Insect Systematics and Diversity, (2021) 5(1): 3; 1–12
doi: 10.1093/isd/ixab002
Research

Methods

Cuticular Hydrocarbon Profile Analyses Help Clarify the Species Identity of Dry-Mounted Cuckoo Wasps (Hymenoptera: Chrysididae), Including Type Material, and Reveal Evidence for a Cryptic Species

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Subject Editor: Elizabeth Jockusch

Received 26 August 2020; Editorial decision 14 December 2020

Abstract

Cuckoo wasps of the *Chrysis ignita* species group are difficult to identify at the species level, and the taxonomic status of various taxa has consequently been controversial. COI barcoding has helped clarify some of the taxonomic problems in this group, but also revealed cryptic diversity at the genetic level that remained difficult to interpret taxonomically. Here we show that analysis of cuticular hydrocarbons (CHCs) clarifies the taxonomic status of cuckoo wasp samples with distinct COI haplotypes. The advantages of studying CHCs in insects for taxonomic purposes reside on the fact that CHC profiles evolve quickly and that all proteins required for CHC biosynthesis are encoded by nuclear genes. Using *Chrysis pseudobrevitarsis* as an example, we show that COI barcoding in combination with analysis of CHCs extracted from freshly collected and from dry-mounted museum specimens (including the lectotype of *C. pseudobrevitarsis*) provides clear evidence for a separate taxon among samples which were previously considered to be conspecific with *C. pseudobrevitarsis*. We describe this taxon as *Chrysis parabrevitarsis* n. sp. and present characters for distinguishing it chemically, genetically, and morphologically (females only) from *C. pseudobrevitarsis*. CHC profile comparison suggests females of *C. pseudobrevitarsis* may chemically mimic females of the vespid wasp *Euodynerus notatus*. Our study demonstrates the value of CHC analyses for supporting taxonomic inferences based on COI barcodes. It additionally underlines the value of dry-mounted collection specimens for chemical analyses and the potential of CHCs for inferring the identity of museum specimens, including type material, in a morphologically noninvasive manner.

Key words: chemical ecology, cryptic diversity, cuticular hydrocarbon, integrative taxonomy

Cuckoo wasps represent a medium-sized family (ca. 3,000 species; Rosa et al. 2017) of Hymenoptera. All of its species seem to have kleptoparasitic or parasitoid life-styles (Kimsey and Bohart 1991). Intriguingly, the species in some of the family's species groups are morphologically very similar to each other and seem to differ primarily ecologically by exploiting different host species (Orlovskytė et al. 2016). The *Chrysis ignita* (Linnaeus,1758) (Hymenoptera: Chrysididae) group is an example of a species group that comprises morphologically very similar taxa that are difficult to separate from each other even by an expert (e.g., Niehuis 2000, Paukkunen et al. 2015, Orlovskytė et al. 2016) (Fig. 1). Recently, phylogenetic analysis of cytochrome c oxidase I (COI) barcodes of species of the *Chrysis ignita* group helped resolve taxonomic controversies regarding European species in this group, but these studies also revealed genetic diversity that is difficult to interpret taxonomically (Soon et al. 2014, Orlovskytė et al. 2016).

While DNA barcoding of the mitochondrial gene COI can help clarify the status of species, especially when distinct COI haplotype groups with large genetic distance correlate with non-mitochondrially inherited traits, it also has a number of downsides, most notably the fact that distinct COI haplotypes are neither necessary nor sufficient to indicate reproductive isolation. In particular,

Version of Record, first published online February 20, 2021 with fixed content and layout in compliance with Art. 8.1.3.2 ICZN.

Fig. 1. Adult female paratype of the cuckoo wasp *Chrysis parabrevitarsis* n. sp. (Germany, Rhineland-Palatinate, Bellheim, 10 June 2012; voucher ID: ZFMK-TIS-36479), Photograph: O. Niehuis.

the circumstance that mitochondrial genes are almost always maternally transmitted compromises attempts to assess reproductive isolation between groups of individuals. For this and other reasons, an extension of the current barcoding system by including multiple single-copy nuclear genes has been suggested (Eberle et al. 2020). Specifically, the authors recommend extending the current barcoding system with a set of nearly universal single-copy nuclear protein-coding genes (USCOs) due to properties of these genes that make them superior over alternative markers. While consideration of USCOs is desirable for clarifying the taxonomic status of potential cryptic species that are otherwise only characterized by unique COI barcode sequences, their analysis is sometimes impossible or at least difficult to achieve. We here apply an alternative method in conjunction with COI barcoding for detecting cryptic species: the analysis of cuticular hydrocarbons (CHCs) via gas-chromatography coupled with mass-spectrometry (GC-MS).

CHCs are long-chain molecules found on the surface of all hexapods (and some other arthropods; Blomquist and Bagnères 2010, Bien et al. 2019). Almost all species investigated so far differ from each other in their CHC profiles (Blomquist and Bagnères 2010), making the analysis of CHCs a promising approach for delineating and identifying even closely related species, a fact that has been demonstrated in various chemotaxonomy studies (Haverty et al. 1990, Page et al. 1997, Akino et al. 2002, Lucas et al. 2002, Schlick-Steiner et al. 2006, Martin et al. 2008, Bagnères and Wicker-Thomas 2010, Guillem et al. 2012, Vaníčková et al. 2014). CHCs are particularly attractive for species delineation, as the proteins required for their biosynthesis are exclusively encoded by nuclear genes (Wicker-Thomas and Chertemps 2010). Finally, CHCs are known to function as semiochemicals (i.e., chemical compounds that transmit information between individuals) and often serve as sex pheromones in mate recognition (Blomquist and Bagnères 2010). However, CHC profiles can be phenotypically plastic (e.g., alter with the insect's food; Liang and Silverman 2000) and intra-specifically polymorphic (Wurdack et al. 2015). CHC profile differences alone are thus—as COI barcode differences—inconclusive to assess reproductive isolation. Simultaneous analysis of COI haplotypes and CHC profiles can be conclusive, because co-segregation of these genetically unlinked traits in a (partially) sympatric situation is most readily explained by reproductive isolation between the investigated groups of individuals (i.e., species). Intriguingly, the analysis of CHC profiles of cryptic cuckoo wasp species has additionally the potential to narrow down the range of historically exploited hosts, because the CHCs of some hosts have been shown to be chemically mimicked by their brood parasitic cuckoo wasps, which thereby escape chemical detection by their host (Strohm et al. 2008, Wurdack et al. 2015). Because GC-MS analysis of CHCs is morphologically non-destructive and is applicable to collection specimens (Martin et al. 2009), it even opens the door to infer the identity of old type material.

Chrysis pseudobrevitarsis Linsenmaier, 1951 (Hymenoptera: Chrysididae) is a trans-Palearctic species of cuckoo wasp, which occurs from Western Europe to Mongolia (Linsenmaier 1997) and is locally common in Central and Northern Europe. A previous study found that samples assigned to this species carry remarkably diverged COI haplotypes (5.2% divergence), with some samples exhibiting a higher similarity (and phylogenetic relationship) to COI haplotypes of other species of the *Chrysis ignita* species group. Soon et al. (2014) consequently speculated that multiple species could have been lumped under the species name *Chrysis pseudobrevitarsis*. However, no morphological trait had been discovered that would have justified concluding that the two haplotypes belong to two biological species (i.e., groups of individuals that are reproductively isolated from each other).

Using the example of *Chrysis pseudobrevitarsis*, we provide the following in the present study: 1) A thorough analysis of COI barcodes and of CHCs extracted from freshly collected as well as from dry-mounted specimens provides evidence for two reproductively isolated taxa among samples previously considered *C. pseudobrevitarsis*; 2) By analyzing the CHC profile of the more than 70-yr-old lectotype of *Chrysis pseudobrevitarsis*, we demonstrate the value of dry-mounted collection specimens for chemical analyses and the potential of CHCs to infer the identity even of old museum specimens (including type material) in a morphologically noninvasive manner; 3) A description of the discovered cryptic species, which we name *Chrysis parabrevitarsis* n. sp.; 4) An outline of chemical, genetic, and morphological (females only) characters by which the two species can be distinguished; and 5) A detailed comparison of the CHC profiles of the two species under consideration with those of reported potential host species.

Material and Methods

Analyzed Samples

We studied the morphology of 107 (17 ♂♂, 90 ♀♀) samples (and COI barcodes of 50 of these; see below) hitherto identified as *C. pseudobrevitarsis* and deposited in the following public or private collections (details given further below): Finnish Museum of Natural History (MZH; University of Helsinki, Helsinki, Finland), NTNU, University Museum (NTNU Trondheim, Norway), Natur-Museum (NMLS; Luzern, Switzerland), private collection of Niklas Johansson (NJ; Habo, Baskarp, Sweden), private collection Oliver Niehuis (ON; Freiburg i. Br., Germany), private collection Paolo Rosa (PR; Bernareggio, Italy), private collection Stefan Tischendorf (TS; Darmstadt, Germany), Natural History Museum of the University of Tartu (TUZ; Tartu, Estonia), Zoological Research Museum Alexander Koenig (ZFMK; Bonn, Germany), and Bavarian State Collection of Zoology (ZSM; Munich, Germany).

To assess whether samples with different COI haplotypes represent reproductively isolated species, we studied the CHCs of 48 (12 ♂♂, 36 ♀♀) samples hitherto identified as *C. pseudobrevitarsis* (28 of which were barcoded). Specifically, we studied the CHCs of 31 samples (6 ♂♂, 25 ♀♀) belonging to *Chrysis parabrevitarsis* n. sp. and the CHCs of 17 samples (6 ♂♂, 11 ♀♀) belonging to *Chrysis pseudobrevitarsis*. To assess whether or not females of either of these species chemically mimic the CHC profile of their hosts, we additionally studied the CHCs of the vespid wasps *Ancistrocerus antilope* (Panzer, 1798) (Hymenoptera: Vespidae)

(23 ♂♂, 17 ♀♀), *Euodynerus notatus* (Jurine, 1807) (Hymenoptera: Vespidae) (13 ♂♂, 11 ♀♀), and *Euodynerus quadrifasciatus* (Fabricius,1793) (Hymenoptera: Vespidae) (14 ♂♂, 14 ♀♀), which have been reported as hosts of *Chrysis pseudobrevitarsis* in the wide sense (Pauli et al. 2019). The sampling locations of all wasps whose CHCs we investigated and related information are given in Supp File 1 (online only).

COI Barcoding

To infer the identity of samples hitherto identified as *C. pseudobrevitarsis*, we applied a COI barcoding approach. For this purpose, we extracted DNA from mesosoma or leg muscle tissue using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) or the Quick-DNA Miniprep Plus Kit (Zymo Research Corporation, Irvine, CA). We subsequently PCR-amplified a section of the mitochondrial gene cytochrome c oxidase subunit I (COI) with the length of ca. 650 bp. PCRs were performed in 20-µl reaction volumes, using the Multiplex PCR Kit by Qiagen and applying the oligonucleotides LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and Nancy (5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′) (Folmer et al. 1994, Simon et al. 2006) or LCO1490-JJ (5′-CHA CWA AYC ATA AAG ATA TYG G-3′) and HCO2198-JJ (5′-AWA CTT CVG GRT GVC CAA ARA ATC A-3′) (Astrin and Stüben 2008) to prime the reactions. We applied the PCR temperature profile provided by Pauli et al. (2019). All PCR products were cleaned with the Illustra ExoProStar Kit (GE Healthcare Life Sciences, Garching, Germany) and then sent for direct, bidirectional Sanger sequencing to Macrogen (Amsterdam, The Netherlands). Forward and reverse strands were assembled, and the resulting contigs were trimmed (i.e., the oligonucleotide primer binding sites were removed) and, if necessary, manually corrected with the software Geneious version 10.2.3 (Kearse et al. 2012). We used the same software to manually align the contigs at the translational level, applying the genetic code for invertebrate mitochondrial DNA. All nucleotide sequences are deposited at GenBank (accession numbers MT888190–MT888216). We additionally included in our analysis the COI barcodes of samples barcoded by the Canadian Centre for DNA Barcoding (Ivanova et al. 2006), or published by Pauli et al. (2019), or inferred by us using the methods described by Soon et al. (2014) (GenBank accession or BOLDSystems sequence ID numbers ACUFI499-13, ACUFI501-13, ACUFI502-13, GBACU2482-13, GBACU2485-13, GBACU2488-13, JX292218, JX292232, JX292239, JX292248, KJ398900, KJ398927, KY430768, LEFIJ6109-17–LEFIJ6118-17, NOCHR073-13). COI haplotype divergence was calculated with the software MEGA version 10.1 (Kumar et al. 2018) applying the Kimura two-parameter (K2P) model (Kimura 1980). We visualized COI haplotype distances by inferring a neighbor joining (NJ) tree, in which we also included COI barcode sequences of *Chrysis brevitarsis* Thomson, 1870, a species closely related to *C. pseudobrevitarsis* (Soon et al. 2014), downloaded from GenBank and Boldsystems Public data portal (accession or sequence ID numbers ACUFI466-12, JX292241, KU854911).

Chemical Analysis

We extracted the CHCs of samples by submerging each of them for 10 min in n-hexane (SupraSolv, Merck, Darmstadt, Germany). The applied extraction volume depended on the size of the studied sample and was chosen to ensure complete submersion of the whole specimen in the solvent. The CHC extracts were subsequently concentrated to ca. 75 µl by evaporating the surplus hexane with a steady stream of CO_2 or N_2. CHC extracts (1 µl per sample) processed in the Niehuis lab were analyzed with an Agilent 7890B gas chromatograph (GC) coupled to an Agilent 5977B quadrupole mass spectrometer (MS; Agilent Technologies Inc., Santa Clara, CA), which was run at 70 eV. The GC was fitted with a DB-5MS column (30 m × 0.25 mm ID; film thickness: 0.25 µm; J&W Scientific, Folsom, USA). The GC injection temperature and the MS transfer line were 250°C. The oven temperature was ramped from 50°C to 300°C by 5°C min^{-1} with a final hold of 10 min at 300°C. CHC extracts processed in the Schmitt lab were analyzed with an Agilent 6890 gas chromatograph coupled to an Agilent 5975 mass selective detector (Agilent Technologies, Waldbronn, Germany). The GC was also operated in splitless injection mode and fitted with a DB-5 Fused Silica capillary column (30 m × 0.25 mm ID; film thickness: 0.25 µm; J&W Scientific, Folsom, United States). The GC injector temperature and the MS transfer line were 300°C. The oven temperature was ramped from 60 to 300°C by 5°C min^{-1} with a final hold of 10 min at 300°C. In both labs, the MS quadrupole temperature was 150°C and the MS source temperature was 230°C. Helium with a constant flow of 1 ml min^{-1} was used as the carrier gas.

To characterize the different CHC profiles, we applied the deconvolution and compound identification procedure described by Stein (1999) and implemented in the software AMDIS (Automated Mass Spectral Deconvolution and Identification System; http://chemdata.nist.gov/mass-spc/amdis/), using a custom mass spectral library containing CHC compounds and their retention indices. AMDIS was run with the following parameter settings: component width = 22, adjacent peak subtraction = 2, resolution = medium, sensitivity = low, and shape requirements = medium. The mass spectra of the identified CHCs (target compounds) in each chromatogram were subsequently imported and curated (e.g., we corrected misidentified compounds) in R, using the flagme package (Robinson and Romoli 2019) for importing the data and the package reshape (Wickham 2007) for data management/table transformation. Methyl-branched alkanes were identified by their diagnostic ions and by calculating their retention indices (Carlson et al. 1998) as well as by using a custom script to calculate the expected diagnostic ions and retention indices of polymethyl-branched alkanes. The double bond positions of alkenes were identified after dimethyl disulfide (DMDS) derivatization of the CHC extracts following the protocol provided by Carlson et al. (1989). Alkadienes were grouped according to their retention indices, as we were unable to determine the positions of the double bonds due to their low amounts. We only considered a compound to be representative of a group (each sex of each species) if it occurred in at least 50% of the analyzed individuals and if its mean relative abundance in the group was above 0.1% of the total abundance of CHCs.

Statistical Analysis of Cuticular Hydrocarbon Profiles

We applied a non-metric multidimensional scaling (NMDS) ordination, based on the Bray-Curtis dissimilarity between the CHC profiles of every pair of samples, to visualize CHC profile differences in a two-dimensional graph (Kruskal 1964a,b). Species-diagnostic CHCs were identified with the aid of a similarity percentage analysis (SIMPER; Clarke 1993), in which we calculated the contribution of individual CHCs to the overall Bray-Curtis dissimilarity between *C. parabrevitarsis* n. sp. and *C. pseudobrevitarsis*. We picked alkenes and methyl-branched alkanes with the strongest average contribution to the overall dissimilarity between the two species and that at the same time differed strongly in their relative amounts (i.e., present exclusively or in large relative amounts in only one of the two

species) between the two species in a given sex as species-diagnostic CHCs. All statistical analyses were conducted in and all graphs were drawn with R version 3.02 (R Development Core Team 2013) using the vegan package version 2.0.10 (Oksanen et al. 2013).

Morphological Analysis

The holotype of *C. parabrevitarsis* n. sp. was photographed with an EOS 5Ds R camera (Canon, Ōta, Tokyo, Japan) attached to an MP-E 2.8/65 mm 1×–5× macro lens (Canon). We used a Wemacro Rail system (Shanghai Macro Photoelectric Technology, Shanghai, China) in combination with the software Helicon Focus version 7.6.1 (HeliconSoft, Kharkiv, Ukraine) to generate a focus-stacked photograph. The image was post-processed with Photoshop CS3 (Adobe, San Jose, CA). SEM micrographs were obtained with the aid of an EVO 15 scanning electron microscope (Carl Zeiss, Jena, Germany).

To describe morphological characters, we followed the terminology given by Kimsey and Bohart (1991) with few exceptions and by The Hymenoptera Anatomy Ontology consortium (Hymenoptera Anatomy Consortium; http://glossary.hymao.org). Specifically, we apply the term mesosoma instead of the term thorax and we apply the term metasoma instead of the term abdomen. Metasomal tergites and sternites are numbered consecutively, starting with 1 at the second abdominal segment (i.e., T1 and S1). Flagellar segments of the antenna are referred to as F1–F11.

Nomenclature

This article and the nomenclatural act(s) it contains have been registered in Zoobank (www.zoobank.org), the official register of the International Commission on Zoological Nomenclature. The LSID (Life Science Identifier) number of the publication is: urn:lsid:zoobank.org:pub:98F149F1-EA69-491C-9984-CEDC200870E0

Results

COI Nucleotide Sequence Divergence

We analyzed a total of 50 COI barcodes of samples hitherto considered *C. pseudobrevitarsis*. The neighbor-joining tree inferred from Kimura-2-parameter-corrected COI nucleotide sequence distances confirms the presence of two distinct haplotype clusters reported by Soon et al. (2014) (Fig. 2). The COI haplotypes of one of these clusters (corresponding to *Chrysis parabrevitarsis* n. sp.) are more similar to haplotypes of *C. brevitarsis*, whose status as a species has been undisputed for decades due to its unique morphological features, than to haplotypes of the second cluster (corresponding to *C. pseudobrevitarsis*). The minimum interspecific genetic distance between the two clusters is 3.6%. For comparison, the minimum interspecific genetic distance between the cluster corresponding to *C. parabrevitarsis* n. sp. and that corresponding to *C. brevitarsis* is 2.3%. COI haplotype diversity within the *C. parabrevitarsis* n. sp. cluster is relatively high (distance values of up to 2.1%).

Cuticular Hydrocarbon Profiles of Cuckoo Wasps

Analysis of the CHCs of samples hitherto considered conspecific with *Chrysis pseuobrevitarsis* (Supp Tables 2 and 3 [online only]) revealed two distinct CHC profiles in each sex (Fig. 3). The two profiles in each sex correspond 1:1 with the two COI haplotype clusters reported. The CHC profile of the female lectotype of *C. pseudobrevitarsis* falls within one of the two groups comprised of the CHC profiles of female samples (Fig. 3), indicating which group the name *C. pseudobrevitarsis* refers to. The other group is subsequently referred to as *Chrysis parabrevitarsis* n. sp. (species description is given below).

We identified a total of 57 different CHC compounds in *Chrysis pseudobrevitarsis* and *C. parabrevitarsis* n. sp. The CHC profiles of *C. pararevitarsis* n. sp. males are characterized by a smaller number of CHC compounds (26) than those of *C. pseudobrevitarsis* males (36). In contrast, the CHC profiles *C. parabrevitarsis* n. sp. females are characterized by a larger number of CHC compounds (33) than those of *C. pseudobrevitarsis* females (21) (Supp Table 3 [online only]). Detailed analysis revealed that females and males of both species can be easily distinguished from each other by the relative abundance of selected diagnostic compounds. Tables 1 and 2 each list eight diagnostic compounds that can be used to distinguish the two species in the female and male sex, respectively. The differences in the relative abundances of the diagnostic compounds are—at least to some extent—already readily visible in the total ion chromatogram (TIC) (Fig. 4).

CHC Profile Similarity Between Cuckoo Wasps and Potential Hosts

When comparing the CHC profiles of females of *C. parabrevitarsis* n. sp. and of *C. pseudobrevitarsis* with those of females of vespid wasps (i.e., *Ancistrocerus antilope*, *Euodynerus notatus*, *Euodynerus quadrifasciatus*) that have been reported to serve as hosts of *C. pseudobrevitarsis* in the wide sense, we found a noteworthy correspondence in the CHC profiles of *C. pseudobrevitarsis* females with those of *Euodynerus notatus* females. In contrast, we did not find any noteworthy similarity between the CHC profiles of *C. parabrevitarsis* n. sp. females and those of the above three host species.

Description of *Chrysis parabrevitarsis* n. sp.

Type Material

9 ♂♂ and 69 ♀♀.—**Holotype.** 1 ♀, Germany, Rhineland-Palatinate, Bellheim; N 49.186309°, E 8.303310°, 19.V.2012, leg. Oliver Niehuis and Manfred Niehuis. The holotype is deposited in the Biobank of the Zoological Research Museum Alexander Koenig in Bonn, Germany (acc. no. ZFMK-TIS-36619, ON3143).—**Paratypes. Estonia** (19 ♀♀), Koitjärve, N 59.41639°, E 25.64833°, 15.–29.VI.2009, window trap, leg. I. Süda, 1 ♀ (TUZ: TUZ102335); Koitjärve 3 km N, N 59.4250000°, E 25.65306°, 5.–17.VII.2011, window trap, leg. I. Süda, 1 ♀ (TUZ: TUZ102383); Koitjärve, N 59.4250000°, E 25.65306°, 15.–29.VI.2009, window trap, leg. I. Süda, 1 ♀ (TUZ: TUZ102334); Pihke, N 58.08333°, E 24.7333°, 8.V.–2.VII.2009, combined flight trap, leg. A. Kraut, 1 ♀ (TUZ: TUZ102402); Restu, N 57.93944°, E 26.38833°, 31.V.–27.VI.2010, combined flight trap, leg. V. Krivtsova, 1 ♀ (TUZ: TUZ102302); Riimaru, N 58.27167°, E 25.18389°, 19.VI.–3.VIII.2007, window trap, leg. I. Süda, 1 ♀ (TUZ: TUZ041328); Ruhnu, N 57.80472°, E 23.26111°, 3–10.VI.2014, Malaise trap, leg. M. Kaljulaid, 1 ♀ (TUZ: TUZ102396); Tõrvase, N 57.58167°, 26.44861°, 31.V.–26.VI.2010, combined flight trap, leg. V. Krivtsova, 1 ♀ (TUZ: TUZ102303); Vehendi, N 58.23°, E 26.15472°, 3.–9.VII.2010, leg. V. Soon, 1 ♀ (TUZ: VS002798; KJ398927); Vormsi, Kärret 2 km S, N 58.96972°, E 23.20583°, 6.VI.–6.VII.2011, window trap, leg. I. Süda 3 ♀♀ (TUZ: TUZ102369; TUZ102371; TUZ102339); 4.VI.–6.VII.2012, window trap, leg. I. Süda, 3 ♀♀ (TUZ: TUZ102386; TUZ102387; TUZ102388); 19.VII.–19.VIII.2011, window trap, leg. I. Süda, 2 ♀♀ (TUZ: TUZ102384; TUZ102382); Võhunõmme, N 59.25056°, E 26.59528°, 8.VII.2001, leg. V. Soon, 1 ♀ (TUZ: TUZ102310).—**Finland** (11 ♀♀), Etelä-Häme, Pälkäne,

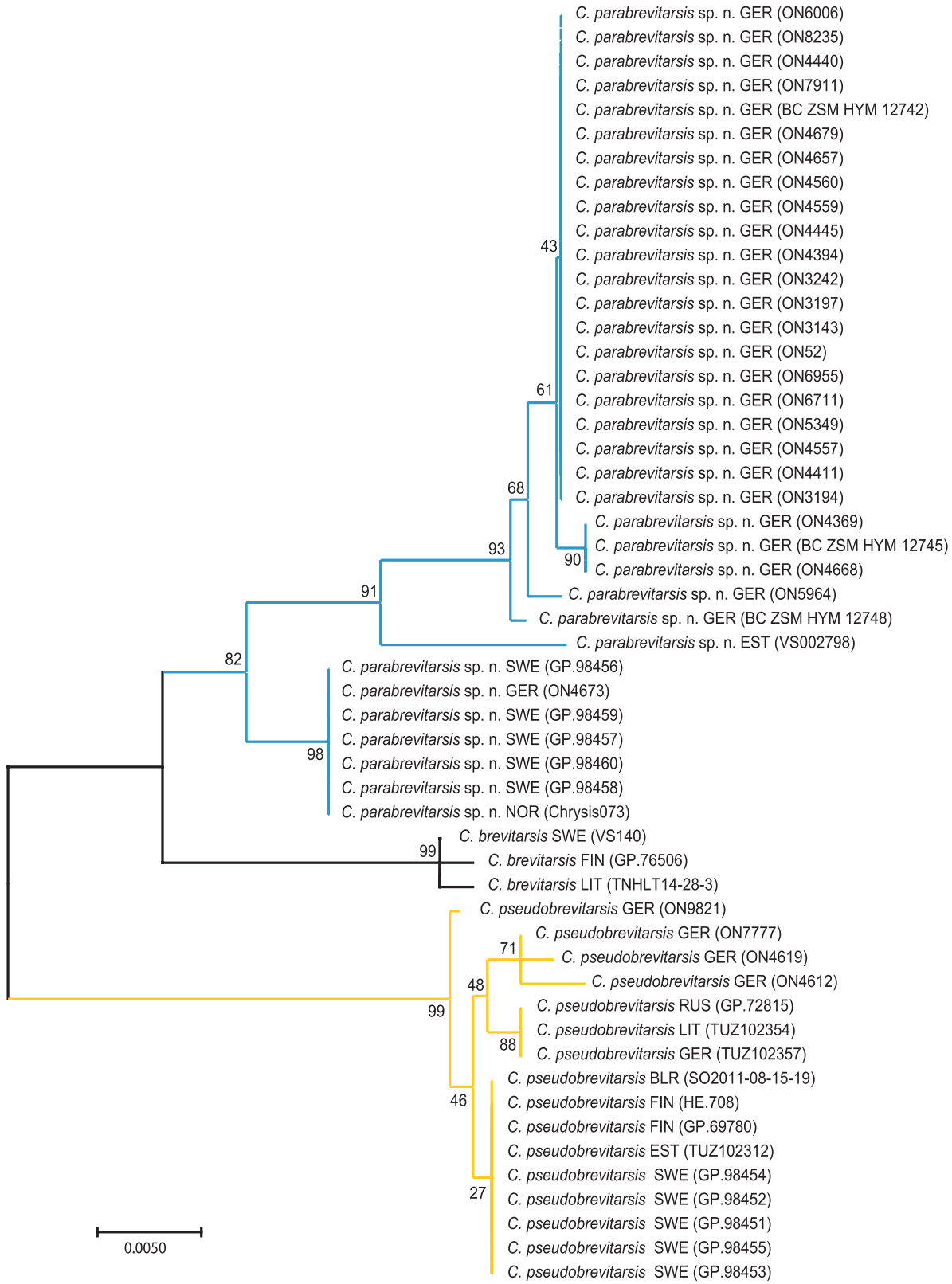

Fig. 2. Neighbor-joining tree inferred from Kimura-2-parameter nucleotide sequence distances between COI haplotypes of *Chrysis parabrevitarsis* n. sp. (blue branches), *Chrysis brevitarsis* and *Chrysis pseudobrevitarsis* (yellow branches). Support values are based on 10 000 bootstrap replicates. Acronyms in capital letters after the species names specify the country of origin: Belorussia (BLR), Estonia (EST), Finland (FIN), Germany (GER), Lithuania (LIT), Norway (NOR), Russia (RUS), and Sweden (SWE). Sample IDs are given in parentheses.

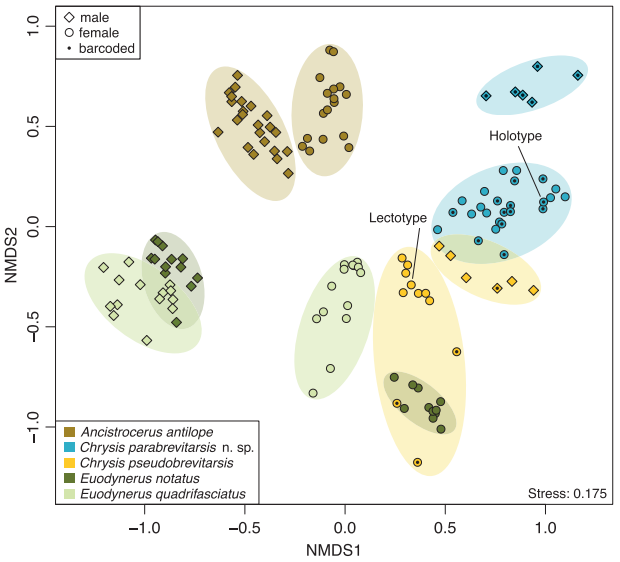

Fig. 3. Two-dimensional non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarities between cuticular hydrocarbon (CHC) profiles of males and females of the cuckoo wasps *Chrysis parabrevitarsis* n. sp. (6 ♂♂, 25 ♀♀) and *Chrysis pseudobrevitarsis* (6 ♂♂, 11 ♀♀) as well as of three vespid wasps known to serve as hosts of these two cuckoo wasps, *Ancistrocerus antilope* (23 ♂♂, 17 ♀♀), *Euodynerus notatus* (13 ♂♂, 11 ♀♀), and *Euodynerus quadrifasciatus* (14 ♂♂, 14 ♀♀). Note that the plot includes the CHC profile data of the holotype of *C. parabrevitarsis* n. sp. and CHC profile data of the lectotype of *C. pseudobrevitarsis*.

19.VI.1960, leg. Y. Ranta, 1 ♀ (MZH: GP.68379); 21.VI.1960, leg. J. Kangas, 1 ♀ (MZH: GP.68382); Varsinais-Suomi, Karjalohja, 20.VII.1964, leg. J. Perkiömäki, 1 ♀ (MZH: GP.4980); Varsinais-Suomi, Rymättylä, 12.VII.1967, leg. A. K. Merisuo, 1 ♀ (MZH: GP.4975); 22.VII.1967, leg. A. K. Merisuo 1 ♀ (MZH: GP.4974); 28.VI.1968, leg. A. K. Merisuo, 1 ♀ (MZH: GP.4976); 18.VII.1968, leg. A. K. Merisuo, 1 ♀ (MZH: GP.4977); 28.VI.1969, leg. A. K. Merisuo, 1 ♀ (MZH: GP.5088); 30.VII.1970, leg. A. K. Merisuo, 1 ♀ (MZH: GP.4978); 22.VIII.1970, leg. A. K. Merisuo, 1 ♀ (MZH: GP.4979); Åland, Finström, leg. W. Hellén, 1 ♀ (MZH: GP.4956).—**Germany** (31 ♀♀, 8 ♂♂), Bavaria, Retzbach, N 49.895260° E 9.838734°, 2012, leg. S. Hopfenmüller, 2 ♀♀ (ON: ON4559; ON4560); 1 ♂ (ON: ON4557); Hesse, Lorch am Rhein, N 50.052812°, E 7.791165°, 09.VII.2013, leg. O. Niehuis, 1 ♂ (ZMFK: ON5349, ZFMK-TIS-38105); 15.VII.2013, leg. O. Niehuis, 1 ♂ (ZFMK: ON4411, ZFMK-TIS-38098); 1 ♀ (ZFMK: ON4394; ZFMK-TIS-38097); 30.V.2014, leg. O. Niehuis, 1 ♂ (ZMFK: ON6955, ZFMK-TIS-38109); Hesse, Lorch am Rhein, Malaise trap #1, N 50.049117°, E 7.79777°, 244 m amsl, 15.–23.VI.2013, leg. O. Niehuis, 1 ♀ (ZMFK: ON4668, ZFMK-TIS-38102); Hesse, Lorch am Rhein, Malaise trap #2, N 50.049767°, E 7.7974°, 261 m amsl, 07.–15.VI.2013, leg. O. Niehuis, 1 ♀ (ZFMK: ON4679, ZFMK-TIS-38104); 09.–15.VII.2013, leg. O. Niehuis, 1 ♀ (ZFMK: ON4657, ZFMK-TIS-38101); 15.–21.VII.2013, leg. O. Niehuis, 1 ♀ (ZFMK: ON4673, ZFMK-TIS-38103); Hesse, Lorch, Nollig (MTB5912 R3414 H5546), N 50.052778°, E 7.790833°, 240 m amsl, 20.V.2004, leg. S. Tischendorf, 1 ♀ (TI: ON10559); 9.VI.2015, leg. O. Niehuis, 1 ♀ (ON: ON10560); Hesse, Lorch, Nollig, N 50.049364°, E 7.797869°, 3.VI.1998, leg. S. Tischendorf, 1 ♀ (TI: ON10561); Hesse, Lorchhausen (TK5912–1248), N 50.054662°, E 7.783803°, 6.VIII.1998, leg. S. Tischendorf, 1 ♀ (TI: ON10558); Hesse, Lorchhausen, nördl. Weinb., MA1045, N 50.054662°, E 7.783803°, 21.VI.1998, leg. M. Niehuis, 1 ♀ (ON: ON9815); Messel, N 49.92694°, E 8.7575°, 4.VI.1998, leg. O. Niehuis, 1 ♀ (ON: ON52, JX292218); Rhineland-Palatinate, Battenberg (e.l., 02.VI.1986), N 49.532045°, E 8.147278°, 1985, leg. M. Beierlein, 1 ♀ (ON: ON9813); Rhineland-Palatinate, Battenberg, N 49.532045°, E 8.147278°, 15.V.1986, leg. M. Beierlein, 1 ♀ (ON: ON9452); Rhineland-Palatinate, Bellheim, N 49.186309°, E 8.303310°, 20.V.2012, leg. O. & M. Niehuis, 1 ♀ (ZFMK: ON3194, ZFMK-TIS-29805); 28.V.2013, leg. O. Niehuis, 1 ♂ (ZFMK: ON4369, ZFMK-TIS-38096); 8.VI.2013, leg. O. Niehuis, 2 ♀♀ (ZFMK: ON4440, ZFMK-TIS-38099; ON4445, ZFMK-TIS-38100); 10.VI.2012, leg. O. & M. Niehuis, 1 ♀ (ZFMK: ON3242, ZFMK-TIS-36479); Rhineland-Palatinate, Bienwald, Forstd. Jakobspfad B, Scheibenhardt, N 48.983083°, E 8.139474°, 25.V.1982, leg. F. Brechtel, 1 ♀ (ON: ON9826); Rhineland-Palatinate, Bienwald, Lauterwiesen, Westl., 6/4, Bienwaldmühle, Lamo 2, N 48.999084°, E 8.070924°, 2.VI.1983, leg. F. Brechtel, 1 ♀ (ON: ON9814); Rhineland-Palatinate, Büchelberg, N 49.027626°, E 8.164353°, 4.V.2014, leg. O. Niehuis, 1 ♂ (ZFMK: ON6711, ZFMK-TIS-38108); 16.V.2015, leg. O. Niehuis, 1 ♀ (ZFMK: ON7911, ZFMK-TIS-38110); Rhineland-Palatinate, Büchelberg (westl.) (MV3530), N 49.027626°, E 8.164353°, 15.VI.1996, leg. M. Niehuis, 1 ♀ (ON: ON9812); Rhineland-Palatinate, Forstd. Borschberg, HB 3, Wörth, 4/2(1), Zucht: 04.08.1983/17.03.1984, 1983, leg. F. Brechtel, 1 ♀ (ON: ON9825); Rhineland-Palatinate, Kaub (MA14NW), Umg. Rhein-Wanderweg, 11.VI.1999, leg. M. Niehuis, 1 ♀ (ON: ON9824); Rhineland-Palatinate, Lauterwisen östl., K, Bienwaldmühle, N 48.999084°, E 8.070924°, 9.VI.1982, leg. F. Brechtel, 1 ♀ (ON: ON9810); Rhineland-Palatinate, Offenbach, N 49.197474°, E 8.227424°, 8.VI.2013, leg. O. Niehuis, 1 ♀ (ZFMK: ON6006, ZFMK-TIS-38107); 30.VI.2013, leg. O. Niehuis, 1 ♀ (ZFMK: ON5964, ZFMK-TIS-38106); Rhineland-Palatinate, St. Goarshausen, N 50.159276°, E 7.714916°, 17.VI.2015, leg. O. Niehuis, 1 ♀ (ZFMK: ON8235, ZFMK-TIS-38112); Rhineland-Palatinate, Vorderweidenthal, N 49.130077°, E 7.884518°, 22.VI.2012, leg. O. Niehuis, 1 ♀ (ZFMK: ON3197, ZFMK-TIS-36441); Thuringia, N 51.323056°, E 11.03194°, 6.VI.2011, leg. F. Burger, 1 ♂ (ZSM: BC ZSM HYM 12742, GBACU2482-13); 1 ♀ (ZSM: BC ZSM HYM 12745, GBACU2485-13); 7.VII.2011, leg. F. Burger, 1 ♂ (ZSM: BC ZSM HYM 12748; GBACU2488-13).—**Hungary** (1 ♀), Veszprém co., Nyirad, N 47.02667°, E 17.45778°, 27.VI.2010, leg. V. Soon, 1 ♀ (TUZ: TUZ102364).—**Norway** (1 ♀), Kviljo, N 58.07556°, E 6.67639°, 14.VIII.2008, leg. F. Ødegaard, 1 ♀ (NTNU: Chrysis073, NOCHR073-13).—**Russia** (2 ♀♀), Leningrad Oblast, Seskar Island (= Seiskari), coll. T. Grönblom, 1 ♀ (MZH: GP.5124); leg. A. K. Merisuo, 1 ♀ (MZH: GP.5074).—**Sweden** (4 ♀♀, 1 ♂), Småland, Kalmar, Igersdela, dry meadow, N 56.65446°, E 16.011613°, 6.VII.2013, leg. N. Johansson, 1 ♀ (NMLS: GP.98458; LEFIJ6116-17); Småland, Nybro, Bäckebo, Bjällingsmåla, deciduous (mixed) forest *Quercus*/*Pinus*/*Populus*, N 56.93157°, E 15.909082°, 12.–24.V.2015, Malaise trap, leg. N. Johansson, 1 ♀ (ZFMK: GP.98460, LEFIJ6118-17); Småland, Nybro, Hornsö, clearcut, N 57.021138°, E 16.126064°, 2013, leg. M. Larshagen, 1 ♀ (MZH: GP.98457, LEFIJ6115-17); Småland, Vetlanda, Skirö, Skärvete, deciduous forest, N 57.355°E, 15.489978°, 25.V.2009, leg. N. Johansson, 1 ♂ (TUZ: GP.98456, LEFIJ6114-17); Småland, Vetlanda, Stenberga, Boda, Djupsgård, grazed meadow, N 57.317068°, E 15.431728°, 22.VI.2009, 1 ♀ (NTNU: GP.98459, LEFIJ6117-17).

Diagnosis

The species belongs to the *Chrysis ignita* species group, showing all general features and the typical habitus of species in this group (Figs. 4 and 5). With stout ovipositor (Fig. 6), short metatarsus (shorter than

Table 1. Diagnostic hydrocarbons for separating *Chrysis parabrevitarsis* n. sp. and *Chrysis pseudobrevitarsis* in the female sex

		C. parabrevitarsis n. sp. (females; *n* = 25)		*C. pseudobrevitarsis* (females; *n* = 11)	
Compound	Kovats retention index (Kováts 1958)	Average rel. amount in % (± std. dev.)	Range of rel. amount in % (min–max)	Average rel. amount in % (± SD)	Range of rel. amount in % (min–max)
9-MeC23	2337	4.7 ± 3.06	1.2–11.1	0.7 ± 0.75	0–2.1
7-C25ene	2482	0 ± 0	0	21.6 ± 17.88	0–59.5
9,13-diMeC25	2564	5.5 ± 2.44	2.9–11.6	0.8 ± 1.12	0–3
13-; 12-; 11-C27ene	2669	7.8 ± 4.7	0.8–18.5	0 ± 0	0
7-C27ene	2683	0.3 ± 0.31	0–1	5.2 ± 4.56	0–15
14-; 13-C29ene	2869	2.4 ± 1.41	0.4–5.9	0 ± 0	0
9-C29ene	2877	1.6 ± 1.42	0–5.5	0 ± 0	0
7-C29ene	2884	4.8 ± 2.66	0.3–13.3	0 ± 0	0

Table 2. Diagnostic hydrocarbons for separating *Chrysis parabrevitarsis* n. sp. and *Chrysis pseudobrevitarsis* in the male sex

		C. parabrevitarsis n. sp. (males; *n* = 6)		*C. pseudobrevitarsis* (males; *n* = 6)	
Compound	Kovats retention index (Kováts 1958)	Average rel. amount in % (±SD)	Range of rel. amount in % (min–max)	Average rel. amount in % (±SD)	Range of rel. amount in % (min–max)
9-MeC23	2337	0 ± 0	0	1.5 ± 1.08	0.3–2.7
7-MeC23	2342	0 ± 0	0	1.4 ± 1.19	0.2–2.7
9-C25ene	2474	0 ± 0	0	1.9 ± 1.53	0.4–4.0
7-C25ene	2482	0 ± 0	0	2.4 ± 1.23	1.3–4.7
13-; 11-MeC25	2534	0 ± 0	0	2.6 ± 0.98	1.6–3.9
7-C29ene	2884	3.2 ± 3.56	0–7.2	0 ± 0	0
13,17-diMeC29	2956	2.9 ± 0.35	2.4–3.4	0.4 ± 0.23	0.1–0.7
15-; 14-; 13-C31ene	3070	18.4 ± 3.82	11.6–21.9	4.4 ± 2.03	2.3–6.9

metatibia), subequal metatibial spurs, and simple toothless mandible, the new species is most similar to *C. pseudobrevitarsis* Linsenmaier, 1951. Use of widely applied identification keys to European cuckoo wasp species (i.e., Linsenmaier 1959, Paukkunen et al. 2015, Wiśniowski 2015) will also lead to this species. Females of *C. parabrevitarsis* can be distinguished morphologically from females of *C. pseudobrevitarsis* by the size and the shape of their patches of uniform sensillae on antennal segments F4–F7 (Fig. 7). In *C. parabrevitarsis*, the patch extends over more than a quarter of the circumference of the flagellomere on F5, while in *C. pseudobrevitarsis*, the patch extends over no more than quarter of the circumference of F5. Differences of the patches on the other flagellomeres are comparable to those on F5. We are unaware of any morphological differences between the males of *C. parabrevitarsis* and *C. pseudobrevitarsis*. However, the two species can be distinguished in both sexes from each other by their species-specific COI nucleotide sequences and their species- and sex-specific CHC profiles (Fig. 4 and Table 2).

Morphological Description

Note that we provide below minimum and maximum values of measurements obtained from a subset of type specimens (*n* = 11; specimens: ZFMK-TIS-36619, TUZ102302, TUZ102303, TUZ102334, TUZ102335, TUZ102383, TUZ102384, TUZ102386, TUZ102387, TUZ102388, TUZ102396) with the measurements of holotype in parentheses.—***Female***. Typical morphology of a species of the *Chrysis ignita* group. Greenish-bluish head and metasoma and golden-reddish mesosoma (Figs. 4 and 5).—**Head**. Height: 1.5–2.1 mm (holotype: 2.0 mm). Width: 2.0–2.7 mm (holotype: 2.5 mm). Length: 0.8–1.1 mm (holotype: 1.1 mm). Frontal carina well developed, pubescence on vertex light brown. Mandibles relatively thick (centrally 0.2–0.4 mm; avg. 0.37 mm) and without subapical tooth. Antennae black, except scapus, pedicellus, and F1, which are all metallic blue. Relative lengths of antennal segments P/F1/F2/F3: 1.0/1.7/1.1/0.9. The patches of uniform sensillae relatively wide, on F5 wider than half the width of the flagellomere (Fig. 7).—**Mesosoma**. Length: 2.6–3.8 mm (holotype: 3.4 mm). Width anterior to tegulae: 1.9–2.9 mm (holotype: 2.5 mm). Length of pronotum medially: 0.5–0.7 mm (holotype: 0.5 mm). Width of pronotum at anterior margin: 1.6–2.3 mm (holotype: 2.2 mm). Pubescence on dorsal mesosoma light brownish. Punctures on scutum comparable in size and density with those on T1, except on scutellum and metanotum, on which punctures are larger and with smaller interstices (Fig. 5). Forewing length: 4.8–7.0 mm (holotype: 7 mm). Metatarsi shortened, shorter than metatibia, which bears subequal spurs.—**Metasoma**. Length: 3.4–4.8 mm (holotype: 4.5 mm). Width: 2.0–2.8 mm (holotype: 2.7 mm). T1 with large and regular punctures comparable in size with those of scutum. T2 with uneven punctures, being dense and big only at the base of the segment and becoming considerably finer and sparser distally (Fig. 5). T3 throughout very finely and densely punctured. Ovipositor short and thick, T4–T7 and S4–S6 illustrated in Fig. 6.—***Male***. Morphologically indistinguishable from the male of *C. pseudobrevitarsis*. We therefore refrain from providing a detailed morphological description.

Distribution

Currently known from Estonia, Finland, Germany, Hungary, Norway, Russia, and Sweden.

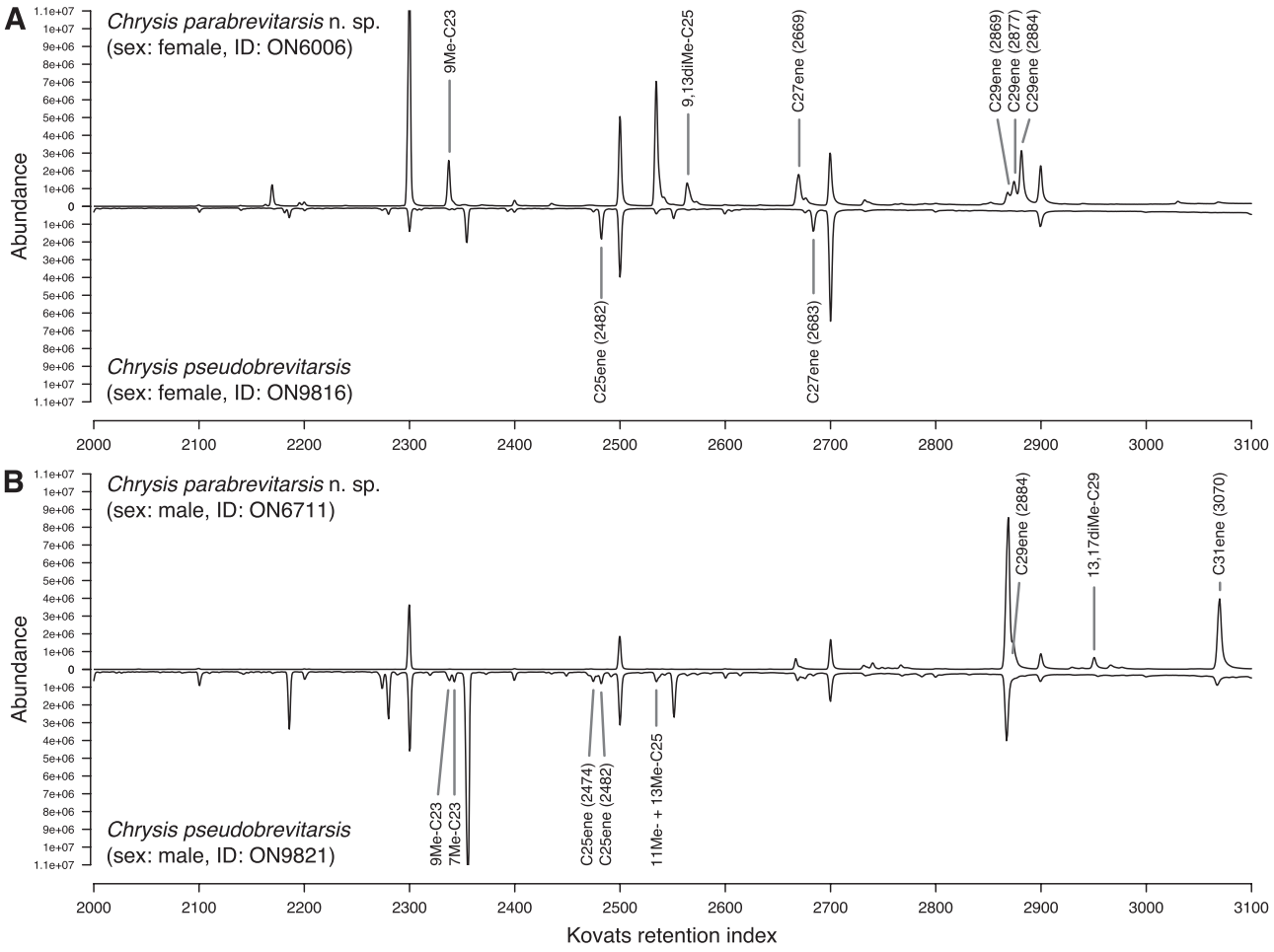

Fig. 4. Exemplar chromatograms showing diagnostic differences (specified in Tables 1 and 2) between the cuticular hydrocarbon profiles of *Chrysis parabrevitarsis* n. sp. and *Chrysis pseudobrevitarsis* in the female (**A**) and male (**B**) sex. The *x*-axis represents the retention time shown in form of Kovats retention indices (Kováts 1958), the *y*-axis shows the total intensity of ions (TIC). Diagnostic alkenes are indicated with their Kovats retention index (Kováts 1958) in parentheses to differentiate them from alkenes with the same chain length but differing in the location of their double bond.

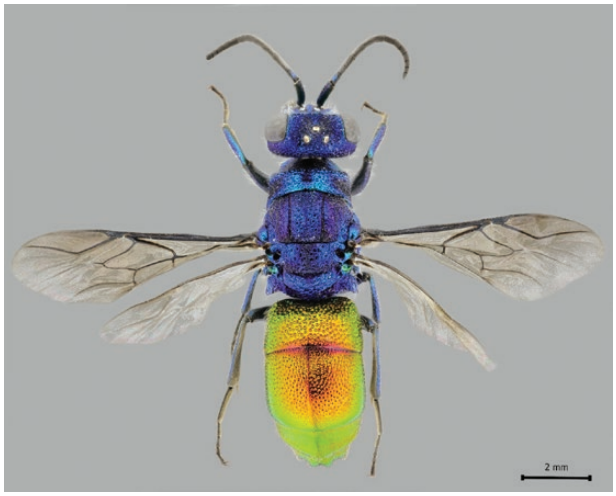

Fig. 5. Holotype (female) of *Chrysis parabrevitarsis* n. sp. (Voucher ID: ZFMK-TIS-36619).

Biology

Due to the fact that previous reports on hosts of *Chrysis pseudobrevitarsis* in the wide sense (i.e., *Ancistrocerus antilope*, *Euodynerus quadrifasciatus*, and *Euodynerus notatus*; summarized by Paukkunen et al. 2015 and Pauli et al. 2019) could refer to either *Chrysis parabrevitarsis* n. sp. or to *Chrysis pseudobrevitarsis*, these records cannot be relied on without verifying the identity of the observed cuckoo wasps. However, multiples paratypes of *C. parabrevitarsis* n. sp. originating from Sweden have been reared from nests of *E. quadrifasciatus*, indicating that this vespid wasp serves as a host of *C. parabrevitarsis* n. sp. Comparison of the CHC profiles of *C. parabrevitarsis* n. sp. females with the CHC profiles of females of the above three reported host species provided no additional clues about host usage. The high similarity between the CHC profiles of female *C. pseudobrevitarsis* and female *E. notatus* could indicate that reports of *E. notatus* as host of *C. pseudobrevitarsis* may indeed refer to this specific cuckoo wasp species and not to *C. parabrevitarsis* n. sp.

Etymology

The species epithet *parabrevitarsis* refers to the names of two closely related species (i.e., *C. brevitarsis* and *C. pseudobrevitarsis*). The name consists of three parts: *para-* is a prefix derived from Greek meaning 'next to', 'abnormal', 'resembling'; *brevis* is the Latin word for 'short', and *tarsus* is the technical term for the last segment of an insect's leg. We use the composed specific epithet *parabrevitarsis* as an adjective in the feminine case.

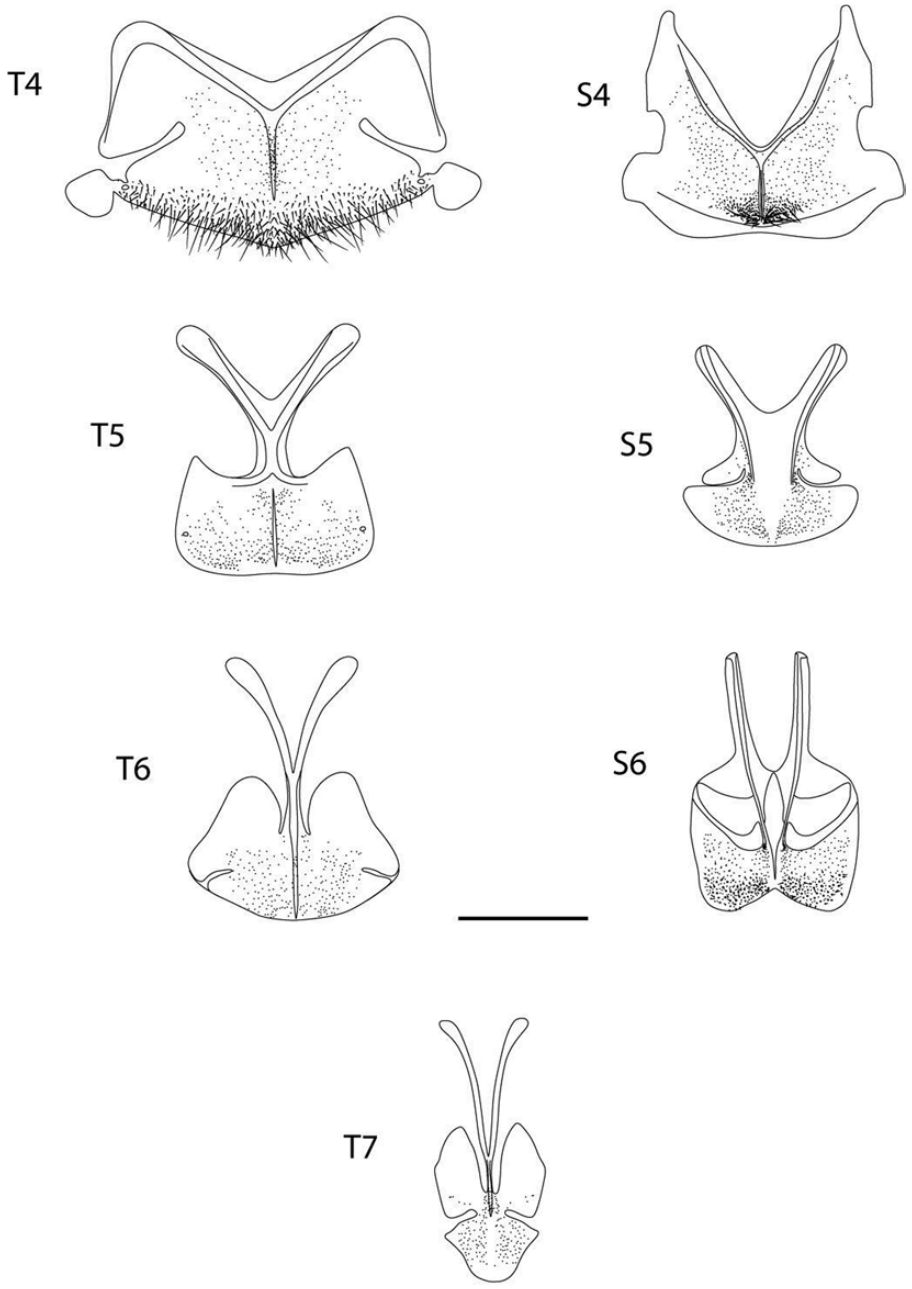

Fig. 6. Shape of the internal metasomal segments (T4–T7 and S4–S6) of female *Chrysis parabrevitarsis* n. sp. (voucher ID: TUZ102402). Scale bar: 1.0 mm.

Discussion

The delineation and description of species and hence of biodiversity has traditionally strongly relied on external morphological characters that are comparably easy to observe. In species that depend on visual cues for mate recognition, it intuitively makes sense to assume that they differ in external morphological characteristics from other species. However, many species are known to exploit other cues than visual ones to identify conspecific mates (Hebets and Papaj 2005, Smadja and Butlin 2008). From a conceptual point of view, biological species do not need to differ from each other in any observable external morphological trait. The taxonomic literature contains a wide array of studies that report and describe cryptic species differing in traits other than external morphological ones from their siblings (e.g., Kurina et al. 2015, Dufresnes et al. 2019). However, no technological progress has had a larger impact on the number of discovered cryptic species than DNA barcoding (e.g., Herbert et al. 2003, Smith and Fisher 2009, Srivathsan et al. 2019).

Animal DNA barcoding currently relies on the nucleotide sequence of a single marker: a specific region of the mitochondrial protein-coding gene COI (Herbert et al. 2003). While COI barcoding works in most instances extremely well for re-identification of known species, it can be misleading when used to delineate species *de novo* (Eberle et al. 2020). Most importantly, because the mitochondrial genome is maternally transmitted without recombination in almost all species, even significant COI haplotype divergence in a group of individuals occurring in sympatry does not necessarily imply that the group contains multiple species. As a result, zoologists are frequently confronted with the challenge of

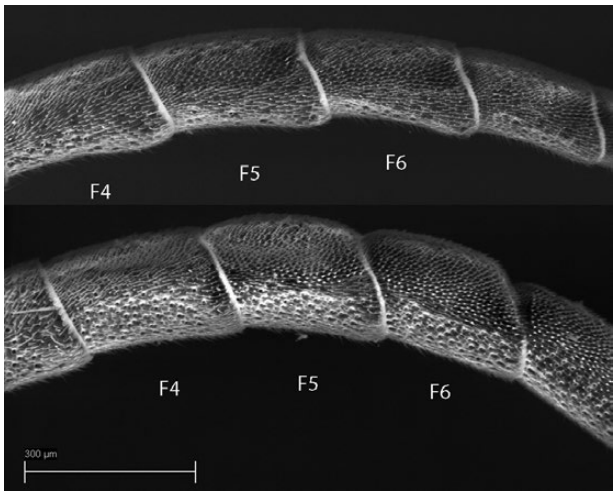

Fig. 7. SEM micrographs of antennal flagellomeres (F) 4–6 in females of *Chrysis pseudobrevitarsis* (top, voucher ID: TUZ117252) and *Chrysis parabrevitarsis* n. sp. (bottom, voucher ID: TUZ102387).

interpreting the taxonomic significance of COI haplotype differences between samples. A highly promising solution suggested by Eberle et al. (2020) is an extension of the traditional barcoding approach by additionally considering nuclear markers. However, the additional analysis of nuclear genes is not always feasible. In fact, any quickly evolving trait entirely encoded by genes of the nuclear genome could alternatively serve as a complementary tool for assessing reproductive isolation.

The cuticle of all insects studied so far is covered by CHCs (Blomquist and Bagnères 2010, Bien et al. 2019). The large number of different CHCs that organisms can synthesize plus the fact that CHC profiles can differ both qualitatively and quantitatively from each other make CHC profiles a high dimensionality trait. Because all proteins required for CHC biosynthesis are encoded by genes of the nuclear genome, CHC profile differences that correspond with distinct COI haplotypes in a sympatric situation are most plausibly explained by reproductive isolation. It must be emphasized, however, that distinct CHC profile differences in a group of individuals alone (i.e., without corresponding COI barcoding clustering) is not sufficient to conclude that reproductively isolated lineages have been sampled, because distinct CHC profiles can also exist in a population of a single species. For example, Wurdack et al. (2015) reported two distinct CHC profiles in the mason wasp *Odynerus spinipes* (Linnaeus,1758) that likely evolved as a counterstrategy to CHC profile mimicry by two kleptoparasitic cuckoo wasps.

We here complemented DNA barcoding with GC-MS analysis of CHCs to assess whether the two distinct haplotype clusters within cuckoo wasps identified as *C. pseudobrevitarsis* (reported first by Soon et al. 2014) represent valid biological species. Since *C. pseudobrevitarsis* with distinct COI haplotypes are found across most of Central and Northern Europe and are thus not allopatric to each other, consistent CHC profile differences between samples carrying distinct COI haplotypes should indicate reproductive isolation. Since we found wasps with distinct COI haplotypes to consistently express different CHC profiles, we consider these wasps to represent different biological species and described the cryptic taxon as a new species. We acknowledge, however, that COI haplotype and CHC profile variation in our sample of *C. parabrevitarsis* and of *C. pseudobrevitrasis* is still high and could indicate the presence of additional cryptic species.

A major advantage that we found of CHCs as a trait in taxonomic work is their long-term stability on dry-mounted samples, at least as long as the sample has not been submersed in a strong CHC solvent (e.g., during the sample killing process). This underlines the value of dry-mounted specimens in private and public collections for chemical analyses. Because the extraction of CHCs with a solvent, such as n-hexane, is—at least from a morphological point of view—noninvasive, it can also be applied to precious type material. CHC extraction with n-hexane also does not seem to damage the sample's DNA (ON, personal observation based on having sequenced target DNA regions as well as whole genomes of hundreds of samples treated with n-hexane). In the present study, doing so enabled us to associate the lectotype of *C. pseudobrevitarsis* with one COI haplotype group long before we became aware of a morphological trait that distinguishes females of *C. parabrevitarsis* n. sp. from those of *C. pseudobrevitarsis*. Had the lectotype been a male, its CHC profile would have been the only available information to us for associating it with a COI haplotype group.

After having gained confidence that the two COI haplotype groups represented distinct species, a thorough search for external morphological differences between the two species revealed subtle differences in the width of a patch of uniform short sensillae on each of the antennal segments F4–F7 (Fig. 7). The surface structure of the antennae of cuckoo wasps of the *Chrysis ignita* species group has been suggested first for taxonomic work by van der Smissen (2010). She described a distinct sensory patch on the antennae of male cuckoo wasps that has species-specific shape and size. While the trait proved to be unreliable to distinguish males of *C. parabrevitarsis* n. sp. from males of *C. pseudobrevitarsis*, we found a comparable character in females that allowed reliable differentiation of the two species. Studying this character requires relatively high optical magnification (>60×) and a diffuse light source.

Due to the high morphological similarity of the two cuckoo wasp species taxonomically disentangled in the present study, it will likely require some time until their distributional and ecological differences are better understood. However, given that the CHCs of dry-mounted museum specimens of these two species can be exploited to infer the samples' identity irrespective of their sex, future studies can capitalize on the large array of precious samples collected during the last two centuries and stored in insect collections.

Supplementary Data

Supplementary data are available at *Insect Systematics and Diversity* online.

Supplementary file 1: Sample information (sampling location, date, and collector, collection) for all wasps chemically studied and/or included in the COI barcode analysis in the present study.

Supplementary file 2: Relative intensity of all analyzed cuticular hydrocarbons in each sampled wasp listed in Supplementary file 1.

Supplementary file 3: Summary statistics for cuticular hydrocarbon intensities in the investigated species.

Acknowledgments

We acknowledge Rainer Blum (Freiburg) and Sandra Kukowka (Bonn) for help with COI barcoding samples and Katharina Christmann and Wolf Haberer (both Freiburg i. Br) for help extracting and analyzing cuticular hydrocarbons. We are indebted to Marco Bernasconi (Luzern) for providing access to the lectotype of *Chrysis pseudobrevitarsis* and for granting permission to extract the lectotype's cuticular hydrocarbons. We thank Manfred Niehuis for help with collecting samples of *Chrysis parabrevitarsis* n. sp. in the field and we are indebted to Ernst

Klimsa and Sebastian Hopfenmüller for sending us samples for chemical analysis. O.N. thanks Hessen Forst, the Struktur- und Genehmigungsbehörde Süd, and the Struktur- und Genehmigungsdirektion Nord (both Rhineland-Palatinate) for granting permission to collect samples. Mariann Külaviir is acknowledged for assistance when generating SEM micrographs. We also acknowledge two anonymous reviewers for constructive advice on improving the manuscript. Finally, O.N. and T.S. acknowledge the German Research Foundation for supporting parts of this study (NI 1387/1-1, SCHM 2645/2-1).

Author Contributions

Project idea: VS, RFC-C, ON; Experimental design: VS, RFC-C, ON, TS; Contributed materials and reagents: VS, NJ, JP, PR, FØ, ON; Molecular procedures: VS, RFC-C, TS, ON; Data analysis: VS, RFC-C, TS, ON; Manuscript preparation: all authors contributed to the writing of the manuscript with VS, RFC-C, and ON taking the lead.

References Cited

Akino, T., M. Terayama, S. Wakamura, and R. Yamaoka. 2002. Intraspecific variation of cuticular hydrocarbon composition in *Formica japonica* Motschoulsky (Hymenoptera: Formicidae). Zool. Sci. 19: 1155–1165.

Astrin, J. J., and P. E. Stüben. 2008. Phylogeny in cryptic weevils: molecules, morphology and new genera of Western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). Invertebr. Syst. 22: 503–522.

Bagnères, A.-G., and C. Wicker-Thomas. 2010. Chemical taxonomy with hydrocarbons, pp. 121–162. *In* G. J. Blomquist and A. G. Bagnères (eds.), Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, United Kingdom.

Bien, T., J. Gadau, A. Schnapp, J. Y. Yew, C. Sievert, and K. Dreisewerd. 2019. Detection of very long-chain hydrocarbons by laser mass spectrometry reveals novel species-, sex-, and age-dependent differences in the cuticular profiles of three *Nasonia* species. Anal. Bioanal. Chem. 411: 2981–2993.

Blomquist, G. J., and A. G. Bagnères. 2010. Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, United Kingdom.

Carlson, D. A., C. S. Roan, R. A. Yost, and J. Hector. 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. Anal. Chem. 61: 1564–1571.

Carlson, D. A., U. R. Bernier, and B. D. Sutton. 1998. Elution patterns from capillary GC for methyl-branched alkanes. J. Chem. Ecol. 24: 1845–1865.

Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18: 117–143.

Dufresnes, C., I. Strachinis, E. Tzoras, S. N. Litvinchuk, and M. Denoël. 2019. Call a spade a spade: taxonomy and distribution of *Pelobates*, with description of a new Balkan endemic. ZooKeys 859: 131–158.

Eberle, J., C. Mayer, D. Ahrens, O. Niehuis, and B. Misof. 2020. A plea for standardized nuclear markers in metazoan DNA taxonomy. Trends Ecol. Evol. 35: 336–345.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3: 294–299.

Guillem, R. M., F. P. Drijfhout, and S. J. Martin. 2012. Using chemo-taxonomy of host ants to help conserve the large blue butterfly. Biol. Conserv. 148: 39–43.

Haverty, M. I., L. J. Nelson, and M. Page. 1990. Cuticular hydrocarbons of four populations of *Coptotermes formosanus* Shiraki in the United States. J. Chem. Ecol. 16: 1635–1647.

Hebets, E. A., and D. R. Papaj. 2005. Complex signal function: developing a framework of testable hypotheses. Behav. Ecol. Sociobiol. 57: 197–214.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci. 270: 313–321.

Ivanova, N. V., J. R. deWaard, and P. D. N. Hebert. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Mol. Ecol. Notes. 6: 998–1002.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, et al. 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649.

Kimsey, L. S., and R. M. Bohart. 1991 (1990). The chrysidid wasps of the world. Oxford Press, New York, NY.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Kováts, E. 1958. Gas-chromatographische charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone. Helv. Chim. Acta. 41: 1915–1932.

Kruskal, J. B. 1964a. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika. 29: 1–27.

Kruskal, J. B. 1964b. Nonmetric multidimensional scaling: A numerical method. Psychometrika. 29: 115–129.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.

Kurina, O., Õunap, E., and Põldmaa, K. 2015. Two new *Neuratelia* Rondani (Diptera, Mycetophilidae) species from Western Palaearctic: a case of limited congruence between morphology and DNA sequence data. ZooKeys 496: 105–129.

Liang, D., and J. Silverman. 2000. "You are what you eat": diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. Naturwissenschaften 87: 412–416.

Linsenmaier, W. 1959. Revision der Familie Chrysididae (Hymenoptera) mit besonderer Brücksichtigung der europäischen Spezies. Mitt. Schweiz. Entomol. Ges. 32: 1–232.

Linsenmaier, W. 1997. Die Goldwespen der Schweiz. Veröff. Natur-Mus. Luzern 9: 1–139.

Lucas, C., D. Fresneau, K. Kolmer, J. Heinze, J. H. Delabie, and D. B. Pho. 2002. A multidisciplinary approach to discriminating different taxa in the species complex *Pachycondyla villosa* (Formicidae). Biol. J. Linn. Soc. 75: 249–259.

Martin, S. J., H. Helanterae, and F. P. Drijfhout. 2008. Evolution of species-specific cuticular hydrocarbon patterns in *Formica* ants. Biol. J. Linn. Soc. 95: 131–140.

Martin, S. J., W. Zhong, and F. P. Drijfhout. 2009. Long-term stability of hornet cuticular hydrocarbons facilitates chemotaxonomy using museum specimens. Biol. J. Linn. Soc. 96: 732–737.

Niehuis, O. 2000. The European species of the *Chrysis ignita* group: revision of the *Chrysis angustula* aggregate (Hymenoptera: Chrysididae). Deut. entomol. Z. 47: 181–201.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. Vegan: community ecology package (version 2.0.10). https://cran.r-project.org/web/packages/vegan/index.html

Orlovskytė, S., E. Budrys, A. Budrienė, R. Radzevičiūtė, and V. Soon. 2016. Sibling species in the *Chrysis ignita* complex: molecular, morphological and trophic differentiation of Baltic species, with a description of two new cryptic species (Hymenoptera: Chrysididae): *Chrysis ignita* complex. Syst. Entomol. 41: 771–793.

Page, M., L. J. Nelson, G. J. Blomquist, and S. J. Seybold. 1997. Cuticular hydrocarbons as chemotaxonomic characters of pine engraver beetles (*Ips* spp.) in the *grandicollis* subgeneric group. J. Chem. Ecol. 23: 1053–1099.

Paukkunen, J., A. Berg, V. Soon, F. Ødegaard, and P. Rosa. 2015. An illustrated key to the cuckoo wasps (Hymenoptera, Chrysididae) of the Nordic and Baltic countries, with description of a new species. ZooKeys. 548: 1–116.

Pauli, T., R. F. Castillo-Cajas, P. Rosa, S. Kukowka, A. Berg, E. van den Berghe, F. Fornoff, S. Hopfenmüller, M. Niehuis, R. S. Peters, et al. 2019. Phylogenetic analysis of cuckoo wasps (Hymenoptera: Chrysididae) reveals a partially artificial classification at the genus level and a species-rich clade of bee parasitoids. Syst. Entomol. 44: 322–335.

R Development Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

Robinson, M., and R. Romoli. 2019. flagme: analysis of metabolomics GC/MS data (version 1.38.1). https://bioconductor.org/packages/release/bioc/html/flagme.html

Rosa, P., A. S. Lelej, S. A. Belokobylskij, V. M. Loktionov, and L. A. Zaytseva. 2017. Family Chrysididae, pp. 126–144. *In* A. S. Lelej, M. Yu. Proshchalykin, and V. M. Loktionov (eds.), Annotated catalogue of the Hymenoptera of Russia. Volume 1. Symphyta and Apocrita: Aculeata. Proceedings of the Zoological Institute RAS, Supplement 6.

Schlick-Steiner, B. C., F. M. Steiner, K. Moder, B. Seifert, M. Sanetra, E. Dyreson, C. Stauffer, and C. Erhard. 2006. A multidisciplinary approach reveals cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). Mol. Phylogenet. Evol. 40: 259–273.

Simon, C., T. R. Buckley, F. Frati, J. B. Stewart, and A. T. Beckenbach. 2006. Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. Annu. *Rev. Ecol. Syst.* 37: 545–579.

Smadja, C., and R. K. Butlin. 2008. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity 102: 77–97.

van der Smissen, J. 2010. Schlüssel zur Determination der Goldwespen der engeren *ignita*-Gruppe (Hymenoptera Aculeata: Chrysididae). Verh. Ver. Naturw. Heimatforsch. Hamburg. 43: 4–184.

Smith, M. A., and B. L. Fisher. 2009. Invasions, DNA barcodes, and rapid biodiversity assessment using ants of Mauritius. Front. Zool. 6: 31.

Soon, V., E. Budrys, S. Orlovskytė, J. Paukkunen, F. Ødegaard, T. Ljubomirov, and U. Saarma. 2014. Testing the validity of Northern European species in the *Chrysis ignita* species group (Hymenoptera: Chrysididae) with DNA Barcoding. Zootaxa 3786: 301–330.

Srivathsan, A., E. Hartop, J. Puniamoorthy, W. T. Lee, S. N. Kutty, O. Kurina, and R. Meier. 2019. Rapid, large-scale species discovery in hyperdiverse taxa using 1D MinION sequencing. BMC Biol. 17: 96.

Stein, S. E. 1999. An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. J. Am. Soc. Mass. Spectrom. 10: 770–781.

Strohm, E., J. Kroiss, G. Herzner, C. Laurien-Kehnen, W. Boland, P. Schreier, and T. Schmitt. 2008. A cuckoo in wolves' clothing? Chemical mimicry in a specialized cuckoo wasp of the European beewolf (Hymenoptera, Chrysididae and Crabronidae). Front. Zool. 5: 2.

Vaníčková, L., M. Virgilio, A. Tomčala, R. Břízová, S. Ekesi, M. Hoskovec, B. Kalinová, R. R. Do Nascimento, and M. De Meyeret. 2014. Resolution of three cryptic agricultural pests (*Ceratitis fasciventris*, *C. anonae*, *C. rosa*, Diptera: Tephritidae) using cuticular hydrocarbon profiling. Bull. Entomol. Res. 104: 631–638.

Wicker-Thomas, C., and T. Chertemps. 2010. Molecular biology and genetics of hydrocarbon production, pp. 53–74. In: G. J. Blomquist and A. G. Bagnères (eds.), Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, United Kingdom.

Wickham, H. 2007. Reshaping data with the reshape package. J. Stat. Softw. 21: 1–20.

Wiśniowski, B. 2015. Cuckoo wasps (Hymenoptera: Chrysididae) of Poland. Diversity, identification, distribution. Ojców National Park, Ojców, Poland.

Wurdack, M., S. Herbertz, D. Dowling, J. Kroiss, E. Strohm, H. Baur, O. Niehuis, and T. Schmitt. 2015. Striking cuticular hydrocarbon dimorphism in the mason wasp *Odynerus spinipes* and its possible evolutionary cause (Hymenoptera: Chrysididae, Vespidae). Proc. Royal Soc. B. 282: 20151777.