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ORIGINAL ARTICLE

Cryptic, sibling or neither of the two? Integrative species delimitation of *Psylliodes* flea beetles with overlapping ranges

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Abstract

Species identification and delimitation are particularly challenging for morphologically similar and geographically overlapping species, such as in the case of Western Palaearctic flea beetle species *Psylliodes kiesenwetteri* Kutschera, 1864 and *Psylliodes ruffoi* Leonardi, 1975. In this study, we implemented an integrative taxonomic approach based on a comprehensive geographic assessment of morphological and genetic variation, including 142 adult specimens from 15 sympatric and allopatric populations. Results of species delimitation methods using the barcode marker COI show that molecular identification and delimitation between *P. kiesenwetteri* and *P. ruffoi* are straightforward. Single-locus and multi-locus phylogenetic analyses indicate these two species have a large genetic divergence and belong to two distinct clades of the *Psylliodes gibbosa* species group. Morphological identification based on qualitative characters shows great differences in identification success depending on the character used. Characters of male and female genitalia perform very well, but can only be assessed on fully sclerified individuals, whereas the colour of antennae was discovered as a new reliable diagnostic character both for teneral and fully sclerified individuals. Morphological identification is particularly sharp when multiple characters are used in combination or when a morphometric approach is performed. In conclusion, despite overall morphological similarity, *P. kiesenwetteri* and *P. ruffoi* are not cryptic species neither they are sibling species, as they belong to distinct clades within the *gibbosa* species group, and they can be reliably distinguished by morphological characters. This study substantiates the relevance of a range-wide assessment of morphological and genetic variation, including individuals from the type locality, and both sympatric and allopatric populations, for taxonomic assessment of morphologically similar species with overlapping ranges.

KEYWORDS

cryptic species, integrative taxonomy, phylogeography, *Psylliodes kiesenwetteri*, *Psylliodes ruffoi*, species identification

1 | INTRODUCTION

Species discovery and description is crucial for documenting biodiversity patterns and understanding processes underlying the diversification of organisms on Earth (Agapow et al., 2004; Schlick-Steiner et al., 2010). For centuries comparative morphology has played a fundamental role in the taxonomy of plants and animals (Padial et al., 2010; Wheeler, 2007). However, relying only on morphological characters presents shortcomings that, in many cases, prevent the delimitation and, consequently, the identification of species (Bickford et al., 2007; Xiao et al., 2010). Indeed, various biological processes such as recent origin of species (Egea et al., 2016) and extensive intraspecific variability (Memon et al., 2006), hybridization (Mallet, 2007) or convergent evolution (Nevo, 2001), make the morphological characters not always predictive of the species boundaries (Bickford et al., 2007). In the last 20 years, the increasing use of integrative taxonomic approaches, which combine different lines of evidence (e.g. genetic, morphological and ecological) and methodologies (e.g. phylogenetic inference and ecological models), has led to significant advances in species discovery and delimitation (Dayrat, 2005; Schlick-Steiner et al., 2010; Sites Jr & Marshall, 2003).

Irrespective of the approach used for species delimitation, species description on the basis of intrinsic characters, typically morphological, is a well-established taxonomic practice (Bauer et al., 2011; ICZN, 1999). This procedure provides a comparative framework for the identification of specimens and is particularly effective when the characters used have a discrete, not-overlapping, variation across closely related species and when the examined material is representative of the intraspecific variation at these characters. However, given the complex biological reality of species these expectations are not always met. For example, complexes of species which are morphologically very similar, challenge this approach due to the limited availability of diagnostic variation to serve for species discrimination (Bickford et al., 2007). This is the case of 'cryptic' species, in which two or more species are, or have been, classified as a single nominal species because they are morphologically indistinguishable, often because they share a very recent common ancestor as in the case of 'sibling' species (i.e. cryptic sister species; Knowlton, 1986; Bickford et al., 2007). In other cases, characters used for the species diagnosis and assumed to have a discrete variation, when further assessed across a wider geographic and population scope, reveal a pattern of continuous variation that is overlapping with closely related species. This is especially likely for wide-ranging species whose descriptions are based on a few individuals from a low number of localities covering a limited portion of the species range (Tessens et al., 2021). This is not an infrequent case for species described in the old times or from poorly sampled areas (Darwell & Cook, 2017; Deng et al., 2019; McBride et al., 2009; Morek et al., 2019). Therefore, to overcome these potential difficulties and uncertainties in species discrimination, not only an integrative taxonomic approach combining independent characters is required but also a comprehensive assessment of the geographic variation of these characters.

However, while the advantages of combining molecular and morphological data are now well established for taxonomic practice, it is less common for these data to be assessed across comprehensive geographical scale. In this respect, a phylogeographic approach combined with morphological assessment offers several advantages as it allows delimiting independent evolutionary lineages and their ecogeographic distributions and define the spatial boundaries of intraspecific morphological variability (Raxworthy et al., 2007), thus offering further support for the delimitation of species (Espíndola et al., 2016; Sites Jr & Marshall, 2003; Struck et al., 2018; Templeton, 2001; Yeates et al., 2011). This approach also allows, through a backward morphological taxonomy procedure, to re-evaluate the morphology of genetically distinct populations and to find morphological differences that have not previously emerged, even in complexes of species considered cryptic (Blanquer & Uriz, 2008; Grebennikov, 2019; Jossart et al., 2021; Leavitt et al., 2016; Martinsson et al., 2015; Ramasindrazana et al., 2011).

The case of flea beetle species *Psylliodes kiesenwetteri* Kutschera, 1864 and *Psylliodes ruffoi* Leonardi, 1975 (Coleoptera, Chrysomelidae, Galerucinae, Alticini) offers an ideal experimental setting for testing the utility of a combined integrative and phylogeographic approach for taxonomic delimitation and identification of morphologically similar and geographically overlapping species. These two species belong to the *Psylliodes gibbosa* species group mainly distributed in the Mediterranean region, which has a long and eventful taxonomic history (Leonardi, 1975; Nadein, 2008). The member species of this complex are generally distinguishable from each other only based on subtle morphological differences, mainly in the shape of the aedeagus and spermatheca and, in general, no single character taken individually can be considered reliable for their identification (Leonardi, 1975). For this reason, the discrimination of species using a morphological approach is challenging, and misidentifications are frequent, especially by inexperienced entomologists. Within this species group, populations belonging to *P. ruffoi* have been confused for a long time with *Psylliodes kiesenwetteri* or *P. gibbosa* Allard, 1860 (Leonardi, 1975). Morphological characters of *P. ruffoi* are somewhat intermediate between *P. kiesenwetteri* and *P. gibbosa*, with

several traits being shared among all three species. Not only morphological variation seems overlapping among these species but also their geographic range show a wide area of overlap in south Italy. Additionally, while recent molecular data support the phylogenetic distinction between *P. gibbosa* and *P. kiesenwetteri* (Gikonyo, 2021), the phylogenetic relationship between *P. kiesenwetteri* and *P. ruffoi* is still unclear. These two species can be found in close sympatry along the southern mountain range of the Apennines in association with semi-arid meadows found in clearings of xerophilous woods. In locality where they are syntopic, it is very common to find individuals of both species on the same plants. Overlapping morphological variation and geographic range with strict syntopy on several mountains, cast the question on whether these taxa represent two species or a single polymorphic species. The clarification of their systematics and distinctiveness also has conservation implications since *P. ruffoi* represents an endemic species to southern Italy with a much narrower range than *P. kiesenwetteri*.

The main aims of this study are to assess genetic and morphological differentiation between the two species in sympatric and allopatric populations, test for the hypothesis of a cryptic (or sibling) species pair and assess the diagnostic value of morphological characters for their discrimination. By extending the taxon set to closely related species of the *P. gibbosa* species group, we also want to provide a multi-locus phylogenetic framework for their systematics and assess whether *P. ruffoi* and *P. kiesenwetteri* are sister species. Finally, the utility of a combined phylogeographic and morphological approach for the systematic assessment of complexes of morphologically similar and geographically overlapping species is discussed.

2 | MATERIALS AND METHODS

2.1 | Study system

The flea beetle genus *Psylliodes* Latreille comprises over 200 species worldwide (Gikonyo et al., 2019). Adults are easily distinguished from other flea beetle genera based on their 10-segmented antennae and tarsi inserted preapically on the metatibia of the hind legs. Most *Psylliodes* species have a restricted host plant range (35% are monophagous and 51% are oligophagous), and only 14% are polyphagous. Of all *Psylliodes* species with known host plants, 50% are associated with Brassicaceae, followed by 13% feeding on Poaceae, 10% on Solanaceae and 10% on Fagaceae (Gikonyo et al., 2019). The *P. gibbosa* species group was proposed by Leonardi (1970) on the basis of the following characters: body shape broadly oval to more or less elongate; punctation of the dorsal integuments rather strongly and densely impressed, especially on frons and labrum; shape of the spermatheca with very elongate basal part and long and coiled ductus. This group currently includes *P. gibbosa*, widely distributed in the Mediterranean area; *P. kiesenwetteri*, occurring in Italy and the Balkan Peninsula; *Psylliodes inflata* Reiche, 1858, from the southern Mediterranean regions; *P. ruffoi*, from southern Italy and Sicily; *Psylliodes gougeleti* Allard, 1859, from the Iberian Peninsula and Algeria; *Psylliodes fageli* Bechyné, 1957, from Algeria; *Psylliodes tenuidentatus* Nadein, 2008, from Israel; and *Psylliodes ridendus* Nadein, 2008 from Turkey (Leonardi, 1975; Nadein, 2008). Gikonyo, (2021), based on molecular data, attributed to this group also *Psylliodes cucullata* (Illiger, 1807), species widespread in the Palaearctic region, morphologically very similar to *P. gibbosa* but with spermatheca of very different shape (Leonardi, 1970). Excluding *P. cucullata*, four species of this group occur in the Italian fauna: *P. gibbosa*, *P. kiesenwetteri*, *P. ruffoi* and *P. inflata* (Figure 1). The range of the endemic species *P. ruffoi* partially overlaps in Sicily with *P. gibbosa* and *P. inflata*, and in the southern part of the Italian peninsula with *P. gibbosa* and *P. kiesenwetteri*.

2.2 | Sampling

Specimens of *P. ruffoi* and *P. kiesenwetteri* were collected from 15 localities (Table S1) in central and southern Italy and Sicily along a transect of about 720 km, covering the entire range of *P. ruffoi* and including both sites of sympatry with *P. kiesenwetteri* and allopatric populations of the two species. Sampling design was based on historical distribution data (Biondi, 2006); material from entomological collections and bibliographic records (Baviera & Biondi, 2015; Biondi, 1988; Leonardi, 1970; Leonardi, 1975; Urbani et al., 2015). Specimens were collected from their host plant by sweep net and the aid of aspirator and then stored in 95% ethanol.

2.3 | Morphological assessment

Morphological identification was performed on the 143 specimens used in molecular analyses based on the diagnostic characters proposed by Leonardi (1975) and Nadein (2008). In particular, for the two species the following characters states were considered: for *Psylliodes kiesenwetteri*, body shape narrow and elongate, with maximal width at the middle; pronotum with more weak shagreened surface; tibial hollow shorter; median lobe of aedeagus shorter and not narrowed preapically, and spermatheca smaller with shorter ductus, complicated by 2 or 3 coils. For *P. ruffoi*, body shape subelliptical

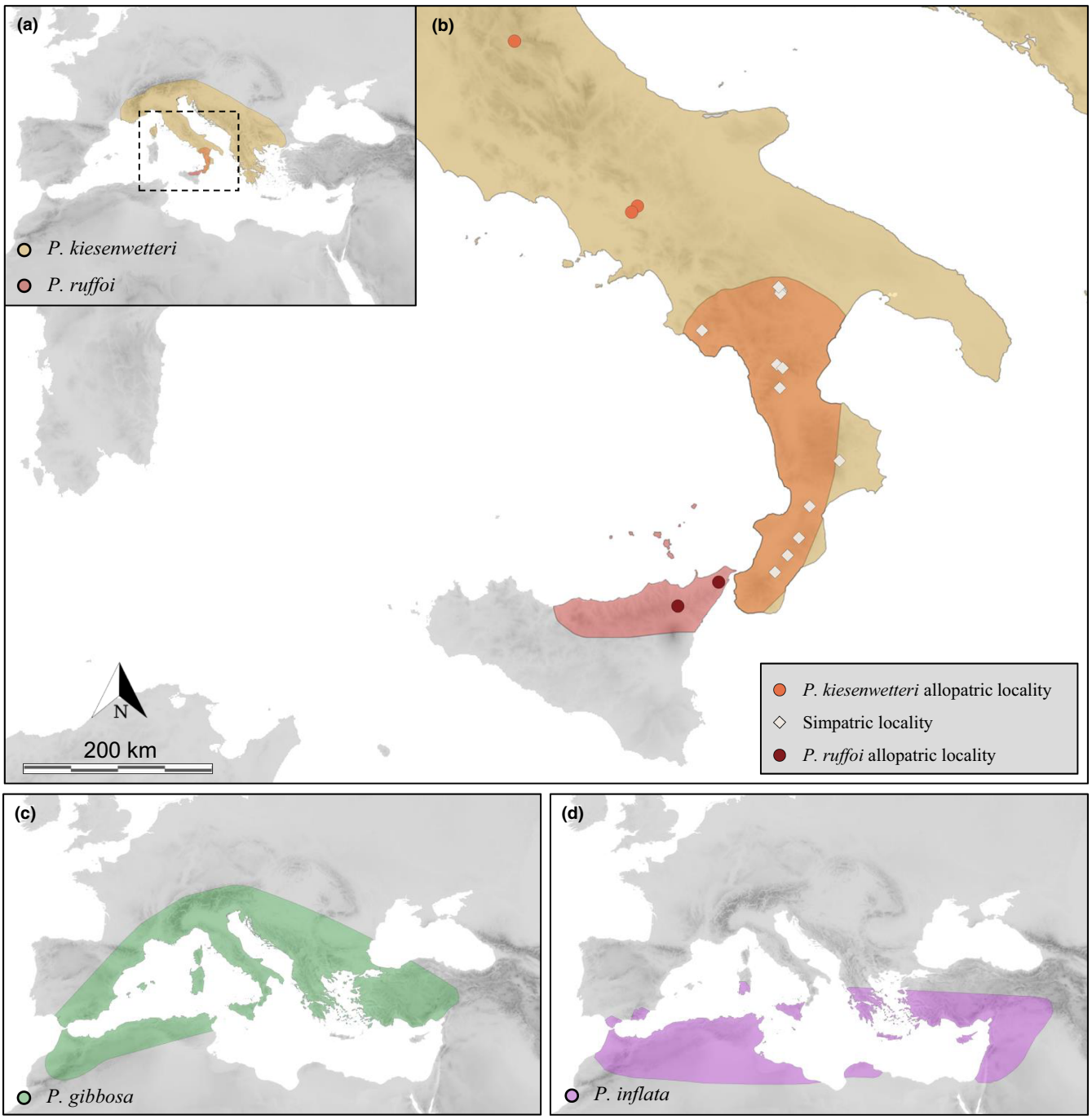

FIGURE 1 Geographical distribution of the four species of the *Psylliodes gibbosa* group that occur in Italy. (a) In yellow the distribution range of *P. kiesenwetteri* and in red that of *P. ruffoi*. The dashed box indicates the study area enlarged in (b), to show the sympatric and allopatric sampling localities for both species. (c) In green, the distribution range of *P. gibbosa* and (d) in purple the distribution range of *P. inflata*

or oval, with maximal width usually at the basal third; pronotum with clearly shagreened surface; tibial hollow more elongate; median lobe of aedeagus longer and slightly narrowed preapically, and spermatheca with longer ductus, complicated at least by 4–5 coils. Furthermore, the colour of the antennae was tested as a further character for the discrimination between *P. ruffoi* and *P. kiesenwetteri*. Indeed, during morphological assessment we noticed two distinct colour patterns of the antennae, one with entirely pale coloration or rarely with slightly darkened distal segments, associated with *P. ruffoi*, and the other one with a distinctly blackened coloration of the distal segments, associated with *P. kiesenwetteri*. Morphological assessment was performed by two of us, MB and PDA, both with long and extensive experience on the Italian flea beetle fauna.

To assess the robustness of morphological characters, we compared, for each specimen, the results of taxonomic identification obtained with each morphological character with the identification based on molecular analyses. The taxonomic robustness of single morphological characters was then expressed as percentage of correct identification (i.e. matching molecular identification).

2.4 | DNA extraction, amplification and sequencing

Total genomic DNA of 143 specimens was extracted using a standard high-salt protocol (Sanbrook et al., 1989) with two different methods to allow further morphological assessment and verification of morphological identification after DNA extraction: (i) using the three left legs of each specimen, and (ii) using the non-invasive method proposed in Salvi et al. (2020). For these specimens we amplified the standard barcode region of the mitochondrial *cytochrome c oxidase I* (*cox1*) gene. For selected individuals of *P. ruffoi* and *P. kiesenwetteri*, one from sympatric and one from allopatric locality, and for one representant of *P. gibbosa* from Morocco, one additional mitochondrial gene fragment, the 3′ end fragment of *cox1* (*cox1*-3′), and five protein-coding single copy nuclear genes from Gikonyo (2021) were amplified: Carbamoylphosphate synthase (*CAD*), Crossveinless 2 (*Cv2*), Methyl methanesulfonate-sensitivity protein 22-like (*MMS22*), Rad 50 protein (*Rad50*) and Short gastrulation (*Sog*). These nuclear markers were selected based on their high evolutionary rate (Gikonyo, 2021). Primers and PCR protocols used for the amplification of the molecularmarkers have been developed either in this study or in previous studies (Gikonyo, 2021; Salvi, D'Alessandro, et al., 2019; Salvi, Maura, et al., 2019; Simon et al., 1994) as reported in Table S2. Successful amplification was determined by gel electrophoresis, and PCR products were purified and sequenced by an external service (Genewitz, UK). The obtained chromatograms of each sequence were manually edited and assembled into a consensus sequence using Geneious Prime 2021 (Biomatters Ltd., Auckland, New Zealand). Heterozygous positions for the nuclear coding gene fragments were identified based on the presence of two peaks at a single site in the chromatograms and were coded in the alignment using IUPAC ambiguity codes. Consensus sequences were deposited in BOLD and GenBank database (BOLD accession number: EABAR001-22 — EABAR142-22; GenBank accession: OP545842 — OP545845, OP548580 — OP548596, OP627912 — OP28053, OP745418).

2.5 | Molecular species delimitation

Newly generated sequences of the standard barcode region of *cox1* were aligned with MAFFT v7.450 using the G-INS-I progressive method algorithm (Katoh et al., 2002) together with one sequence of *P. kiesenwetteri* from Croatia with GenBank accession number MW254865 (Gikonyo, 2021).

First, we construct a neighbour-joining (NJ) tree using MEGAX (Kumar et al., 2018) with Kimura-two parameters (K2P) (Kimura, 1980) to identify principal groups with a distance-based approach. The node support for the NJ tree was estimated through 1000 bootstrap replicates. *Psylliodes vehemens* Wollaston, 1854 was used as outgroup based on phylogenetic affinity (Gikonyo, 2021). Then we performed two species delimitation analyses to infer the number of putative species and their correspondence to morphospecies: (i) nucleotide distance with thresholds estimated ad hoc on the data set and (ii) Assemble Species by Automatic Partitioning (ASAP; Puillandre et al., 2021). Nucleotide distance-based methods resulted in high efficiency for species delimitation on Chrysomelidae (Magoga et al., 2021). The pairwise nucleotide distance matrices required for ad hoc nucleotide distance threshold methods were estimated using the R library *ape* v5.3 (Paradis & Schliep, 2019). A pairwise distance matrix of intraspecific and interspecific genetic distance was calculated using the K2P substitution model with the pairwise deletion option. With the R package *spider* v1.5.0 (Brown et al., 2012) we performed a threshold optimization analysis using the *localMinima* function. Finally, we cluster nucleotide sequences with the ad hoc threshold using the function *tclust*. ASAP analyses were run using the program web-interface (https://bioinfo.mnhn.fr/abi/public/asap); K2P was selected as nucleotide substitution model and other parameters were left as default.

2.6 | Morphometric assessment

Morphometric assessment was performed on a set of specimens collected in 13 locality both from areas of sympatry between *P. kiesenwetteri* and *P. ruffoi* and allopatric areas (Table S1). Examined material consists of fully sclerified specimens, 20 ♂♂ and 20 ♀♀ for each species, previously extracted for DNA. These samples were dissected and subsequently dried and mounted on an entomological card with visible median lobe of the aedeagus or spermatheca, this last one included in Euparal. The specimens were examined, measured and dissected using a Leica M205C stereomicroscope. Photographs were taken using a Leica DMC5400 camera and composed using Zerene Stacker software version 1.04. Scanning electron micrographs

were taken using a Hitachi TM-1000. We performed a comprehensive morphometric analysis based on a wide set of morphometric characters in order to assess (i) the morphological variation of the target species and (ii) the diagnostic power of character-sets to correctly assign specimens to molecularly delimited species. Fifteen morphometric variables were selected as predictors: length of elytrae (LE), width of elytrae (WE), length of pronotum (LP), width of pronotum (WP), length of antennae (LAN), length of median lobe of aedeagus (LAED); length of spermathecal capsule (LSP); LE/LP; WE/WP; WP/LP; WE/LE; LE + LP; LAN/(LE + LP); LE/LAED; LE/LSP. No data standardization or normalization were performed for these measures. Terminology follows D'Alessandro et al. (2016) for the median lobe of aedeagus and Furth and Suzuki (1994) for the spermatheca. A forward stepwise discriminant function analysis was performed separately on males and females (Tabachnick & Fidell, 1989), using Mahalanobis distances and setting α threshold for type I error to 0.05. To assess how the 15 morphometric variables discriminate the four groups analysed (*kiesenwetteri* ♂♂, *kiesenwetteri* ♀♀, *ruffoi* ♂♂, *ruffoi* ♀♀) and to compute the relative discriminant functions, a canonical analysis was performed using the package NCSS version 11 for Windows.

2.7 | Phylogenetic analyses

To investigate the phylogenetic relationship between the South-European species *P. kiesenwetteri* and the Italian endemic *P. ruffoi* and with the other representatives of the *P. gibbosa* group, we used a multi-locus approach based on DNA sequences of the 7 gene fragments amplified on representative individuals of each species or retrieved from GenBank from Gikonyo, 2021 (Table S3).

Sequences of each gene were aligned separately with MAFFT v7.450 using the G-INS-I progressive method algorithm. Then, we build a concatenated sequence alignment and inferred phylogenetic relationships using both maximum likelihood (ML) and Bayesian inference (BI) methods using *P. vehemens* Wollastonas an outgroup (Gikonyo, 2021). ML trees were inferred in IQ-TREE 1.6.12 (Nguyen et al., 2015) using the W-IQ-TREE webserver (Trifinopoulos et al., 2016). The best substitution models for each gene partition in our concatenated matrix were determined by the ModelFinder module, including flexible rate heterogeneity across sites models (Kalyaanamoorthy et al., 2017), based on the Bayesian information criterion (BIC) (Table S4). We used the Edge Linked partition model to allow each partition to have its own evolutionary rate. Branch support was assessed by 1000 replicates of ultrafast bootstrapping (UFboot) (Hoang et al., 2018; Minh et al., 2013) and SH-like approximate likelihood ratio test (SH- aLRT) (Guindon et al., 2010). BI analyses were performed with two independent MCMC runs, with four chains each, on Mr Bayes v3.2.6 (Ronquist et al., 2012) for 10 million generations with a burn-in of 1 million generations (10%). Trees were sampled every 1000 generations and Tracer v1.7 (Rambaut et al., 2018) was used to assess convergence. FigTree v1.3.1 (Rambaut & Drummond, 2009) was used to depict the trees.

3 | RESULTS

3.1 | Molecular species delimitation

We obtain 142 *cox1* standard barcode region sequences, 89 sequences for *P. kiesenwetteri* and 53 sequences for *P. ruffoi*. The NJ tree inference produced a topology with two distinct clades (both with 100% bootstrap support) which correspond to the two groups recovered by the species delimitation analysis (Figure 2a). The frequency distributions of pairwise K2P distances estimated using the R library *ape* v5.3 and ASAP highlighted the existence of a clear barcoding gap in the *cox1* data set (Figure S1a,b). Intraspecific K2P distance values ranged from 0 to 4.2% (mean = 0.6%) and interspecific K2P distances ranged from 14.3% to 16.8% (mean = 15.6%). The optimal distance threshold identify by *localMinima* function was estimated at 9%. With these threshold value set as distance cut-off for clustering, *tclust* function divided the sequences into two clusters, coherently with the two clusters delimited by the best ASAP-score partitions (asap-score: 1.00; *p*-val (rank): 1.00 e-05 (1); W (rank): 1.02 e-03 (1)).

3.2 | Morphological identification and morphometry

The taxonomic assignment of *P. kiesenwetteri* and *P. ruffoi* specimens based on the evaluation of each of the characters proposed by Leonardi (1975) and Nadein (2008), revealed a varying percentage of correct species assignment (relative to the molecular identification) across the analysed characters (Table 1). Overall, aedeagal and spermathecal characters perform very well, with percentage of correct species assignment ranging from 96 to 100%. However, these characters can only be assessed in fully sclerified specimens. Teneral specimens, due to the absence of sclerification in reproductive organ, cannot be reliable identified through these characters. In our dataset, about one quarter of specimens (24%) were teneral. Other characters, such as body shape and pronotal surface, perform relatively well, with percentage of correct species

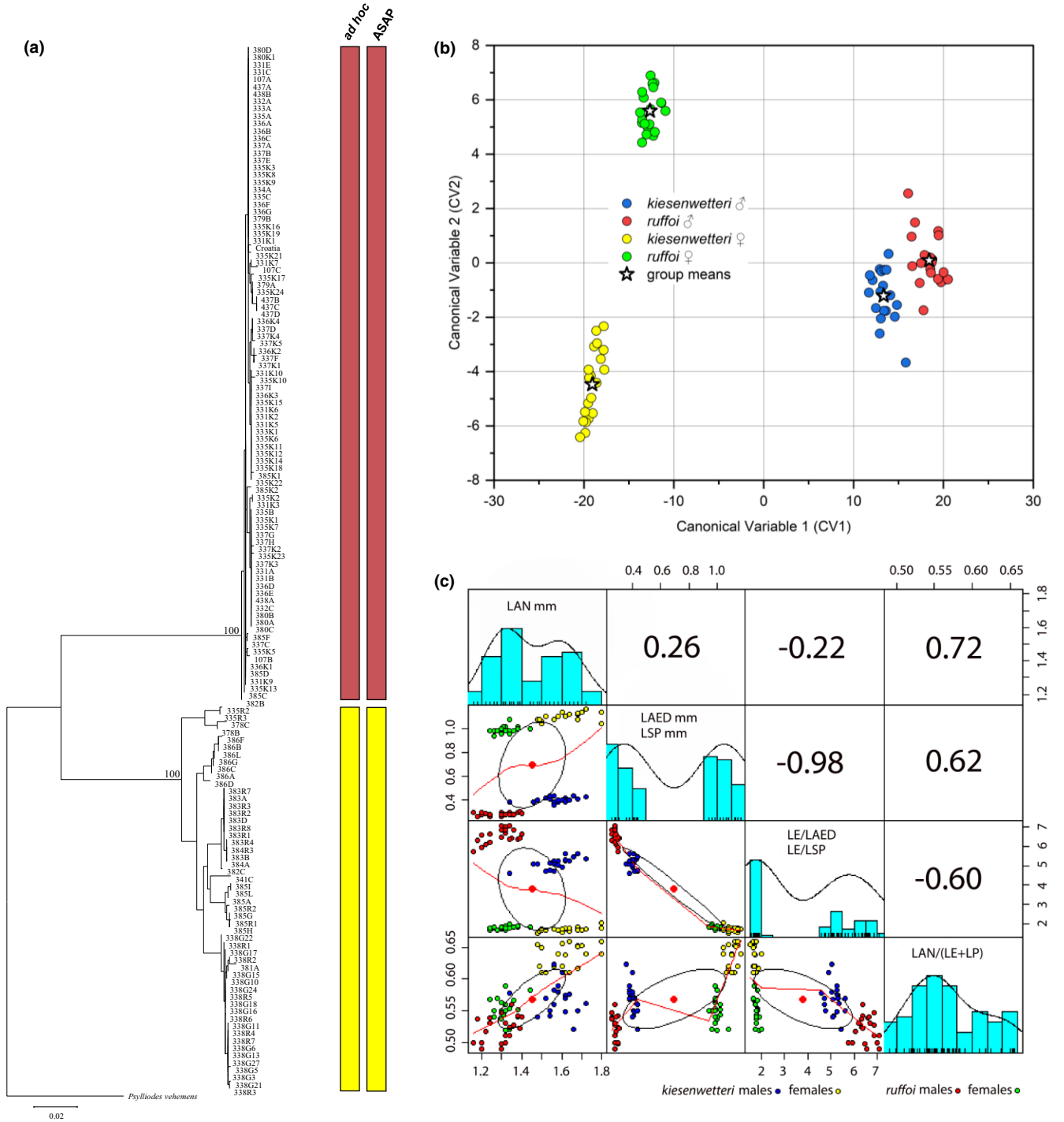

FIGURE 2 NJ Tree based on *cox1* sequences of 131 analysed specimens, with vertical bars representing results of species delimitation methods (ASAP, ad hoc) (a). In yellow the *Psylliodes ruffoi* group and in red the *P. kiesenwetteri* group identified by the two species delimitation methods. Scatterplots (CV1 by CV2) of the canonical analysis for males and females of *Psylliodes kiesenwetteri* and *P. ruffoi* (b) and scatterplot matrix of the variables identified with discriminant stepwise analysis (c): On diagonal the distribution of the single variables, with Pearson correlation coefficients (above) and scatterplots (below) relative to each couple of variables

assignment ranging from 71 to 85%. On the contrary, the tibial hollow is unreliable for species identification, with percentage of correct species assignment ranging from 54 to 61%. Finally, the character of the colour of the antenna proved to be very reliable (correct species assignment ranging from 94 to 100%) for the discrimination and its assessment is not dependent on the state of sclerification of the specimen.

Morphometric analyses based on the discriminant function identified six variables, LAED (for ♂♂), LSP (for

TABLE 1 Reliability percentage for each of the diagnostic characters used in the morphological assessment of *Psylliodes kiesenwetteri* and *P. ruffoi* specimens. (*: Not applicable to immatures)

	Body shape	Pronotal surface	Aedeagus*	Spermatheca*	Tibial hollow	Colour of antennae
P. kiesenwetteri	71%	77%	96%	100%	54%	100%
P. ruffoi	85%	74%	98%	100%	62%	94%

*Not applicable to immatures

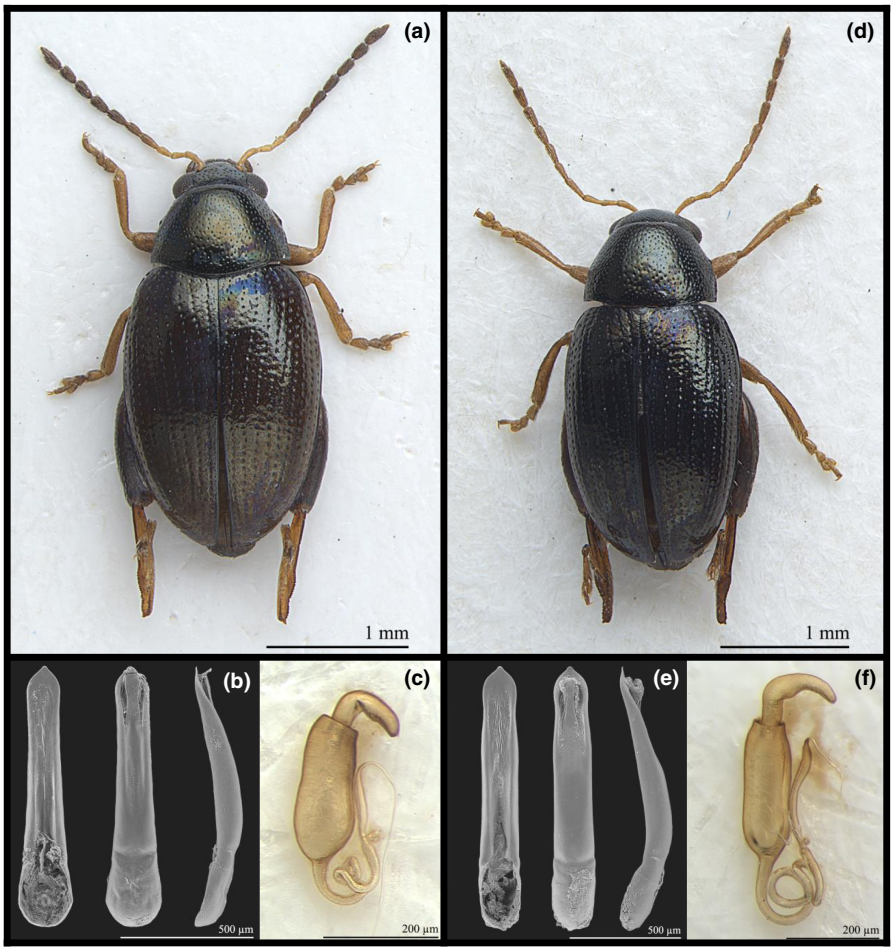

FIGURE 3 *Psylliodes kiesenwetteri*: (a) habitus, (b) median lobe of the aedeagus and (c) spermatheca; *Psylliodes ruffoi*: (d) habitus, (e) median lobe of the aedeagus and (f) spermatheca

♀♀), LE/LAED (for ♂♂), LE/LSP (for ♀♀), LAN, and LAN/(LE+LP), that show highly significant differences between the two species (Figures 2b,c and 3; Table 2). The classification matrix based on these variables returns 100% of corrected species attributions for the specimens analysed (Table 3). In addition, squared Mahalanobis distances matrix (SMD) suggests that the following pairs are well discriminated: ♂♂ *ruffoi* - ♂♂ *kiesenwetteri* (SMD = 52.46), and ♀♀ *ruffoi* - ♀♀ *kiesenwetteri* (SMD = 115.66). The first two functions identified by the Canonical Analysis (CV1 and CV2), representing 98.6% of total explained variance (EV), were considered, and the group centroids are reported in Figure 2. The CV1 accounts for 94.3% of EV and allows discriminating the males of *kiesenwetteri* from those of *ruffoi*. The CV2 (4.3% of EV) allows to discriminate the females of the two species.

3.3 | Phylogenetic relationship

Phylogenetic analyses based on ML and BI methods gave consistent results and identified two highly supported clades (BS = 100, PP = 1) within the *P. gibbosa* complex: clade A including *P. ruffoi*, *P. gibbosa* and *P. inflata*, and clade B composed by *P. cucullata* and *P. kiesenwetteri* (Figure 4). Within clade A, two specimens of *P. ruffoi*, one from Sicily and one from Calabria, form a supported clade (clade A2; BS = 100, PP = 1), that is sister to the clade A1

of *P. gibbosa* and *P. inflata* (BS = 100, PP = 1). Within Clade B, the three specimens of *P. kiesenwetteri* from Calabria, Abruzzo and Croatia formed a well-supported clade (clade B1; BS = 100, PP = 1) (Figure 4).

TABLE 2 Discriminant stepwise analysis for males and females: variables in the model, Wilk's lambda, *F* to enter and *p*-level

	Lambda	*F*	*p-value*
LSP	91.03	385.75	<.01
LE/LSP	81.91	172.1	<.01
LAN/(LE + LP)	81.36	110.62	<.01
LAN	79.11	95.96	<.01
LAED	33.14	18.34	<.01
LE/LAED	9.27	10.24	<.05

4 | DISCUSSION

In many animal groups, the identification and description of species is based on morphological characters. The exclusive use of morphological characters can, however, lead to wrong delimitation of species when intraspecific morphological variability overlaps with the interspecific variability, either because of recently diverged species (within the so-called 'grey zone' [De Queiroz, 2005, 2007]) or for long-lasting morphological stasis as observed in some cryptic species. To achieve a more robust species delimitation, it is necessary to integrate different kinds of characters and to perform a wide-range assessment of their variability. The case study of *P. kiesenwetteri* and *P. ruffoi* provided and emblematic example on the benefits of using an integrated taxonomic approach supported by exhaustive geographical sampling in systematic assessments of

TABLE 3 Discriminant stepwise analysis: Classification matrix for males and females

	Correct classification (%)	*P. kiesenwetteri* ♂ (*N*)	*P. kiesenwetteri* ♀ (*N*)	*P. ruffoi* ♂ (*N*)	*P. ruffoi* ♀ (*N*)
P. kiesenwetteri ♂	100	20	0	0	0
P. kiesenwetteri ♀	100	0	20	0	0
P. ruffoi ♂	100	0	0	20	0
P. ruffoi ♀	100	0	0	0	20

Note: Percentage of correct classification and observed classification (*N*) for each group.

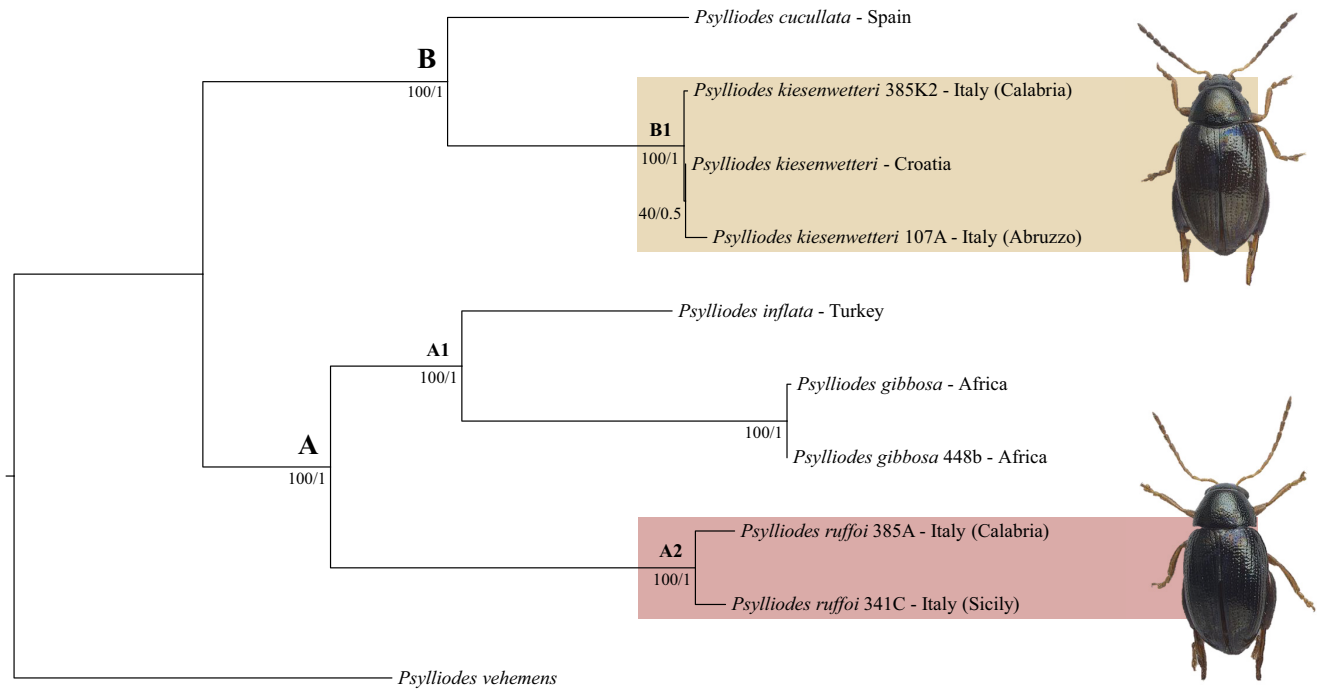

FIGURE 4 Maximum likelihood phylogenetic tree of the *Psylliodes gibbosa* complex based on concatenated DNA sequences of *cox1* and 5 nuclear genes. Bootstrap support from maximum likelihood analyses (left) and posterior probability from Bayesian analysis (right) are reported in correspondence of the nodes. Voucher code and geographic origin is reported for each specimen on the right of the species name

morphologically similar and geographically overlapping species. Such an approach allowed answering both taxonomic questions on species delimitation and identification, and evolutionary questions on their relationships and time since diversification.

Molecular identification and delimitation between *P. kiesenwetteri* and *P. ruffoi* are straightforward. These two species are recovered as two well-supported clades in the NJ tree based on *cox1* distances and are supported by the species delimitation analyses, with a clear gap between the intra and the interspecific levels of divergence at the *cox1* barcoding fragment. The genetic distance threshold at *cox1* between *P. kiesenwetteri* and *P. ruffoi* was estimated at 9%. This value is much higher than that found between related species of chrysomelids using either the same threshold estimation function (genus *Longitarsus*, *localminima* value: 5.4%, Salvi et al., 2020) or different threshold estimation functions (genera *Chaetocnema* and *Phyllotreta*, *threshVal* value: 1.5%, Coral Şahin et al., 2019; European Chrysomelids genera, *threshVal* value: 1%, Magoga et al., 2018). Indeed, the maximum intraspecific distance observed within *P. kiesenwetteri* and *P. ruffoi* was 4.5%, whereas their minimum interspecific distance was 14.3%. These comparisons indicate that *P. kiesenwetteri* and *P. ruffoi* are clearly separated evolutionary units with a large genetic divergence underlying an old diversification.

On the contrary, the morphological identification of these two evolutionary and taxonomic units based on a classical qualitative evaluation of the characters proposed by Leonardi (1975) and Nadein (2008) shows great differences in identification success depending on the character used. Characters of male and female genitalia perform very well, in line with previous studies on insects (Simmons, 2014; Tuxen, 1970). The spermatheca shows the maximum discrimination capacity (100%): the difference in the length of the duct and the presence of a different number of complications of the coils makes the spermatheca the only character capable of discriminating all the female specimens of *P. kiesenwetteri* and *P. ruffoi* consistently with the molecular methods. Characters of the aedeagus also show a high capacity of discrimination between the two species, with an identification between 96% and 98%. However, genital organ characters can only be assessed in fully sclerified individuals. Teneral specimens do not present sclerification in the reproductive organs, and it is therefore not possible to reliably assess the shape of genitalia. Within our data set, about a quarter of the samples were teneral specimens. Perhaps such a high proportion of teneral specimens is due to the sampling time that in some localities might have been associated with the time of appearance of a new generation. However, given the wide geographical and temporal scope of our sampling, we can reasonably assume that proportions of young, teneral, individuals are relatively high in ordinary range-wide samplings of *P. kiesenwetteri* and *P. ruffoi*, therefore, limiting the use of genitalia characters for the identification of these species. Non-genital morphological characters such as the shape of the body and the pronotal surface have lower discrimination capacity (from 71 to 85%) but are still valuable, whereas the tibial cavity is of limited use for the identification of *P. kiesenwetteri* and *P. ruffoi* with a high proportion of incorrect assignments (up to 46%). Remarkably, the colour of antenna was found in this study as a new promising character for species discrimination, with correct identification rates comparable to those of aedeagus and spermatheca. However, contrary to the latter, the assessment of the colour of the antenna is applicable both to teneral and fully sclerified individuals. The discovery of this character was possible by re-evaluating the qualitative morphological characters after the molecular identification of the evolutionary units, in a typical process of 'reverse taxonomy' (Kanzaki et al., 2012).

Despite a varying performance of single morphological characters for the discrimination between *P. kiesenwetteri* and *P. ruffoi*, the morphological identification became particularly sharp when multiple characters are used in combination or when a morphometric approach is performed. The results of morphometric analyses performed on fully sclerified specimens of *P. kiesenwetteri* and *P. ruffoi* from both sympatric and allopatric populations, are fully consistent with molecular identification results. A set of six morphometric variables allowed 100% of correct identification between males and females of the two species (Tables 2 and 3). Also the morphometric approach indicates the importance of genital characters and the antennae in discriminating between the two species. However, unlike qualitative analysis, in morphometric assessments the discrimination capacity of the aedeagus characters is equal to that of the spermatheca (in both cases 100%). These results corroborate the hypothesis that a quantitative morphometric analysis, which further investigates the possible morphological differences between different species, is a more reliable method than a classic qualitative morphological assessment (Mutanen & Pretorius, 2007).

Furthermore, by extending the single-locus molecular approach to multiple unlinked loci, we were able to clarify the phylogenetic relationships between *P. ruffoi* and *P. kiesenwetteri* and within the *gibbosa* species group. *Psylliodes ruffoi* has long been confused with *P. gibbosa* or *P. kiesenwetteri* (Leonardi, 1975) since the morphological characters of *P. ruffoi* are somewhat intermediate between those of *P. kiesenwetteri* and *P. gibbosa*, with several traits shared between all three species. The intermediate position both at morphological and geographical distribution level between these two species has also led to the speculation

that *P. ruffoi* could be a hybrid between *P. kiesenwetteri* and *P. gibbosa* (Nadein, 2008). Phylogenetic analyses allow to falsify either the hypothesis on the hybrid origin of *P. ruffoi* and the assumption of its sister relationships with *P. kiesenwetteri*. Bayesian and maximum likelihood analyses showed that *P. ruffoi* is sister to the pairs *P. gibbosa* and *P. inflata*, whereas *P. kiesenwetteri* is sister to *P. cucullata* (Figure 4). The close relationship between these latter species is quite surprising given the very different shape of the spermatheca of *P. cucullata* compared with *P. kiesenwetteri* and may warrant further investigation on the systematics of the *P. cucullata* complex. Therefore, answering the main questions of this study, we can conclude that, despite overall morphological similarity, *P. kiesenwetteri* and *P. ruffoi* are not sibling species, as they belong to distinct clades within the *gibbosa* species group, neither they are cryptic species, because they can be reliable distinguished by using morphological characters such as the colour of the antennae, the shape of genitalia or a combination of characters such as the shape of the body and the pronotal surface.

The results of this study show how the strength of the integrative taxonomy approach relies not only on the comparisons between species delimitation results based on different types of characters but also on their integration. In our case, it was possible to identify morphological characters useful for species discrimination thanks to a posteriori analysis of the variability found in molecularly based units. These findings corroborate the notion that in many cases species considered as cryptic have not been tested under careful morphological analyses, that in combination with genetic, ecological or behavioural differences can establish morphological characters sufficient for distinct identification (Cardoso et al., 2009; Dayrat, 2005; Lajus et al., 2015; Sáez & Lozano, 2005; Will et al., 2005).

Moreover, this study substantiates the relevance of a range-wide assessment of morphological and genetic variation, including populations from the type locality, the entire overlapping area between the species, as well as allopatric populations of both species. Such an exhaustive sampling makes it less likely the presence of well-known artefacts derived from a partial analysis of genetic and morphological variability (Bergsten et al., 2012; Furfaro et al., 2021; Meyer & Paulay, 2005; Phillips et al., 2019; Puillandre et al., 2012; Tessens et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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