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Molecular Identification of Western-Palaearctic Leaf Beetles (Coleoptera, Chrysomelidae)

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ABSTRACT

Due to the difficulties associated with morphological identification of insects, it became necessary to resort to other identification tools, such as DNA barcoding, where the mitochondrial cytochrome *c* oxidase subunit I (COI) molecular marker is commonly used. The effectiveness of DNA-based identification of species relies on the availability of sequences in public databases for comparison. Nevertheless, there is still a large number of non-sequenced species in these databases, preventing a molecular identification. In this study, we generate COI barcode sequences, with a total of 658 bp, for the six studied Chrysomelidae species. Phylogenetic and sequence divergence analyses were also performed, which allowed the discrimination of all species under study, supporting once again the suitability of this genetic marker. The obtained sequences were added to BOLD and GenBank databases, contributing to the increase of records in online databases and making the identification of some Chrysomelidae species easier.

Key words: Coleoptera, Chrysomelidae, DNA barcoding, Cytochrome *c* oxidase I, databases.

INTRODUCTION

Chrysomelidae (leaf beetles) is one of the richest families of Coleoptera, including over 40 000 described species, divided into 19 subfamilies and over 2 500 genera (Futuyma, 2004). These insects are commonly round and highly convex, almost exclusively phytophagous and have been associated with host plants in a large number of Angiosperm families (Flinte *et al*., 2011; Petitpierre, 2011).

The importance of Chrysomelidae in pure and applied entomology has stimulated a considerable interest in taxonomic and systematic studies (Hsiao, 1994). An accurate species-level identification is essential to research in several fields, namely ecology, evolutionary biology, biodiversity and conservation biology (Monaghan *et al*., 2005). Insect identification is commonly based on morphological characters. This methodology often requires entomological experts with specialized taxonomic knowledge, insect

collections and appropriate identification keys, which are often incomplete or only effective for a particular life stage or gender (Valentini *et al*., 2008; Raupach *et al*,. 2010). The effectiveness of morphological keys may also be affected by geographic variations or by the loss of some morphological characters, such as colour patterns, as a result of preservation processes (Wells and Stevens, 2008; Buhay, 2009).

In order to overcome these difficulties, Hebert *et al*. (2003) proposed a molecular identification system, known as DNA barcoding, which provides a fast and more accurate identification by using short standardized gene regions, known as DNA barcodes, of the target specimen (Hebert and Gregory, 2005; Hajibabaei *et al*., 2007). The DNA barcode itself consists of a 658 bp region of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) (Nelson *et al*., 2008). The effectiveness of DNA-based identification relies on the availability of sequences in public databases for comparison (Levkanicova and Bocak, 2009). However, there is still a large number of species that are not included in these databases, most likely due to the existence of such diverse groups, which may constrain the representation of an acceptable proportion of the total world biodiversity (Frézal and Leblois, 2008; Virgilio *et al*., 2010). In addition, owing to difficulties in morphological identification, many of the reference sequences are only identified to family or genus level, preventing the identification to species level.

Several studies concerning the identification of Coleoptera species through DNA barcoding have emerged in the last years. Nevertheless, these studies are mainly focused on North and Central Europe, and lack some information about the western part of Europe (Kubisz *et al*., 2012; Hendrich *et al*., 2015; Mazur *et al*., 2014; Pentinsaari *et al*., 2014).

Following our laboratory experience with the molecular marker COI in other insects (Ferreira *et al*., 2011; Oliveira *et al*., 2011; Rolo *et al*., 2013; Farinha *et al*., 2014), in this study, we generated new COI barcode data for six Chrysomelidae species collected in Aveiro, Portugal, contributing to the increase of records in online databases. Intra- and interspecific divergence were determined, as well as the phylogenetic relationship among the species.

MATERIAL AND METHODS

Sampling

All specimens were collected in Aveiro, Portugal, using pitfall traps and were then stored in 96% ethanol. Morphological identification was performed by one of the authors (IFS), an experienced coleopterologist, resorting to genitalia analysis whenever possible. Nomenclature follows Kippenberg (2010) and Döberl (2010). Once identified, each specimen was separated individually, labelled, stored in absolute ethanol and maintained at 4ºC. A total of six species belonging to Chrysomelidae family were collected and followed for subsequent analyses: *Chrysolina bankii* (Fabricius, 1775), *Chrysolina peregrina* (Herrich-Schäffer, 1839), *Chrysolina diluta* (Germar, 1824), *Chrysolina haemoptera* (Linnaeus, 1758), *Apteropeda ovulum* (Illiger, 1807) and *Psylliodes cucullata* (Illiger, 1807) (Table 1).

Table 1. Species of leaf beetles (Chrysomelidae) and outgroup species (*C. maxillosus*, Staphylinidae) analysed in this study, their chorology and database accession number. Chorological categories adapted from Vigna-Taglianti *et al*. (1999).

Genus	Species	Chorology	No. of specimens	Sample ID	BIN	GenBank accession numbers
Chrysolina	*Chrysolina bankii* (Fabricius, 1775)	W-Mediterranean	3	*C.bankii*_1	BOLD:ABA4528	KJ840835
				*C.bankii*_2	BOLD:ABA4479	KJ840836
				*C.bankii*_3	BOLD:ABA4479	KJ840834
	Chrysolina peregrina (Herrich-Schäffer, 1839)	W-Mediterranean	2	*C.peregrina*_1	BOLD:ABA8916	KJ840842
				*C.peregrina*_2	BOLD:ABA8916	KJ840841
	Chrysolina diluta (Germar, 1824)	Gallo-Iberian	3	*C.diluta*_1	BOLD:ABV0244	KJ840839
				*C.diluta*_2	BOLD:ABV0244	KJ840838
				*C.diluta*_3	BOLD:ABV0244	KJ840837
	Chrysolina haemoptera (Linnaeus, 1758)	European	1	*C.haemoptera*	BOLD:ABV0229	KJ840840
Apteropeda	*Apteropeda ovulum* (Illiger, 1807)	W-Mediterranean	3	*A.ovulum*_1	BOLD:ACM3858	KJ840833
				*A.ovulum*_2	BOLD:ACM3858	KJ840832
				*A.ovulum*_3	BOLD:ACM3858	KJ840831
Psylliodes	*Psylliodes cucullata* (Illiger, 1807)	Asiatic-European and Nearctic	2	*P.cucullata*_1	BOLD:ACM3254	KJ840845
				*P.cucullata*_2	BOLD:ACM3254	KJ840844
Creophilus	*Creophilus maxillosus* (Linnaeus, *1758)*	Semi-cosmopolitan	1	*Cr.maxillosus*	BOLD:ACD0973	KJ840843

DNA Extraction, Amplification and Sequencing

Genomic DNA was extracted from 2-3 legs of each specimen, using DNeasy® Blood and Tissue (Qiagen) extraction kit, following the manufacturer's protocol, with an overnight incubation step. COI amplification was performed using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') in a total reaction volume of 25 µl, containing 1x Colorless GoTaq® Flexi Reaction Buffer (Promega), 100 µM of dNTPs (Fermentas), 2 mM $MgCl_2$, 0.2 µM of each primer, 0.16 µg/µl of BSA, 0.02 U GoTaq® Flexi DNA Polymerase (Promega) and 3 µl of DNA extract. COI amplification consisted in an initial denaturation step at 94ºC for 1 minute, followed by 5 cycles of 94ºC for 30 seconds, 45ºC for 1 minute, 72ºC for 1 minute, then 35 cycles of 94ºC for 1 minute, 50ºC for 1 minute and 30 seconds, 72ºC for 1 minute and a final extension step of 72ºC for 5 minutes. PCR bands were detected by 1% agarose gel electrophoresis, stained with RedSafe (iNtRON Biotechnology, Korea) and visualized under ultraviolet light. PCR products were purified using the commercial SureClean kit (Bioline, UK) according to manufacturer's instructions. Sequencing reactions were performed in both directions, using the amplification primers, by a specialized company (Macrogen Inc., The Netherlands).

Data Analysis

Sequences were analysed and edited in Sequencer® v4.0.5 (Gene Codes Corporation, USA) and BioEdit Sequence Alignment Editor v7.0.5.3 (Hall, 1999).

The obtained sequences were compared with reference sequences deposited in the online databases GenBank (produced and maintained by the National Center

for Biotechnology Information - NCBI) and BOLD (Barcode of Life Data Systems), in order to corroborate the morphological identification, whenever possible.

Sequence matrices were constructed in Bioedit, aligned with ClustalX v2.0.12 (Thompson *et al*., 1997) and were then converted into a .NEXUS format using the software Concatenator v1.1.0 (Pina-Martins and Paulo, 2008) in order to allow subsequent analyses.

With the purpose of studying intra- and interspecific divergence, Kimura's 2-parameter genetic distances were determined in PAUP* v4.0d99 (Swofford, 2002). Phylogenetic analyses were performed in RaxML v7.2.8 (Stamatakis, 2006), using a Maximum Likelihood (ML) method and on MrBayes v3.2.1 (Ronquist *et al*., 2012) for a Bayesian Inference (BI) analysis. For the ML analysis, 1 000 replicates were performed. In BI analysis, 1 500 000 generations were sampled, every 1 000 generations.

Another specimen collected in the present study, the rove beetle *Creophilus maxillosus* (Linnaeus, 1758) (Coleoptera, Staphylinidae), was used as outgroup to root the trees.

RESULTS

Molecular Identification

A total of 658 bp for COI gene were obtained for the 15 studied specimens. The alignment was straightforward and no insertions or deletions were found. Well defined peaks in chromatograms and the absence of premature stop codons indicated that amplification of pseudogenes did not occur.

The obtained sequences were compared with reference sequences of known species contained in the online databases GenBank (www.ncbi.nlm.nih.gov) and BOLD (www.boldsystems.org). These databases allow species identification when our obtained sequence matches the available reference sequence with an identity value higher than 97%, given that intraspecific genetic distance should not exceed 3%, according to the standard criteria (Hebert *et al*., 2003). Our sequences matched, with high identity values to other reference sequences identified only at order, family or genus level. This did not allow us to corroborate the previous identification, based on morphological criteria, emphasizing the enormous need to increase the records in the mentioned databases, particularly those identified at the species level.

Intraspecific and Interspecific Variation

In order to study intra- and interspecific divergence, a distance matrix was calculated in PAUP*, using Kimura 2-parameter (K2P).

Percentages of nucleotide divergence values and standard deviation for K2P are presented in Table 2. In one case, only one specimen of *Chrysolina haemoptera* was present in the matrix, preventing the determination of intraspecific variation.

Concerning the intraspecific divergence values, these were all below the 3% threshold, confirming species association according to the standard criteria. Intraspecific distances ranged from no intraspecific distance in *Apteropeda ovulum* to a maximum of 1.977% (±0.989) in *Chrysolina bankii*.

Analysing the results present in Table 2, it is easily perceivable that interspecific genetic distances have smaller values when the comparisons include two species belonging to the same genus, as expected. Interspecific variation ranged from a minimum of 10.993% (±0.240) to a maximum of 25.179% (±0.115). The minimum value was found between congeneric species *Chrysolina diluta* and *C. haemoptera*. *Chrysolina bankii* and *Apteropeda ovulum,* the species with the highest interspecific divergence value, belong to different genera, thus, they are expected to be genetically more distant.

According to Frézal and Leblois (2008), interspecific variation values should be ten times higher than the mean value of intraspecific variation. Although the values of interspecific variation were higher than ten times the mean value of intraspecific variation, we observed that those values were lower among the species belonging to the same genus.

Table 2. Summary of intra- and interspecific genetic distances (Kimura's 2-parameter) obtained for COI data of six species. Standard deviation values are shown in brackets. C= Chrysolina, A= Apteropeda, P= Psylliodes.

	C. bankii	*C. diluta*	*C. peregrina*	*C. haemoptera*	*A. ovulum*	*P. cucullata*
C. bankii	**1.977** (±0.989)	**17.161** (±0.182)	**17.455** (±0.099)	**17.367** (±0.140)	**25.179** (±0.115)	**23.168** (±0.105)
C. diluta		**0.923** (±0.462)	**16.666** (±0.096)	**10.993** (±0.240)	**22.224** (±0.002)	**19.754** (±0.084)
C. peregrina			**0.152** (±0.000)	**14.904** (±0.096)	**23.028** (±0.000)	**22.327** (±0.137)
C. haemoptera				- -	**21.161** (±0.000)	**19.151** (±0.201)
A. ovulum					**0.000** (±0.000)	**20.749** (±0.047)
P. cucullata						**0.458** (±0.000)

Phylogenetic Analysis

Phylogenetic analyses were performed in order to distinguish monophyletic groups and consequently allow species delimitation. Sometimes, an oversimplified or inappropriate method may fail to distinguish monophyletic groups, compromising all the analysis. Therefore, it is advisable to make a comparison of tree-building methods in order to achieve better results (Nelson *et al*., 2007). Thus, two methodologies were chosen for phylogenetic reconstruction: Maximum Likelihood (ML) and Bayesian Inference (BI).

Fig. 1 shows the phylogram obtained by ML for COI gene, which presents a similar topology to BI method but with different support values in some nodes.

Here, it is possible to observe that despite the small number of samples, we were able to separate the three genera, being that Psylliodes and Apteropeda are phylogenetically closer to each other than to Chrysolina. In addition, specimens from the same species cluster together, as expected, with high support values. Although there is only one specimen of Chrysolina haemoptera, it is possible to observe that it clusters more closely with the other species belonging to the same genus.

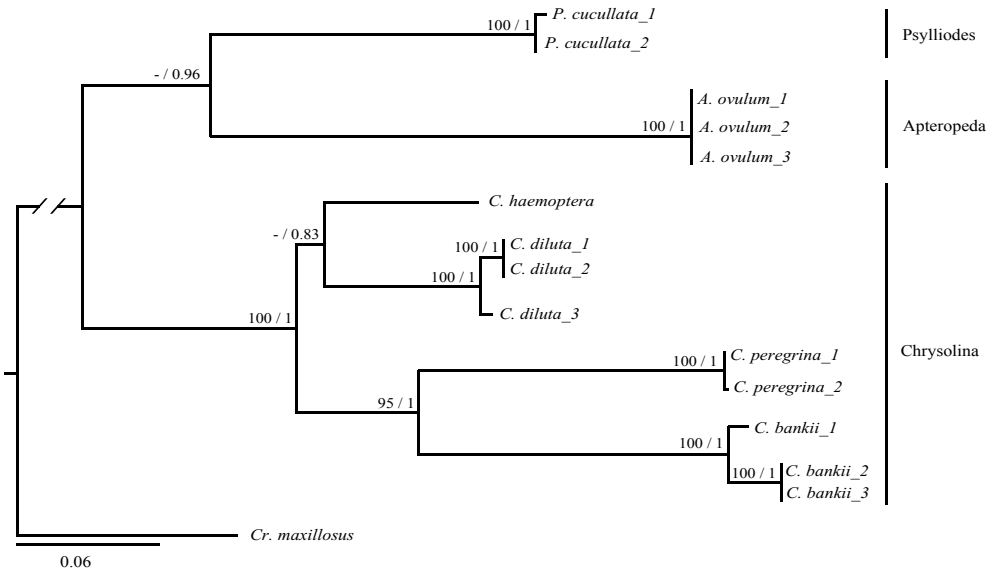

Fig. 1. Maximum likelihood phylogram (1,000 bootstrap replicates) based on 15 sequences of cytochrome c oxidase I (COI) gene from 6 species and 1 outgroup (*Creophilus maxillosus*). Numbers above branches represent Maximum Likelihood and Bayesian Inference support for nodes (bootstrap and posterior probability values, respectively). C = Chrysolina, P = Psylliodes, A = Apteropeda and Cr = Creophilus.

DISCUSSION AND CONCLUSIONS

Over the past years, molecular identification has become an important tool to identify Coleoptera species. Successful amplification of COI confirms that it is a robust molecular marker to be used in the identification of Chrysomelidae species. However, the effectiveness of this technique depends on the availability of sequences in online databases. Due to a poor knowledge of species catalogue in some regions, there is a lack of reference sequences and, as a result, many specimens are identified only to genus, family or even order level. This constrain was noticed in this work, since we were not able to corroborate the morphological identification at species level for none of our specimens.

The existence of a threshold value to discriminate species is one criterion used in the DNA barcoding approaches (Hebert *et al*., 2003). In this study, these thresholds were achieved, given that intraspecific values were all below the 3% threshold and that high interspecific divergence values allow the differentiation between all of the presented species, with values above the 3% threshold. Nevertheless, it is important to remember that it is necessary to use threshold values carefully, especially in our case, since the number of specimens is limited, as well as the geographic area.

Despite the fact that the aim of the DNA Barcode Project is not to resolve phylogenetic relationships, phylogenetic analysis should be done in order to distinguish monophyletic groups and consequently permit species delineation (Nelson *et al*.,

2007). Here, we performed a phylogenetic analysis employing the Maximum Likelihood (ML) and the Bayesian Inference (BI) methods; the obtained trees show similar topologies with different support values. Even with a small number of species, it was possible to cluster together the specimens from the same species, with high support values, allowing species discrimination.

The obtained sequences were added to BOLD and GenBank databases (Table 1). As previously referred, the number of barcoded species is still rather small and the proportion of unassigned sequences and unidentified species in these online databases is quite considerable, limiting specimen identification through this approach. Nevertheless, although this work presents a restricted number of specimens and species, it represents an important contribution to increase the knowledge on Chrysomelidae species, making it easier to identify and corroborate the morphological identification of these species.

Moreover, all Chrysolina species collected in this study belong to different subgenera: *Chrysolina (Sulcicollis) peregrina, Chrysolina (Chrysolina) bankii, Chrysolina (Palaeosticta) diluta, and Chrysolina (Colaphodes) haemoptera*. Since the sub-generic division of Chrysolina is quite controversial, the obtained COI barcode data can provide interesting information.

Further studies are still required in order to increase the knowledge on the richness of Coleoptera as well as to test new molecular markers, as there are still many unstudied coleopteran species. Additionally, those experiments should be settled in different regions to evaluate geographical and climatic differences. This is already being performed and the results are currently under analysis.

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REFERENCES

Buhay, J. E., 2009, “COI-like” sequences are becoming problematic in molecular systematic and DNA barcoding studies. *Journal of Crustacean Biology*, 29(1): 96-110.

Döberl, M., 2010, *Chrysomelidae Alticinae. In*: Löbl, I., Smetana, A. (Eds.). Catalogue of Palaearctic Coleoptera. Apollo Books, 491-563.

Farinha, A., Dourado, C. G., Centeio, N., Oliveira, A. R., Dias, D., Rebelo, M. T., 2014, Small bait traps as accurate predictors of dipteran early colonizers in forensic studies. *Journal of Insect Science,* 14(77): 1-16.

Ferreira, S., Oliveira, A. R., Farinha, A., Rebelo, M. T., Dias, D., 2011, Forensic Entomology: Nuclear and Mitochondrial Markers for Diptera and Coleoptera identification. *Forensic Science International: Genetics Supplement Series*, 3: 174-175.

Flinte, V., de Freitas, S., de Macedo, M. V., Monteiro, R. F., 2011, Altitudinal and temporal distribution of *Plagiometriona* Spaeth, 1899 (Coleoptera, Chrysomelidae, Cassidinae) in a tropical forest in southeast Brazil. *ZooKeys*, 31(157): 15-31.

Frézal, L., Leblois, R., 2008, Four years of DNA barcoding: Current advances and prospects. *Infection, Genetics and Evolution*, 8: 727-736.

Futuyma, D. J., 2004, *Preface. In:* Jolivet, P., Santiago-Blay, J. A., Schmitt, M. (Eds.). New Developments in the Biology of Chrysomelidae. SPB Academic Publishing, The Hague, 19-20.

Hajibabaei, M., Singer, G. A. C., Clare, E. L., Hebert, P. D. N., 2007, Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. *BioMed Central Biology*, 5*:* 24-30.

Hall, T. A., 1999, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41: 95-98.

Hebert, P. D. N., Cywinska, A., Ball, S. L., deWaard, J. R., 2003, Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B*, 270: 313-321.

Hebert, P. D. N., Gregory, T. R., 2005, The Promise of DNA Barcoding for Taxonomy. *Systematic Biology*, 54(5): 852-859.

Hendrich, L., Morinière, J., Haszprunar, G., Hebert, P. D., Hausmann, A., Köhler, F., Balke, M., 2015, A comprehensive DNA barcode database for Central European beetles with a focus on Germany: adding more than 3500 identified species to BOLD. *Molecular Ecology Resources*, 15(4): 795-818.

Hsiao, T., 1994, *Molecular Techniques for Studying Systematics and Phylogeny of Chrysomelidae. In:* Jolivet, P. H., Cox, M. L., Petitpierre, E. (Eds.). Novel Aspects of the Biology of Chrysomelidae. Springer Netherlands, 237-248.

Kippenberg, H., 2010, *Chrysomelidae, Chrysomelinae. In:* Löbl, I., Smetana, A. (Eds.). Catalogue of Palaearctic Coleoptera. Apollo Books, 390-443.

Kubisz, D., Kajtoch, L., Mazur, M. A., Rizun, V., 2012, Molecular barcoding for central-eastern European Crioceris leaf-beetles (Coleoptera: Chrysomelidae). *Central European Journal of Biology*, 7(1): 69-76.

Levkanicova, Z., Bocak, L., 2009, Identification of net-winged beetle larvae (Coleoptera: Lycidae) using three mtDNA fragments: a comparison of their utility. *Systematic Entomology*, 34: 210-221.

Mazur, M. A., Kubisz, D., Kajtoch, L., 2014, Restricted geographic distribution and low genetic distinctiveness of steppic *Crioceris quinquepunctata* (Coleoptera: Chrysomelidae) populations in Central East Europe. *Entomologica Fennica*, 25: 102-110.

Monaghan, M. T., Balke, M., Gregory, T. R., Vogler, A. P., 2005, DNA-based species delineation in tropical beetles using mitochondrial and nuclear markers. *Philosophical transactions of the Royal Society of London B*, 360(1462): 1925-33.

Nelson, L. A., Wallman, J. F., Dowton, M., 2007, Using COI barcodes to identify forensically and medically important blowflies. *Medical and Veterinary Entomology*, 21: 44-52.

Nelson, L. A., Wallman, J. F., Dowton, M., 2008, Identification of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2). *Forensic Science International*, 177: 238-247.

Oliveira, R., Farinha, A., Rebelo, M. T., Dias, D., 2011, Forensic entomology: Molecular identification of blowfly species (Diptera: Calliphoridae) in Portugal. *Forensic Science International: Genetics Supplement Series,* 3: 439-440.

Pentinsaari, M., Hebert, P. D. N., Mutanen, M., 2014, Barcoding beetles: a regional survey of 1872 species reveals high identification success and unusually deep interspecific divergences. . PLoS ONE, 9(9): e108651.

Petitpierre, E., 2011, Cytogenetics, cytotaxonomy and chromosomal evolution of Chrysomelinae revisited (Coleoptera, Chrysomelidae). *ZooKeys*, 79(157): 67-79.

Pina-Martins, F., Paulo, O. S., 2008, Concatenator: sequence data matrices handling made easy. *Molecular Ecology Resources*, 8: 1254-1255.

Raupach, M. J., Astrin, J. J., Hannig, K., Peters, M. K., Stoeckle, M. Y., Wägele, J. W., 2010, Molecular

species identification of Central European ground beetles (Coleoptera: Carabidae) using nuclear rDNA expansion segments and DNA barcodes. *Frontiers in Zoology*, 7:26.

Rolo, E., Oliveira, A. R., Dourado, C. G., Farinha, A. C., Rebelo, M.T., Dias, D., 2013, Identification of sarcosaprophagous Diptera species through DNA barcoding in wildlife forensics. *Forensic Science International,* 228: 160-164.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., Huelsenbeck, J. P., 2012, MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Systematic Biology*, 61(3): 539-542.

Stamatakis, A., 2006, RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22: 2688-2690.

Swofford, D. L., 2002, *PAUP*: Phylogenetic analysis using parsimony (*and other methods).* Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G., 1997, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25(24): 4876-4882.

Valentini, A., Pompanon, F., Taberlet, P., 2008, DNA barcoding for ecologists. *Trends in Ecology & Evolution*, 24(2): 110-117.

Vigna-Taglianti, A., Audisio, P. A., Biondi, M., Bologna, M. A., Carpaneto, G. M., De Biase, A., Fattorini, S., Piattella, E., Sindaco, R., Venchi, A., Zapparoli, M., 1999, A proposal for a chorotype classification of the Near East fauna, in the framework of the Western Palearctic region. Biogeographia, 20, 31-59

Virgilio, M., Backeljau, T., Nevado, B., Meyer, M. D., 2010, Comparative performances of DNA barcoding across insect orders. *BioMed Central Bioinformatics*, 11: 206-215.

Wells, J. D., Stevens, J. R., 2008, Application of DNA-Based Methods in Forensic Entomology. *Annual Review of Entomology*, 53: 103-120.

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