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Delimiting species in the taxonomically challenging orchid section *Pseudophrys*: Bayesian analyses of genetic and phenotypic data

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Accurate species delimitation is critical for biodiversity conservation. Integrative taxonomy has been advocated for a long time, yet tools allowing true integration of genetic and phenotypic data have been developed quite recently and applied to few models, especially in plants. In this study, we investigated species boundaries within a group of twelve *Pseudophrys* taxa from France by analyzing genetic, morphometric and chemical (*i.e.*, floral scents) data in a Bayesian framework using the program integrated Bayesian Phylogenetics and Phylogeography (iBPP). We found that these twelve taxa were merged into four species when only genetic data were used, while most formally described species were recognized as such when only phenotypic (either morphometric or chemical) data were used. The result of the iBPP analysis performed on both genetic and phenotypic data supports the proposal to merge *Ophrys bilunulata* and *O. marmorata* on the one hand, and *O. funerea* and *O. zonata* on the other hand. Our results show that phenotypic data are particularly informative in the section *Pseudophrys* and that their integration in a model-based method significantly improves the accuracy of species delimitation. We are convinced that the integrative taxonomic approach proposed in this study holds great promise to conduct taxonomic revisions in other orchid groups.

KEYWORDS

integrative taxonomy, species delimitation, iBPP, floral scents, orchids

Introduction

Accurately delimiting species is of critical importance for many fields of research in biology, including conservation biology. Species are commonly defined as independently evolving lineages that can be delimited using various criteria (Hey, 2006; De Queiroz, 2007). As any single line of evidence may fail at detecting species

boundaries (Knowles and Carstens, 2007), many authors have advocated the use of an integrative approach combining several lines of evidence, both genetic and phenotypic (Dayrat, 2005; Will et al., 2005; Padiál et al., 2010; Pires and Marinoni, 2010). However, until recently, genetic and phenotypic data were almost always integrated in a purely qualitative way, as no quantitative methods were available for processing simultaneously both data types (Schlick-Steiner et al., 2010; Yeates et al., 2011). Fortunately, model-based species delimitation methods, which were originally developed for DNA sequences (Fujita et al., 2012; Naciri and Linder, 2015), were later extended to integrate quantitative traits (Guillot et al., 2012; Solís-Lemus et al., 2015), thereby improving objectivity and repeatability of integrative species delimitation. Such methods have been applied to various animal (Huang and Knowles, 2016; Pyron et al., 2016; Olave et al., 2017; Núñez et al., 2022) and plant (Yang et al., 2019; Zhang et al., 2020) clades and have proven useful in several cases. Because model-based species delimitation methods may cause oversplitting when solely based on genetic data (Sukumaran and Knowles, 2017; Mason et al., 2020), combining the latter with phenotypic data may provide more conservative estimates of species numbers (e.g., Pyron et al., 2016). Conversely, in recently radiating clades, in which species often lack clear genetic differentiation, integrating morphological or ecological data may increase the power to detect species boundaries (e.g., Edwards and Knowles, 2014; Solís-Lemus et al., 2015).

Hyperdiverse clades deserve particular conservation attention but may be taxonomically challenging. This is, for example, the case of the Orchidaceae family, which comprises more than 30,000 named species [Plants of the World Online [POWO], 2022], including some of the most threatened species in the world (Fay, 2018), but in which species boundaries are sometimes blurred (Barrett and Freudenstein, 2011; Pessoa et al., 2012). Within this family, the Mediterranean genus *Ophrys* L. is of particular interest, due to its high level of ecological specialization and endemism rate, but it is also considered as a textbook example of taxonomic confusion (Bertrand et al., 2021; Cuyppers et al., 2022), which may affect its conservation (Agapow et al., 2004; Pillon and Chase, 2007; Vereecken et al., 2010; Schatz et al., 2014). Some of this confusion arises from conflicting views on which operational criteria should be used to delimit species in this genus. Specifically, some authors support that taxa should have achieved reciprocal monophyly (Devey et al., 2008; Bateman et al., 2011) to be considered as “good” species, while others argue that interactions between *Ophrys* and pollinators are more informative than neutral markers due to their key role in speciation (Schiestl and Ayasse, 2002; Ayasse et al., 2011; Vereecken et al., 2011; Baguette et al., 2020). Indeed, *Ophrys* species attract one or a few pollinator species (Joffard et al., 2019; Schatz et al., 2020) using sex pheromones-mimicking floral scents (Schiestl et al., 1999; Ayasse et al., 2003). In these species, changes in

floral scents may cause pollinator shifts, which may in turn mediate reproductive isolation between conspecific populations and drive speciation (Sedeeq et al., 2014). Distinct views on which criteria should be used to delimit species has led to the recognition of dozens (Devey et al., 2008) versus hundreds (Paulus, 2006) of *Ophrys* species. In addition, even authors who favor the same criteria sometimes disagree on where along the speciation continuum independently evolving lineages should be recognized as species, i.e., “splitters” (e.g., Devillers and Devillers-Terschuren, 1994; Delforge, 2016) versus “lumpers” (e.g., Pedersen and Faurholdt, 2007; Kühn et al., 2020). In this context, model-based species delimitation methods integrating genetic and phenotypic data could be particularly helpful.

In this study, we aim at delimiting species through the integration of molecular markers, morphometric characters and floral scents in a group of twelve *Pseudophrys* taxa. We compare species boundaries based on genetic and phenotypic data alone or in combination and we discuss the potential of integrative taxonomy in solving long-standing debates about *Ophrys* taxonomy.

Materials and methods

Studied species and populations

The monophyletic section *Pseudophrys* Godfrey comprises twelve groups, each of them including one to twelve taxa (Delforge, 2016). Here, we focused on the twelve *Pseudophrys* taxa that are described in France (Table 1 and Figure 1; Bournérias and Prat, 2005). Among them, eight belong to the *O. fusca* group (namely *O. bilunulata*, *O. delforgei* subsp. “*O. forestieri*” sensu neotypus 1999, *O. funerea*, *O. lupercalis*, *O. marmorata*, *O. peraiolae*, *O. sulcata*, and *O. zonata*), one to the *O. iricolor* group (*O. eleonora*), two to the *O. lutea* group (namely *O. corsica* and *O. lutea*) and one to the *O. omegaifera* group (*O. vasconica*). These twelve taxa differ in their geographical distribution, some of them being widely distributed (e.g., *O. bilunulata*, *O. lupercalis*, and *O. lutea*), while others have restricted distribution areas, e.g., in South-eastern France (*O. delforgei*), South-western France and Northern Spain (*O. vasconica*) or Corsica and Sardinia (*O. corsica*, *O. eleonora*, *O. funerea*, *O. marmorata*, *O. peraiolae*, and *O. zonata*). By contrast, these twelve taxa do not strongly differ in their flowering phenology or habitats: except for *O. sulcata* and *O. vasconica*, they all flower in early spring and grow in open, dry habitats typical of the Mediterranean region (Bournérias and Prat, 2005). Among them, *O. lupercalis*, *O. lutea*, *O. sulcata*, and *O. vasconica* are regionally protected in France (Bournérias and Prat, 2005) and several of them are currently considered as threatened at the national or regional level, such as *O. eleonora* (considered as endangered at the national level and as critically endangered in the Corsican region) and

TABLE 1 Number of populations and individuals sampled for molecular, morphometric, and chemical data.

Taxon	Population	Molecular data	Morphometric data	Chemical data
<i>Ophrys bilunulata</i> Risso (1844)	Gruissan, 11, France	3 (1)	15	10
	Clapier, 34, France	3 (1)	10	5
	La Gaude, 06, France	2 (1)	–	–
<i>Ophrys corsica</i> Soleirol ex Foelsche and Foelsche (2002)	Bonifacio, 2A, France*	1	25	15
<i>Ophrys delforgei</i> Devillers-Terschuren and Devillers (2006)	Martigues, 13, France*	2	25	20
<i>Ophrys eleonorae</i> Paulus and Gack (2004)	Antisanti, 2B, France	1	–	2
<i>Ophrys funerea</i> Viviani (1824)	Palasca, 2B, France	3 (2)	20	9
	Corte, 2B, France	3 (2)	15	9
	Laconi, Sardinia, Italy	2	–	–
<i>Ophrys lupercalis</i> Devillers and Devillers-Terschuren (1994)	Armissan, 11, France*	2	0	0
	Saint Bauzille de Montmel, 34, France	2 (1)	20	15
	Saint-Florent, 2B, France	–	5	5
<i>Ophrys lutea</i> Cavanilles (1793)	Montferrier sur Lez, 34, France	3 (1)	10	10
	Montarnaud, 34, France	3 (1)	20	10
	Cassis, 13, France	1	–	–
	Maala, Kabylia, Algeria	1	–	–
	Benicolet, Valencian community, Spain	1	–	–
	Sempere, Valencian community, Spain	1	–	–
<i>Ophrys marmorata</i> Foelsche and Foelsche (1998)	Bonifacio, 2A, France*	5 (3)	20	8
<i>Ophrys peraiolae</i> Foelsche et al. (2000)	Palasca, 2B, France*	3	15	8
<i>Ophrys sulcata</i> Devillers and Devillers-Terschuren (1994)	Lapanouse, 12, France	3 (2)	25	10
	Oléron, 17, France*	2	–	–
	Vence, 06, France	1	–	–
<i>Ophrys vasconica</i> Delforge (1991)	Belpech, 11, France	1	20	15
<i>Ophrys zonata</i> Devillers and Devillers-Terschuren (1994)	Saint-Florent, 2B, France	3 (1)	25	15

*: Populations located at the locus classicus. (): Number of newly-published sequences.

O. marmorata (considered as vulnerable in the Corsican region) (IUCN et al., 2010; Delage and Hugot, 2015).

Four hundred ninety individuals belonging to one to six populations per taxon were selected and sampled for molecular, morphometric, or chemical analysis between 2013 and 2016 (Table 1). Within populations, molecular, morphometric, and chemical data were not collected on the same individuals as the iBPP program (see below) requires independence of genetic and phenotypic data. Molecular data were collected in one to six populations per taxon, distributed over most of their geographic range, in up to five individuals per population. Morphometric and chemical data were collected in one or two of these populations only, but on up to 25 individuals per population. One population was sampled at the *locus classicus* (i.e., site where the species was described for the first time) for six of these twelve taxa.

Genetic data collection and analysis

One leaf of one to five individual(s) per population were collected between 2014 and 2017 and dried in silica gel for a

few days. DNA extraction was performed with a Plant Minikit (®Quiagen). Three genes were amplified and sequenced in 52 individuals: the internal transcribed spacers (ITS) 1 and 2, the first intron of the beta-galactosidase-like (BGP) gene and the first intron of the LEAFY/FLORICULA (LFY) gene. For 36 individuals, sequences have been published in Joffard et al. (2020), while for 16 individuals, sequences are published for the first time in this study (Supplementary Table 1).

Polymerase chain reaction (PCR) and sequencing were carried out as described in Joffard et al. (2020). Sequences were edited using CodonCode Aligner v.4.2.7 (CodonCode Corporation). Uncertainties and alleles from heterozygous individuals were merged into consensus sequences using International Union for Pure and Applied Chemistry (IUPAC) coding. Consensus sequences were aligned using the Muscle algorithm (Edgar, 2004) as implemented in SeaView v.4.4.2 (Gouy et al., 2010) prior to concatenation.

A phylogenetic analysis was performed on the concatenated alignment using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). *Ophrys cinerophila* from Samos (Greece) was used as outgroup based on Joffard et al. (2020). The best partitioning scheme and the best model for each partition was chosen

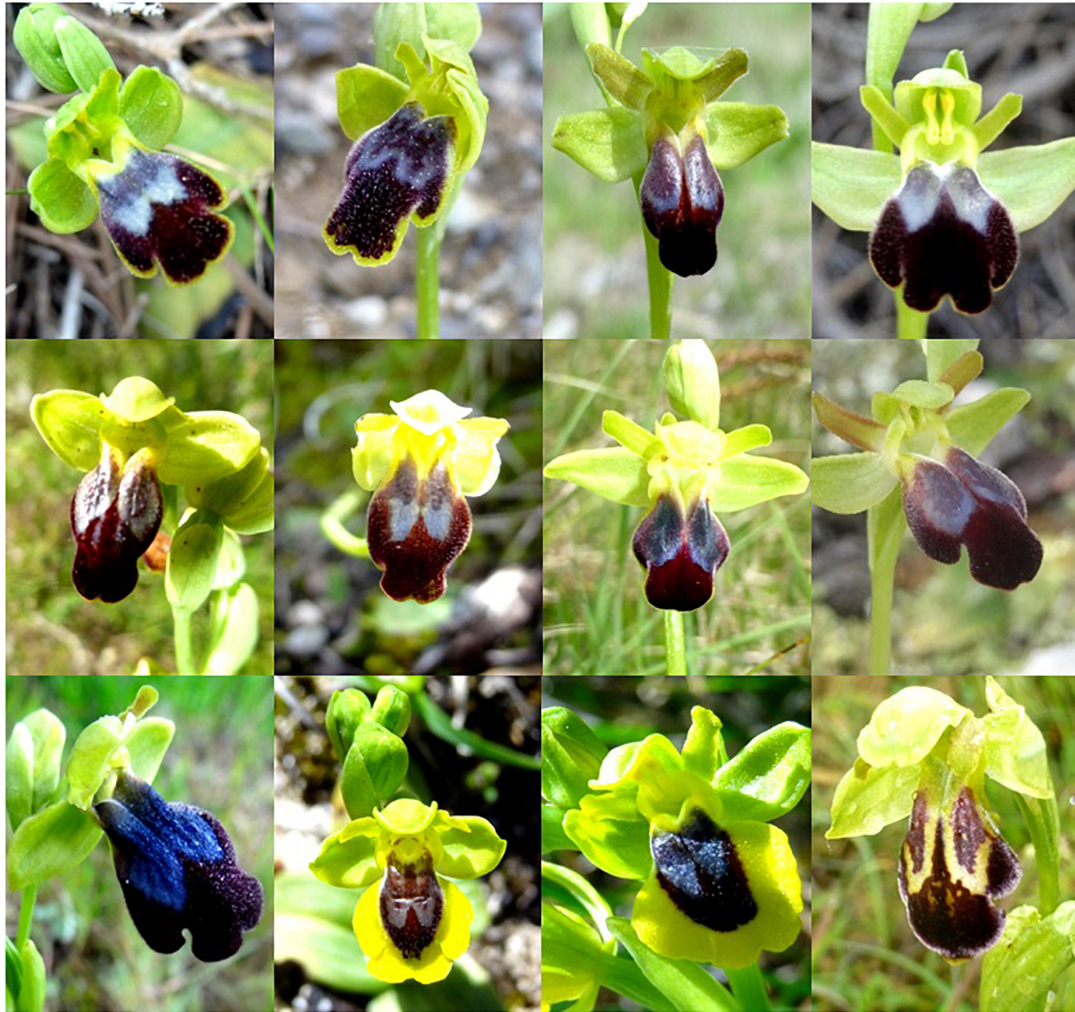


FIGURE 1

Photographs of the 12 French *Pseudophrys* taxa sampled for molecular, morphometric, and chemical data. From left to right and top to bottom: *Ophrys bilunulata*, *O. delforgei*, *O. funerea*, *O. lupercalis*, *O. marmorata*, *O. peraiolae*, *O. sulcata* and *O. zonata* (*O. fusca* group), *O. eleonorae* (*O. iricolor* group), *O. corsica*, *O. lutea* (*O. lutea* group), and *O. vasconica* (*O. omegaifera* group). © N. Joffard and B. Schatz.

using the Bayesian Information Criterion (BIC) as estimated by PartitionFinder v.1.1.1 (Lanfear et al., 2012). Bayesian analysis was conducted with two separate runs of four Markov chain Monte Carlo (MCMC) chains for 10 million generations with tree sampling every 1,000 generations. 25% of the sampled trees were discarded as burn-in, and the 75% best scoring trees were used to calculate the consensus tree.

A DNA barcoding analysis was performed on the concatenated alignment using the Automatic Barcode Gap Detection (ABGD) website (Puillandre et al., 2012). ABGD is a tool designed to infer species hypotheses based on automatized identification of barcode gaps between intra- and interspecific pairwise distances. It aims at revealing a significant barcoding gap in the distribution of pairwise genetic distances, reflecting a discontinuity between intra- and interspecific distances among individuals. ABGD partitions individuals into groups in a

recursive manner until no further splits are possible, while integrating priors on maximum and minimum intraspecific differentiation and barcode gap width. In this study, pairwise distances were computed as K2P-corrected distances. We left the default values of 10 steps from $P_{min} = 0.001$ to $P_{max} = 0.1$ for number of steps and intraspecific differentiation, and the default value of 1.5 for barcode gap width.

Phenotypic data collection and analysis

For morphometric data, fifteen to thirty-five individuals per taxon were sampled in 2015 and 2016 (Table 1). *Ophrys eleonorae* was not sampled for morphometric data as no flowering individuals were found in 2015 nor in 2016. However,

this species is known to be morphologically distinct from the eleven other taxa in that it has a particularly long (~15 to 25 mm) and wide (~10 to 20 mm) labellum (Bournérias and Prat, 2005). In each individual, twelve morphometric characters were measured to the nearest 0.01 mm in the field using a digital caliper. Four of these characters concerned the labellum, whose size and shape are important because they must match those of the pollinator, but the length and/or width of the stigmatic cavity, lateral petals, and sepals were also measured (Supplementary Figure 1).

For chemical data, eight to twenty individuals per taxon were sampled for floral scents in 2014 and 2015 (Table 1) using solid phase microextraction (SPME) (except in the case of *O. eleonora* in which only two individuals were sampled) as described in Joffard et al. (2016). Floral scents were then analyzed by GC-MS using a Shimadzu QP2010 Plus gas chromatograph-mass spectrometer with an OPTIMA[®] 5-MS capillary column (30 m × 0.25 mm × 0.25 μm, Macherey-Nagel, Düren, Germany) and helium as carrier gas with the method described in Joffard et al. (2016). Retention times of a series of *n*-alkanes (Qualitative retention time mix, ASTM, Sigma Aldrich[®]) were used to convert retention times into retention index. Compounds were identified based on retention index and mass spectra which were compared to those recorded in databases (NIST, 2007, Wiley Registry 9th) and in the literature (Adams, 2007) and, for some of them, to retention index and mass spectra of analytical standards. Peak areas were measured with the software GCMSsolution (4.11) (Shimadzu[®]).

Two partial least square discriminant analyses (PLS-DA) were performed, one for morphometric characters and one for floral scents. PLS-DA was chosen over Principal Component Analysis (PCA) because it is suitable for data that are non-independent (due to allometry in the case of morphometric characters and shared biosynthetic pathways in the case of floral scents). Because variances were non-homogenous among compounds, floral scents data were centred log-ratio-transformed prior to analysis. Statistical analyses were performed in R version 3.1.2 (R Development Core Team, 2008).

IBPP species delimitation

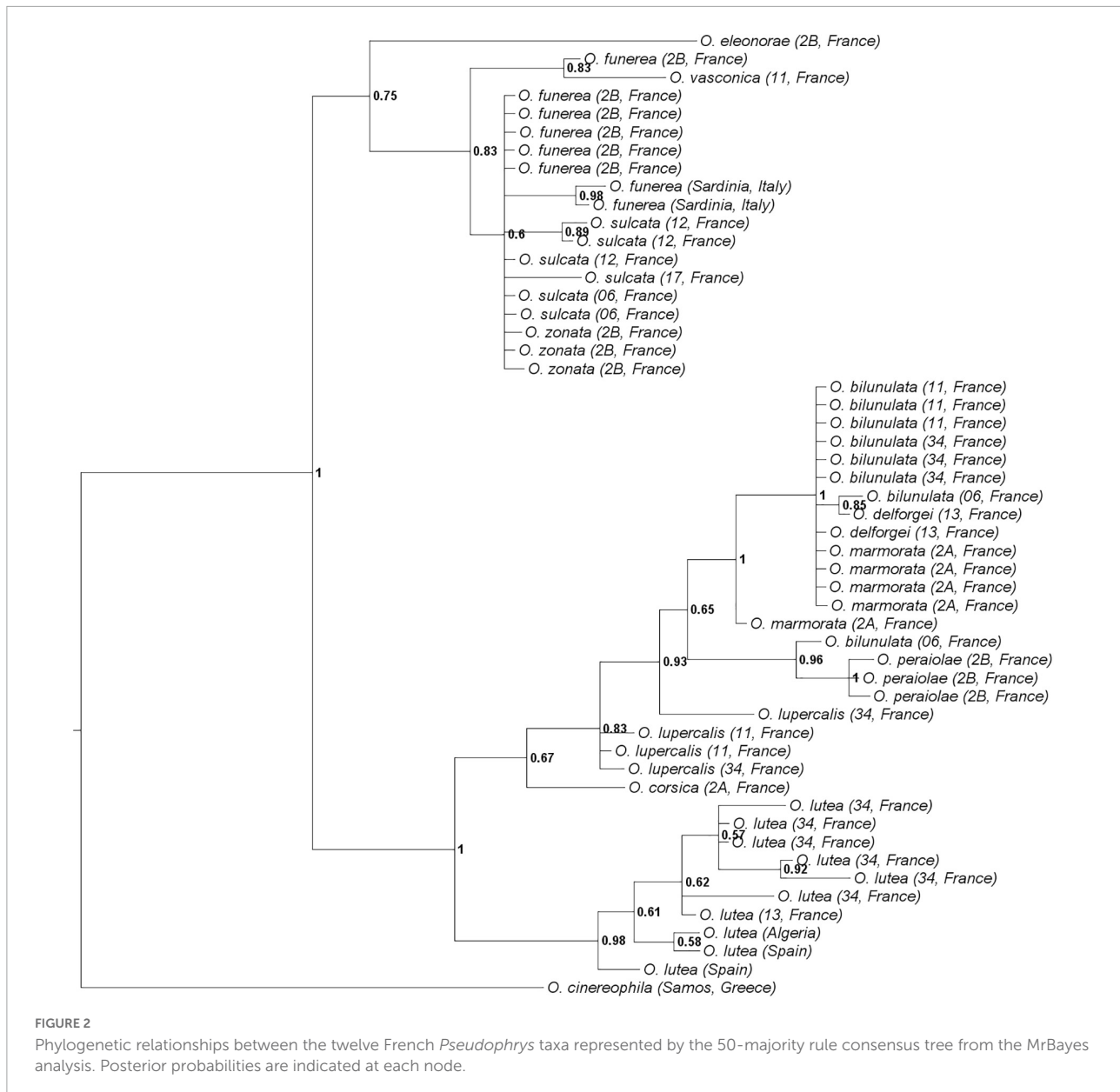
A joint Bayesian inference based on genetic and phenotypic data was used to delimit species using the program iBPP v.2.1.3 (Solís-Lemus et al., 2015). This program is an extension of the multispecies coalescent model-based program BPP (Rannala and Yang, 2003; Yang, 2015) which includes models of evolution for phenotypic data under a Brownian Motion (BM) process. Because the program assumes independence of phenotypic data, scores on the first two (for morphometric characters) and five (for floral scents) components resulting from two preliminary PCA were included in the analysis. Note that given the role of floral scents in pollinator attraction, these scents may not

have evolved according to the assumptions of a BM process, but the results of the program iBPP have been shown to be robust to such a violation (Solís-Lemus et al., 2015). The program begins with a strictly bifurcating guide tree, which in our case was constructed with the software MrBayes (see above), and collapses internal nodes sequentially. We used a prior gamma distribution $G(2, 2,000)$ for τ (branch lengths) and θ (product of N_e the population size and μ the mutation rate) for genetic data and left the default values of 0 for σ^2 (variance) and λ (within/between species ratio) for phenotypic data (non-informative priors). A reversible MCMC analysis was ran over 1,000,000 generations, sampled every ten generations, with 1,000 generations (10%) discarded as burn-in. Seven analyses were performed: (i) with genetic data only, (ii) with morphometric data only, (iii) with chemical data only, (iv) with both genetic and morphometric data, (v) with both genetic and chemical data, (vi) with both morphometric and chemical data, and (vii) with the entire dataset. Because phylogenetic relationships between the taxa *O. bilunulata*, *O. delforgei*, and *O. marmorata*, as well as between the taxa *O. funerea*, *O. sulcata*, and *O. zonata* could not be resolved, several alternative topologies were tested for the guide tree and the topology that gave the most conservative species delimitation model for these two triplets was retained. The robustness of the results was tested by analyzing the data with both the fine tune settings of zero and one (Yang and Rannala, 2010), and by repeating each analysis five times.

Results

Genetic data

ITS, BGP, and LFY sequences were obtained for 52, 49, and 52 individuals, respectively (153 sequences, including 45 that are newly published). Sequences were obtained for at least three individuals per taxon, except for *O. corsica*, *O. eleonora*, and *O. vasconica* (sequences obtained for one individual only). ITS, BGP, and LFY sequences contained 73, 562, and 603 parsimony-informative sites on 809, 948, and 2,210 sites, respectively. The phylogenetic tree (Figure 2) was congruent with the one described in Joffard et al. (2020), with two well-supported clades, one comprising the taxa *O. lutea*, *O. corsica*, *O. lupercalis*, *O. peraiolae*, *O. delforgei*, *O. bilunulata*, and *O. marmorata* and one comprising the taxa *O. eleonora*, *O. vasconica*, *O. sulcata*, *O. funerea*, and *O. zonata*. On the nine taxa for which several individuals were sampled for molecular analyses, only two - namely *O. lutea* and *O. peraiolae* - were found to be monophyletic with a posterior probability of 0.98 and 1, respectively. The AGBD method detected three species only: it recognized *O. eleonora* as a species but merged *O. lutea*, *O. corsica*, *O. lupercalis*, *O. peraiolae*, *O. delforgei*, *O. bilunulata*, and *O. marmorata* on the one hand, and *O. vasconica*, *O. sulcata*,



O. funerea, and *O. zonata* on the other hand. Mean K2P-corrected distances between individuals were of 2.38×10^{-3} substitutions per site within and 7.09×10^{-3} substitutions per site between these species. The barcoding gap was located between 3.00×10^{-3} and 4.00×10^{-3} substitutions per site ([Supplementary Figure 2](#)).

Morphometric data

Labellum length ranged from 6.11 to 14.35 mm, with a mean of $9.11 (\pm 1.45)$ mm, and labellum width from 5.88 to 14.23 mm, with a mean of $8.98 (\pm 1.51)$ mm. *Ophrys lupercalis* and *O. vasconica* were characterized by large sepals, petals, and

labella compared to other species, while *O. sulcata*, *O. funerea*, and *O. zonata* were characterized by long petals and sepals but a relatively small labellum, with a high length/width ratio. By contrast, *O. bilunulata*, *O. marmorata*, and *O. peraiolae* were characterized by larger labella with a lower length/width ratio. Finally, the yellow-flowered *O. corsica* and *O. lutea* were characterized by short sepals and petals and a short but wide labellum ([Figure 3](#) and [Supplementary Table 2](#)).

Chemical data

Over one hundred VOCs were detected in the blends of the twelve studied taxa, mostly alkanes (19), alkenes and alkadienes

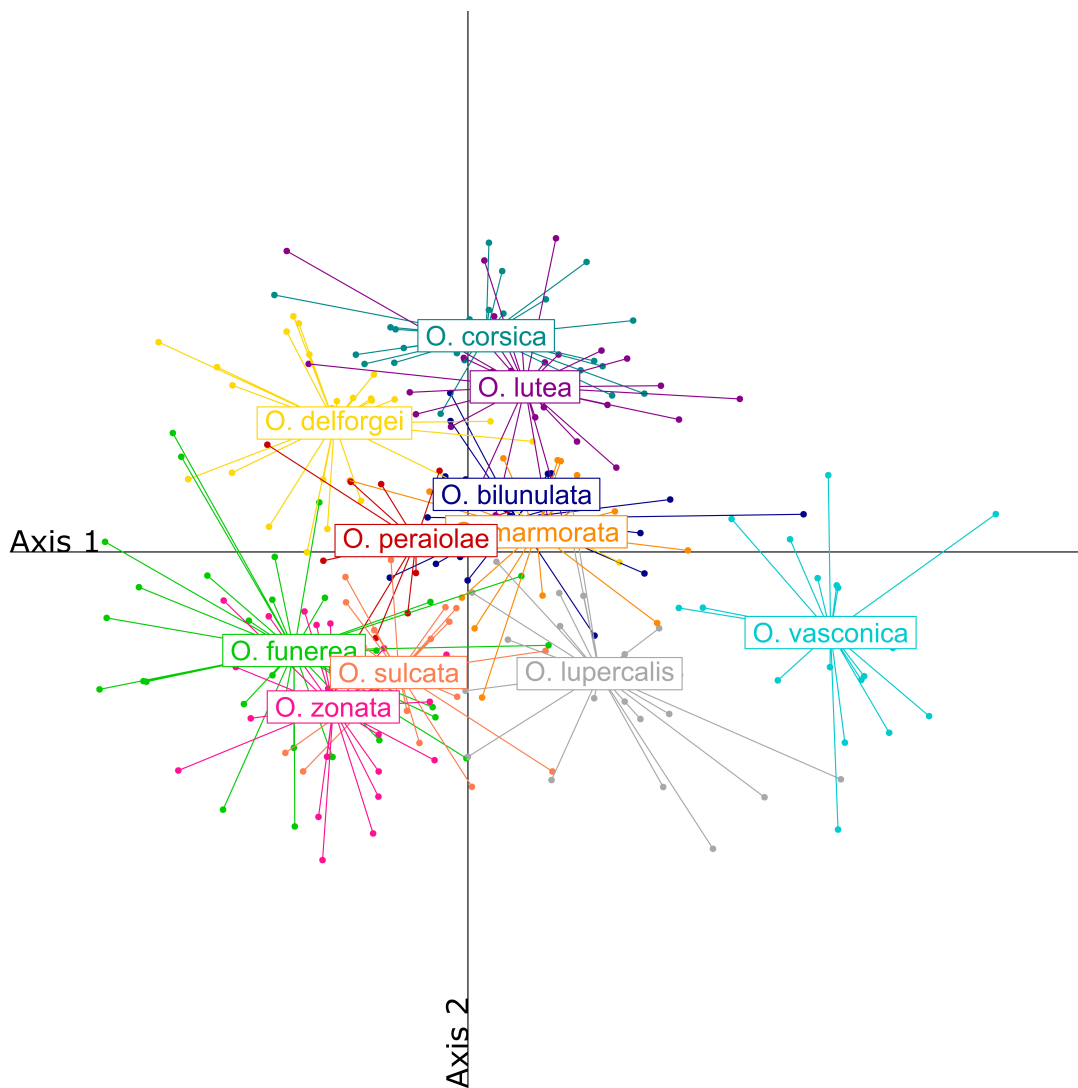


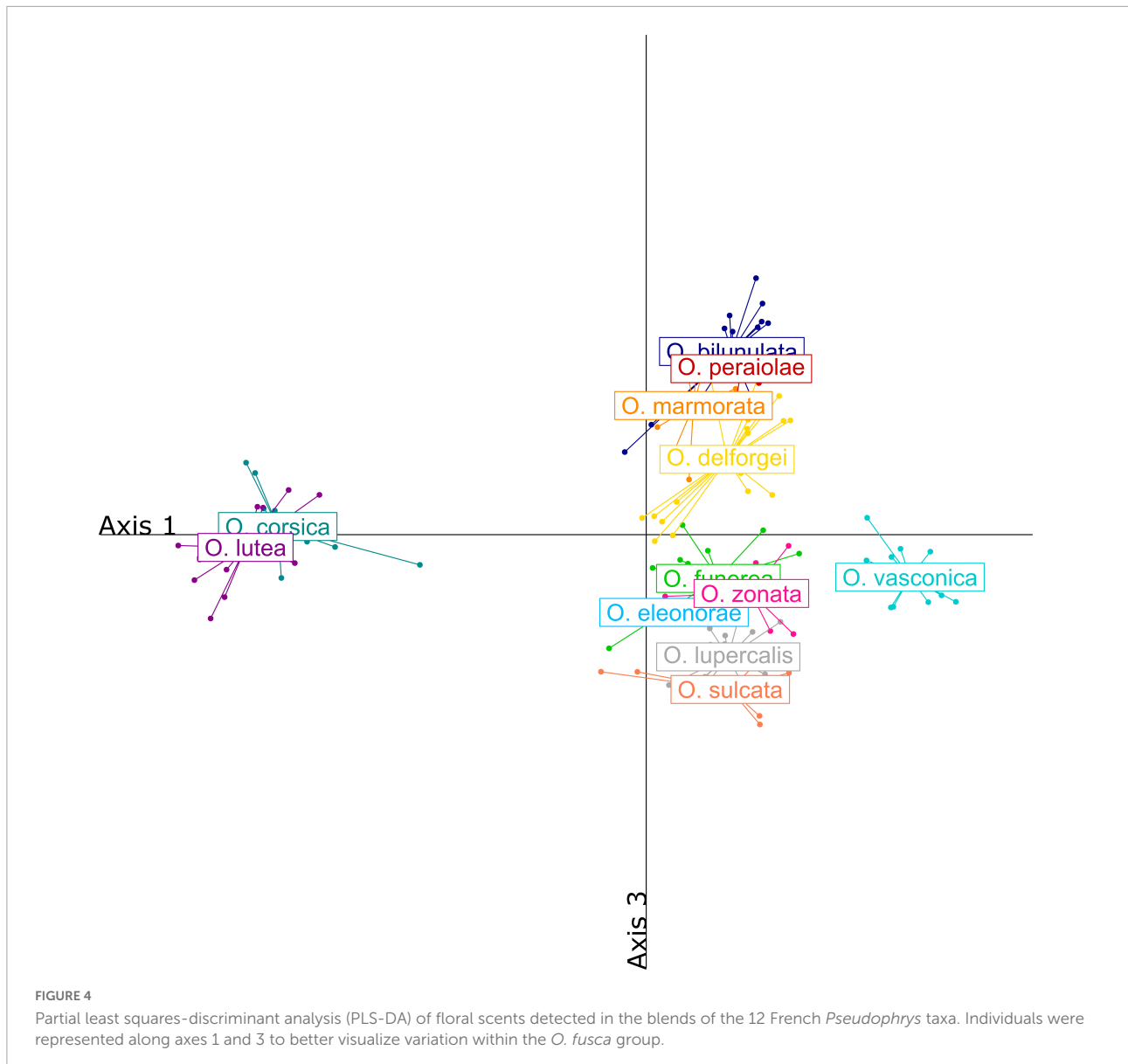
FIGURE 3
Partial least squares-discriminant analysis (PLS-DA) of morphometric characters measured on the twelve French *Pseudophrys* taxa.

(29), aldehydes (23), acids (13) and fatty acid esters (24) (Supplementary Table 3). Blends were dominated by alkenes and alkadienes (58.6%) as well as alkanes (28.2%), but aldehydes and fatty acid esters both accounted for more than 5% of the blends. The blends of the *O. fusca*, *O. iricolor*, *O. omegaifera*, and *O. lutea* groups were well differentiated, both qualitatively and quantitatively (Figure 4). More precisely, taxa from the *O. fusca* group generally did not produce any fatty acid esters, while taxa from the *O. iricolor*, *O. omegaifera*, and *O. lutea* groups produced significant amounts of nonyl, decyl, and octyl esters, respectively. Within the *O. fusca* group, some species also had well-differentiated blends, although this differentiation was often quantitative rather than qualitative. By contrast, several taxa, such as the *O. funerea*/*O. zonata* pair, produced very similar floral scents.

IBPP species delimitation

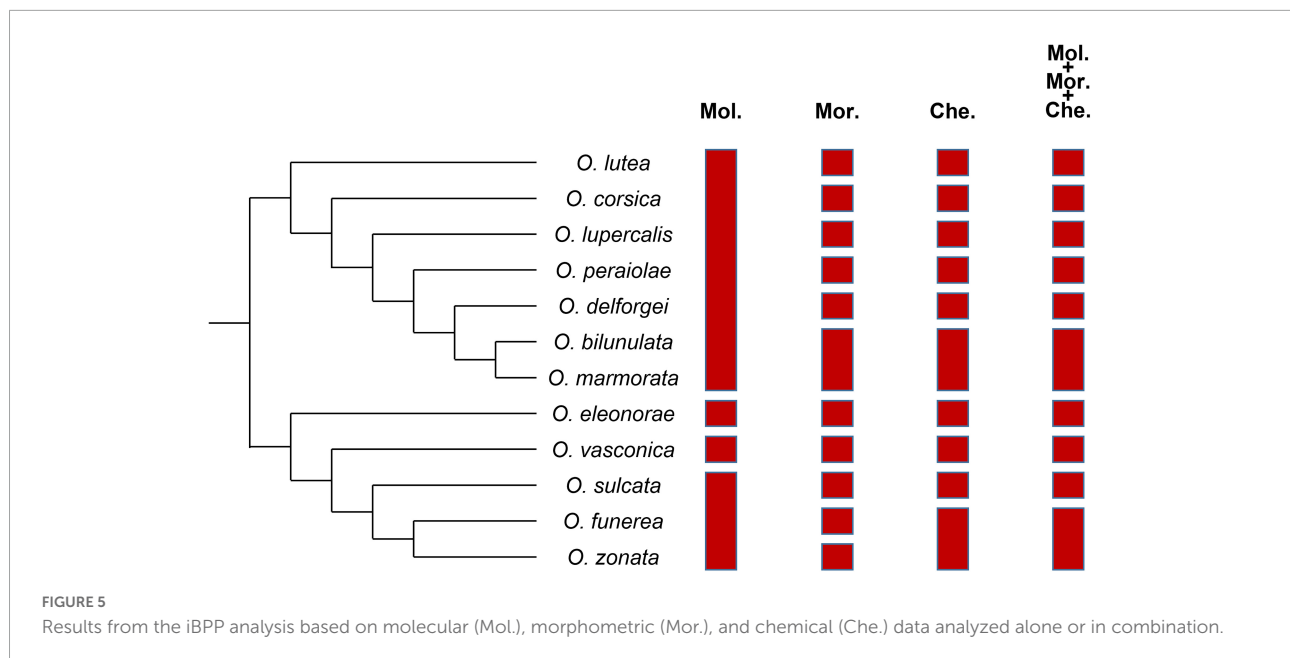
Results of iBPP analyses (best species delimitation models, with their respective species numbers and posterior probabilities) are summarized Figure 5 and detailed Supplementary Table 4.

Whatever the type of data included in the analysis, the posterior probability of the best model never exceeded 70%, showing relatively weak support for this model compared to the next best ones. When only genetic data were considered, the three best models (*i.e.*, those for which the sum of posterior probabilities exceeded 80%) all recognized *O. lutea*, *O. corsica*, *O. lupercalis*, *O. peraiolae*, *O. delforgei*, *O. bilunulata*, and *O. marmorata* as a single species. They also all considered *O. eleonora* as a genuine species and merged *O. funerea*



and *O. zonata*. The best model (PP = 61.86%) was a four-species model recognizing *O. vasconica* as a species but merging *O. sulcata* with the *O. funerea/O. zonata* pair. By contrast, when only morphometric data were considered, the two best models recognized most taxa as genuine species: the first one (PP = 67.10%) merged *O. bilunulata* and *O. marmorata*, while the second one (PP = 21.98%) delimited twelve species. The same results were obtained when molecular data were included in the analysis, but the posterior probabilities of these two best models decreased, while that of a ten-species model merging both *O. marmorata* with *O. bilunulata* and *O. zonata* with *O. funerea* increased (**Supplementary Table 4**). Likewise, when only chemical data were considered, the two best models were the ten-species model merging both pairs, and an eleven-species model merging *O. zonata* with *O. funerea*

only, with comparable posterior probabilities (PP = 47.63 and 44.84%, respectively). Including molecular data in the analysis increased support for the first model (PP = 53.59%) compared to the second one (PP = 39.50%). When only phenotypic (morphometric + chemical) data were considered, the three best models either suggested to merge both the *O. bilunulata/O. marmorata* and *O. funerea/O. zonata* pairs (PP = 44.83%) or one only (PP = 29.04% for the model merging *O. marmorata* with *O. bilunulata* and 16.02% for the one merging *O. zonata* with *O. funerea*). Finally, the same results were obtained when genetic and phenotypic data were combined, with only slight differences in the posterior probability attributed to each of these three best models compared to the previous analysis (PP = 49.88, 27.63, and 14.19%, respectively).



These results were robust to the algorithm that was used to collapse internal nodes, and repetitions of each analysis gave similar results. By contrast, these results varied depending on the topology of the guide tree. More specifically, when *O. bilunulata* and *O. marmorata* on the one hand, and *O. funerea* and *O. zonata* on the other hand were not considered as sister-species in the guide tree, the best model was the twelve-species model, because these two taxa were distinct from *O. delforgei* and *O. sulcata*, respectively.

Discussion

Our study aimed at comparing species boundaries drawn from molecular, morphometric, and chemical data alone or in combination in a group of twelve *Pseudophrys* taxa. Our results showed that including phenotypic data in the analysis helped being more accurate when delimiting species in this group. Based on this integrative taxonomic approach, eight formally described species were recognized as such, while the best model suggested merging two pairs of taxa into one species each.

Integration of genetic and phenotypic data in the section *Pseudophrys*

Our results showed that genetic differentiation between the twelve studied taxa was often limited and that species delimitations drawn from genetic data only (using either ABGD or iBPP) were thus very conservative, with only a few taxa recognized as genuine species. Such a limited genetic differentiation could result from incomplete lineage sorting or

hybridization (Soliva et al., 2001; Soliva and Widmer, 2003), that are both likely given the recent diversification of the section *Pseudophrys* (Breitkopf et al., 2014; Baguette et al., 2020) and the weakness of post-zygotic barriers between sympatric species (Cortis et al., 2009). Although the markers used in this study were selected because of their high resolution at the scale of the genus, they may not be informative enough to discriminate between such closely related taxa. Just like other non-model organisms, the *Ophrys* genus will likely benefit from the democratization of high-throughput sequencing technics allowing to develop more resolutive markers (e.g., Bateman et al., 2018).

By contrast, phenotypic differentiation between the twelve studied taxa was often significant, perhaps because morphometric characters and floral scents are selected by pollinators and may thus evolve faster and be less affected by hybridization than neutral markers (Seddeek et al., 2014). Indeed, the size and shape of the labellum are likely to be selected to match those of the pollinator's body (Triponez et al., 2013), and floral scents to match sex pheromones of female insects (Schiestl et al., 1999; Ayasse et al., 2003). Interestingly, our results show that morphometric characters are as informative as floral scents to discriminate between *Pseudophrys* species. Both are classically used as criteria to delimit *Ophrys* species (Bernardos et al., 2005; Mant et al., 2005), but in the past decades, much more emphasis has been put on chemical signals (Schiestl et al., 1999; Ayasse et al., 2003; Stöckl et al., 2005; Véla et al., 2007). However, our results suggest that using morphometric characters for taxonomic purposes is relevant in the section *Pseudophrys* and emphasize the potential role of orchid enthusiasts in providing valuable data for taxonomic research (Véla et al., 2015). As in the case of molecular markers,

more informative morphometric or chemical markers could be developed using more sophisticated techniques, such as geometric morphometrics (Rakosy et al., 2017; Gibert et al., 2022). More importantly, it would be interesting to distinguish between selected (*i.e.*, functionally significant) and neutral phenotypic traits through electrophysiological and/or behavioral studies (Schiestl et al., 1999; Rakosy et al., 2017). The distinction between biologically active and non-active floral scents, in particular, would likely provide further insights into the taxonomy of the section *Pseudophrys* (Stöckl et al., 2005, 2009), as shown in the section *Euophrys* (*e.g.*, Mant et al., 2005).

Because of these heterogeneous levels of resolution between molecular, morphometric and chemical data, species boundaries drawn from genetic *versus* phenotypic data were not congruent. Such an incongruence mirrors disagreements between authors favoring phylogenetic distinctness *versus* reproductive isolation through attraction of distinct pollinator species as a criterion to delimit *Ophrys* species (Paulus, 2006; Devey et al., 2008; Bateman et al., 2010). Methods based on genetic data are often judged more reliable than methods based on phenotypic data because they are not subject to investigator bias, nor affected by environmental or maternal conditions (Fujita et al., 2012). However, speciation sometimes leaves no signature at the level of neutral markers, especially when it is recent and when only a few loci mediate reproductive isolation, as it is assumed to be the case in the genus *Ophrys* (Xu and Schlüter, 2015). In this case, neutral markers-based methods may fail at detecting species boundaries by putting aside the data that are the most informative. Our results also show that integrating several phenotypic traits (in our case, morphometric and chemical) in the analysis may be helpful. For example, in our study, two taxa were slightly distinct morphologically, but strictly similar from a chemical point of view. When only morphometric characters were analysed, these two taxa were recognized as species, whereas when both morphometric characters and floral scents were analysed, they were merged. This shows that integrating new data types – either new molecular markers or new phenotypic traits – may challenge previous taxonomic inferences, species boundaries being hypotheses which should be tested using many data types to increase their robustness (Padial et al., 2010).

Taxonomy and conservation of the section *Pseudophrys*

The section *Pseudophrys* is known to be taxonomically challenging, due to the lack of resolution of classic molecular markers in this section (Schlüter et al., 2007; Devey et al., 2008) and to the striking morphological similarity between its members (Bernardos et al., 2005). Ninety-seven *Pseudophrys* species are described across the Mediterranean region (Delforge, 2016), but the taxonomic rank of many of them has been

questioned when confronted to molecular, morphometric or chemical evidence (*e.g.*, *O. arnoldii*: Bernardos et al., 2005; *O. vallesiana*: Gögler et al., 2016). Our analysis supports most *Pseudophrys* species that are described in France, with two remarkable exceptions: on the one hand, the first and second best models suggested to merge *O. marmorata* with the previously described species *O. bilunulata*, and on the other hand, the first and third best models suggested to merge *O. zonata* with the previously described species *O. funerea*. The similarity within these two pairs of taxa has been emphasized before (Bournérias and Prat, 2005; Tison and de Foucault, 2014), *O. marmorata* being sometimes called “*O. bilunulata* from Corsica” (Bournérias and Prat, 2005). Tison and de Foucault (2014) also suggested merging *O. delforgei* with the *O. bilunulata/O. marmorata* pair, and *O. sulcata* with the *O. funerea/O. zonata* pair, but our analysis does not support these proposals, since we found that both *O. delforgei* and *O. sulcata* were morphologically and chemically distinct from their closest relatives. The continental *O. bilunulata* and the Corsican *O. marmorata* were found to be genetically and phenotypically similar, whereas the Cyrno-Sardinian *O. funerea* and *O. zonata* were found to be slightly distinct morphologically but similar both genetically and chemically. Interestingly, recent records suggest that both *O. bilunulata* and *O. marmorata* are pollinated by *Andrena flavipes* (Schatz et al., 2021), which supports our proposal to merge these two taxa. Likewise, both *O. funerea* and *O. zonata* are pollinated by this species (Foelsche et al., 2000; Schatz et al., 2021), suggesting that these two taxa are not reproductively isolated and should be considered as conspecifics. The proposal to merge *O. marmorata* with *O. bilunulata* does not imply that this taxon should not be considered as vulnerable in Corsica anymore; however, it implies that it should not be considered as threatened at the national level. Another important conclusion of our study is the fact that *O. peraiolae* – which is sometimes described as a morph of *O. marmorata* (Delforge, 2005) and sometimes merged with *O. funerea* and *O. zonata* (Delage and Hugot, 2015) – likely corresponds to a genuine species, although it may be of hybrid origin (Tison and de Foucault, 2014). More generally, our analysis supports many species that are not recognized in the latest version of the European Red List (*e.g.*, *O. lupercalis*, *O. bilunulata*, etc.), in which they are all referred to as “*O. fusca sensu lato*” (Rankou, 2011). We hope that this study will prompt the reassessment of their IUCN status and the implementation of appropriate conservation actions, especially for species with extremely restricted distribution areas and declining population sizes such as *O. peraiolae*. We encourage the use of the integrative taxonomic approach proposed in this study to other orchid groups in which species boundaries are blurred, as it provides a framework to interpret patterns of genetic and phenotypic divergence among taxa and would speed up taxonomic revisions that are urgently needed for defining conservation priorities.

Data availability statement

The data presented in the study are deposited in the NCBI GenBank repository, and accession numbers are provided in the [Supplementary material](#).

Author contributions

NJ, EV, CM, and BS conceived and designed the analysis. NJ, EV, and BS collected the data. NJ, BB, VA, and CM performed the analysis. All authors discussed the results and contributed to the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.1058550/full#supplementary-material>

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