 or 8/8	8/7	8/7 and 8/8	6/7	no data	no data
Number of dorsal annuli	79–87	60–68	68–74	56–63	about 62	no data
Number of ventral annuli	70–81	61–69	72–79	59–64	no data	no data
Host plant	Inside buds of *Cedrus deodara*				Inside buds of *Cedrus atlantica*	

Host plant and relation to host

Mites live inside buds of *Cedrus deodara* (Roxb. ex D. Don) G. Don (Pinaceae), causing their enlargement, partial necrosis of internal tissues and death of buds (Fig. 13).

Material examined

Adults and immatures in slide series E4498 and E4499 collected in ABKHAZIA: Tzandripsh, City Park, 08 July 2018, coll. G. Yu. Konechnaya; adults and immatures in slides E4550, E4551, and E4552 collected in SOUTH AFRICA: NW Province, Buffelspoort, 10 km WSW of Buffelspoort Dam, Barlett Farm, 25°50′00.7″S 27°23′53.4″E, 10 October 2018, coll. S. Neser. Material has been deposited in Acarological collection of ZIN RAS, Saint-Petersburg, Russia.

Remarks

Up to now, *T. cedri* is known from three localities (Fig. 7). It was first collected by R. Maire from buds of aboriginal *Cedrus atlantica* in Atlas de Blidah (Algeria) in 1913, transferred by Mr. C. Houard to Austria, and described by A. Nalepa in 1920. Since then, no records of this mite species have been published. We found *T. cedri* in buds of another cedar species, *C. deodara*, Himalayan cedar, in Abkhazia and South Africa. In both localities, *C. deodara* is not native. In Russia it appeared first in 1842 when it was introduced in Crimea in Nikitsky Botanical Garden (Gulisashvili 1959, p. 129), from where it could later be transferred to Abkhazia. However, it could be that it was brought to Abkhazia directly from Asia in the first third of the 20th century by Dr. V.V. Markovich (1865–1942), when he was a director of the Sukhumi Botanical Garden in Abkhazia and was responsible for plant introduction in the Southern part of the former USSR (Dr. I.G. Chuhina, Vavilov Institute of Plant Industry, Russia, personal communication, May 2019). The South African introduction of *C. deodara* most probably came from a nursery or a botanical garden of England, where it had arrived during the 19th century from the natural distribution area of *C. deodara* in India or Pakistan.

FIGURE 10. DIC microphotographs of *Trisetacus cedri* (female). A—prodorsal shield; B—tarsal appendages; C—dorsal view of rear part of opisthosoma; D—anal lobe and telosoma (ventral view). Scale bar: A = 10 µm; B = 5 µm; C, D = 15 µm.

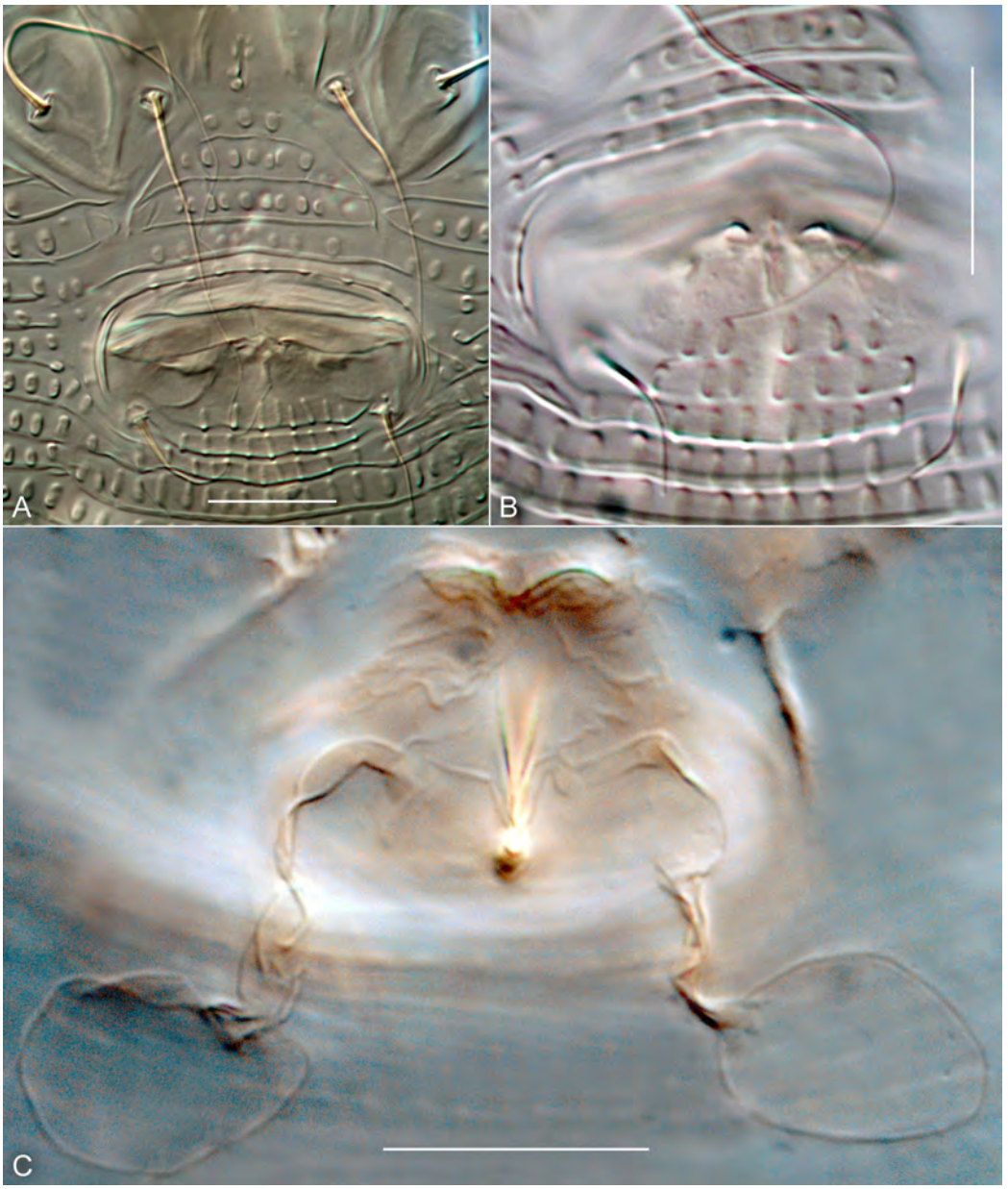

FIGURE 11. DIC images of genital structures of *Trisetacus cedri*. A,B—male external genitalia; C—female internal genitalia. Scale bar: A, B = 10 µm; C = 15 µm.

According to Farjon & Filer (2013) and The Gymnosperm database (*www.conifers.org*), the genus *Cedrus* includes four highly geographically isolated species: *C. atlantica* (Endl.) G.Manetti ex Carrière (grows in Atlas Mountains of Algeria and Marocco, Northn Africa), *C. brevifolia* Elwes et Henry (Troodos Mountains, Cyprus), *C. libani* A. Rich. (Lebanon Mountains, Syria and Southern Turkey), and *C. deodara* (Himalayas). Fossil records suggest that the genus *Cedrus* originated in the high latitude area of Eurasia by the Early Paleocene (Qiao *et al.* 2007). Later, *Cedrus* migrated to Europe and North Africa, where it diverged into several species as a result of vicariance caused by climate oscillations (Farjon & Filer 2013). A molecular phylogenetic study resulted in the following

tree: *C. deodara* (*C. atlantica* (*C. brevifolia* + *C. libani*)) (Qiao *et al.* 2007). The same study estimated the time of divergence between *C. brevifolia* and *C. libani* as 6.56±1.2 mya and between *C. atlantica* and *C. brevifolia* + *C. libani* as 18.81 ± 1.25 mya.

It could be hypothesized that the distribution of *T. cedri* coincides with that of the genus *Cedrus* if the host plant and its pest mites have coexisted for a long period. However, the results of our recent field surveys contradict this hypothesis: in 2018 and 2019 we sampled *C. libani* in Turkey (natural cedar forest near village Goltarla, Antalya region, 36°33′25.8″N 29°57′03.7″E) and *C. brevifolia* in Cyprus (Troodos Mountains, Cedar valley, 34°59′30.5″N 32°41′17.7″E); about 100 buds from about 20 trees of *C. libanoni* and *C. brevifolia* were examined, and no *Trisetacus* mites were found. Therefore, the estimation of the natural distribution of *T. cedri* remains problematic and a further search for bud mites on *Cedrus* spp. is necessary to determine the distribution and origin of *T. cedri*.

GenBank data

MN022222 and MN025334 (Abkhazian population); MN022223 and MN025335 (South African population).

Remarks

Sequences of D1–D2 28S of *T. cedri* from Abkhazian and South African populations were 100% identical. Sequences of COI from different populations differ in only one synonymous substitution (AAC in Abkhazian sample vs AAT in South African sample, asparagine). A blast search of the COI sequence (South African population) showed the highest similarity with sequences KY922366.1 (*Trisetacus piceae*, 100% cover, 84.16% identity) and KY922367.1 (*Trisetacus pini*, 99% cover, 83.56% identity). A blast search for D1–D2 28S showed the highest values for sequences of the same two *Trisetacus* species: KY921990.1 (*T. piceae*, 99% cover, 94.12% identity) and KY921991.1 (*T. pini*, 99% cover, 93.87% identity).

FIGURE 12. DIC images of long and short forms of *Trisetacus cedri* from buds of *Cedrus deodara* introduced in Abkhazia.

Type material

Type material of *T. cedri* is probably lost, but topotypes can be found in the Nalepa collection of the Natural History Museum (Vienna Austria). According to Chetverikov *et al.* (2016), two vials (#742 and #742a) from box C1 contain remnants of damaged buds of *Cedrus atlantica* collected in 1914 from an unknown locality (possibly from the type locality in Algeria). In November 2014 we briefly examined part of the material from these vials and did not find good mite specimens for morphological investigation. But still, this material may provide a source for recovering topotypes and designating a neotype.

FIGURE 13. Damages caused by *Trisetacus cedri* on *Cedrus deodara* introduced in Pretoria, South Africa. A—infested bud (cut open), showing partial tissue necrosis and dead new leaves; B—a group of mites inside the bud (same view as Fig. A, enlarged); C—shoot with non-infested axillary bud (above) and short side shoot with first infested bud from which a new short shoot grew with its infested terminal bud; D—non-infested axillary bud (below), and enlarged, infested bud on short side shoot (above); E—same as C, enlarged bud with flared bracts and dead new leaves; F—dying, pendulous shoot with infested buds on short side shoots; G—non-infested young buds with appressed bracts on young shoot. Scale bars: A,C,D,E,F,G = 2 mm; B = 200 µm.

Emendation to differential diagnosis

The morphology of *T. cedri* closely resembles that of several *Trisetacus* species from North American and European Pinaceae. It is close to *T. grosmanni* Keifer 1959, which typically inhabits the buds of *Abies* spp. in North America (Smith 1984b). However, *T. grosmanni* has sharp and conical tubercles lining its opisthosomal annuli, whereas *T. cedri* has rounded tubercles. *T. cedri* also resembles *T. ucluelentensis* Smith 1984a, known from foliage of *Picea sitchensis* (Bong) from Canada, but in *T. ucluelentensis* the lateral fields of prodorsal shield are smooth whereas in *T. cedri* the lateral fields are ornamented with short ridges and sparse microgranulations. *T. cedri* is also similar to *T. halepensis* Castagnoli 1973, described from Italy from needle bases of *Pinus halepensis* Mill., but in *T. halepensis* the admedian lines of the prodorsal shield are shorter, forming a V-shaped figure, and no microtubercles are situated behind the *sc* tubercles, whereas in *T. cedri* the admedians are longer, form a horseshoe-like figure, and a group of 4–8 microtubercles is present behind each *sc* tubercle.

Discussion

Dimorphism in Trisetacus abietis and T. cedri

A remarkable sexual dimorphism was found in *T. abietis*. Females of this species have long, asymmetrical 8/7-rayed empodia, whereas males have shorter, symmetrical 6/6-rayed empodia and shorter solenidia ω I. The same differences in the shape and size of empodia and solenidia are known in *Novophytoptus*, endoparasitic mites living in air cavities under the epidermis of herbaceous monocots of the order Poales (Chetverikov 2015; Chetverikov & Petanović 2016a). Empodia and solenidia ω I are both tarsal appendages that contact the surrounding surfaces when a mite moves through or penetrates plant tissue. We do not have data on the biology of *T. abietis*, but our observations on *Novophytoptus* behavior suggest that in enodoparasitic phytoptids, males can be found only in subepidermal tissues where the mites reproduce. Females can also be found on the plant surface when they migrate and search for new sites to penetrate beneath the epidermis (Chetverikov & Petanović 2016a). Therefore, males possibly do not move far from the place where they hatched from the egg stage, and so they do not need to channel or squeeze through the epidermis (like migrating females have to do). This may be the reason why the males of *T. abietis* have less developed apical tarsal appendages than females.

Two forms of males and females were found in *T. cedri*. These forms differ in body length, hence the terms “long form” (LF) and “short form” (SF). Such bisexual dimorphism was described before only in two *Trisetacus* species, *T. kirghisorum* Shevchenko 1962 and *T. piceae* Roivainen 1951 (De Millo 1967; Shevchenko & De-Millo 1968; Bagnyuk 1976), and it was recently also detected in the putatively relictual genus *Pentasetacus* Schliesske (Chetverikov *et al.* 2019). The presence of LF and SF in one mite population suggests a complex life cycle with morphologically different seasonal generations of mites, the phenomenon known as deuterogeny (Putman 1939, Keifer 1942). Although it is more common that only dimorphic females are present in a complex eriophyoid life cycle (Hall 1967, Manson & Oldfield 1996), we predict more examples of bisexually dimorphic species will be discovered in Eriophyoidea, especially among phytoptids. Such data would be important for testing the hypothesis on the complexity of the ancestral life cycle in Eriophyoidea, which was previously suggested based on observations of a laboratory population of pentasetacids (Chetverikov & Petanović 2016b).

Remarks on the evolution of Trisetacus

Molecular phylogenetics suggest that the divergence between the main lineages of *Cedrus* (hosts of *T. cedri*) happened about 20 mya (Qiao *et al*. 2007), and the major groups of *Abies* (hosts

of morphologically close species *T. abietis* and *T. neoabietis*) originated during the Miocene and Pliocene; the crown group of *Abies* diversified approximately 14–16 mya (Semerikova *et al*. 2018, p. 21). These dates may serve as rough estimates for divergence times within *Trisetacus* lineages associated with the pinacean genera *Abies* and *Cedrus*. In two recent molecular phylogenetic studies, the divergence of *Trisetacus* species was also estimated to have arisen during the Miocene (Li *et al*. 2016; Skoracka *et al*. 2018), which is in accordance with the aforementioned divergence of *Cedrus* and *Abies*. Remarkably, the divergence between the main lineages of "*Aceria tosichella* Keifer", a complex of cryptic eriophyoid species from grasses, also arose during the Miocene (Skoracka *et al*. 2018). Paradoxically, the cryptic species from the "*A. tosichella*" complex are more morphologically homogenous and inhabit shorter lived hosts (grasses *vs* conifers) than *Trisetacus*. It has been shown that rates of evolution are slower in trees and shrubs than in herbs (Smith & Donoghue 2008), and this may also be true for eriophyoids associated with arborous *vs* herbaceous plants (Boczek & Shevchenko 1996). The morphological homogeneity of the "*A. tosichella*" complex could be explained by the phenomenon known as "morphological stasis" (Lidgard & Hopkins 2015), but it is not clear why "*A. tosichella*" appears to be evolving so slowly compared to *Trisetacus*. This question could be answered by well-resolved, time-calibrated phylogenies of *Trisetacus* and associated conifers, allowing the evolution of morphological characters to be traced on the co-phylogenies.

Acknowledgements

DNA extraction, PCR, conventional light microscopy and comparative morphology were performed by PEC, PGE, and MSB and were supported by the Russian Foundation for Basic Research (RFBR grant # 19-04-00127 A) and ZIN RAS (project AAAA-A19-119020790133-6). These procedures were undertaken at the Laboratory of Parasitology of ZIN RAS. Sequencing and confocal microscopy studies were performed by PEC and SSP and were partially supported by the Russian Science Foundation (RSF grant 16-16-10011) and were carried out using the equipment of the "Development of Molecular and Cellular Technologies" Resource Center (project #28463067) and "Center for Microscopy and Microanalysis" at St. Petersburg State University (project #39273635). We are grateful to Drs F. Beaulieu and W. Knee (Agriculture and Agri-Food Canada) for the microphotographs of *T. neoabietis,* to Dr. G. Konechnaya (Komarov Botanical Institute of the Russian Academy of Sciences) for collecting mites from conifers in Abkhazia, and to Dr. Alexey Masharskiy (Saint-Petersburg State University) for their assistance in sequencing and PCR optimization. PBK was supported by Russian Science Foundation (RSF grant 19-14-00004). We also thank the Florida Department of Agriculture and Consumer Services – Division of Plant Industry for supporting author SB.

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Submitted: 6 Jun. 2019; accepted by Xiao-Yue Hong: 23 Jul. 2019; published: 13 sep. 2019