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Research

Morphology, Systematics, Evolution

Molecular Characterization of Mosquito Diversity in the Balearic Islands

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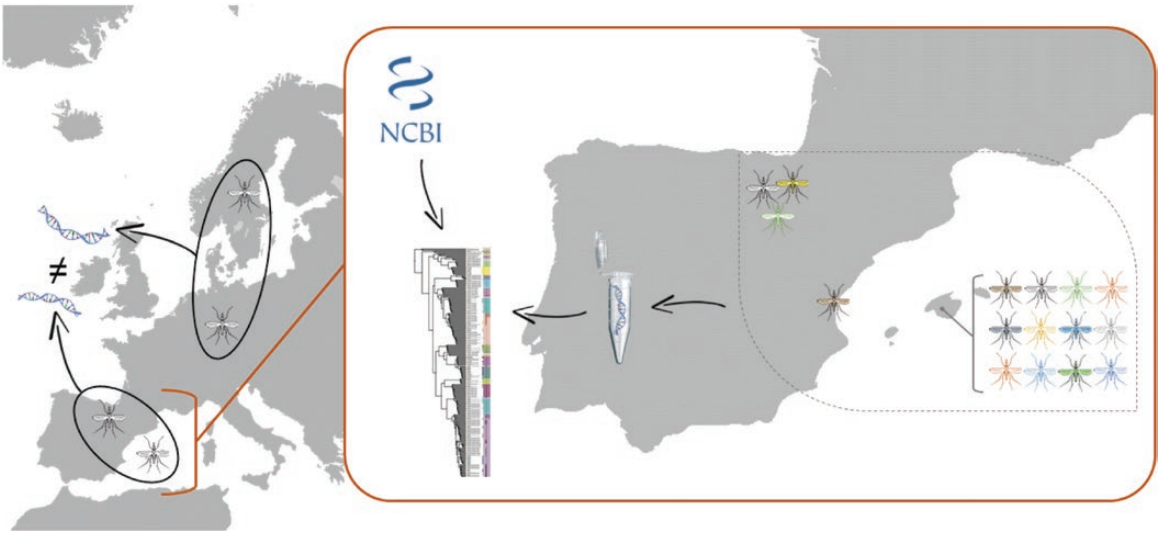
Abstract

Several outbreaks of mosquito-borne diseases have taken place in Europe in recent years. In Spain, both active and passive surveillance have demonstrated that dengue and West Nile viruses are currently circulating, and seven autochthonous dengue cases have been reported in the last 2 yr. The effectiveness of vector control programs largely depends on the accuracy of the taxonomic identification of the species. However, in Spain, identification almost completely relies on the use of morphological keys to characterize the mosquito fauna. This study investigates the congruence between molecular and morphological species boundaries in 13 Spanish mosquito taxa. The Cytochrome *c* oxidase subunit I (*COI*) gene region was sequenced from 60 adult specimens collected in Mallorca, plus several representatives from other Spanish regions for comparative purposes. Phylogenetic relationships were established using Bayesian and maximum-likelihood approaches. Using three species delimitation algorithms (ABGD, mPTP, and GMYC), we found strong evidence for cryptic speciation within *Anopheles algeriensis* Theobald, a widespread mosquito in the Mediterranean basin. We also delimited the Mallorcan rock pool mosquito *Aedes mariae* (Sergent & Sergent), from mainland European populations. Finally, we found difficulties in the use of wing characters in species keys to distinguish *Culiseta annulata* (Schrk) from *Culiseta subochrea* (Edwards). Given that these species are vectors of pathogens of medical relevance and have veterinary importance, their accurate taxonomic identification is essential in European vector surveillance programs.

Graphical Abstract



Mosquitoes are responsible for the deaths of millions of people each year due to their role as vectors of pathogens which cause diseases such as malaria, dengue, and yellow fever. Although the majority of the cases are reported from tropical countries, several outbreaks have also taken place in the Northern hemisphere demonstrating that mosquito-borne diseases are not geographically restricted to tropical regions. This is partly related to the rapid expansion of vectors of human diseases. In 2007, the rapidly spreading invasive species *Aedes albopictus* (Skuse) was implicated in the Chikungunya fever outbreak that affected 205 people in Italy (Rezza et al. 2007) and was also identified as the vector of two dengue fever cases in France (Gould et al. 2010). It is also the most likely vector of the seven native dengue cases reported in Spain in the last 2 yr (ECDC 2019). Although there is little doubt that invasive species represent a serious Public Health threat, the role of native mosquitoes should not be underestimated. Recent outbreaks of West Nile virus (WNV) in Romania (Campbell et al. 2001) and in the United States (Nash et al. 2001) were attributed to native species of Culex mosquitoes (Diptera:Culicidae). In the south of Spain, WNV has been found circulating in humans and horses in recent years and it may be expanding northwards (Busquets et al. 2019), although vector species are currently unknown.

Correct identification of vector species is a critical step in the design of effective control strategies. Mosquito identification has been traditionally carried out using dichotomous keys that describe the morphological characters of a particular life stage. Although this technique has proved invaluable and is still widely used to distinguish many mosquito species, it has limitations; the technique requires taxonomic experts to perform accurate identifications and it can be prone to misidentifications due to the loss of key morphological characters during collection and preservation of specimens. Furthermore, morphology-based taxonomy cannot differentiate between members of species complexes, which frequently differ in their ecology, host preferences, and behavioral patterns and as a result in their role as disease vectors which has implications for the design of effective control strategies (Paredes-Esquivel et al. 2009)

The field of mosquito taxonomy has been renewed with the development of new tools for accurate identification (Cywinska et al. 2006). Molecular-based techniques are now widely used not only to identify sibling species but also to solve phylogenetic relationships (Bourke et al. 2013). The Cytochrome *c* oxidase subunit I (*COI*) gene region of the mitochondrial genome is the gold standard for barcode identification of species (Hebert et al. 2003) and has proved invaluable for distinguishing between mosquito species (Cywinska et al. 2006, Wang et al. 2012, Ashfaq et al. 2014, Bourke et al. 2018, Hernandez-Triana et al. 2019). This approach is particularly important to establish species delimitations in unexplored groups (Puillandre et al. 2012). Although *COI* barcoding has been reported to fail to differentiate some species of mosquitoes of the Anopheles and Culex genera (Cywinska et al. 2006, Wang et al. 2012, Bourke et al. 2013, Laurito et al. 2013), it is clear that it can be an important first step for mosquito species discovery (Bourke et al. 2018). In Spain, the diversity and distribution of mosquito species rely almost completely on morphological identifications. However, there are almost no studies that test the validity of this approach. Here, we aim to explore mosquito diversity in the Balearic Islands, using molecular procedures. We have conducted phylogenetic analyses of *COI* gene sequences coupled with three species delimitation approaches to solve the mosquito species boundaries. This study demonstrates that there is need for better approaches to characterize the mosquito diversity in Spain, since its understanding is essential in vector surveillance and control programs.

Material and Methods

Mosquito Collection and Morphological Identification

Sixty mosquito specimens belonging to 13 species were collected between 2010 and 2018 from 12 coastal sites in the Island of Mallorca, the largest of the Balearic archipelago, located in the Western region of the Mediterranean Basin. For comparison purposes, we also included representative mosquitoes from mainland Spain: five specimens of *Aedes mariae* from the Mediterranean coastal region of Peñiscola, and 17 specimens of *Culiseta annulata* (7), *Culiseta subochrea* (4), and *Anopheles algeriensis* (6) from La Rioja and Navarra, in the Northern central area of the Iberian Peninsula (Fig. 1).

Adult mosquitoes were collected using BG sentinel and MiniCDC light traps containing CO_2 as the bait. Sampling was carried out weekly and each trap was set at sunset. On the following morning, specimens were transported in vivo to the laboratory and then frozen at –20°C for molecular analysis. Larvae were also collected from

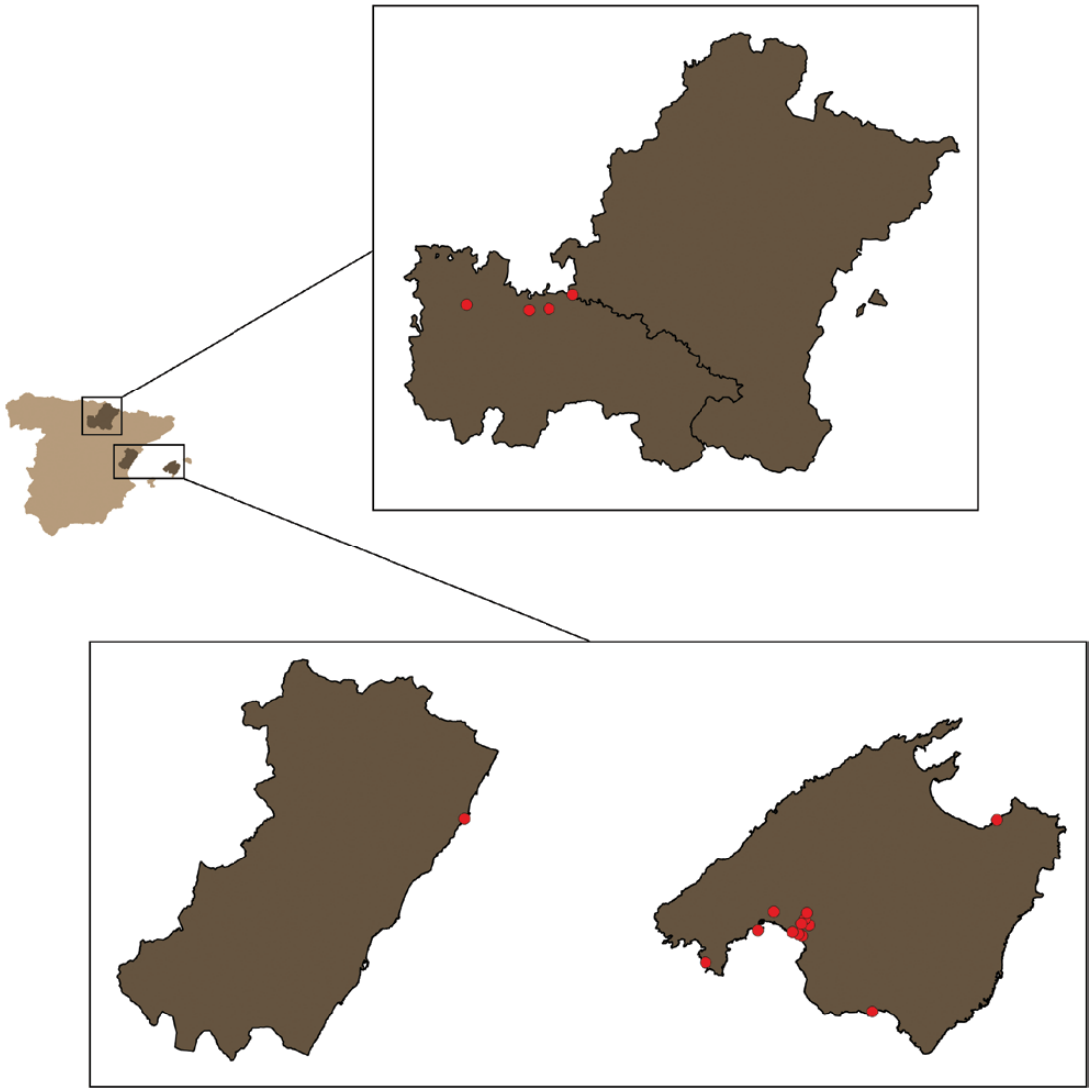


Fig. 1. Location map and mosquito collection sites in Spain: La Rioja-Navarra (above) and Castellón-Mallorca (below).

breeding sites, transported to the laboratory and kept alive at 25°C until adults emerged. Female mosquitoes were identified to species level using the keys of Becker et al. (2010).

DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from individual mosquitoes, using DNeasy Blood & Tissue Kit (QIAGEN, GmbH, Hilden, Germany). The manufacturers specifications were followed except for the final elution step, where a 100-µl elution buffer was used instead of 200 µl. DNA was quantified with a NanoDrop 1000 spectrophotometer Thermo Scientific (Saveen Werner ApS, Denmark) and stored at –20°C. Polymerase chain reaction (PCR) was performed using the thermal cycler GeneAmp PCR System 2400 (Perkin Elmer) using primers (LCO1490 5′-GGTCAACAAATCA TAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGA CCAAAAAATCA-3′; Folmer et al. 1994). If amplifications failed, the primers C1-J-1718 forward 5′-GGAGGATTTGGAAATTGAT TAGTGCC-3′ and C1-N-2191 reverse 5′-CCCGGTAAAATTAA AATATAAACTTC-3′ (Simon et al. 1994) were used to repeat the PCR. The 25 µl PCR reaction consisted of 1 µl of DNA template, Bioline 10× PCR reaction buffer, 3.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 1 unit of Bioline Taq DNA polymerase, and 0.2 µM of each primer. The PCR conditions consisted of an initial denaturation step at 94°C for 4 min; followed by 35 cycles at 94°C 30 s, 50°C 30 s, and 72°C 45 s, and a final extension step of 72°C for 10 min. PCR products were visualized on 1.5% agarose gel electrophoresis stained with ethidium bromide. Samples were purified using the MSB Spin PCRapace (Invitek, Berlin, Germany) according to the manufacturer's specifications. PCR products were sequenced using the same primers as above and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

DNA-Based Taxonomic Identifications

The taxonomic identification of the sequences was carried out under a phylogenetic framework as described in Jurado-Rivera et al. (2009). The newly generated sequences were edited using CodonCode Aligner (CodonCode Corporation, Dedham, MA) and used individually to perform BLAST searches in the NCBI database retrieving the 300 best hits. The DNA sequences from each search were dereplicated and filtered by length (minimum length = 400 bp) using USEARCH 6.1.544 (Edgar 2010). The top 15 sequences of each filtered BLAST output were merged into a combined dataset, and

duplicated sequences were removed with USEARCH 6.1.544. The resulting DNA dataset represented an accurate compilation of the most closely related sequences to our mosquito *COI* reads available in GenBank. Both datasets (GenBank selection plus our mosquito sequences) were combined with three Sphaeroceridae sequences that were used as outgroups (KM633419, KM633426, and KM648404). Sequences were aligned using MAFFT 7 (Katoh and Standley 2013), and the best partitioning strategy and evolutionary models were selected under the Bayesian Information Criterion in IQTREE 1.6.12. The phylogenetic analyses were performed using both maximum likelihood with IQTREE (1,000 ultrafast bootstrap replicates) and Bayesian inference in MrBayes 3.2 (Ronquist et al. 2012) specifying two independent runs of four MCMC chains each, assessing MCMC convergence in Tracer v1.7.1 by ensuring effective sample size values higher than 200 (Rambaut et al. 2018) and implementing a 10% burnin fraction.

Species Boundaries

We used the results obtained from the phylogenetic inferences to perform molecular species delimitation analyses using the Multirate Poisson Tree Processes method (mPTP) (Kapli et al. 2017), the Generalized Mixed Yule Coalescent model (GMYC) (Pons et al. 2006, Fujisawa and Barraclough 2013), and the Automatic Barcode Gap Discoverer (ABGD) (Puillandre et al. 2012). The mPTP method fits the branching events of each delimited species to a distinct exponential distribution and does not require any similarity threshold as input (Kapli et al. 2017). We constructed an IQ-TREE ML phylogenetic tree, using two independent MCMC chains with 100 million generations each sampling every 1,000. On the other hand, GMYC performs a likelihood ratio test to compare a single coalescent branching rate across a clock-like tree as a null hypothesis with a model including both coalescent and Yule branching tree-bifurcating patterns. We used the R library SPLITS v1.0–19 (Ezard et al. 2009) under a single threshold on the ultrametric tree estimated with BEAST 1.10.4 (Suchard et al. 2018) implementing two independent runs of 10 million generations each and a 10% burnin fraction. Finally, ABGD is independent of tree typology and split the dataset into candidate species, based on the existence of barcode gap (interspecific divergence higher than intraspecific ones) and a prior intraspecific divergence (*p*) (Puillandre et al. 2012). ABGD was carried out applying the K2P model, using default parameters, with the exception of the relative gap width, which was defined as 1.4. Results of a recursive and initial partition analysis were compared.

Finally, Kimura two-parameter (K2P) genetic distances (minimum interspecific and maximum intraspecific distances) were calculated in Mega6 (Tamura et al. 2013). Molecular operational taxonomic units (MOTUs) were defined as the consensus of the results obtained from the different species delimitation approaches.

Results

Sequence Analysis

In total, 82 *COI* mosquito consensus sequences were generated in this study and deposited in GenBank under accession codes (MH348258-MH348270, MH370023-MH370049, and MT192942-MT192983). The BLAST search generated an additional dataset consisting of 205 unique closely related GenBank sequences (excluding duplicated) greater than 400 bp in length (see accession numbers in Supp Table S1 [online only]). The combined DNA dataset of newly generated, GenBank and outgroup sequences had an aligned length of 658 bp, including 245 parsimony-informative, 14 singleton and 399 constant sites. Neither insertion nor deletion events were observed in the dataset, and no stop codons were detected after translation.

Phylogenetic Analysis and the Species Delimitation Approach

The best partitioning scheme and evolutionary models selected under the Bayesian Information criterion in IQTREE consisted into partitioning the dataset by codon position with a TN+F+I+G4 model for the first, a HKY+F+I for the second, and a HKY+F+I model for the third coding positions of the alignment, respectively. Both the ML and Bayesian trees showed clearly defined and highly supported clusters in all MOTUs identified (Supp Figs. S1 and S2 [online only]). The GMYC method reported the higher number of putative species (39), while mPTP and ABGD showed similar results (29 and 28, respectively).

The phylogenetic analyses showed that the *Ae. mariae* complex is paraphyletic, with species *Ae. mariae*, and *Aedes phoeniciae* Coluzzi & Sabatini intermingled with *Aedes caspius* (Pallas), which also appears paraphyletic. The ABGD approach clustered the morphospecies *Ae. caspius* and members of the *Ae. mariae* complex (*Ae. mariae, Aedes zammitii* (Theobald), and *Ae. phoeniciae*) into a single MOTU. Similar results were obtained with the mPTP approach that considered *Ae. zammitii* as a different species. On the other hand, the GMYC algorithm allowed for the separation of all members of the *Ae. mariae* complex but also included the Majorcan *Ae. mariae* as a different species (Fig. 2) from the continental Europe ones (including specimens from Peñiscola). The minimum interspecific distances obtained when comparing *Ae. mariae* mosquitoes from Mallorca with those from Peníscola and Italy were 1.62% and 1.35%, respectively. In contrast, this same parameter was 0% when comparing *Ae. mariae* sequences from Peníscola and those from Italy. In Mallorca, the minimum interspecific distance between *Ae. mariae* and *Ae. capius* was 1.35%, and the maximum intraspecific distance was 0.27% for *Ae. mariae* and 2.99% for *Ae. caspius*. The monophyly of these clades was strongly supported by both posterior probability and bootstrap values (1 BPP; bootstrap 100 and 1 BPP; bootstrap 99, respectively). On the other hand, the morphospecies *Ae. caspius* was split into four different molecular species in the GYMC delimitation analysis.

Two strongly supported monophyletic clades (1 BPP) were also found within the morphospecies *An. algeriensis* (Fig. 2), with a clade formed by specimens from Spain (1 BPP; bootstrap 100) and another one formed by specimens of *An. algeriensis* from Germany and Sweden. These two clades showed a minimum interspecific distance of 4.99%. All delimitation methods agreed on the subdivision of the morphospecies *An. algeriensis* into these two separate molecular species.

The molecular analyses showed the placement of seven specimens from Mallorca morphologically identified as *Cs. annulata* within the *Cs. subochrea* clade. The morphological examination of the specimens reported that the Balearic specimens showed wing characters compatible with *Cs. annulata* ('cross veins r-m and m-cu aligned'), but abdominal scales like *Cs. subochrea* 'terga with indistinct pale basal bands formed by yellowish scales (not white), pale scales are also present among the dark scales in the apical half of the terga'. Some specimens from La Rioja and Navarra were also included (Cs.a_21_LR, Cs.a_18_LR, Cs.a_19_LR, Cs.a_20_LR, Cs.a_52_LR, Cs.s_37_LR, Cs.s_36_LR, and Cs.s_43_N, Cs.s42_N) and the opposite situation was observed in two specimens (Cs.s/a_93_LR and Cs.s/a_093_LR) showing wings-like *Cs. subochrea* but terga-like *Cs. annulata*. When characters located in the wings were

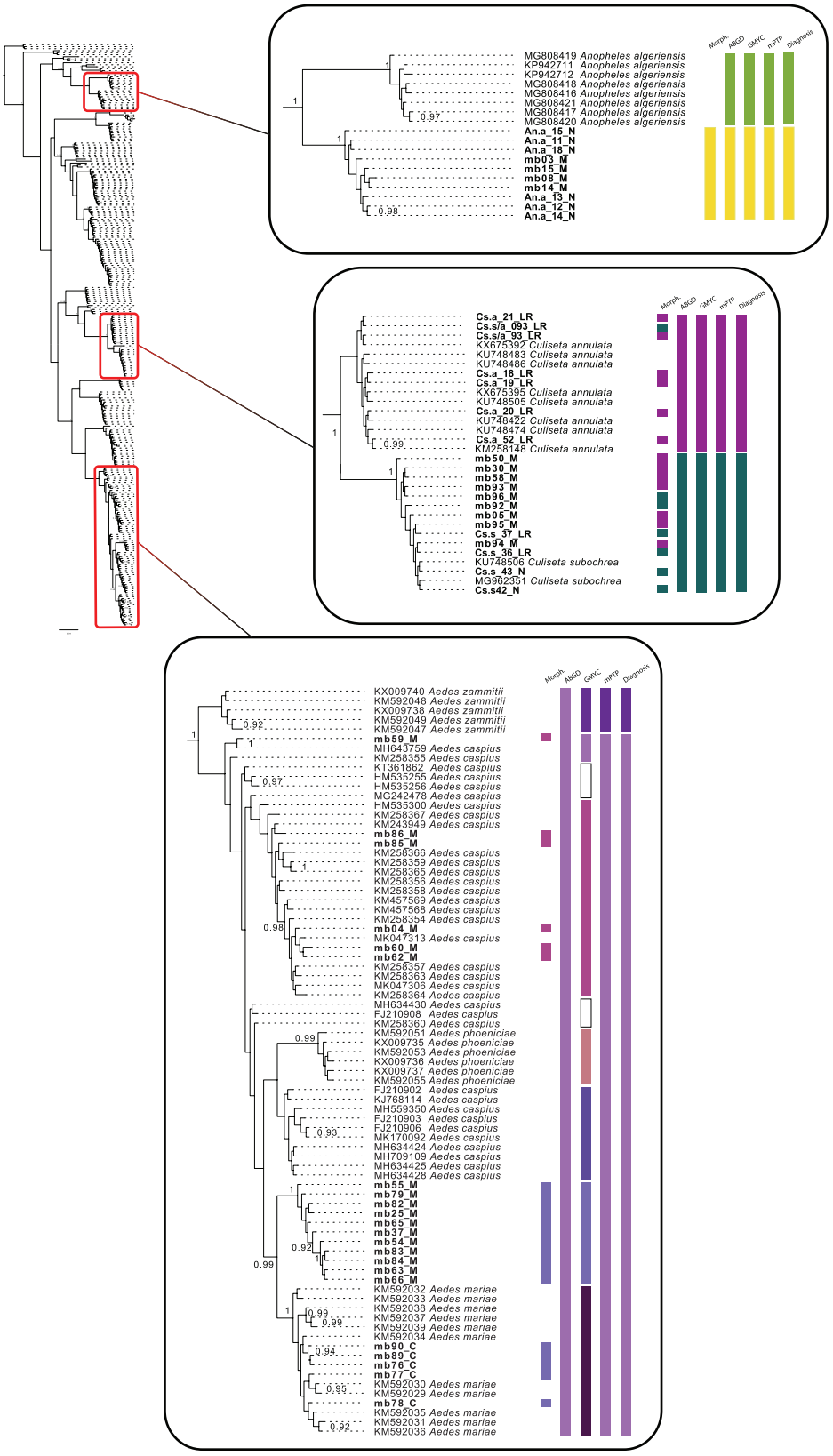


Fig. 2. Bayesian phylogenetic tree based on *COI* DNA sequences and species delimitation results. Main figure panels are zoom-ins of the original three (in Annex Fig. 2) to depict the groups: *Anopheles algeriensis*, *Culiseta subochrea* and *Culiseta annulata*, and the *Aedes mariae* complex.

excluded, the morphological identification was in agreement with the molecular one.

The Balearic morphospecies *Aedes vexans* (Meigen), *Ae. albopictus*, *Culiseta longiareolata* (Macquart), *Culex theileri* Theobald, *Coquilletidia richiardi* (Ficalbi), *Aedes detritus* (Halliday), and *Uranotaenia unguiculata* Edwards each formed highly supported monophyletic clades with GenBank sequences from the same species (Supp Fig. S2 [online only]) and their identity was also confirmed by all species delimitation methods included in this study. On the other hand, delimitation analyses combined species *Culex pipiens s.l.* (L.) and *Culex quinquefasciatus* Say.

Discussion

This study demonstrates that molecular taxonomy is an effective strategy for diversity assessment and mosquito identification in Spain. These results are consistent with reports based on DNA barcoding in other regions of the world, where congruence with the morphological identifications has ranged from 82 to 98% (Wang et al. 2012, Ashfaq et al. 2014, Chan et al. 2014, Rozo-Lopez and Mengual 2015, Versteirt et al. 2015). Incongruence between morphology and molecular data may be related to errors in the initial morphological identification, leading to incorrectly named sequences in the public databases used for sequence comparisons (Krzywinski and Besansky 2003).

We found disagreement among all species delimitation approaches used in this study. Methods such as ABGD allow the user to set the initial parameters; however, results may be considered subjective. According to Puillandre et al. (2012), a $P = 0.01$ is the closest to the species defined by authors when testing ABGD in four datasets. However, this value did not allow us to separate members of the *Ae. mariae* complex and *Ae. caspius* that appeared together in the same MOTU. On the other hand, GYMC was able to separate members of the *Ae. mariae* complex, while also splitting *Ae. caspius*. We found disagreement between the DNA-based identification and morphological identification based on the use of keys to separate *Cs. annulata* from *Cs. subochrea*. Furthermore, the sequence analysis also showed preliminary evidence of cryptic speciation in *An. algeriensis* and in *Ae. mariae s.s.* It is important to emphasize that the results obtained in this study are not definitive, as they only include one molecular marker. To confirm these findings, it would also be necessary to increase sample size, as the current sample size is small. A combination of several markers would also be required to arrive at any firm conclusion on the status of these potentially cryptic species, in order to better represent the genome and to determine the occurrence of incomplete lineage sorting, evolutionary history and introgression. Nevertheless, the use of mitochondrial markers has served as a first step in identification of cryptic species within Culicidae (Ruiz-Lopez et al. 2012), and we hope that this will open a new avenue of research in this field in Spain, where identifications to date rely almost entirely on morphology.

Preliminary Evidence for Cryptic Speciation in *An. algeriensis*

All species delimitation approaches supported in the existence two species within *An. algeriensis*. This is formed by two highly supported monophyletic clades separated by a high genetic divergence (minimum interspecific distance = 4.99%). The first *An. algeriensis* group is formed only by the Spanish specimens from Navarra and Mallorca, while the second is formed by specimens from Germany and Sweden. *Anopheles algeriensis* is widely distributed in the Palearctic region, with Yemen as the easternmost limit (Al-Eryani et al. 2016). This species is scarce in most areas where it has been reported (Krüger and Tannich 2013), including the Balearic Islands and this led to the conclusion that *An. algeriensis* is a malaria vector of secondary importance (Becker et al. 2010). However, this is an abundant species (23.3% of all collected mosquitoes) in the wetland of La Grajera in la Rioja (Ruiz-Arrondo et al. 2017). Although *Anopheles algeriensis* has been found naturally infected with *Plasmodium vivax* in Yemen (Al-Eryani et al. 2016), its capacity as an effective vector is largely unproven. The findings of our study will prompt questions on its role in malaria transmission within endemic areas. In Spain, this species displays a high degree of variation in epidemiologically important life history traits, such as host feeding and resting preference (Bueno Marí et al. 2011). Previous studies have shown such variation in behavior may be an indication of cryptic species diversity (Paredes-Esquivel et al. 2009). A correct characterization of the vector species of this complex is of public health importance in Europe as this information is essential to design effective vector control programs.

Incipient Speciation in the Balearic Rock Pool Mosquitoes?

Few mosquito species can survive in the highly saline conditions of coastal rock pools. The *Ae. mariae* complex, which includes three sibling species *Ae. mariae s.s.*, *Ae. phoeniciae*, and *Ae. zammitii* (Coluzzi and Sabatini 1968) can be found in such habitats in the Mediterranean region. The *Aedes* genus has undergone various taxonomic revisions in recent years (Reinert et al. 2009), but here, we have included these species within the genus *Aedes* as in Soghigian et al. (2017). Interestingly, members of the *Ae. mariae* complex could only be separated as different species by the GMYC approach, while ABGD, mPTP failed to do so. The inference of the latter methods is not consistent with the current knowledge of these species. The *Ae. mariae* complex has been subjected to broad analysis, which included morphological, genetic, and reproduction studies of the group (Coluzzi and Sabatini 1968, Mastrantonio et al. 2016). However, it is known that they have a parapatric distribution with incomplete reproductive isolation (Mastrantonio et al. 2016). Interestingly, the GYMC approach also distinguished the Mallorcan clade as a different species. It has been hypothesized that the *Ae. mariae* complex split during the Pleistocene and that further fragmentation events resulted from the retreat and advance of the coastal line (Mastrantonio et al. 2015). Whether or not this was the reason behind the separation of the Mallorcan clade remains unclear. Further analysis and experimental crosses in the laboratory would be necessary to establish the species status of the Mallorcan *Ae. mariae*, and its potential role as vector of pathogens should be determined through vector incrimination studies.

The consensus criteria included *Ae. caspius* and the *Ae. mariae* complex members in the same MOTU. This does not seem reasonable if we consider that both taxa have a very distinctive ecology. While the former is a salt-marsh mosquito, the latter are rock pool mosquitoes with the ability to tolerate high salinity values (up to 87.5 pp). In contrast, the GYMC approach gave more consistent results with the knowledge of this species than other delimitation methods. The minimum interspecific distance between *Ae. mariae s.s.* and *Ae. caspius* was 1.35%. Interestingly, this value is lower than the typical species-level difference (Hebert et al. 2003). We found a higher value (1.62%) when comparing *Ae. mariae s.s.* sequences from Mallorca with the same species from continental Spain, supporting our hypothesis of the speciation of the Mallorcan *Ae. mariae*. The limitation

of *COI* to resolve well-supported mosquito species (Bourke et al. 2018) has to be taken into consideration, while introgressive hybridization between *Ae. mariae* and *Ae. capius* cannot be ruled out and would require further studies. Information obtained from other loci may provide better support for the delimitation of these species. *Aedes caspius* has been considered a complex of species (Becker et al. 2010), and the high intraspecific variation (maximum distance = 2.99%) found in Mallorcan populations of this species is perhaps suggestive of more than one species present in the island.

Current Morphological Keys Cannot Always Distinguish *Cs. annulata* from *Cs. subochrea* in Spain

The species *Cs. annulata* and *Cs. subochrea* are morphologically similar but can be differentiated by the presence of white (*Cs. annulata*) or yellow (*Cs. subochrea*) basal bands and the absence or presence of pale scales in the apical half of terga, respectively. In addition, the alignment of veins r-m and m-c is supposed to be effective to distinguish both species, being totally aligned in *Cs. annulata* and slightly separated in the case of *Cs. subochrea* (Becker et al. 2010). The phylogenetic analysis and the species delimitation methods agree in the clustering of two distinctive groups. As these groups contained mixed membership (Fig. 2), we rechecked the morphology and found that the character in the wings could not always separate both species, while the 'the absence or presence of pale scales in the apical half of terga' was in clear agreement with molecular identification. Conspicuous characters are frequently used by field workers as a quick method of species differentiation as in-depth examination of specimens can be difficult and time consuming. This may lead to misidentification of these vectors. It is crucial to include other molecular markers to confirm these findings and redesign the morphological keys for these species in Spain. Although *Cs. subochrea* is not known to be a vector of human pathogens, *Cs. annulata* is a competent vector of the Tahyna virus vector (Bárdos et al. 1975). Therefore, correct identification among these species is crucial in case of outbreaks in the country. This is the first record of *Cs. subochrea* in the Balearic Islands.

Implications for Disease Transmission

Nine out of 11 morphospecies collected from Mallorca have been recorded as vectors of human diseases. The remaining two are *Culiseta longiareolata* and *Uranotaenia unguiculata*, which rarely feed on humans but are considered vectors of veterinary importance (Becker et al. 2010, Camp et al. 2018). In recent years, there has been an increase in awareness of mosquitoes and the diseases they transmit in Europe. Changes in climatic conditions together with globalization have already resulted in active transmission of pathogens in new areas. The relative importance of potential vectors depends on their feeding behavior, longevity, and other life history traits that vary within species complexes (Gendernalik et al. 2017). It is therefore crucial to know which vector species are currently circulating in the continent. Cryptic speciation in mosquitoes is still an underexplored topic across Europe, and the findings of the current study demonstrate that species diversity continues to be underestimated.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Table S1. List of all specimens included in this study. Sequences in bold correspond to those sequenced in this study; the rest are those retrieved from GenBank for comparison purposes.

Fig. S1. Maximum-likelihood tree based on the analysis of the *COI* gene region. Node numbers represent bootstrap support values. Only support values > 99 are shown.

Fig. S2. Bayesian phylogenetic tree based on *COI* DNA sequences and species delimitation results. Node numbers represent Bayesian posterior probability values. Only support values > 0.9 are shown.

Sequences retrieved from GenBank are represented by the accession numbers. Sampling sites are indicated as follow: M (Mallorca), C (Castellón), LR (La Rioja) and N (Navarra). Numbers above nodes are posterior probabilities. Columns represent results from different species delimitation approaches: Morphology (Morph); Automatic Barcode Gap Discovery (ABGD); Generalized Mixed Yule Coalescent (GMYC); Multi-rate Poisson Tree Processes method (mPTP) and the consensus diagnosis based on all species delimitation approaches (Diagnosis).

Acknowledgments

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