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High genetic variation within mitochondrial CO1 in Middle European *Thanasimus formicarius* (Linné, 1758) (Coleoptera: Cleridae)

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Abstract

The aim of this study is to assess the species status of the Middle-European *Thanasimus* Latreille, 1806 species using mitochondrial CO1 sequence data. Molecular biological results clearly support the synonymy of *T. pectoralis* (Fuss, 1863) and *T. rufipes* (Brahm, 1797) with *T. femoralis* (Zetterstedt, 1828) as already proposed by Kolibáč (1992). Results of the present study indicate high genetic variation within *T. formicarius* (Linné, 1758) and emphasize the study of population dynamics of *T. formicarius* within Europe. Furthermore, preliminary screening of all available *T. formicarius* sequences on BOLD and Genbank (shorter than 500bp) indicates the presence of a "Continental" and a more "Atlantic" clade in *T. formicarius*. To support our hypothesis of a probably cryptic species among *T. formicarius*, more studies, with more specimens from different populations, especially from southern England, northern France and the northern part of the Iberian Peninsula, will be necessary.

Key words: DNA barcoding, Germany, Clerinae, Cleroidea

Introduction

Species of the genus *Thanasimus* Latreille, 1806 (the ant beetles) are important predators of bark and ambrosia beetles (scolytine weevils), usually hunting their prey on the surface of conifer trunks. Ant beetles are commonly captured in pheromone traps targeting bark beetles and are attracted to aggregation sites of their prey (Bussler 1986; Heidger 1994). Scolytine aggregation pheromones therefore produce a kairomone response in *Thanasimus* species (Bakke & Kvamme 1981; Billings & Cameron 1984; Tommeras 1988; Erbilgin & Raffa 2001). Rettelbach (1994) demonstrated that male and female adults of *Thanasimus femoralis* (Zetterstedt, 1828) have a predation capacity of 100 adult spruce bark beetles (*Ips typographus* (L.)), their larvae of 450 spruce bark beetle stages; this reflects their enormous importance as natural spruce bark beetle antagonists. Prolonged development time of *Thanasimus formicarius* (Linné, 1758) in relation to its prey species *Tomicus piniperda* (Linné, 1758) and *Ips typographus* (Linné, 1758) was investigated by Schroeder (1999).

Currently, many coleopterists treat the Middle-European *Thanasimus* species as three distinct species, i.e., *T. formicarius*, *T. pectoralis* (Fuss, 1863), and *T. rufipes* (Brahm, 1797), using their differing biology as an argument. *Thanasimus pectoralis* mainly overwinters beneath the bark of sycamore (*Acer pseudoplatanus* L.) and sometimes in holm oak (*Quercus ilex* L.) while *T. rufipes* can be found at fallen pines or beaten from green needles of pines. Kolibáč (1992) synonymized *T. pectoralis* and *T. rufipes* with *T. femoralis* using male aedeagi and female copulatory organs as evidence, leaving only *T. formicarius* and *T. femoralis* as the Middle-European *Thanasimus* species. Distribution, bionomy and phenology for these species are described in detail by Niehuis (2013).

The aim of this investigation is to use molecular evidence to assess the species status of the Middle-European *Thanasimus* using mitochondrial CO1 sequence data. The results of this study focus on data obtained during four major European DNA barcoding campaigns: 'Barcoding Fauna Bavarica' (BFB, www.faunabavarica.de; Haszprunar 2009), 'German Barcode of Life' project (GBOL, www.bolgermany.de; Geiger *et al.* 2016), 'PASSIFOR' (Rougerie

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et al. 2015) and the 'Finish Barcode of Life' (FINBOL; Pentinsaari *et al.* 2014). Since its onset in 2009, DNA barcodes from over 23,000 German species (and/or Barcode Index Numbers, BINs) of Metazoa have been assembled, reflecting the analysis of nearly 250,000 specimens in the Barcode of Life Data System (BOLD, www.boldsystems.org; Ratnasingham & Hebert 2007), that are now curated at the SNSB-Zoologische Staatssammlung München (ZSM, see www.barcoding-zsm.de). Because BINs correspond closely to biological species (e.g. Ratnasingham & Hebert 2013; Hausmann *et al.* 2013), they enable the creation of an interim taxonomic system in a structured, transparent, and sustainable way and thus become a valuable foundation for subsequent detailed, integrative taxonomic studies. Furthermore, the BIN system enables analyses that are equivalent to studies based on named species, i.e. where the underlying specimens are identified by specialists using traditional methods (i.e. morphology). The assembled DNA barcode reference library is especially comprehensive for Coleoptera (Hendrich *et al.* 2015; Raupach *et al.* 2016, 2018; Rulik *et al.* 2017), Diptera (Morinière *et al.* 2019), Ephemeroptera, Plecoptera and Trichoptera (Morinière *et al.* 2017), Heteroptera (Raupach *et al.* 2014; Havemann *et al.* 2018), Hymenoptera (Schmidt *et al.* 2015, 2017; Schmid-Egger *et al.* 2018), Lepidoptera (Hausmann *et al.* 2011a, 2011b), Neuroptera (Morinière *et al.* 2014), Orthoptera (Hawlitschek *et al.* 2016), Araneae and Opiliones (Astrin *et al.* 2016), and Myriapoda (Spelda *et al.* 2011; Wesener *et al.* 2015).

Material and methods

For this study, we used CO1 DNA barcode data available on the Barcode of Life Database (BOLD, www.boldsystems.org), which was previously released in the context of DNA barcoding reference libraries focusing on European Coleoptera (Hendrich *et al.* 2015; Rougerie *et al.* 2014; Pentinsaari *et al.* 2014; Rulik *et al.* 2017), as well as some new specimens from the ongoing DNA barcoding projects ran at the SNSB-ZSM and entries, which were publicly available on BOLD.

Molecular biology. A tissue sample was removed from each specimen and transferred into 96-well plates at the SNSB-ZSM for subsequent DNA extraction. Metadata Voucher information such as locality data, habitat, altitude, collector, identifier, taxonomic classifications, habitus images, DNA barcode sequences, primer pairs and trace files are publicly accessible in the "DS-THANASI" dataset in BOLD (http://www.boldsystems.org—dataset DOI; dx.doi.org/10.5883/DS-THANASI).

Once tissue samples were taken, material was sent to the Canadian Center for DNA Barcoding (CCDB, Guelph, Canada) where they were processed using standard barcoding protocols. All samples were PCR amplified with modified Folmer primers CLepFolF (5'—ATT CAA CCA ATC ATA AAG ATA TTG G) and CLepFolR (5'—TAA ACT TCT GGA TGT CCA AAA AAT CA) for the barcoding fragment (5' mitochondrial cytochrome oxidase subunit 1—CO1), and the same primers were employed for subsequent Sanger sequencing reactions (see also Ivanova *et al.* 2006; deWaard *et al.* 2008). The sequence data and trace files were uploaded to BOLD and subsequently also to GenBank (accession numbers are available under dx.doi.org/10.5883/DS-THANASI).

Data analysis. Sequence divergences for the COI-5P barcode region (mean and maximum intraspecific variation and minimum genetic distance to the nearest-neighbor species) were calculated using the "Barcode Gap Analysis" tool on BOLD, employing the Kimura-2-Parameter (K2P) distance metric (Puillandre *et al.* 2012). MUSCLE was applied for sequence alignment restricting analysis to sequences with a minimum length of 500bp. Neighbor joining (NJ) trees were calculated following alignment based on K2P distances. The "BIN Discordance" analysis on BOLD was used to reveal cases where specimens assigned to different species shared a BIN, and those cases where a particular species was assigned to two or more BINs. Sequences are grouped into clusters of closely similar COI barcode sequences, which are assigned a globally unique identifier, termed a "Barcode Index Number" or BIN (Ratnasingham & Hebert 2013). This system enables tentative species identifications when taxonomic information is lacking. The BIN System involves a 3-step online pipeline, which clusters similar barcode sequences algorithmically into operational taxonomic units (OTUs) being "named" by a number. Specimens sharing a BIN very often represent a close species-proxy as delineated by traditional taxonomy (Hausmann *et al.* 2013).

Results

Comparison of the CO1 DNA barcode sequences within a NJ tree (Fig. 1) and in the Barcode Gap Analysis (Table 1), reveals that the specimens of *Thanasimus femoralis* (*T. pectoralis* and *T. rufipes*) cannot be discriminated into clear single species clusters. All studied specimens of the species-cluster in question appear dispersed in the same BIN (BOLD:ABW4499). However, the specimens of *T. formicarius*, used as outgroups in this study, exhibit high genetic variation and are distributed within 5 BINs (Fig. 1).

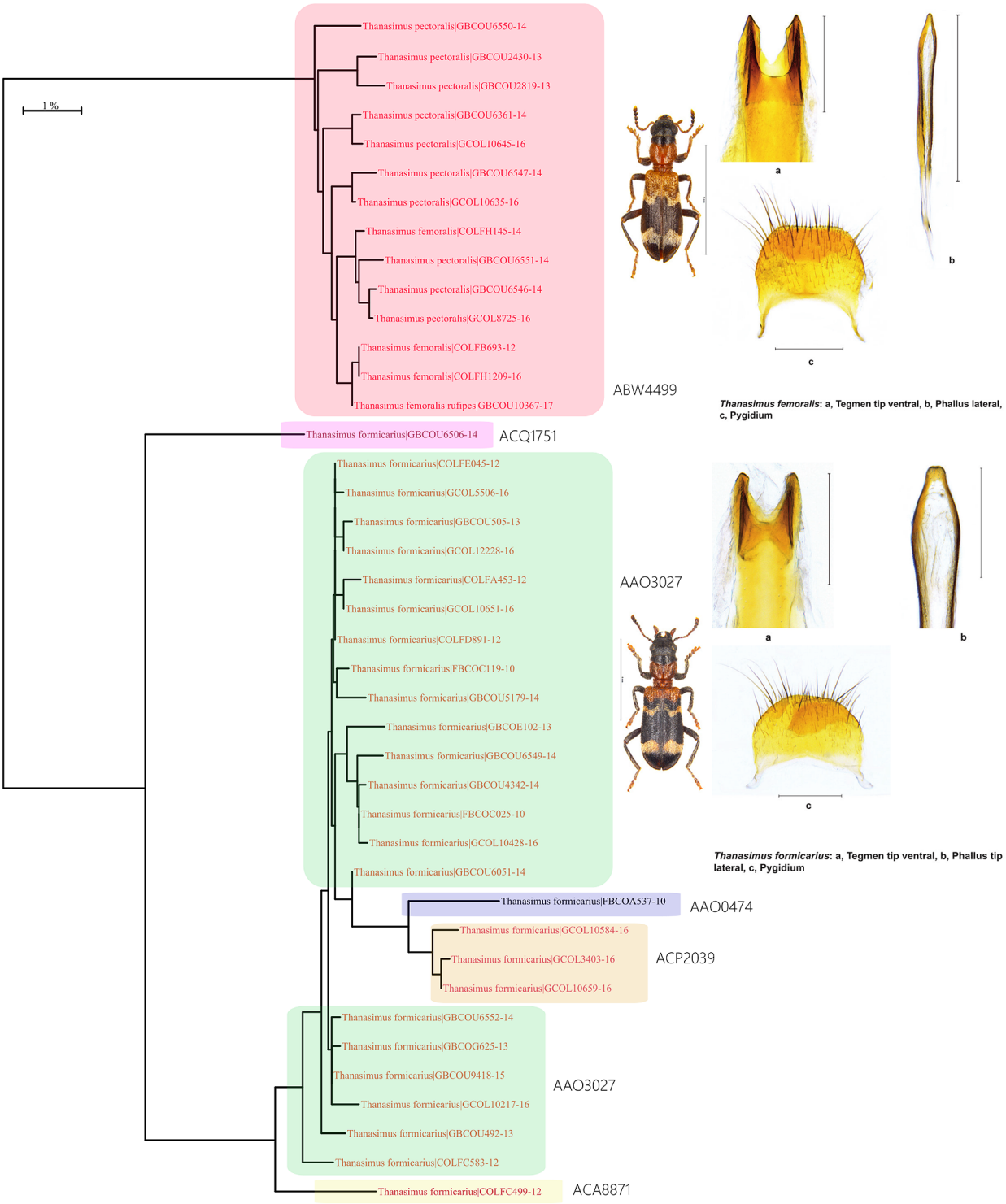

FIGURE 1. Neighbour-joining tree of all *Thanasimus* mentioned in this study.

TABLE 1. Barcode Gap analysis of *Thanasimus* species studied. For each species, the mean and maximum intra-specific values are compared to the nearest neighbour distance in the table below. Where the species is a singleton, N/A is displayed for intra-specific values. Distances are highlighted if the nearest neighbour is less than 2% divergent, or when the distance to the nearest neighbour is less than the max intra-specific distance.

Species	Mean Intra-Sp	Max Intra-Sp	Nearest Species	Distance to NN
Thanasimus femoralis	0.51	0.77	*Thanasimus femoralis rufipes*	0.15
Thanasimus femoralis rufipes	N/A	0	*Thanasimus femoralis*	0.15
Thanasimus formicarius	1.9	8.03	*Thanasimus pectoralis*	10.94
Thanasimus pectoralis	1.49	2.34	*Thanasimus femoralis*	0.31

Our molecular biological results clearly support the synonymy of *T. pectoralis* and *T. rufipes* with *T. femoralis* as already proposed by Kolibáč (1992). Whereas CO1 Barcoding has certain limitations in discriminating some closely related species (*Wolbachia* effects, e.g.), it supports findings based on morphology (Dobson 2004; Duron *et al.* 2008; Jiggins *et al.* 2001). We therefore propose further studies which should implement more genes (whole mitochondrial genomes and/or nuclear markers) to eliminate any remaining doubts on the species status of *T. femoralis*.

The indication of a high genetic variation within *T. formicarius* emphasize the study of population dynamics of *T. formicarius* within Europe (Fig. 2). Furthermore, preliminary screening of all available *T. formicarius* sequences on BOLD and Genbank (shorter than 500bp) indicates the presence of a "Continental" and a more "Atlantic" clade in *T. formicarius*, the latter including our single specimen from southern England [ACQ1751] (Fig. 1). This unusual distribution is also known in the beetle family Dytiscidae, in the species *Hydroporus necopinatus* Fery, 1999 with its three subspecies *H. necopinatus necopinatus* Fery, 1999 from Portugal and northern Spain, *H. necopinatus robertorum* Fery, 1999 from western France, and *H. necopinatus roni* Fery, 1999 from southern England (*H. necopinatus*-complex; see Fery 1999).

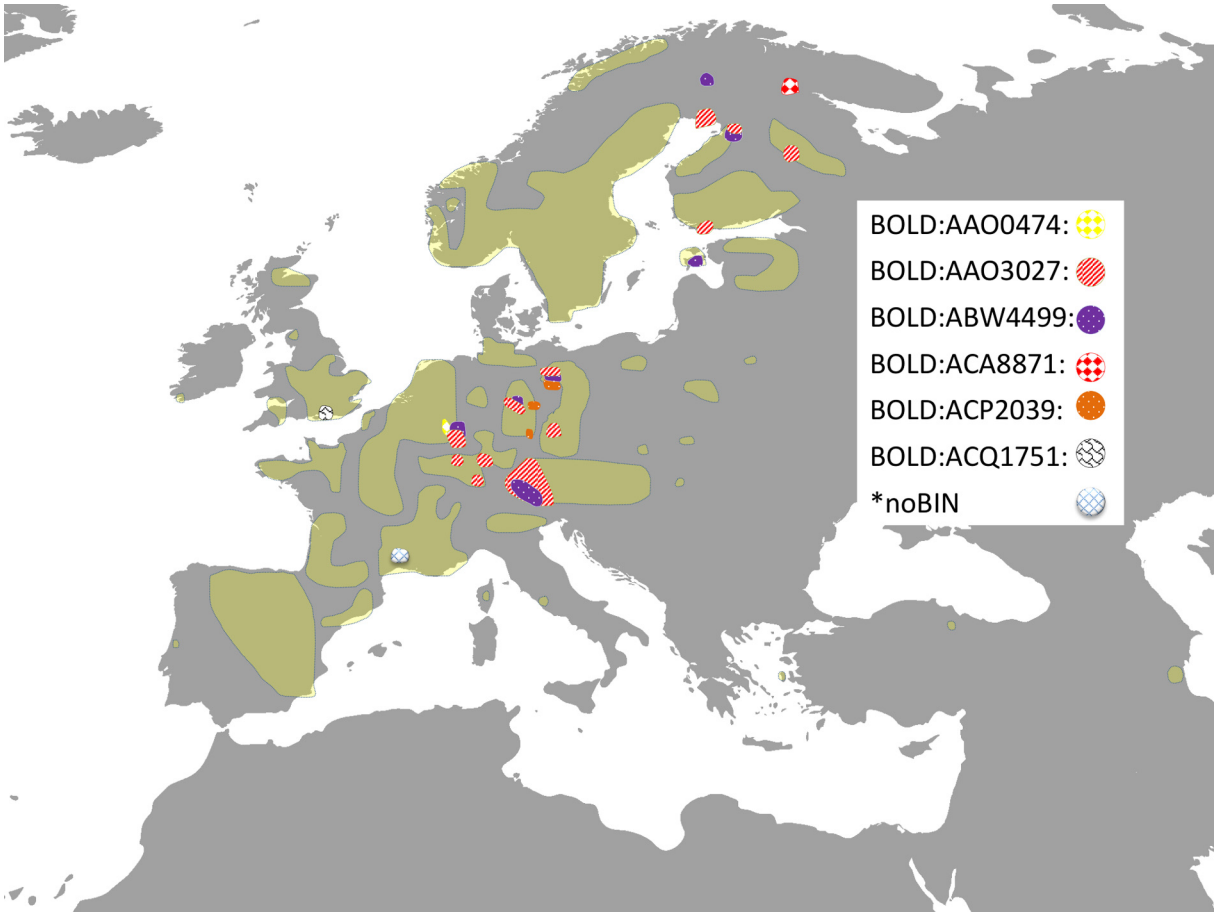

FIGURE 2. Distribution of all *Thanasimus* clades in Europe. The light green areas are based on GBIF distributional data [https://www.gbif.org/] of both species.

To support our hypothesis of a probable cryptic species among *T. formicarius*, more studies with more specimens from different populations, especially from southern England, northern France and the northern part of the Iberian Peninsula, will be necessary.

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