

Too strict or too loose? Integrative taxonomic assessment of Bombus lapidarius complex (Hymenoptera: Apidae)

Thomas Lecocq, Paolo Biella, Baptiste Martinet, Pierre Rasmont

▶ **To cite this version:**

Thomas Lecocq, Paolo Biella, Baptiste Martinet, Pierre Rasmont. Too strict or too loose? Integrative taxonomic assessment of Bombus lapidarius complex (Hymenoptera: Apidae). Zoologica Scripta, 2020, 49 (2), pp.187-196. 10.1111/zsc.12402 . hal-02501060

HAL Id: hal-02501060
https://hal.univ-lorraine.fr/hal-02501060

Submitted on 6 Mar 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Too strict or too loose? Integrative taxonomic assessment of *Bombus lapidarius* complex (Hymenoptera: Apidae)

Thomas Lecocq[1,2*], Paolo Biella[3], Baptiste Martinet[1], Pierre Rasmont[1]

1: Laboratory of Zoology, Research institute of Biosciences, University of Mons, Place du Parc 20, 7000 Mons, Belgium

2: Université de Lorraine, Inra, URAFPA, F-54000 Nancy, France

3: University of South Bohemia, Department of Zoology, Faculty of Science, Branišovská 31, 37005;

4: Biology Centre, Czech Academy of Sciences, Institute of Entomology, Branišovská 31, 37005, České Budějovice, Czech Republic

*Corresponding author:

Thomas Lecocq, Unit research animal and functionality of animal products, Université de Lorraine, Boulevard des Aiguillettes, B.P. 70239, 54506 Vandœuvre-lès-Nancy, France. Tel: +33 3 72 74 56 96. E-mail: thomas.lecocq@univ-lorraine.fr

Short running title: *Bombus* integrative taxonomy by evidence cumulation

Abstract

Lecocq et al. (2019) Too strict or too loose? Integrative taxonomic assessment by multi-evidence cumulation of *Bombus lapidarius* complex (Hymenoptera: Apidae). *Zoologica Scripta*, 00, 000-000.

The latest progress of the taxonomy is the use of integrative approach for species delimitation based on a multisource dataset. However, the taxonomic decision that should be made when convergence between the different lines of evidence is not observed remains debated. Here, we investigate the consequences of the application of an "integration by cumulation" approach on the taxonomic statuses within the *Bombus lapidarius* complex when using an integrative taxonomic framework (i.e. nuclear and mitochondrial markers along with reproductive traits) compared to a strict "integration by congruence" method. Our results show similar taxonomic conclusions whatever the decision-making approach used except for one taxon. According to the differentiation observed in our integrative taxonomic framework, recent divergence time, and other field observations for this taxon, we assume that a too strict decision-making method could fail to detect recently diverged species. This is exemplified by the new species *Bombus bisiculus* sp. n. occurring in South Italy and Sicily. *Thomas Lecocq, Université de Lorraine, Inra, URAFPA, F-54000 Nancy, France. E-mail thomas.lecocq@univ-lorraine.fr*

Introduction

Since the beginning of the Linnaean nomenclature, taxonomists have described and named an ever increasing number of species thanks to the perpetual integration in taxonomy of new analytic tools or methods (e.g. population genetics, phylogeography, phylogeny, or chemical ecology) (Padial & De La Riva, 2010; Padial et al., 2010). The latest culmination of the taxonomy progress is the species delimitation based on a multisource approach to gather different lines of evidence of speciation (Dayrat, 2005; Schlick-Steiner et al., 2010). This integrative taxonomy is a framework that unifies new conceptual and methodological developments in qualitative/quantitative assessment of species status (Schlick-Steiner et al., 2010). The approach's paradigm is strongly related to the unified species concept (USC) (De Queiroz, 2007). The USC states authors nearly universally acknowledge that species are separately evolving lineages of population or meta-populations but diverge in criteria for delimiting species (De Queiroz, 2007; Padial et al., 2010). The concept maintains the numerous delimiting species criteria as operational criteria (De Queiroz, 2007). Separation of metapopulation lineages could thus be inferred from evidence for reproductive isolation, phylogenetic divergence, or ecological differentiation. Integrative taxonomy considers these operational criteria to be separate lines of evidence when assigning species status (Schlick-Steiner et al., 2010). This approach aims at proposing strongly supported taxonomic statuses, but it has not led to standardized species delimitation. Indeed, the degree of congruence between operational criteria required to regard a taxon as a distinct species remains debated (De Queiroz, 2007; Padial et al., 2010), although species diagnose is more likely in multiple evidence detection. There are two conflicting decision frameworks: the "integration by congruence" and the "integration by cumulation" (Padial et al., 2010). The integration by congruence defines species through the convergence of divergence in many or all operational criteria. This a restrictive approach that recognizes only the strongly supported species (Padial et al., 2010). However, this could underestimate the number of species because the speciation process is not always accompanied by change of all diagnostic characters (Adams et al., 2009). Moreover, another risk could be that species that diverged in the distant past could have a increasing probability of divergence in all diagnostic traits resulting in a bias toward uncovering older species (Padial et al., 2010). In contrast, the integration by cumulation considers that divergence in any or few operational criteria can suggest speciation (Padial et al., 2010). This allows uncovering recently diverged and poorly differentiated species but the uncritical use of a single line of evidence can lead to overestimation of species number (Padial et al., 2010).

Recently, the confusing taxonomy of bumblebees (Hymenoptera: Apidae: *Bombus*) has reaped the benefits of integration of several independent lines of evidence in the species delimitation framework (Bertsch, 1997; Bertsch et al., 2005; Lecocq et al., 2015c, 2015a). While the efficiency of commonly used taxonomic diagnostic characters as components of integrative taxonomy approach has been recently assessed (Lecocq et al., 2015c, 2015a), the suitability of alternative decision frameworks (i.e. integration by congruence *versus*

integration by cumulation) has not been investigated to date. Indeed, published studies have used an integration by congruence approach leading to only strongly supported species status (Bertsch, 1997; Bertsch et al., 2005; Lecocq et al., 2015c, 2015a). However, the recent bumblebee evolutionary radiation (i.e. from the late Miocene or even during the Quaternary for some species groups) linked to last climatic oscillations (Hines, 2008; Duennes et al., 2012; Lecocq et al., 2013; Dellicour et al., 2017) could theoretically lead to recently diverged species where complete gene lineage sorting in all genomes (i.e. mtDNA and nclDNA), reciprocal monophyly for many loci, or phenotypic differentiation are not yet observed. Therefore, taxonomic conclusions based on integration by cumulation approach could be more suitable for bumblebee taxonomy, especially for most recently diverged taxa.

In this study, we investigate the taxonomic status within the *Bombus lapidarius* complex (Lecocq et al., 2013, 2015c). According to the latest taxonomic revision based on a multisource approach along with an integration by congruence decision framework (Lecocq et al., 2015c), the *B. lapidarius* complex includes (i) two species (*B. caucasicus* Radoszkowski 1859 and *B. lapidarius* (L.)), (ii) three poorly differentiated subspecies (*B. lapidarius atlanticus* Benoist, 1928 from Maroccan Atlas, *B. lapidarius decipiens* Pérez, 1890 from Iberian peninsula, and *B. lapidarius lapidarius* (L.) from the Mainland of Europe and W. Turkey), and (iii) one highly differentiated taxon currently included in *B. lapidarius* (S. Italian and Sicilian *decipiens*-like). *Bombus lapidarius* includes another subspecies (*B. lapidarius eriophorus*) in the Caucasus but it has not been revised with a multisource approach. *Bombus caucasicus* is a species from the North East Anatolia and Caucasus while *B. lapidarius* occurs in most of the West-Palearctic regions. Molecular clock and phylogeographic analyses showed that the divergence between each *B. lapidarius* subspecies and S. Italian-Sicilian *decipiens*-like occurred during Quaternary climatic oscillations (Lecocq et al., 2013). In contrast, the divergence of *B. caucasicus* happened likely earlier but cannot be currently related to a particular past biogeographic event (Lecocq et al., 2013). Here, we (i) assess the taxonomic status of taxa included in the *Bombus lapidarius* complex using an integration by cumulation decision framework, (ii) highlight the taxonomic changes compared to an integration by congruence decision framework, and (iii) provide the description of *Bombus bisiculus* sp. n.

Material and methods

Sampling

Two hundred seventy seven specimens from the *B. lapidarius* complex were previously sampled for other studies (Lecocq et al., 2013, 2015c): *B. lapidarius lapidarius* (genetic analyses [GA] = 196, CLGS analyses [CA] = 174, morphological comparisons [MC] = 196), *B. lapidarius decipiens* (GA = 23, CA = 17, MC = 20), *B. lapidarius atlanticus* (GA = five, CA = zero, MC = 10), *B. caucasicus* (GA = 10, CA = three, MC = 13), and *decipiens*-like from South Italy (GA = 20, CA = 35, MC = 35). Specimens were killed by freezing at -

20°C. Further 282 specimens of *decipiens*-like were added for taxon description purpose (see supporting information File S1). For these specimens, a map was prepared with Carto Fauna Flora version 2.0 (Barbier & Rasmont, 2000)

Genetic divergence

Two gene fragments were sequenced: mitochondrial cytochrome oxidase 1 (COI) and phosphoenolpyruvate carboxykinase (PEPCK). Four closely related species included as outgroup were analyzed (Cameron et al., 2007): *B. alagesianus* Reinig 1930, *B. incertus* Morawitz 1882, and *B. sichelii* Radoszkowski 1859. The genetic dataset was previously published (Lecocq et al., 2015c).

First, we performed pairwise analyses of molecular variance (AMOVA) in Arlequin ver. 3.5. (Excoffier & Lischer, 2010) with 100,000 random permutations to assess potential genetic differentiation between taxa. Second, we performed phylogenetic analyses to highlight monophyletic lineages among *B. lapidarius* taxa. Each gene was analyzed independently using maximum likelihood (ML) and Bayesian methods (MB). Trees were rooted with outgroup species. For all kind of analyses, the genetic datasets were partitioned according to (i) each gene according to intron and exon positions and (ii) each exon by base position (1^{st}, 2^{nd}, and 3^{rd}) to explore the best substitution model. The best fitting substitution models for each genetic markers were chosen through the Akaike information criteria corrected for small sample sizes (Hurvich & Tsai, 1989) implemented in jModeltest (Posada, 2008).

Ten independent runs of analyses in GARLI 2.0 (Zwickl, 2006) for each gene were performed for ML analyses with a random starting tree and the automated stopping criterion (stop when the ln score remained constant for 20,000 consecutive generations). The topology and −ln L were nearly identical among replicates; the tree with the highest likelihood of one of those runs was retained. The statistical confidence in nodes was evaluated with 15,000 non-parametric bootstrap replicates (Felsenstein, 1985) and using the automated stopping criteria set at 10,000 generations. Topologies with bootstrap values ≥ 70% were considered well supported (Hillis & Bull, 1993).

MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003) was used to performed MB analyses. For each gene, ten independent analyses were carried out using 600 million generations, four chains with mixed-models, default priors, and saving trees every 100 generations. The analyses were stopped after checking convergence between chains using the average standard deviation of split frequencies and by plotting likelihood values across generations with Tracer 1.4 (Rambaut & Drummond, 2007). The first 20 million generations (200,000 first trees saved) were discarded as burn-in. The phylogeny and posterior probabilities were then estimated from the remaining trees and a majority-rule 50% consensus tree was constructed. Topologies with posterior probabilities ≥ 0.95 were considered as well supported (Wilcox et al., 2002).

Reproductive trait divergence

A commonly used as chemical markers for resolving species status (Rasmont et al., 2005; Bertsch & Schweer, 2012), the male cephalic labial gland secretions (CLGS) were analyzed. The CLGS are species-specific secretions synthesized *de novo* by cephalic labial glands (Žáček et al., 2013). They consist of a complex mixture of mainly aliphatic compounds (Coppée et al., 2008; Lecocq et al., 2011), with several main components (Lecocq et al., 2015c).

Chemical dataset was previously published (Lecocq et al., 2015c). The chemical dataset were transformed (log (x+1)) to reduce the great difference of abundance between compounds in highly and low concentration. This dataset included 63 compounds detected in the CLGS of *B. lapidarius*. The five main compounds were fatty acid derivatives: hexadecen-1-ol (Δ9 and Δ7, $C_{16}H_{32}O$), hexadecan-1-ol ($C_{16}H_{34}O$), hexadecenoic acid (Δ9 and Δ7, $C_{16}H_{30}O_2$), hexadecenyl hexadecenoate ($C_{32}H_{60}O_2$), and hexadecyl hexadecenoate ($C_{32}H_{62}O_2$). CLGS differentiations among *B. lapidarius* taxa were assessed through principal component analyses (PCA; R-package MASS, Venables and Ripley, 2002) using R 3.5.1 (R Development Core Team, 2018). Groups detected in PCA analysis were assessed by performing multiple response permutation procedure (MRPP) (R-package vegan, Oksanen *et al*. 2011).

Data integration and decision framework

We assumed that (i) the species status attribution is more likely when divergences in most of operational criteria, (ii) the strict integration by congruence decision framework could lead to an underestimating of the species diversity, and (iii) the species delimitation based some small differentiations in one operational criterion could lead to deleterious taxonomic inflation. Therefore, we developed a balanced integration by cumulation approach to integrate these three statements.

In genetic part of the framework, concordance of mitochondrial and nuclear differentiations (i.e. genetic divergence with or without reciprocal monophyly) was regarded as a strong evidence of speciation between taxa (Lecocq et al., 2015c). Although a reciprocal monophyly between taxa in mitochondrial and nuclear phylogeny is a stronger evidence of potential speciation, it was not considered as minimal condition to detect speciation process because a recent speciation event can result in an incomplete lineage sorting (Weber et al., 2019). Moreover, mitochondrial and nuclear differentiations between geographically overlapping taxa (when physical barrier cannot explain the genetic distinctness) were considered as a more conclusive evidence of speciation process than the same differentiated between allopatric taxa. Indeed, differentiations between parapatric taxa could be fostered by reinforcement process triggered by a speciation event.

In reproductive traits, the differentiation of main compounds in CLGS was regarded as a strong indicator of ethologic consequences that potentially alters the pre-mating recognition because of most of bumblebee

species diverge in CLGS main compounds (Lecocq et al., 2015c). Since conclusive bioassays demonstrating that an observed CLGS differentiation leads to pre-mating isolation between taxa (Lecocq et al., 2015b) remain difficult to achieve (Lhomme et al., 2013; Lecocq et al., 2015c, 2015a), the interpretation of CLGS differentiation was coupled with an estimation of the gene flow between overlapping putative pre-mating isolated taxa (i.e. AMOVA test).

Overall, we considered that a taxon can be regarded as species if it displays both genetic (i.e. mtDNA and nDNA) and CLGS differentiations that suggest as speciation process as previously mentioned. We regarded these species statuses as further supported when reciprocal monophyly is also observed. Otherwise, we (i) attributed the subspecies status when differentiation is observed in one operational criterion (Hawlitschek et al., 2012; Torstrom et al., 2014; Lecocq et al., 2015c, 2015a; Miralles et al., 2017) or (ii) established the conspecificity with the senior synonym taxon when no differentiation is observed.

Results

Genetic differentiation

Genetic analyses showed differentiations between most of taxa in all genetic markers. ML and MB analyses performed on the same dataset led to similar tree topologies and to identical relationships within the *B. lapidarius*-complex. Mitochondrial and nuclear datasets produced different tree topologies.

Genetic analyses based on PEPCK detected genetic differentiations between (i) *B. caucasicus* (Group [G] A), (ii) S. Italian-Sicilian *decipiens*-like (GB), (iii) *B. lapidarius decipiens* + *B. lapidarius atlanticus* (GC), and (iv) *B. lapidarius lapidarius* (GD). The AMOVA tests confirmed these differentiations (GA *versus* GB: Fst = 0.68, p-value < 0.01; GA *versus* GC: Fst = 0.73, p-value < 0.01; GA *versus* GD: Fst = 0.64, p-value < 0.01; GB *versus* GC: Fst = 0.59, p-value < 0.01; GB *versus* GD: Fst = 0.72, p-value < 0.01; GC *versus* GD: Fst = 0.80, p-value < 0.01). However, PEPCK phylogenetic analyses did not highlight reciprocal monophyly between taxa (Fig. 1).

Genetic analyses based on COI detected three genetically differentiated groups: (i) *B. caucasicus* (GA), (ii) S. Italian-Sicilian *decipiens*-like (GB), (iii) *B. lapidarius lapidarius* + *B. lapidarius decipiens* + *B. lapidarius atlanticus* (GC) (Fig. 1). The AMOVA tests confirmed these results (GA *versus* GB: Fst = 0.93, p-value < 0.01; GA *versus* GC: Fst = 0.95, p-value < 0.01; GB *versus* GC: Fst = 0.81, p-value < 0.01). COI phylogenetic trees showed reciprocal monophyly between GA, GB, and GC (Fig. 1).

CLGS differentiation

Analyses of chemical dataset showed a CLGS differentiation between some taxa. PCA revealed three different groups: (i) *B. caucasicus* (G1), (ii) S. Italian-Sicilian *decipiens*-like (G2), (iii) *B. lapidarius*

lapidarius + *B. lapidarius decipiens* (G3) (Fig. 2). Global MRPP tests confirmed these divergences (T= 0.32, A=0.13, *p*-value <0.01). Pairwise MRPP confirmed divergences between these three groups: G1 *vs.* G2 (T=0.27, A=0.18, *p*-value <0.01), G1 *vs.* G3 (T=0.34, A=0.02, *p*-value <0.01), G2 *vs.* G3 (T=0.33, A=0.11, *p*-value <0.01). The three different groups displayed differences in main compounds. The hexadec-7-enoic acid was detected in S. Italian-Sicilian *decipiens*-like only while hexadec-9-enoic acid was detected in *B. caucasicus*, *B. lapidarius decipiens*, and *B. lapidarius lapidarius*. Samples of *B. caucasicus* displayed the hexadec-7-enol while the hexadec-9-enol was detected in *decipiens*-like, *B. lapidarius decipiens*, and *B. lapidarius lapidarius*.

Taxonomic status

Our integrative decision framework indicated that the *B. lapidarius* complex gathers three species: *B. caucasicus*, *B. lapidarius*, and S. Italian-Sicilian *decipiens*-like. Nuclear and mitochondrial differentiations, reciprocal monophyly with other taxa in COI, and the significant CLGS differentiation involving main compounds confirmed the current species status of *B. caucasicus* (Table 1). Subspecies statuses of *B. lapidarius atlanticus*, *B. lapidarius decipiens*, and *B. lapidarius lapidarius* included in *B. lapidarius* were confirmed according to their genetic differentiation and their shared CLGS composition (Table 1). *Decipiens*-like displayed a level of nuclear differentiation similar to *B. lapidarius* subspecies but formed a monophyletic well supported group in COI and displayed a CLGS differentiation involving main compounds. Therefore, our integrative decision framework suggested that *decipiens*-like deserved a species status (Table 1). Moreover, our AMOVA analyses suggested a restricted gene flow between S. Italian-Sicilian *decipiens*-like and *B. lapidarius* despite their overlapping distribution in Central Italy.

The name *decipiens* was first used by Pérez (1890) to describe a Pyrenean color form of *B. lapidarius*; corresponding to *B. lapidarius decipiens*. This name was used subsequently by most authors for the Iberian subspecies (e.g. Reinig 1935; Tkalců 1962). The authors also used this name for the South Italian *B. lapidarius* (e.g. Reinig 1935, 1969; Tkalců 1960). Therefore, *decipiens*-like had not available name. Therefore, we here described it as a new species (see argumentation for species status in discussion) as *B. bisiculus* sp. n. (Figs 3-5, see description in supporting information File S1)

Discussion

Our integration by cumulation decision method leads to quite similar results than the integration by congruence approach previously applied on same species group (Lecocq et al., 2015c) (Table 1). The species status of *B. caucasicus* is assessed as well as the subspecies status of *B. lapidarius atlanticus*, *B. lapidarius decipiens*, and *B. lapidarius lapidarius*. The only exception is the status of *B. bisiculus* considered as a subspecies by cumulation decision method and as species by congruence approach. Since the two conclusions are based on the same dataset and statistical approaches, the discordance with previous taxonomic status

(Lecocq et al., 2015c) of *B. bisiculus* is only explained by the two alternative decision methods. Although, this first comparison of outcomes provided by alternative decision methods in bumblebees does not allow examining the efficiency of each approach in details, the conflicting species status of *B. bisiculus* can be used to underlines practical consequences of decision-making procedure.

Beside the simple impact on taxonomical nomenclature, the choice of one decision approach can deeply affect further developments in other biology fields (e.g. conservation biology or evolutionary biology). For instance, new original assessment for red list will be needed for *B. bisiculus* due to the present taxonomic conclusion (IUCN Species Survival Commission, 2012). Moreover, considering *B. bisiculus* with a species status would be one more evidence that South Italy acted as a speciation area for bumblebees, paving the way to detailed taxonomic assessment of Italian taxa (see also Martinet et al., 2018). These examples show how new taxonomic conclusions can trigger further research which can be possibly unnecessary or false if the new species status is unsupported. Since the use of a too loose decision-making procedure can lead to such a problematic taxonomic inflation (Isaac et al., 2004), assigning subspecies status to taxa for which the species status is proposed only by an integration by congruence remains the most conservative solution (Lecocq et al., 2015c, 2015a). This means that this solution should be favored in most of differentiated taxa with conflicting taxonomic statuses according to loose and strict decision methods. Nevertheless, we agree with Valdecasas *et al.* (2008) about that the taxonomy standard should be excellence rather than the creation of sets of rules that impose narrow practices and paradigms. Taking this into account, we consider that a taxon should be assigned to species status when (i) species status is assigned by loose decision method, (ii) strict decision approach results in subspecies status near the species threshold, and (iii) additional pieces of evidence (not included in commonly used integrative taxonomy framework because they are rarely available for the studied species group) suggest a reproductive isolation. *Bombus bisiculus* exemplifies this kind of taxon. First, the integration by cumulation approach highlight it species status. Second, an integration by congruence approach show that its differentiation degree is near the species-level differentiation (Lecocq et al., 2015c). Third, additional information tends to support its species status: (i) there is a persistence of the genetic divergence through time despite the current secondary contact zone with *B. lapidarius* (Lecocq et al., 2013), (ii) few putative/doubtful hybrids *B. bisiculus* X *B. lapidarius* are known (Reinig, 1935, 1970), and (iii) a potential reinforcement process acting on CLGS of *B. bisiculus* and *B. lapidarius* has been suggested in the contact zone (Lecocq et al., 2013). One could argue that such a species delimitation approach could lead to a dramatic taxonomic inflation in bumblebees. However, an overview of already published integrative taxonomic revision of bumblebee species (review in Lecocq *et al.* 2015b) shows that such taxa are not commonly met; making this risk limited. Moreover, the conservative solution of attributing the infra-specific rank to these kinds of taxa could hide a recent speciation process useful for other biology facets. Indeed, the long-standing debate on the subspecies usefulness in living world (Ebach & Williams, 2009; Patten, 2009), that fosters a trend to eliminate the trinomial designation in species names (Manier, 2004; Mulcahy, 2008), could lead to disregard such taxa.

Therefore, we assign the species status to *B. bisiculus*. This result is conflicting with all previous studies only based on color patterns (Reinig, 1935, 1970; Tkalců, 1960; Rasmont, 1983). Further analyses (e.g. check potential hybridization in the contact zone and fitness of offspring, ecological niche differentiation in overlapping area) should be performed in order to assess or falsify this status.

Acknowledgements

We gratefully acknowledge I. Valterová and K. Urbanová (Prague, Czech Republic) for their help in chemical analyses. Computational resources have been provided by the *Consortium des Équipements de Calcul Intensif* (CÉCI) funded by the Belgian F.R.S.-FNRS.

References

Adams, D.C., Berns, C.M., Kozak, K.H., & Wiens, J.J. (2009). Are rates of species diversification correlated with rates of morphological evolution? *Proceedings of The Royal Society B*, 276, 2729–38.

Barbier, Y., & Rasmont, P. (2000). *Carto Fauna-Flora 2.0. Guide d'utilisation*. Mons, Belgium, Université de Mons-Hainaut.

Bertsch, A. (1997). Discrimination of the bumblebee species *Bombus cryptarum* and *B. lucorum* by means of male labial gland secretions and morphological characters (Hymenoptera : Apidae). *Entomologia Generalis*, 22, 129–145.

Bertsch, A., & Schweer, H. (2012). Cephalic labial gland secretions of males as species recognition signals in bumblebees: are there really geographical variations in the secretions of the *Bombus terrestris* subspecies? *Beiträge zur Entomologie*, 62, 103–124.

Bertsch, A., Schweer, H., Titze, A., & Tanaka, H. (2005). Male labial gland secretions and mitochondrial DNA markers support species status of *Bombus cryptarum* and *B. magnus* (Hymenoptera, Apidae). *Insects Sociaux*, 52, 45–54.

Cameron, S.A., Hines, H.M., & Williams, P.H. (2007). A comprehensive phylogeny of the bumble bees (Bombus). *Biological Journal of the Linnean Society*, 91, 161–188.

Coppée, A., Terzo, M., Valterová, I., & Rasmont, P. (2008). Intraspecific variation of the cephalic labial gland secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Chemistry & Biodiversity*, 5, 2654–2661.

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85, 407–415.

Dellicour, S., Kastally, C., Varela, S., Michez, D., Rasmont, P., Mardulyn, P., & Lecocq, T. (2017). Ecological niche modelling and coalescent simulations to explore the recent geographical range history of five widespread bumblebee species in Europe. *Journal of Biogeography*, 44, 39–50.

Duennes, M.A., Lozier, J.D., Hines, H.M., & Cameron, S.A. (2012). Geographical patterns of genetic

divergence in the widespread Mesoamerican bumble bee *Bombus ephippiatus* (Hymenoptera: Apidae). *Molecular phylogenetics and evolution*, 64, 219–31.

Ebach, M.C., & Williams, D.M. (2009). How objective is a definition in the subspecies debate? *Nature*, 457, 785.

Excoffier, L., & Lischer, H.E.L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10, 564–567.

Felsenstein, J. (1985). Phylogenies and the comparative method. *The American Naturalist*, 125, 1–15.

Hawlitschek, O., Nagy, Z.T., & Glaw, F. (2012). Island evolution and systematic revision of Comoran snakes: why and when subspecies still make sense. *PloS one*, 7, e42970.

Hillis, D.M., & Bull, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology*, 42, 182–192.

Hines, H.M. (2008). Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: *Bombus*). *Systematic biology*, 57, 58–75.

Hurvich, C.M., & Tsai, C.-L. (1989). Regression and time series model selection in small samples. *Biometrika*, 76, 297–307.

Isaac, N.J.B., Mallet, J., & Mace, G.M. (2004). Taxonomic inflation: Its influence on macroecology and conservation. *Trends in Ecology and Evolution*, 19, 464–469.

IUCN Species Survival Commission (2012). *Guidelines for application of IUCN red list criteria at regional and national levels Version 4.0.* Gland, Switzerland, IUCN.

Lecocq, T., Brasero, N., Meulemeester, T. De, Michez, D., Dellicour, S., Lhomme, P., … Rasmont, P. (2015a). An integrative taxonomic approach to assess the status of Corsican bumblebees : implications for conservation. *Animal Conservation*, 18, 236–248.

Lecocq, T., Coppée, A., Mathy, T., Lhomme, P., Cammaerts-Tricot, M.-C.C., Urbanová, K., … Rasmont, P. (2015b). Subspecific differentiation in male reproductive traits and virgin queen preferences, in *Bombus terrestris*. *Apidologie*, 46, 595–605.

Lecocq, T., Dellicour, S., Michez, D., Dehon, M., Dewulf, A., De Meulemeester, T., … Rasmont, P. (2015c). Methods for species delimitation in bumblebees (Hymenoptera, Apidae, *Bombus*): towards an integrative approach. *Zoologica Scripta*, 44, 281–297.

Lecocq, T., Dellicour, S., Michez, D., Lhomme, P., Vanderplanck, M., Valterová, I., … Rasmont, P. (2013). Scent of a break-up: phylogeography and reproductive trait divergences in the red-tailed bumblebee (*Bombus lapidarius*). *BMC evolutionary biology*, 13, 263.

Lecocq, T., Lhomme, P., Michez, D., Dellicour, S., Valterová, I., & Rasmont, P. (2011). Molecular and

chemical characters to evaluate species status of two cuckoo bumblebees: *Bombus barbutellus* and *Bombus maxillosus* (Hymenoptera, Apidae, Bombini). *Systematic Entomology*, 36, 453–469.

Lhomme, P., Sramkova, A., Kreuter, K., Lecocq, T., Rasmont, P., & Ayasse, M. (2013). A method for year-round rearing of cuckoo bumblebees (Hymenoptera: Apoidea: Bombus subgenus *Psithyrus*). *Annales de la Société Entomologique de France*, 49, 117–125.

Manier, M.K. (2004). Geographic variation in the long-nosed snake, *Rhinocheilus lecontei* (Colubridae): beyond the subspecies debate. *Biological Journal of the Linnean Society*, 83, 65–85.

Martinet, B., Lecocq, T., Brasero, N., Biella, P., UrbanovÁ, K., ValterovÁ, I., … Rasmont, P. (2018). Following the cold: geographical differentiation between interglacial refugia and speciation in the arcto-alpine species complex *Bombus monticola* (Hymenoptera: Apidae). *Systematic Entomology*, 43, 200–217.

Miralles, A., Macleod, A., Rodríguez, A., Ibáñez, A., Jiménez-Uzcategui, G., Quezada, G., … Steinfartz, S. (2017). Shedding light on the Imps of Darkness: an integrative taxonomic revision of the Galápagos marine iguanas (genus *Amblyrhynchus*). *Zoological Journal of the Linnean Society*, 181, 678-710.

Mulcahy, D.G. (2008). Phylogeography and species boundaries of the western North American Nightsnake (*Hypsiglena torquata*): revisiting the subspecies concept. *Molecular phylogenetics and evolution*, 46, 1095–115.

Oksanen, F.J., Blanchet, G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., … Wagner, H. (2011). Vegan: Community Ecology Package. R package version 2.5-2. Available from https://CRAN.R-project.org/package=vegan.

Padial, J.M., & De La Riva, I. (2010). A response to recent proposals for integrative taxonomy. *Biological Journal of the Linnean Society*, 101, 747–756.

Padial, J.M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in zoology*, 7, 16.

Patten, M.A. (2009). "Subspecies" and "race" should not be used as synonyms. *Nature*, 457, 147.

Posada, D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, 25, 1253–1256.

De Queiroz, K. (2007). Species concepts and species delimitation. *Systematic Biology*, 56, 879–886.

R Development Core Team (2018). *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.R-project.org/

Rambaut, A. & Drummond, A.J. (2007). Tracer. V1.5.0 Available from http://beast.bio.ed.ac.uk/Tracer.

Rasmont, P. (1983). Catalogue commenté des Bourdons de la région ouest-paléarctique (Hymenoptera,

Apoïdea, Apidae). *Notes fauniques de Gembloux*, 7, 1–72.

Rasmont, P., Franzen, M., Lecocq, T., Harpke, A., Roberts, S., Biesmeijer, K., … Schweiger, O. (2015). Climatic Risk and Distribution Atlas of European Bumblebees. *BioRisk*, 10, 1–236.

Rasmont, P., Terzo, M., Aytekin, A.M., Hines, H., Urbanova, K., Cahlikova, L., & Valterova, I. (2005). Cephalic secretions of the bumblebee subgenus *Sibiricobombus* Vogt suggest *Bombus niveatus* Kriechbaumer and *Bombus vorticosus* Gerstaecker are conspecific (Hymenoptera, Apidae, *Bombus*). *Apidologie*, 36, 571–584.

Reinig, W.F. (1935). On the variation of *Bombus lapidarius* L. and its cuckoo, *Psithyrus rupestris* Fabr., with notes on mimetic similarity. *Journal of Genetic*, 30, 321–356.

Reinig, W.F. (1970). Bastardierungszonen und Mischpopulationen bei Hummeln (*Bombus*) und Schmarotzerhummeln (*Psithyrus*) (Hymenopt., Apidae). *Mitteilungen der Münchener entomologischen Gesellschaft*, 59, 1–89.

Ronquist, F., & Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E., & Crozier, R.H. (2010). Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, 55, 421–438.

Tkalců, B. (1960). Zur Hummelfuna Der Apenninen. *Memorie del Museo Civico di Storia Naturale di Verona* 8, 23–68.

Tkalců, B. (1962). Sur la faunistique des Bourdons d'Espagne. *Bulletin de la Société Entomologique de Mulhouse*, 18, 14–16.

Torstrom, S.M., Pangle, K.L., & Swanson, B.J. (2014). Shedding subspecies: The influence of genetics on reptile subspecies taxonomy. *Molecular phylogenetics and evolution*, 76, 134–43.

Valdecasas, A.G., Williams, D., & Wheeler, Q.D. (2008). "Integrative taxonomy" then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, 93, 211–216.

Venables, W.N., & Ripley, B.D. (2002). *Modern Applied Statistics with S.* New York, NY, Springer.

Weber, A.A.-T., Stöhr, S., & Chenuil, A. (2019). Species delimitation in the presence of strong incomplete lineage sorting and hybridization: Lessons from *Ophioderma* (Ophiuroidea: Echinodermata). *Molecular Phylogenetics and Evolution*, 131, 138–148.

Wilcox, T.P., Zwickl, D.J., Heath, T.A., & Hillis, D.M. (2002). Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. *Molecular Phylogenetics and Evolution*, 25, 361–371.

Žáček, P., Prchalová-Hornákov, D., Tykva, R., Kindl, J., Vogel, H., Svatoš, A., … Valterová, I. (2013). *De Novo* Biosynthesis of Sexual Pheromone in the Labial Gland of Bumblebee Males. *ChemBioChem*, 14, 361–371.

Zwickl, D.J. (2006). *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criteria.* P-D Thesis, The University of Texas at Austin, Austin, Texas.

Table

Table 1. Assessment of genetic and reproductive trait operational criteria for the *Bombus lapidarius* complex and resulting taxonomic conclusions. Genetic differentiation indicates if the taxon is significantly differentiated according to AMOVA analyses. +/-means that the taxon is/ is not differentiated. When the taxon is not differentiated from other ones, the numbers group together undifferentiated taxa. Reciprocal monophyly indicates if the taxon forms a monophyletic group strongly supported and distinct from other taxa. +/-means that the taxon is/is not a monophyletic group. CLGS indicates if the taxon has/has not specific composition of cephalic labial gland secretions (CLGS) including in main compounds. +/-means that the taxon has/ has not a specific CLGS composition. When the taxon shares CLGS composition with other ones, the numbers group together taxa that share similar CLGS. ? means that the CLGS composition is unknown.

Taxa	**Genetic criteria**				**Reproductive trait criterion**	**Taxonomic conclusion**	
	Genetic differentiation		**Reciprocal monophyly**		**CLGS**	**Integration by congruence (Lecocq et al. 2015c)**	**Integration by cumulation**
	COI	PEPCK	COI	PEPCK			
caucasicus	+	+	+	- [1]	+	*B. caucasicus*	*B. caucasicus*
bisiculus	+	+	+	- [1]	+	*B. lapidarius bisiculus*	*B. bisiculus*
decipiens	+	+	- [1]	- [1]	- [2]	*B. lapidarius decipiens*	*B. lapidarius decipiens*
atlanticus	+	+	- [1]	- [1]	?	*B. lapidarius atlanticus*	*B. lapidarius atlanticus*
lapidarius	+	- [3]	- [1]	- [1]	- [2]	*B. lapidarius lapidarius*	*B. lapidarius lapidarius*

Figure

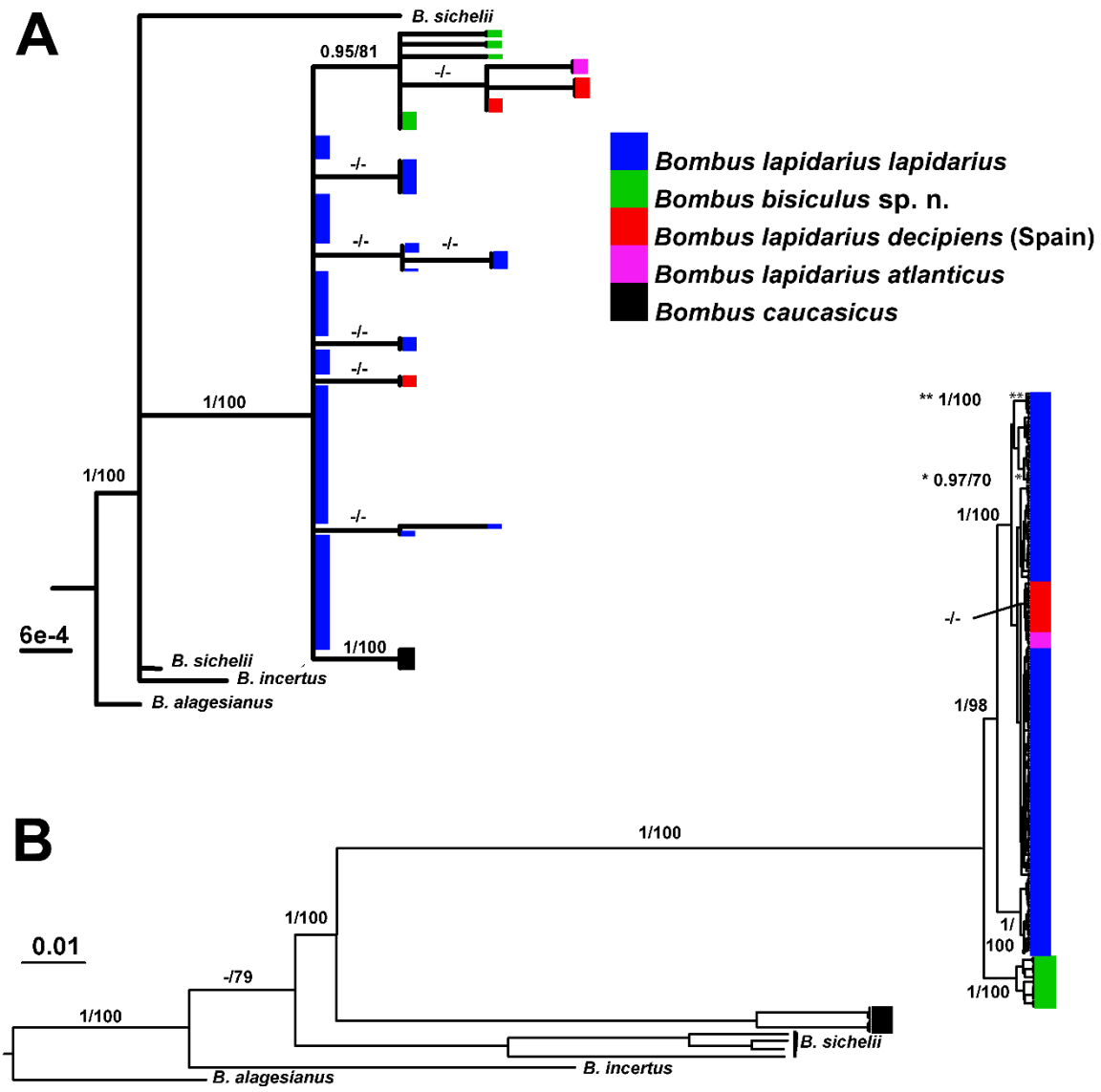


Figure 1. Majority rule (50%) consensus tree of *Bombus lapidarius, B. caucasicus* and *B. bisiculus* sp. n. based on Bayesian analyses based on: A. PEPCK data matrix; B. COI data matrix. Values above branches are Bayesian posterior probabilities / maximum likelihood bootstrap values. Only posterior probabilities >0.95 and maximum likelihood bootstrap values > 70% are showed.

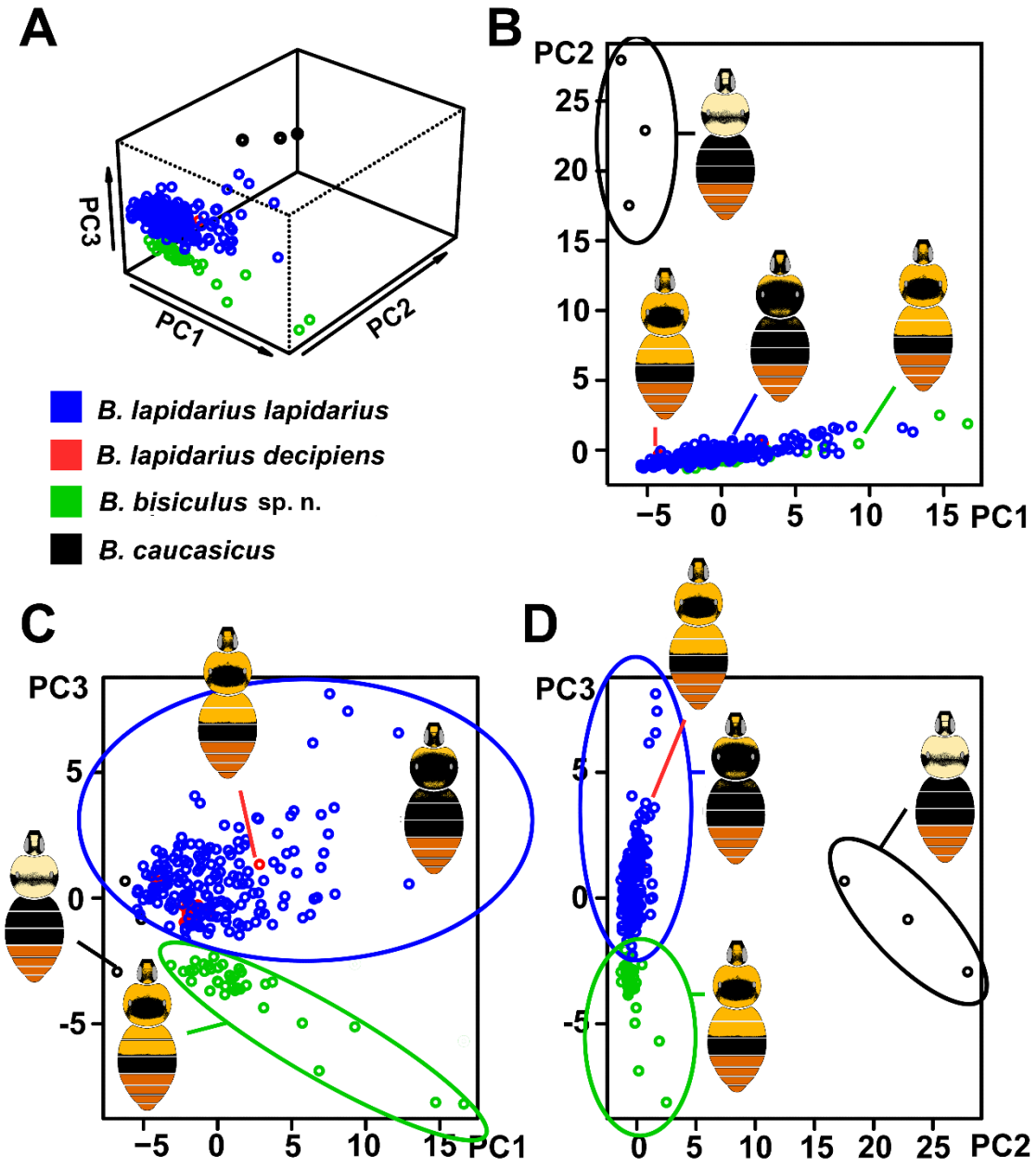


Figure 2. Principle components analyses based on cephalic labial gland secretions of *Bombus lapidarius, B. caucasicus* and *B. bisiculus* sp. n. PC1, PC2 and PC3 are first, second, and third axes of the analyses.

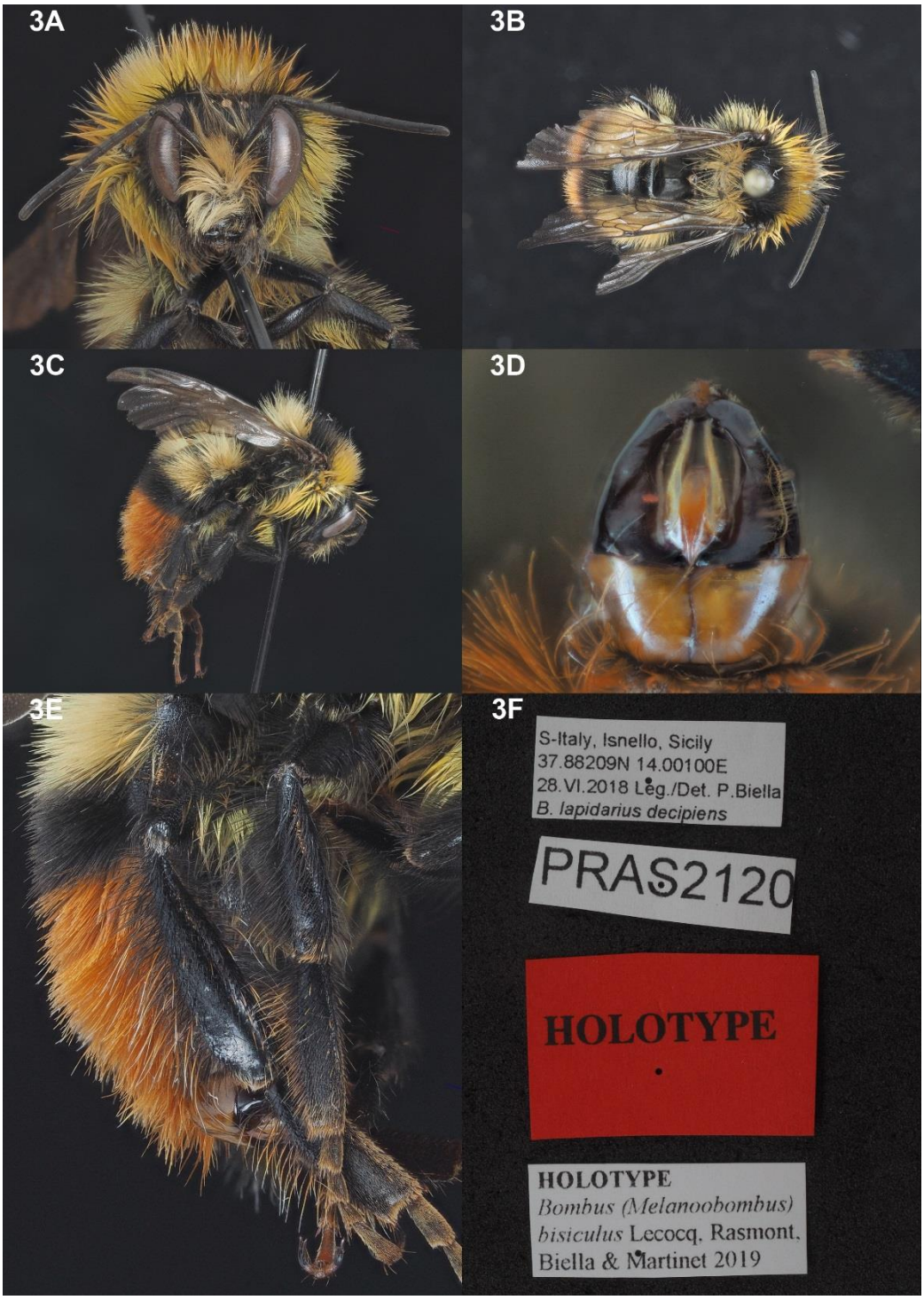


Figure 3. Photographs of *Bombus bisiculus* sp. n. (male, holotype, PRAS2120). A: face; B: dorsal view; C: profile; D: genitalia, E: zoom on median and posterior legs, F: labels associated to the holotype (Photographs by P. Rasmont.).

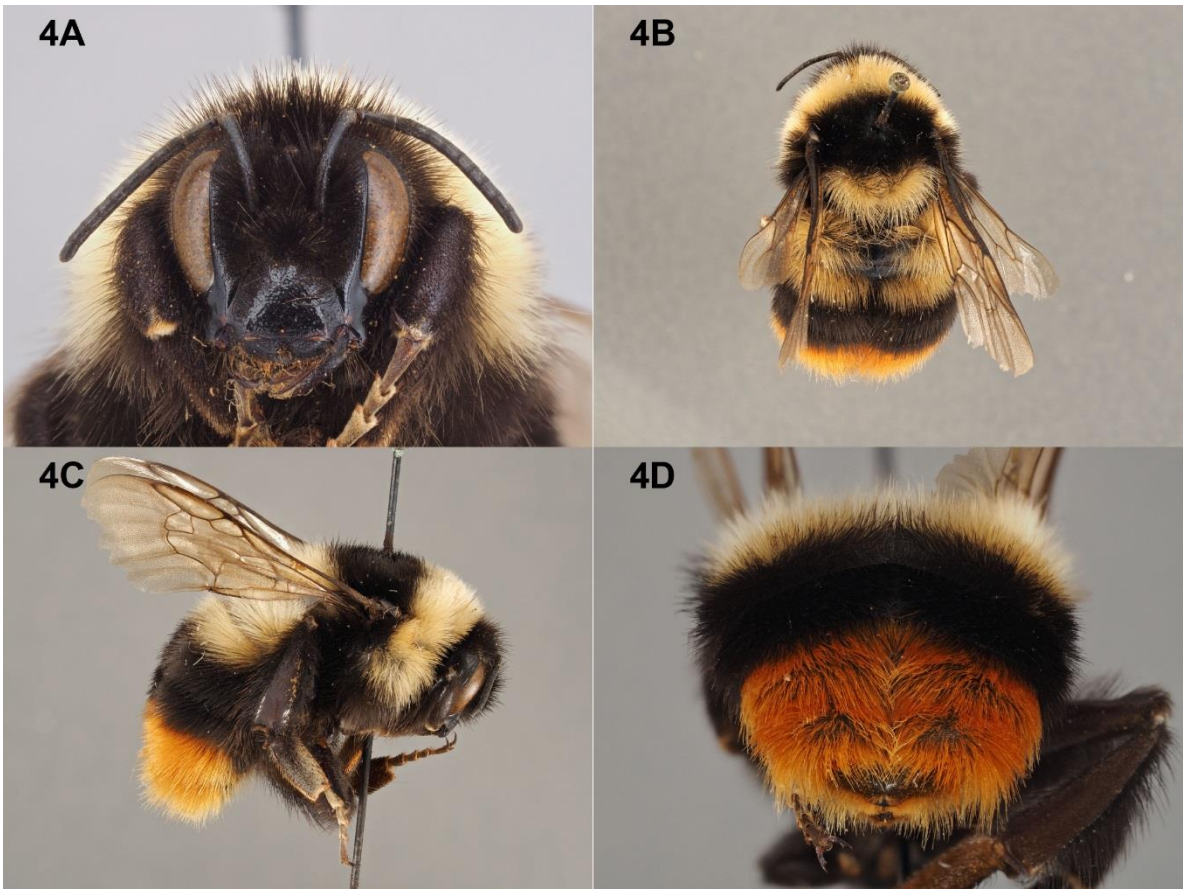


Figure 4. Photographs of *Bombus bisiculus* sp. n. (female, paratype 1). A: face; B: dorsal view; C: profile; D: abdomen (Photographs by P. Rasmont.).

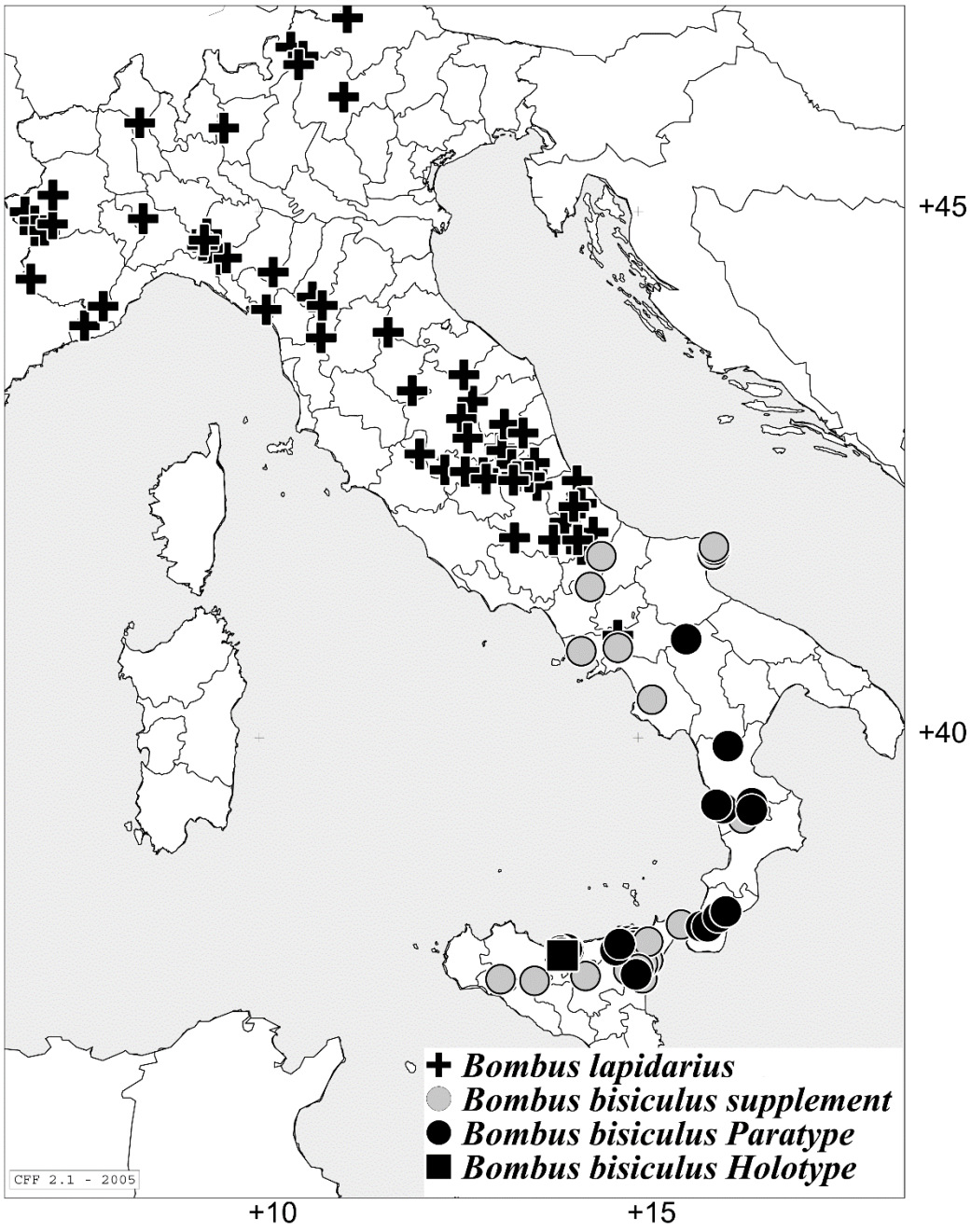


Figure 5. Distribution map of *Bombus bisiculus* sp. n. and *B. lapidarius lapidarius* in Italy. The map display locations of holotype, paratypes, and supplementary materials used for the description of *Bombus bisiculus* sp. n. The occurrence data of *B. lapidarius lapidarius* are extracted from the database *Base de données fauniques Gembloux-Mons* (BDFGM; Rasmont et al., 2015).

Supporting information

File S1. Species descriptions and diagnose of *Bombus bisiculus* sp. n.