The European Zoological Journal

ISSN: 2475-0263 (Online) Journal homepage: www.tandfonline.com/journals/tizo21

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To cite this article: M. Kasparek, P. Biella, F. Ramazzotti & A. Galimberti (2025) Integrative taxonomy reveals cryptic speciation in the anthidiine wool carder bee *Anthidium loti* Perris, 1852 (Hymenoptera: Megachilidae) in the Western Palaearctic, The European Zoological Journal, 92:1, 1680-1694, DOI: 10.1080/24750263.2025.2594898

To link to this article: https://doi.org/10.1080/24750263.2025.2594898

THE EUROPEAN ZOOLOGICAL JOURNAL
2025, VOL. 92, NO. 1, 1680–1694
https://doi.org/10.1080/24750263.2025.2594898

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Integrative taxonomy reveals cryptic speciation in the anthidiine wool carder bee *Anthidium loti* Perris, 1852 (Hymenoptera: Megachilidae) in the Western Palaearctic

M. Kasparek[a], P. Biella[b], F. Ramazzotti[b,c] and A. Galimberti[b,c]

[a]Heidelberg, Germany; [b]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milano, Italy; [c]National Biodiversity Future Center, Palermo, Italy

ABSTRACT

The Mediterranean, the Irano-Anatolian and the Caucasus regions are known as major biodiversity hotspots with high speciation dynamics in many groups of plants and animals. In order to better understand underlying processes, the situation of the wool carder bee *Anthidium loti*, a pollinator whose distribution extends from the Mediterranean into Central Asia and Pakistan, was examined. Despite a high level of geographic variation in the colour pattern, it was found that colouration does not correspond to distinct population groups. However, genetic examination of the COI mitochondrial marker and the LW-rhodopsin nuclear one from populations of the entire distribution range revealed that there are two distinct groups: eastern and western. Phylogenetic analysis showed that these two groups belong to monophyletic clades and multiple species delimitation analyses confirmed the presence of two well distinct species within *A. loti* s.l. Further evidence for the presence of distinct taxa was obtained by a multivariate Discriminant Function Analysis of nine morphometric parameters, which allowed us to correctly assign 93.8% of all specimens to the respective species. Based on these results and the examination of the historical type material, the western population group was assigned to *Anthidium loti* Perris 1852, and the eastern population group to *Anthidium regulare* Eversmann 1852, which has previously been regarded as a synonym. The taxonomic status is hereby re-established (stat. resurr.), and a lectotype is designated. This study adds novel evidence to the growing body of knowledge demonstrating a high cryptic diversity in the Mediterranean region.

ARTICLE HISTORY
Received 28 July 2025
Accepted 20 November 2025

KEYWORDS
Cryptic species; pollinator; speciation; allopatry; DNA barcoding

Introduction

The Mediterranean Basin and the neighbouring areas are renowned major biodiversity hotspots. The Mediterranean, the Irano-Anatolian and the Caucasus regions are characterized by exceptionally high species diversity and a high rate of endemism (Myers et al. 2000; Thompson 2020). The reasons for this diversity are multifaceted. Located at the crossroads between Europe, Asia and Africa, the Mediterranean region has been described as a centre of diversification for plant lineages of various biogeographic origins, as well as an area where many species differentiated *in situ* (Blondel & Aronson 1999). The same principle holds true for animals. One of the major drivers for the high biodiversity in the Mediterranean appears to be the fact that Quaternary climatic oscillations were less severe there than in more northern regions. Consequently, the Mediterranean region could host multiple refugia during the various phases of the glacial periods (Nieto-Feliner 2011).

For wild bees (Hymenoptera: Anthophila), a high species diversity in the Mediterranean Basin and eastward to Central Asia has been confirmed in many studies (Michener 2007; Lhomme et al. 2020; Orr et al. 2021; Ghisbain et al. 2023; Reverté et al. 2023; Cornalba et al. 2024). This includes species richness within the tribe Anthidiini, where the number of species in Mediterranean countries is several times higher than in central and north European countries (Warncke 1980; Kasparek 2022). Yet, the specific evolutionary trajectories that led to this high diversity are little understood.

CONTACT P. Biella paolo.biella@unimib.it ZooPlantLab, Department of Biotechnology and Bioscience, University of Milano-Bicocca, Piazza della Scienza 2, Milano 20126, Italy

Supplemental data for this article can be accessed online at https://doi.org/10.1080/24750263.2025.2594898

Profound analyses of geographic variation patterns are largely lacking for bees in the Mediterranean. To gain a better understanding of bee populations in this region, we chose the wool carder bee *Anthidium loti* Perris 1852 s.l. for our investigation, as this species exhibits, on the one hand, a wide distribution spanning from the Iberian Peninsula across the entire northern Mediterranean, the Anatolian landmass, and the Caucasus into Central Asia (Warncke 1980) and, on the other hand, it is known for its high phenotypic variation (Kasparek 2022). A comprehensive DNA barcoding overview of Central European bees (Schmidt et al. 2015) found that specimens of *A. loti* from the northern Italian peninsula were assigned to a different Barcode Index Number (BIN) than specimens from southern France. This finding suggests either substantial regional variation in DNA sequences or the existence of cryptic species overlooked by current taxonomy. To clarify this situation, we undertook a comprehensive morphological, biogeographic and genetic analysis of a wide sampling from across the species' entire distribution range. The present study thereby aims to enhance our understanding of morphological and genetic diversity within anthidiine bees and may also assist in resolving taxonomic discrepancies in other taxa where genetic and phenotypic variation are incongruent.

Materials and methods

Material for species distribution assessment

A comprehensive literature survey on the occurrence of *Anthidium loti* s.l. was undertaken (Supplementary Appendix S1). In most cases, only general toponyms were available and their localities were georeferenced using Google Earth Pro, version 7.3.6. Locations that could not be determined with Google Earth or not within an estimated area of approximately 5–10 km were ignored. In a next step, the records available through the Global Biodiversity Information Facility (GBIF) were screened (www.gbif.org/species/1334967, downloaded on 1 September 2023). Records with incomplete and uninformative metadata were discarded, while the geographic coordinates were determined and added where these had not been available but the toponym was clear. Additional records were retrieved from the author M.K. collection (acronym CMK), from specimens received for examination mostly from colleagues in various countries and from MIBZPL (Collection of the ZooPlantLab of the University of Milano-Bicocca, Milano, Italy). Finally, an additional set of records, mainly from France and Spain, were obtained from Jan Smit (Duiven, The Netherlands).

Morphological diagnosis

The basis for the morphological examination were 130 specimens collated in CMK. The examination included the type material of *Anthidium loti* Perris 1852, loaned from the Centre de Biologie pour la Gestion des Populations (CBGP), Montferrier-sur-Lez (France), and the type of *A. regulare* Eversmann, 1852, loaned from the Institute of Systematics and Evolution of Animals of the Polish Academy of Sciences (Kraków, Poland) (Figure 1). A selection of male specimens was softened to stretch the abdomen for examining the sterna and for extracting the genitalia.

For a morphometric analysis, 72 specimens from European countries were selected (Supplementary Appendix S2). Material from non-European countries, particularly material from Turkey, was excluded to avoid the influence of clinal variation. The following nine measurements were used for the analysis: clypeus width at the suture between the supraclypeal area and the paraocular area (anterior clypeus width); clypeus width at the widest point (lower clypeus width), clypeus length (along the midline excluding the black apical rim), eye distance (at the height of the antennal sockets), head width (maximum distance at the outer edge of the compound eye in frontal view), distance between the posterior ocellus and the compound eye; inner distance between the posterior ocelli; distance between the posterior ocellus and the median point of the preoccipital ridge, and length of the marginal cell. The methodological approach for taking the measurements and for evaluating them with multivariate statistics is described in detail in Kasparek (2018, 2020a, 2020b).

Multivariate statistical procedures were applied for analysing morphometric data. A Principal Component Analysis (PCA) was performed to reveal patterns in morphological variation and a Discriminant Function Analysis (DFA) (= Canonical Variates Analysis) was performed to determine whether the set of body

Figure 1. *Anthidium regulare* Eversmann, 1852, male, lectotype, from Kazakhstan (Deposited in the Institute of Systematics and Evolution of animals, Polish Academy of Sciences, Kraków, Poland). The species had previously been recognized as synonym of *Anthidium loti* Perris, 1852, but is re-established herein as valid species. A. Habitus; B. face; C. Apical terga.

measurements is effective in predicting category membership, that means the western or eastern population groups of *A. loti* s.l. The statistical tests were performed with PAST (PAleontological STatistics), Version 4.15 (Hammer et al. 2001).

Material for genetic analysis

We selected 57 specimens of *A. loti* s.l. for molecular analysis, from 13 countries covering as much as possible the known distribution range of the species to maximize the chance of observing intraspecific geographic variation. Only material collected during the last 10–15 years was used. In addition, six specimens of *A. manicatum*, four *A. cingulatum*, two *A. oblongatum* and one *A. florentinum* from Europe were added to the molecular analysis. For the genetic analyses between and within *Anthidium* species, we further downloaded DNA sequences of various species publicly available from GenBank and BOLD databases. Detailed information is provided in Supplementary Appendix S3.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from a mid-leg using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Before proceeding with the extraction phases, the specimens were rinsed and rehydrated in physiological solution (NaCl 0.9%) for 15 min.

Concerning DNA barcoding characterization, PCRs were carried out using PuReTaq Ready-To-Go™ PCR Beads from GE Healthcare in 25 µl final reaction volumes containing 22 µl of sterile water, 1 µl of each primer and 1 µl of gDNA (10 ng). For each sample, the standard DNA barcode 5'-end region of the cytochrome c oxidase subunit 1 gene (COI) gene (658 bp) was amplified using the LCO1490-HCO2198 primer pair (Folmer et al. 1994). In case of unsuccessful amplification, the alternative COI primer pairs LepF1–LepR1 (Hebert et al.

2004) and Ap1851–Ap2154R (Pisanty et al. 2022) were adopted to amplify two shorter but partially overlapping regions covering the full barcode one. In both cases, PCR amplification conditions were as follows: 94°C for 5 min, 5 cycles at 94°C for 60 s, 45°C for 90 s, and 72°C for 90 s followed by 35 cycles at 94°C for 60 s, 50°C for 90 s, and 72°C for 60 s and a final extension at 72°C for 7 min. This process was performed on 52 specimens. Moreover, 12 samples were processed by the Canadian Centre for DNA Barcoding (CCDB), Guelph, using standardized high-throughput protocols described in http://ccdb.ca/resources.

In addition to the COI, a region from the nuclear DNA, LW-rhodopsin gene, was amplified and sequenced for 14 *A. loti s.l.* to confirm possible differences noticed in the COI marker, as well as for one *A. florentinum*, *A. oblongatum* and four *A. cingulatum* for use in subsequent analyses. Laboratory procedures were conducted according to Litman et al. (2016) using Opsin fora and Opsin rev3y primers with the following thermal profile: 94°C for 5 min, 35 cycles at 94°C for 45 s, 54/58°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 7 min. After amplicon purification, the sequencing was performed bidirectionally at Eurofins Genomics (Milan, Italy) with the same oligos used for the amplification step.

Consensus sequences were obtained by editing the electropherograms with BioEdit 7.2 (Hall 1999). After primer trimming, the presence of an open reading frame was verified for the obtained consensus sequences by using the online tool EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq). Specimen sampling metadata and consensus sequences were deposited in the BOLD System (project ZPLAL, and see Supplementary Appendix S3 for process ID codes).

Haplotype analysis and genetic distances

Orthologous COI and LW-Rhodopsin sequences belonging to *Anthidium* were downloaded from BOLD Systems and GenBank in June 2023. After alignment with MAFFT 7.110 (Katoh & Standley 2013) using the E-INS-I option, sequences showing insertions or deletions (with the exception of LW-Rhodopsin), those missing more than 1% of sites, or those overlapping less than 75% in length with the region amplified and sequenced for the samples in this study were discarded. After this filtering, COI and LW-Rhodopsin sequences were combined with the genetic data obtained in this study to constitute a single dataset for each marker for a total of 244 COI and 36 LW-Rhodopsin sequences, respectively, comprehensively belonging to 23 and 13 *Anthidium* species (see Supplementary Appendix S3).

We calculated the uncorrected pairwise genetic distances (p-distance) per marker between and within groups using MEGA X (Kumar et al. 2018). Haplotype and nucleotide diversity were calculated for COI sequences using dnaSP 6.0 (Rozas et al. 2017). Since many *Anthidium* species were represented by single LW-Rhodopsin sequences, haplotype structure indexes were not calculated for this marker.

For both the sequenced markers (i.e., COI and LW-rhodopsin), the aligned nucleotide sequences were collapsed into unique haplotypes using FaBox 1.5 (Villesen 2007). To investigate the frequency and geographic distribution of mtDNA and nDNA haplotypes, median-joining networks, encompassing all the investigated *Anthidium loti s.l.* populations, were built using the median-joining algorithm implemented in PopART v1.7 (Leigh & Bryant 2015). Separate haplotype networks were generated for each marker and colour-coded according to taxonomy.

Phylogenetic and species delimitation analysis

Phylogenetic analysis was conducted on the sequences of the COI marker used for the genetic distances analyses; LW-Rhodopsin was excluded because only a small set of sequences was available for this marker and usually from specimens and localities different from those used in the COI. Three sequences belonging to *Apis mellifera*, *Bombus terrestris* and *Trachusa interrupta* were added to the analysis as outgroups.

One sequence per haplotype was included in the analysis. The best model for sequence evolution was obtained with IQ-Tree 2.2.2.6 (Minh et al. 2020) to avoid *a priori* models, which indicated that the model was GTR+F+I+G4 from both AIC and BIC values. This model was set in MrBayes 3.2.7a (Ronquist et al. 2012) to run phylogenetic analyses with 1,000,000 generations, sampling every 1,000 generations, with four chains, 10% of generations were cut as burn in. The convergence of the runs was verified to make sure that the effective sample sizes (ESS) were all above 200 by examining the likelihood plots using TRACER v.1.7.1 (Rambaut et al. 2018).

We performed species delimitation using PTP (Poisson Tree Processes) since it outperforms GYMC (Zhang et al. 2013). To do so, we conducted bPTP analyses on the web server https://species.h-its.org/

ptp. We used the phylogeny of the COI tree and both mIPTP and hsPTP algorithms (i.e. maximum likelihood and heuristic search) with options as 500,000 MCMC generations, a thinning value of 100, a burn in of 10%. Outgroups were kept when conducting the PTP analyses. An additional method for species delimitation was performed with ASAP – Assemble Species by Automatic Partitioning (Puillandre et al. 2021) and it was run through the web-based interface https://bioinfo.mnhn.fr/abi/public/asap using default parameters and the simple-distance model (p-distances) to calculate the most supported barcoding gaps for partitioning the dataset into putative species, using the COI dataset as for the PTP.

The Relative Taxonomic Resolving Power Index (Rtax) and the Taxonomic Index of Congruence (Ctax) between the two species delimitation algorithms was calculated as in Miralles and Vences (2013).

Results

Morphological peculiarities

In our study, we aimed to identify potential geographic morphological differences and focused on possible differences between the western and the eastern population groups of *A. loti* s.l. In terms of colouration, specimens from the southern part of the distribution area were found to exhibit in general a richer yellow hue compared to those from further north (Figure 2). However, no significant differences in colouration were observed along the east–west axis of the distribution. One prominent feature of variation was the punctation of the terga, particularly in males. For example, most specimens exhibited a dense punctation on T2, but there were also some individuals with interstices up to one puncture diameter. While the spaces between the punctures were typically smooth, some showed a rugulose texture. Overall, the punctation was less dense in males compared to females. However, our analysis did not reveal a significant correlation between this punctation pattern and the geographic origin of the specimens.

A detailed comparison of male genitalia also did not reveal geographic differences between the eastern and western population groups of *A. loti* s.l. (Figure 3). Similarly, the hidden sternum 8 (S8), which often exhibits species-specific characteristics in *Anthidium* species, did not show distinct differences between representatives of the two population groups of *A. loti* s.l. The only clear geographic differentiation was observed in the size and shape of sternum 6 (S6) in males. In the eastern population group of *A. loti* s.l., S6 was conspicuously longer with a truncate apex, whereas in the western population group of *A. loti* s.l., it was relatively shorter with rounded edges (Figures 4). Additionally, S6 was found to be at least one-fourth as long as it is wide in the eastern population group of *A. loti* s.l., while it was usually shorter than one-fourth its width in the western population group. These differences also align with the type specimens of *A. regulare* stat. resurrect. and *A. loti* (see below). Nevertheless, the shape and size of S6 shows some variation, and less experienced researchers may find it challenging to correctly assign some intermediate specimens. No external morphological traits were found that would allow for distinguishing the females of the eastern and western population group of *A. loti* s.l.

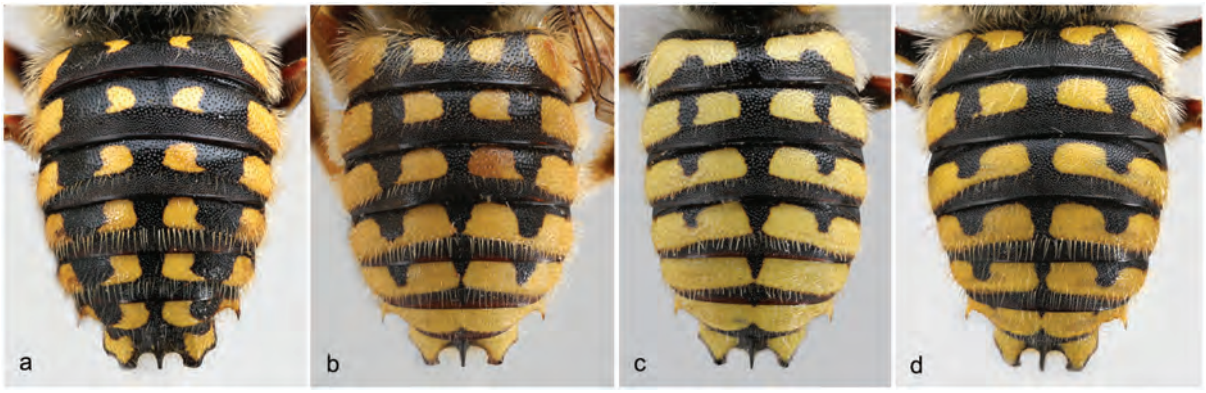

Figure 2. Variation in the colour pattern of the metasomal terga in male *Anthidium loti* s.l. a. Croatia; b. Turkey; c. Iran; d. Spain. a–c were assigned here to *Anthidium regulare* Eversmann, 1852, d to *Anthidium loti* Perris, 1852.

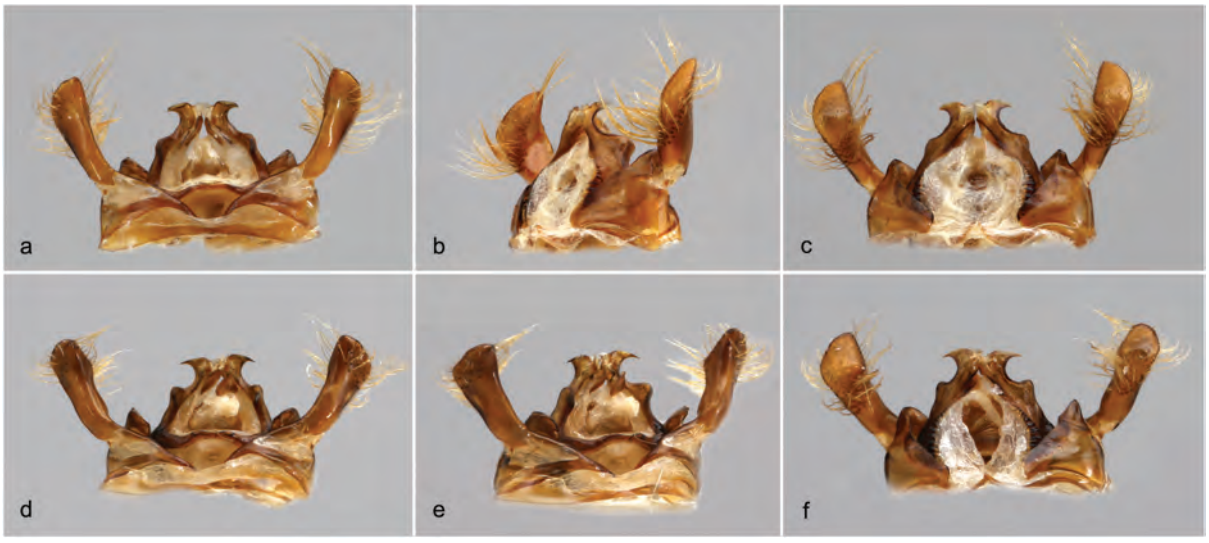

Figure 3. The male genitalia of the eastern population group (a–c) and the western population group (d–f) of *Anthidium loti* s.l. (a: dorsal, b: oblique, c: ventral; a–c from Israel, d–f from Spain).

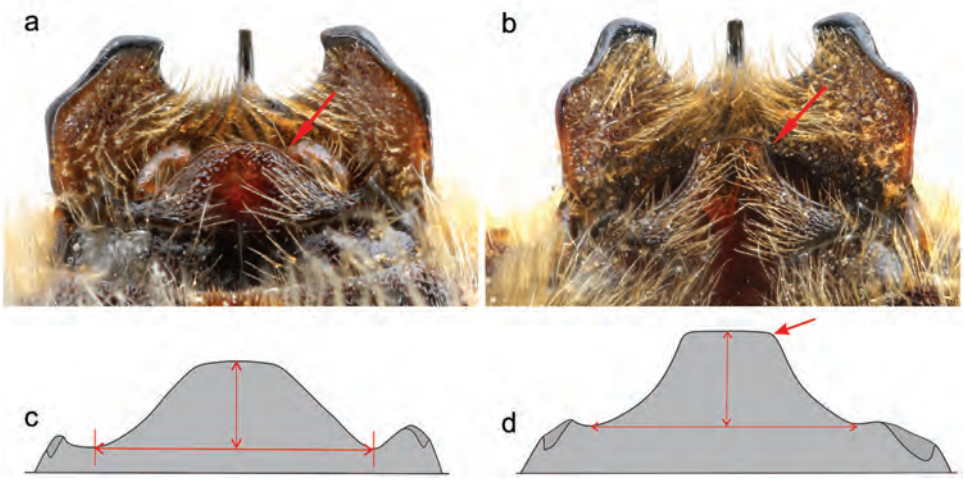

Figure 4. Sternum 6 (S6) of the male of the western population group that has been assigned to *A. loti* (a) and the eastern population group that has been assigned to *A. regulare* (b). The relative length and shape of S6 is the only external character known to distinguish these two species (c, d). In *A. loti* (c), S6 is less than four times longer than wide and has a rounded apex, while it is at least four times wider than long and the apex is truncate in *A. regulare* (d).

Morphometric peculiarities

Applying Principal Component Analysis (PCA) on nine morphological measurements revealed two factors with eigenvalue > 0.01, collectively explaining 93.2% of the variance. The first principal component (PC1) accounted for 84.5% of the variation, and the second (PC2) for 8.6%. The highest loading characters were head width and length of the marginal cell for PC1, while the highest loading for PC2 was eye distance.

A Discriminant Function Analysis (DFA) was applied to the nine morphometric parameters, and it was tested whether there are differences between the eastern and the western OTUs. In fact, these two population groups of *A. loti* s.l. formed in the DFA separate clusters, both for males and females (Figure 5). The two population groups of *A. loti* s.l. are thus well separated by morphometric traits. A confusion matrix confirms that all females (100%) were correctly assigned to their respective population groups of *A. loti* s.l., and, in males, 93.8% were accurately assigned.

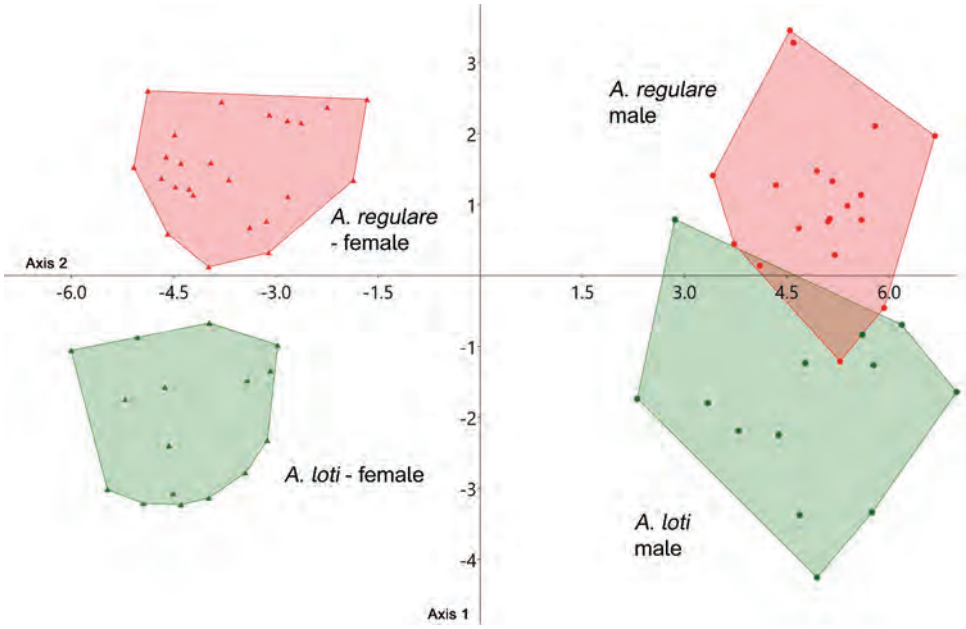

Figure 5. Results of a Discriminant Function Analysis for male and female specimens of the eastern population group (*Anthidium regulare*) and the western population group (*A. loti*), using nine morphometric characters.

Genetic analyses

The interspecific average p-distances between various *Anthidium* species examined ranged from 2.8 to 16.7%, with an average of 10.92% (±1.96%) in COI and from 0.76 to 9.35% with an average of 4.80% (±2.26%) in LW-Rhodopsin. Between the two groups in the *Anthidium loti* s. l. (i.e., eastern and western populations), the p-distance value was 2.80% (±0.66%) in COI and 0.76% (±0.36) in LW-Rhodopsin (Supplementary Appendix S3). Moreover, the barcoding gap analysis conducted with ASAP identified a threshold p-distance value of 1.79%.

In total, 86 haplotypes were identified in the COI dataset, corresponding to an average value of 3.6 ± 2.5 haplotypes per species within the *Anthidium* genus. The estimated genetic diversity indices calculated in the total dataset for each analysed marker are shown in Table 1. In the COI marker, the overall haplotype diversity (H) in the *Anthidium* genus was 0.943 ± 0.007; it was 0 in *A. loti* and 0.609 ± 0.086 in *A. regulare*. The highest value of nucleotide diversity (π) was found in *A. loti* s.l. (0.01267 ± 0.00129) and *A. jocosum* (0.02614 ± 0.01307). The haplotype networks demonstrated the presence of two well distinct haplogroups within *Anthidium loti* s.l. in both markers with one exclusive haplotype for *A. loti* and nine distinct haplotypes for *A. regulate* in COI, while one haplotype for *A. regulare* and one for *A. loti* were consistently found across specimens in the rhodopsin marker (Figure 6).

In the phylogenetic tree of the COI marker, all nodes at the species level were generally well-resolved and received strong support. Monophyly of the *Anthidium* genus received strong support as did the two haplogroups of *A. loti* s.l. (Figure 7).

The species delimitation analyses using mlPTP, hsPTP, and ASAP algorithms confirmed the presence of two well distinct putative species within *A. loti* s.l. (Figure 7). The bPTP analyses resulted in a high resolving power because the Taxonomic Index of Congruence (Ctax) between mlPTP and hsPTP resulted as 1, as the set of species supported is identical with both algorithms. The Ctax between mlPTP and ASAP was 0.95 and likewise between hsPTP and ASAP (only one taxon recognized by mlPTP and hsPTP is not recognized by ASAP, overall). The Relative Taxonomic Resolving Power Index (Rtax) value of bPTP was of 1 since the maximum number of recognized species by bPTP is identical to the overall maximum number of species by any algorithm; conversely, Rtax for ASAP was 0.95 because this algorithm recognizes one species less than the overall maximum. The only taxa recognized by bPTP that ASAP did not separate are the two clades within *A. jocosum* that are considered as one species by ASAP.

Table 1. Table with intraspecific genetic distances of the two genetic regions investigated (COI, LW-Rhodopsin).

	COI				LW-Rhodopsin			
Species	No. haplotypes	Haplotype diversity (H) (SD)	Nucleotide diversity (π) (SD)	Intraspecific p-distance (SE)	No. haplotypes	Haplotype diversity (H) (SD)	Nucleotide diversity (π) (SD)	Intraspecific p-distance (SE)
regulare stat. resurrect.	9	0.609 (0.086)	0.00127 (0.00025)	0.0013 (0.0004)	1	0 (0)	0 (0)	0.0017 (0.0012)
loti s.str.	1	0 (0)	0 (0)	0 (0)	1	0 (0)	0 (0)	0 (0)
loti s.l.	10	0.723 (0.042)	0.01267 (0.00129)	0.0127 (0.0028)	2	0.440 (0.112)	0.00264 (0.00067)	0.0042 (0.0018)
chilense	0	_	_	_	1	0 (0)	0 (0)	0 (0)
cingulatum	2	0.4 (0.237)	0.00065 (0.00039)	0.0006 (0.0007)	2	0.5 (0.265)	0.00105 (0.00056)	0.0011 (0.0010)
clypeodentatum	5	0.505 (0.158)	0.00186 (0.00072)	0.0023 (0.0008)	0	_	_	_
cockerelli	0	_	_	_	1	0 (0)	0 (0)	0 (0)
collectum	2	1 (0.5)	0.00162 (0.00081)	0.0016 (0.0017)	0	_	_	_
colliguayanum	0	_	_	_	1	0 (0)	0 (0)	0 (0)
cordiforme	0	_	_	_	1	0 (0)	0 (0)	0 (0)
deceptum	0	_	_	_	1	0 (0)	0 (0)	0 (0)
edwardsii	2	1 (0.5)	0.00325 (0.00162)	0.0032 (0.0022)	0	_	_	_
emarginatum	2	0.333 (0.215)	0.00054 (0.00035)	0.0005 (0.0005)	0	_	_	_
florentinum	1	0 (0)	0 (0)	0 (0)	1	0 (0)	0 (0)	0 (0)
formosum	1	0 (0)	0 (0)	0 (0)	0	_	_	_
gratum	0	_	_	_	1	0 (0)	0 (0)	0 (0)
illustre	1	0 (0)	0 (0)	0 (0)	1	0 (0)	0 (0)	0 (0)
jocosum clade I	1	0 (0)	0 (0)	0 (0)	0	_	_	_
jocosum clade II	3	0.833 (0.022)	0.00433 (0.00124)	0.0043 (0.0019)	0	_	_	_
jocosum s.l.	4	0.900 (0.161)	0.02614 (0.01307)	0.00261 (0.0042)	0	_	_	_
maculifrons	1	0 (0)	0 (0)	0 (0)	0	_	_	_
maculosum	5	1 (0.126)	0.00747 (0.00220)	0.0075 (0.0024)	0	_	_	_
manicatum	8	0.559 (0.0690)	0.00234 (0.00047)	0.0023 (0.0009)	1	0 (0)	0 (0)	0 (0)
montanum	3	1 (0.272)	0.00216 (0.00072)	0.0022 (0.0016)	0	_	_	_
mormonum	3	1 (0.272)	0.00541 (0.00153)	0.0054 (0.0024)	0	_	_	_
oblongatum	4	0.373 (0.120)	0.00079 (0.00030)	0.0008 (0.0004)	1	0 (0)	0 (0)	0 (0)
palmarum	2	1 (0.5)	0.00170 (0.00085)	0.0017 (0.0018)	0	_	_	_
porterae	2	0.667 (0.314)	0.00216 (0.00102)	0.0022 (0.0015)	1	0 (0)	0 (0)	_
punctatum	4	0.575 (0.115)	0.00170 (0.00039)	0.0017 (0.0011)	1	0 (0)	0 (0)	0 (0)
septemspinosum	4	0.900 (0.161)	0.00227 (0.00062)	0.0023 (0.0014)	0	_	_	_
tenuiflorae	5	0.562 (0.143)	0.00148 (0.00052)	0.0015 (0.0006)	0	_	_	_
unicum	0	_	_	_	1	0 (0)	0 (0)	0 (0)
utahense	5	0.857 (0.137)	0.00216 (0.00057)	0.0022 (0.0011)	0	_	_	_

"n.c". denotes uninformative cases either without sequences or with only one available. SD = standard deviation; SE = standard error.

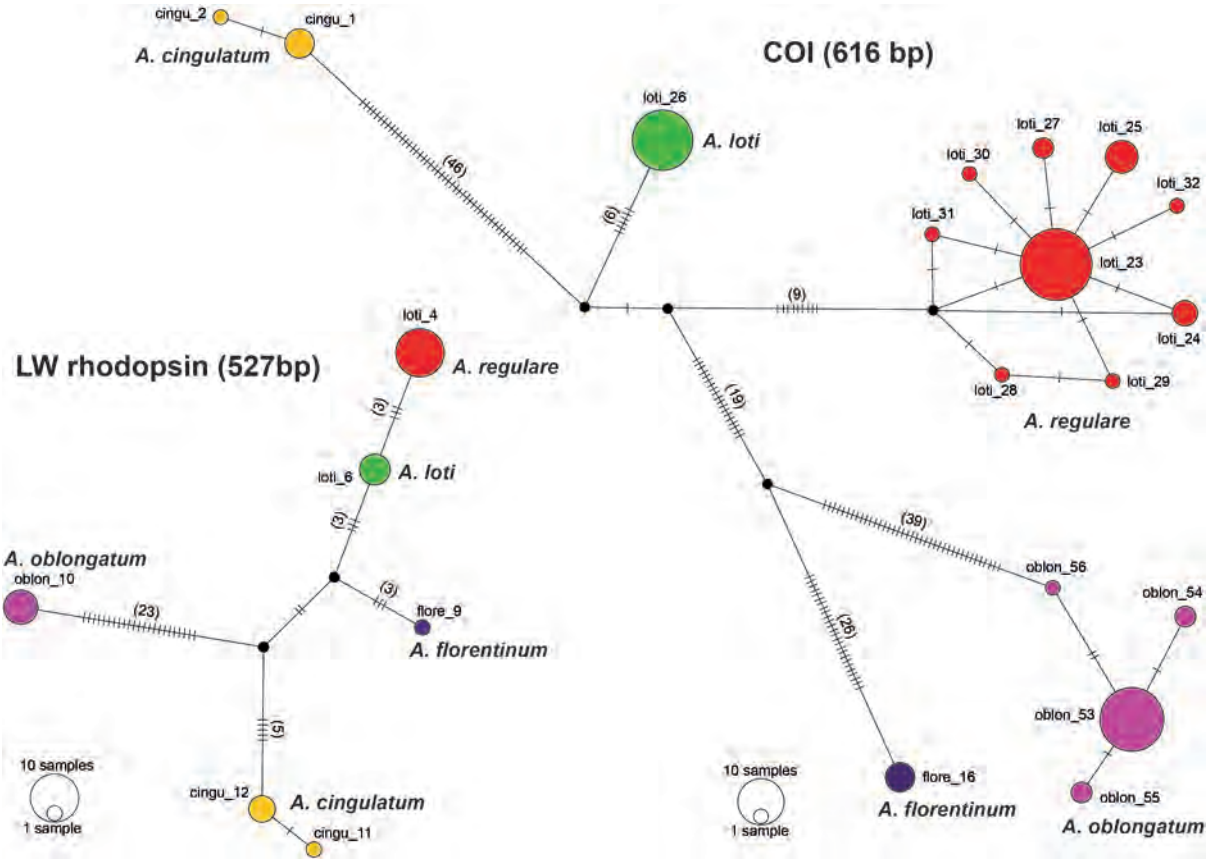

Figure 6. Median-joining network of COI DNA barcode and LW-rhodopsin haplotypes of five *Anthidium* species, including *A. regulare* stat. resurrect. (see Table 1 for the associated genetic indices). Each circle represents a haplotype, and circle size is proportional to haplotype frequency. Colours indicate different taxa. Small black dots represent possible median vectors, while dashes represent substitutions (also indicated within brackets when >2).

Distribution

Overall, 614 occurrence records were obtained from 30 countries (Supplementary Appendix S1), including some duplicates (e.g. from multiple collection specimens from the same locality, or locality data from specimens which have been published before). In total, 151 occurrence locations were obtained from the literature, 229 distribution records from GBIF which were reduced to 190 records after cleaning and 334 were previously unpublished.

The distribution of *Anthidium loti* s.l. extends from the Iberian Peninsula across the northern Mediterranean and the Balkans into the Anatolian landmass, the Caucasian region and further into Iran, Kazakhstan, Turkmenistan and Pakistan (Figure 8). The taxon has not been reported from northern Africa. It also appears to be absent from Corsica (Le Divelec et al. 2024) and Sardinia but it is present in Sicily. In the eastern Mediterranean, it has been found in Cyprus and its distribution in the Levant extends to northern Israel and Palestine but does not appear to extend further south. While the presence in Kazakhstan is well-documented through the description of *A. regulare* (type material examined), its occurrence in Pakistan relies solely on historical literature data (Dover 1925) and awaits further confirmation (Kasparek & Ahmed, accepted).

These two population groups of *A. loti* s.l. as defined by the genetic and morphometric analysis come into contact in the area to the south of the Maritime Alps, with a distinct, non-overlapping border. The delineation between these two forms roughly aligns with the political border between France and Italy (except West Liguria close to the border with France). In the north, a male from the Aosta Valley in northwestern Italy (CMK collection) was morphologically classified as part of the eastern population group of *A. loti* s.l., while material from West Ligurian populations genetically belong to the western group. No material was available from neighbouring Switzerland, but it is likely that records from southern Canton Ticino (Praz et al. 2023) refer to the eastern population group, while historical records to the west of the watershed of the Saint-Gotthard Massif around Lake Geneva (Frey-Gessner 1912) belong to the western population group.

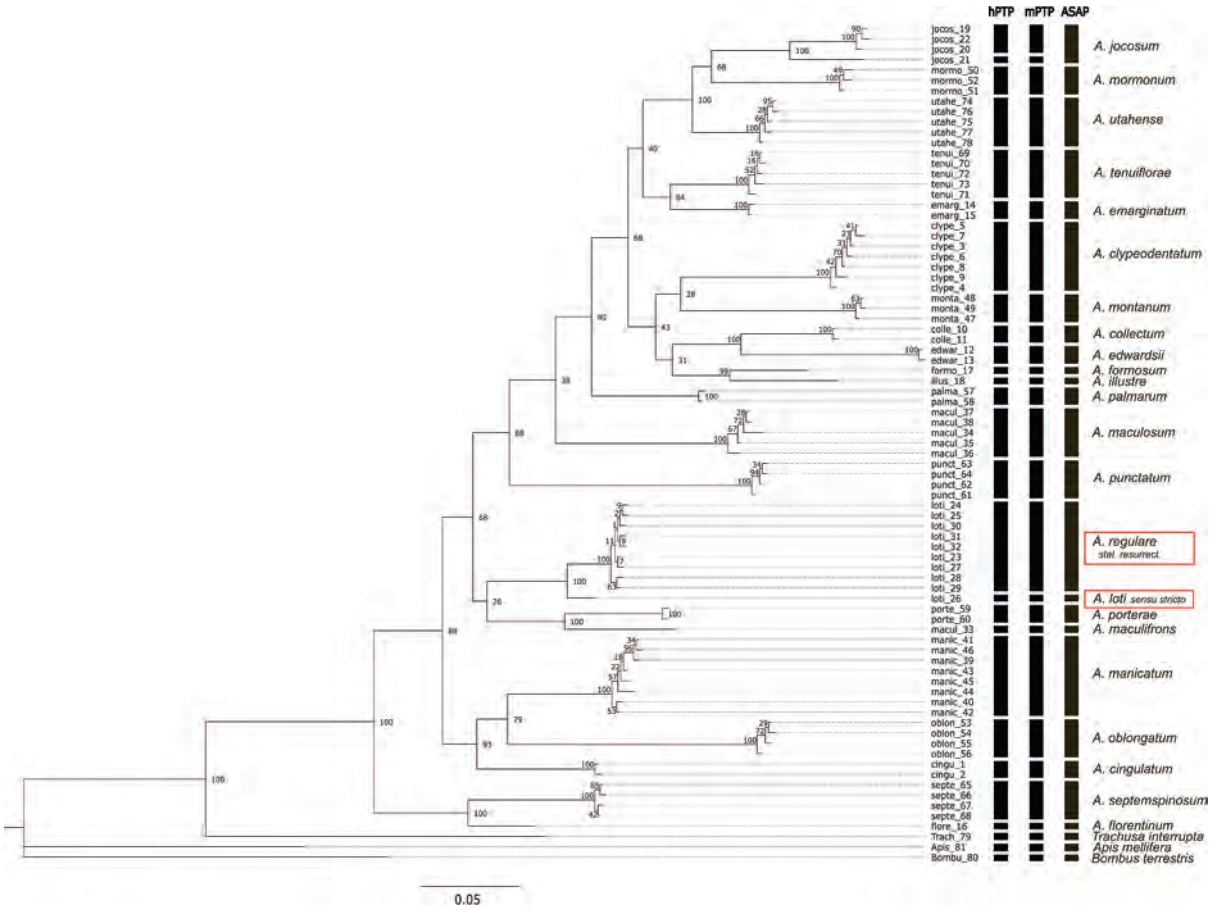

Figure 7. Consensus phylogenetic tree within the *Anthidium* genus using the COI marker and the output of the species delimitation analysis (indicated by the algorithm names above the black polygons).

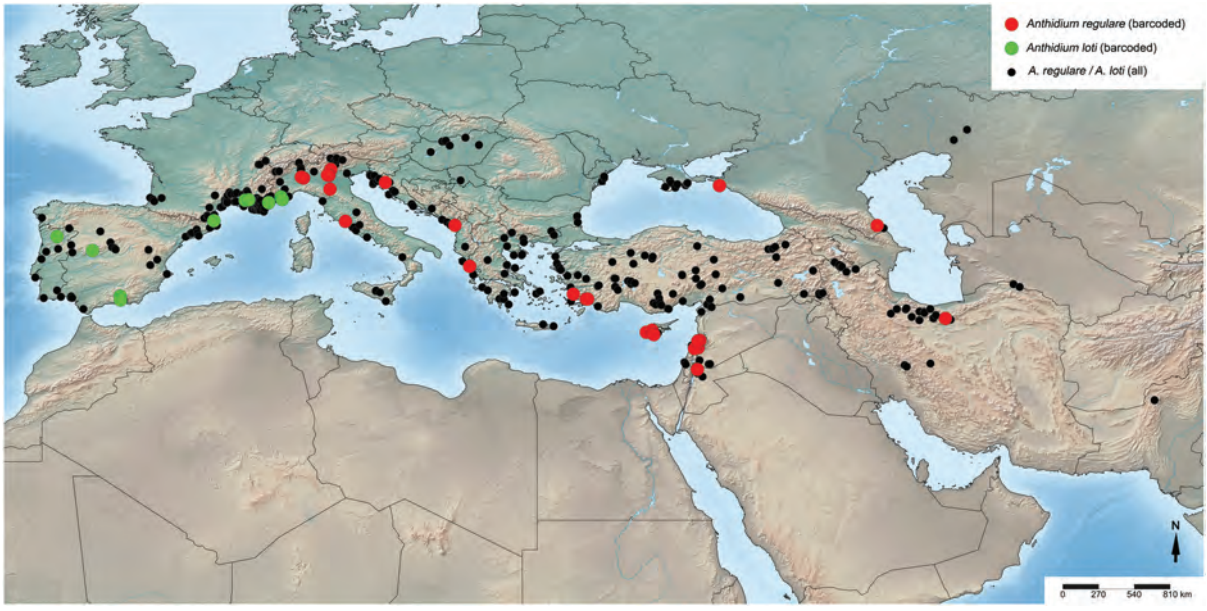

Figure 8. Distribution of *Anthidium loti* Perris, 1852 and *A. regulare* Eversmann, 1852. Black dots show distribution data from literature and other sources. Green dots show presence records of *A. loti*, red dots presence records of *A. regulare*, all confirmed by genetic analysis.

Taxonomy

Both population groups of *A. loti* s.l. have clearly defined distribution areas which allows to attribute them to available names: The oldest available name for the western population group of *A. loti* s.l. is *Anthidium loti* Perris 1852, the oldest available name for the eastern population group of *A. loti* s.l. is *Anthidium regulare* Eversmann 1852. This attribution was confirmed by an examination of the historical type specimens. Thus, the following synonymy can be established (Friese 1917; Warncke 1980; Kasparek 2022):

Anthidium loti Perris 1852 (France)

Apis variegata Fabricius, 1781. – Italy (Homonym).
Anthidium variegatum (Fabricius, 1781). – Friese (1898)
Apis varia Gmelin, 1790 (Replacement name, which itself turned out to be a homonym, see Warncke 1980).
Anthidium sinuatum Lepeletier de Saint Fargeau, 1841. – Spain (Nomen oblitum, see Litman et al., 2021).
Barcode Index Number (BIN): BOLD:ACG1108

Anthidium regulare Eversmann 1852 (Kazakhstan, type locality part of Russia at the time of the description) stat. resurrect.

Anthidium mosaicum Costa, 1863 (Italy)
Anthidium meridionale Giraud, 1863 (Italy)
Anthidium quadriseriatum Kriechbaumer, 1873 (Greece or Italy)
Anthidium quadriseriatum var. *circumcinctum* Kriechbaumer, 1873 (Greece or Italy)
Anthidium variegatum var. *incisum* Friese 1917 (Turkey)
Barcode Index Number (BIN): BOLD:ABU8896

Perris (1852) described *A. loti* from Landes, a department in the Nouvelle-Aquitaine region, southwestern France. The specific collecting site and date are unavailable, both in the specimen label and Perris' original notebook housed at Centre de Biologie pour la Gestion des Populations (CBGP), Montferrier-sur-Lez cedex, France (INRAE-UMR). The type locality of *A. regulare* was given by Eversmann (1852) in his "Fauna Hymenopterologica Volgo-Uralensis" as "Hab. in prov. Orenburg. australi, circa Indersk". Although Orenburg (Оренбу́рг) is currently located within the Russian Federation, Indersk belongs today to the Atyrau Province of Kazakhstan. A male deposited in the Institute of Systematics and Evolution of Animals of the Polish Academy of Sciences (ISEZ), Kraków, Poland, labelled as "Indersk, Anthidium regulare Evm. [golden disc]" (Figure 1) is designated herein as the lectotype. No additional material from the type locality is available. Warncke (1980) noted an occurrence in Kalmykovo, north of today's Orenburg in Russia; however, it cannot be conclusively determined whether he intended to reference the actual type locality. Apparently based on this record, the species has been included in the list of the bees of Russia (Proshchalykin et al. 2023).

Discussion

We identified two distinctive population groups of *A. loti* s.l. in the Western Palaearctic region: a western one confined to the Iberian Peninsula, France and the Liguria region of Italy (W Italy), and an eastern one widely distributed across the rest of Italy, the Balkans and across Greece and Turkey into Russia and Iran. Each of the two groups are in clearly defined distribution areas, with no known overlap. Both groups are phenotypically very similar and the males can be distinguished only by the shape of the sternum 6, while no traits are known to distinguish females by external characters. However, both population groups of *A. loti* s.l. are clearly defined by the combined evaluation of nine morphometric character traits of the head and the wing, and by genetic data. The combination of morphometric, genetic and distributional data enables recognizing these lineages as distinct species under the principle of taxonomy by congruence (Padial et al. 2010).

Based on the concordant evidences, these taxa are revised as follows. The oldest available name for the western population group is *Anthidium loti*, which had been described from western France, and the oldest available name for the eastern population group, which is *A. regulare*, and which had

been described from Kazakhstan. A morphological comparison (sternum 6) of the material examined herein with the historical type specimens confirmed the correct assignment of the population group of *A. loti* s.l. to these species. The two species can be distinguished by male characters, and in fact an illustrated description of the external characters of *Anthidium loti* s.l. including the truncate S6 was recently provided by Kasparek (2022). Notably, the figures presented in that work actually correspond to the eastern population group of *A. loti* s.l. As for females, single characters cannot discriminate the two species, as also noted in some other *Anthidium* bees (Litman et al. 2022), but combining several morphological features did distinguish them in a multivariate ordination space (Figure 4).

The coherence among multiple lines of evidence provides strong support for the recognition of two distinct species in the case studied here. The genetic markers used here agreed with each other showing marked differences between *loti* and *regulare*. A situation of agreement between nuclear and mitochondrial markers occurred in other studies focusing on bees, such as in *Megachile* (e.g., Soltani et al. 2017), in *Bombus* (e.g., Martinet et al. 2018), but also other animal groups such as dragonflies (e.g., Dijkstra et al. 2023). So far, the nuclear region we used has already been studied in the Megachilidae family and even in the *Anthidium* genus (Litman et al. 2016), proving to be quite efficient in clarifying the taxonomy and phylogenetic relationships when in interaction with mitochondrial genes. Conversely, we found no evidence of mito-nuclear discordance in the species studied, in contrast to some other bee species groups where incoherence between mitochondrial and nuclear DNA has been reported (Gueuning et al. 2020).

Allopatric speciation is well-known in many groups of animals and is considered the dominant form of speciation in organisms that engage in sexual reproduction (Mayr 1963; Coyne & Orr 2004). Secondary contact after allopatric divergence is also a recurring theme in literature (e.g., Schield et al. 2019; Dong et al. 2020). For anthidiine bees, allopatric speciation followed by renewed contact has been suggested for various species in the genus *Eoanthidium* (Kasparek 2020b) and *Trachusa* (Kasparek 2018) in the Mediterranean basin. It is a possibility that this scenario also occurred in the *A. loti* s.l. The realization that what was once classified as *A. loti* actually comprises two distinct species presents a significant challenge for the classification of wild bee diversity, both in the Mediterranean and beyond. *Anthidium diadema* Latreille, 1809, *A. taeniatum* Latreille, 1809, and *A. punctatum* Latreille, 1809 are pan-Mediterranean candidates worthy to be studied in detail, just to mention a few.

Conclusions

The case of *Anthidium loti/Anthidium regulare* shows that we are still far from a complete understanding of bee diversity in the Mediterranean. In this study, we integrated several approaches to demonstrate the existence of two different species within a taxon once believed to be a unique entity. Similar cases of new species awaiting to be characterized are likely hidden in several other animal and plant groups in the Mediterranean region. Taking advantage of existing and future collaborative and coordinated efforts, it will likely be possible to shed additional light on biodiversity and even help its conservation.

Acknowledgments

We would like to thank Maximilian Schwarz (†), Ansfelden (Austria) for making his rich collection of Anthidiini bees available to M. K. For the loan of museum materials, we thank Martin Husemann, Zoological Museum Hamburg, University of Hamburg, Hamburg, Germany (ZMH) (now: Karlsruhe), Joseph Monks, Natural History Museum, London, United Kingdom (NHMUK), Esther Ockermüller, Biodiversity Centre Upper Austria, Linz, Austria (OLL), Eric Pierre, Centre de Biologie pour la Gestion des Populations, Montferrier-sur-Lez cedex, France (CBDP), Łukasz Przybyłowicz, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences Kraków, Poland (DZPAS), and Villu Soon, Natural History Museum, University of Tartu, Estonia (TUZ). The following persons provided data and material from their private collections: Leander Bertsch, Munich (Germany), Petr Bogusch, Hradec Králové (Czech Republic), Mira Boustani, Hérouville-Saint-Clair (France), Jakovos Demetriou, Athens (Greece), Alexander Fateryga, Feodosiya (Russia), Markus Fuhrmann, Kreuztal (Germany), Sirio Gamba, San Biagio della Cima (Italy), Paul Geisendörfer, Würzburg (Germany), Martin Hauser, Sacramento, CA (USA), Gerhard Herb, Mauerstellen (Germany), Gerald Hölzler, Vienna (Austria), Hanno

Korten, Würzburg (Germany), Wolf-Harald Liebig, Bad Muskau (Germany), Pierre Rasmont, Mons (Belgium), Christian Schmid-Egger, Berlin (Germany), Marco Selis, Viterbo (Italy), Jan Smit, Duiven (The Netherlands), and Thomas J. Wood, Mons (Belgium). We are very grateful to all of them.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

We thank the funding agencies; the funder had no role in conducting the research and/or during the preparation of the article: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 5, Project title "National Biodiversity Future Center—NBFC".

Data availability statement

DNA data are deposited in an online repository (BOLD, project ZPLAL, and see Supplementary Appendix S3 for process ID codes); all the other relevant data are included in the article.

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