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A DNA barcode reference library of Portuguese mosquitoes

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Abstract

Mosquitoes are important biological vectors of pathogens and species identification in all life stages is the first step for effective monitoring and control of mosquito-borne diseases. Molecular methods for species identification have been developed over the last years to overcome the limitations of the taxonomic identification based on morphology. DNA barcoding, using a fragment of the mitochondrial cytochrome oxidase I (COI) gene, can be used for species identification but a reliable and comprehensive reference database of verified sequences is required. In this study, we aimed to generate a DNA barcode reference library for the identification of mosquito species from Portuguese mosquito fauna, including most relevant vector species. Mosquitoes captured under the National Vector Surveillance Program (REVIVE) were processed for DNA extraction, COI gene fragment amplification and sequencing. Nighty-eight barcode sequences were obtained, representing 26 species and 6 genera. Sequences were submitted to GenBank and BOLD and were used for validation of phenetic classification. Barcode Index Number (BIN) assignment and Automatic Barcode Gap Discovery (ABGD) were used and clustered COI sequences into twenty-five molecular operational taxonomic units (MOTUs). This is the first comprehensive study that combines morphological and molecular identification of most mosquito species present in Portugal aiming to offer a reliable framework for mosquito species identification.

KEYWORDS
culicidae, cytochrome oxidase I (COI), DNA barcoding, mosquito surveillance, Portugal, species identification

1 | INTRODUCTION

Mosquito-borne diseases are amongst the most deadly and important emerging and re-emerging diseases worldwide (Dahmana & Mediannikov, 2020). These diseases affect primarily tropical and sub-tropical regions, but in recent years, several arboviruses have been circulating and invading Europe, such as West-Nile (in Greece, Germany, Netherlands and Spain) (Bakonyi & Haussig, 2020), dengue (European Centre for Disease Prevention and Control, 2021; Lazzarini et al., 2020), chikungunya (Calba et al., 2017; Lindh et al., 2019) and Zika (Martinet et al., 2019) viruses, being responsible for autochthonous human outbreaks. The role of mosquitoes as vectors of pathogens that drive human and animal disease underscores the importance of mosquito surveillance programmes. The National Vector Surveillance Network—REVIVE (REde de VIgilância de VEctores), in place since 2008, has already identified 28 of the 40 described species for Portugal (Centro de Estudos de Vetores e Doenças Infeciosas Doutor Francisco Cambournac Departamento de Doenças Infeciosas, 2019; Ribeiro et al., 1988), including the invasive species *Aedes aegypti* and *Ae. albopictus*. Several of these

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species are potential vectors of pathogens affecting human and animal health.

Mosquito diversity is high, with a total of 3,574 valid species, in 113 genera divided in two subfamilies *Anophelinae* and *Culicinae* (Harbach, 2018; Strickman et al., 2020). Rapid and accurate identification of all life-stage mosquito specimens is essential for mosquito vectors and mosquito-borne disease surveillance. Mosquito identification has historically relied on morphology, using dichotomous keys. This method is time-consuming, depends on expert knowledge, and may be problematic in specimens that were damaged during collection and storage (Beebe, 2018). Life-stages of a certain number of species can be extremely challenging to identify. In some cases, morphology cannot also distinguish between identical members of species complexes, sibling species or biotypes/ecoforms of some species that can have different roles as vectors of disease (Beebe, 2018). In these cases, several markers have been used to achieve a species identification such as acetylcholinesterase (Ace2) or internal transcribed spacer 2 (ITS2) (Smith & Fonseca, 2004; Vinogradova & Shaikevich, 2007).

Alternative methods based on molecular tools have been also used in the last years to overcome morphology-based identification limitations. Since 2006, DNA barcoding has been used to identify mosquito species. This technique consists of the amplification and sequencing of a fragment of the mitochondrial gene Cytochrome C Oxidase subunit I (COI) (658 base pairs), which is known as the universal barcoding region (Hebert et al., 2003). After amplification and sequencing of the DNA fragment, nucleotide sequences need to be compared against a reference database of previously identified mosquitoes to achieve species identification. Available databases, such as GenBank or Barcode of Life Database (BOLD), are being greatly expanded, but in order to have reliable information, useful for surveillance purposes but also for diversity investigation, it is important to have a local reference library of sequences of local mosquito populations. Libraries where each species is represented by multiple, geographically distinct populations that capture maximal intraspecific variation and that help define the boundaries between species are of great utility and have strong reliability (deWaard et al., 2019).

The current work aimed to develop a DNA reference barcode library for the Portuguese mosquito fauna. The number of barcode sequences for the Portuguese mosquito fauna in BOLD is limited, with only 80 sequences belonging to eight species available. In this way, these results contribute to a reference mosquito DNA barcode library publicly available. For the validation and assessment of the taxonomic reliability of the resulting library, two types of species delimitation algorithms were used, to test whether specimens assigned to a morphospecies can be found within the same cluster of molecularly similar sequences (MOTU). The development and implementation of a validated in-house barcode library of the mosquitoes that are commonly found in Portugal will allow DNA barcoding to become a useful tool for the surveillance of mosquitoes and mosquito-borne diseases. Furthermore, mosquito COI sequence diversity is assessed, allowing a greater insight into the composition of genera, and mosquito diversity that would be unrecognizable when using only morphological methods, disclosing the presence of cryptic species complexes.

Impacts

- A reference DNA barcode library for 26 mosquito species reported for Portugal was generated.
- The results contribute to the international mosquito barcode initiative, with less common species, that will help on the surveillance and control of mosquitoes and mosquito-borne diseases.
- DNA barcoding can be used for specimen identification and species discrimination of Portuguese mosquitos.

2 | MATERIALS AND METHODS

2.1 | Mosquito collection and a priori identification

Specimens used for DNA barcoding were collected under the Portuguese National Vector Surveillance Program (REVIVE—REde de VIgilância de VEtores). Adult mosquitoes were collected using BG-sentinel (Biogents AG, Regensburg, Germany) and Centers for Disease Control and Prevention (CDC) traps (John W. Hock Company, Florida, USA) baited with CO_2, and immature stages collections were performed by using a dipper for larvae and ovitraps for eggs (Osório et al., 2014). Collections were performed throughout Portugal in the period 2008–2019. Information regarding date and local of capture can be found in Table S1.

Mosquitoes were morphologically identified using the identification keys of Ribeiro and Ramos (Ribeiro & Ramos, 1999) and Schaffner et al. (Schaffner et al., 2001). After morphological identification, mosquitoes were stored at −20°C. Specimens belonging to species complexes (that include species that are not morphologically distinguishable) such as *Anopheles maculipennis* complex (Proft et al., 1999), *An. claviger* complex (Kampen et al., 2003) or *Aedes detritus* complex (Brengues et al., 2014) were molecularly identified by the amplification of the internal transcribed spacer 2 (ITS2) region from the nuclear ribosomal DNA (results not presented). The species *Culex pipiens* and *Cx. torrentium* were also distinguished by the use of acetylcholinesterase gene (Ace2 assay) and biotypes of the *Culex pipiens* species were identified using a multiplex PCR to detect a polymorphism in the flanking region of the CQ11 microsatellite (Osório et al., 2013).

Taxonomic classification and species nomenclature are according to Harbach (Harbach, 2018) (http://mosquito-taxonomic-inventory.info/aedini-classification). The tribe *Aedini*, that contains the genus Aedes has gone through several taxonomic revisions in the last years; however, they are not commonly applied (Versteirt

et al., 2015). In this study, we use the species names provided in Table 1, where we have also included the names according to the recent revisions (Reinert et al., 2009).

2.2 | DNA extraction and amplification

Mosquitoes previously identified belonging to the REVIVE collection were selected for molecular identification. Three representatives of each of the species and species complexes reported for Portugal, were selected for COI marker amplification. *Culex theileri* was an exception as only one male was available.

DNA was extracted from the whole mosquito, except for male specimens, where genitalia was dissected for further analysis. DNA extraction was performed using a commercial kit (Nzytech) or automated extraction (NucliSENS easyMag—bioMérieux) following manufacturer's protocol.

Amplification of the 658 bp COI gene fragment was done using the primers LCO1490 and HCO2198 designed by Folmer et al., 1994 and using an adapted PCR protocol from the same authors. Amplification was done using the following cycling conditions: a first step of 95°C for 1 min followed by 39 cycles of 95°C for 30 s; 40°C for 1 min and 72°C for 1 min. A final extension step of 72°C for 5 min was then performed. PCR products were visualized in a 1.5% agarose gel. Samples with the expected band size were then purified using JETquick PCR Product Purification Spin kit (GENOMED GmbH, Löhne, Germany) and sequenced in both directions using the amplification primers and ABI Prism 3,130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or by a commercial company (STAB VIDA, Lda, Caparica, Portugal).

TABLE 1 Species names of the tribe *Aedini* used in this study and the names according to most recent revisions

Species names used in this study	Name according to Reinert 2009
Aedes (Ochlerotatus) berlandi (Seguy, 1921)	*Ochlerotatus berlandi* (subgenus uncertain)
Aedes (Ochlerotatus) caspius (Pallas, 1771)	*Ochlerotatus caspius* (subgenus uncertain)
Aedes (Ochlerotatus) detritus (Haliday, 1833)	*Ochlerotatus detritus* (subgenus uncertain)
Aedes (Finlaya) eatoni (Edwards, 1916)	*Ochlerotatus* (*Finlaya*) *eatoni*
Aedes (Finlaya) geniculatus (Olivier, 1791)	*Dahliana geniculata* (first renamed as *Ochlerotatus geniculatus*)
Aedes (Stegomyia) albopictus (Skuse, 1895)	*Stegomyia albopicta* (subgenus uncertain)
Aedes (Stegomyia) aegypti (Linnaeus, 1762)	*Stegomyia (Stegomyia) aegypti*
Aedes (Fredwardsius) vittatus (Bigot, 1861)	*Fredwardsius vittatus*

2.3 | Data analysis

Individual chromatograms were analysed using FinchTV 1.4.0 (Geospiza, PerkinElmer) and forward and reverse sequences were trimmed, edited and assembled to produce a consensus sequence, using BioEdit 7.0.5.3 software (Hall, 1999). Sequence alignment was performed in MAFFT (Rozewicki et al., 2019) available at (Madeira et al., 2019).

All COI sequence data have been submitted to the GenBank database under accession numbers [MW961258—MW961355], and to BOLD platform, together with bi-directional trace files as well as specimen details. All information is available in the 'DNA barcoding of Portuguese mosquito species (Diptera: Culicidae)' project (PTMOS) on BOLD (Ratnasingham & Hebert, 2007), with the Digital Object Identifier (DOI) dx.doi.org/10.5883/DS-PTMOS.

A bootstrap neighbour joining tree (1,000 replicates) based on genetic distances using the K2P substitution model (Kimura, 1980) was created using MEGA 6 (Tamura et al., 2013). This model was used because it is the standard model for DNA barcoding data sets, enabling direct comparisons to results from other studies. The tree was created to provide a graphic representation of the relationship amongst different sequences and was analysed in terms of pattern of terminal branching clustering. All records were analysed directly on BOLD platform, using several tools available. 'Sequence composition' tool was used and calculation of within species and within genus genetic K2P pairwise distances was performed with the 'Distance Summary' tool. For these calculations, this tool does not include singletons, that is, species represented by a single sequence, or, in case of within genus divergence, genus represented by a single species. 'Barcode Gap Analysis' tool was used to compute and compare the distance of each sequence to its furthest conspecific (the maximum intraspecific genetic distance) and to its nearest non-conspecific, that is, nearest neighbour (NN), in order to assess the existence of a barcode gap (Ratnasingham & Hebert, 2007).

Molecular species delimitation can be achieved through several approaches. We used two different methods, Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) and Barcode Index Number (BIN) assignment tool (Ratnasingham & Hebert, 2013). Both methods are independent of prior taxonomic assignment and cluster COI sequence data into Molecular Operational Taxonomic Units (MOTUs) based on sequence similarity. ABGD method is independent of tree typology and splits the data set into candidate species (i.e. MOTUs), 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, except for the relative gap width, which was defined as 1.2.

BOLD automatically attributes a BIN to all records meeting required quality criteria (>507 bp, <1% Ns, no stop codon or contamination flag) (Ratnasingham & Hebert, 2013). Basically, it consists in a cluster algorithm that compares each record under study with all sequences produced from other barcoding projects or mined from

TABLE 2 COI K2P sequence divergence at each taxonomical level for the mosquitoes from Portugal

	n	Taxa	Comparisons	Min dist. (%)	Mean dist. (%)	Max dist. (%)
Within species	96	24	205	0.00	0.28	2.17
Within genus	91	4	1,082	1.23	10.48	17.22
Within family	98	1	3,466	10.60	14.87	19.95

Note: Within species (intraspecific) divergence was calculated for mosquito species represented by more than one specimen; within genus (congeneric) divergence was calculated for genus with more than one species. Computed on BOLD.

TABLE 3 Mean and maximum intraspecific distance between individuals of the same species and distance to nearest neighbour specimen (i.e. non-conspecific specimen with the lowest interspecific distance)

Species	BIN	n	Mean intra-Sp (%)	Max intra-Sp (%)	Nearest species	Distance to NN (%)
Aedes aegypti	BOLD:AAA4210	3	1.45	2.17	*Aedes vittatus*	11.67
Aedes albopictus	BOLD:AAA5870	3	0	0	*Aedes aegypti*	12.98
Aedes berlandi	BOLD:ACY5176	3	0.3	0.46	*Aedes detritus*	11.66
Aedes caspius	BOLD:AAB7911	4	1.16	1.7	*Aedes detritus*	7.51
Aedes detritus	BOLD:AAM2826	4	1.31	2.17	*Aedes caspius*	7.51
Aedes geniculatus	BOLD:AEE7172	3	0.66	0.92	*Aedes caspius*	10.1
Aedes vittatus	BOLD:AAV4160	1	N/A	0	*Aedes caspius*	9.04
Anopheles algeriensis	BOLD:ACY6163	3	0.51	0.77	*Anopheles petragnani*	11.3
Anopheles atroparvus	BOLD:ABY9583	5	0.74	1.23	*Anopheles maculipennis*	2.32
Anopheles claviger	BOLD:AAM4220	2	0.46	0.46	*Anopheles petragnani*	6.36
Anopheles maculipennis	BOLD:AEA0105	4	0.1	0.15	*Anopheles atroparvus*	2.32
Anopheles petragnani	BOLD:AAA9648	5	0.34	0.61	*Anopheles claviger*	6.36
Coquillettidia richiardii	BOLD:AAS0072	3	0.61	0.92	*Culex mimeticus*	14.01
Culex hortensis	BOLD:AAI5767	3	1.23	1.54	*Culex theileri*	9.73
Culex impudicus	BOLD:AAB6945	2	0	0	*Culex territans*	1.23
Culex laticinctus	BOLD:ABZ7976	3	0.42	0.63	*Culex theileri*	2.02
Culex mimeticus	BOLD:AAM3149	3	0.1	0.15	*Culex torrentium*	6.85
Culex modestus	BOLD:AAJ7317	2	0.61	0.61	*Culex pipiens*	5.52
Culex pipiens	BOLD:AAA4751	13	0.09	0.46	*Culex torrentium*	2.8
Culex territans	BOLD:AEE2449	6	0.31	0.76	*Culex impudicus*	1.23
Culex theileri	BOLD:AAA4752	1	N/A	0	*Culex laticinctus*	2.02
Culex torrentium	BOLD:AAA4751	2	0	0	*Culex pipiens*	2.8
Culex univittatus	BOLD:ADJ6510	7	0.04	0.15	*Culex laticinctus*	7.39
Culiseta annulata	BOLD:AAD6954	5	0.06	0.15	*Culiseta longiareolata*	11.31
Culiseta longiareolata	BOLD:AAP0901	4	0.08	0.15	*Culex pipiens*	10.94
Uranotaenia unguiculata	BOLD:ADI8313	4	0.15	0.3	*Culex mimeticus*	13.12

Note: Distances were calculated as K2P genetic distances. NN - Nearest neighbour.

GenBank, present in the BOLD database. Ultimately, it assigns a BIN to each record, either an existent one or creating a new one. A BIN is an analogue of MOTU and can work as a proxy for species (Hebert et al., 2016).

Concordance between BINs assignment and species identification by classical taxonomy was analysed by the 'BIN Discordance Report', which compares the taxonomy on selected records against all others in the BINs they are associated with.

Reliability of the generated library was evaluated by comparing the MOTUs count and boundaries amongst the different species delimitation methods and their congruence with species designations achieved by taxonomy. The rationale behind this is that if several distinct algorithms have the same clustering pattern, resulting in the definition of the same number and 'pattern' of MOTUs, and additionally these MOTUs are congruent/concordant with taxonomic classification, then the reference library is reliable (Knebelsberger et al., 2014).

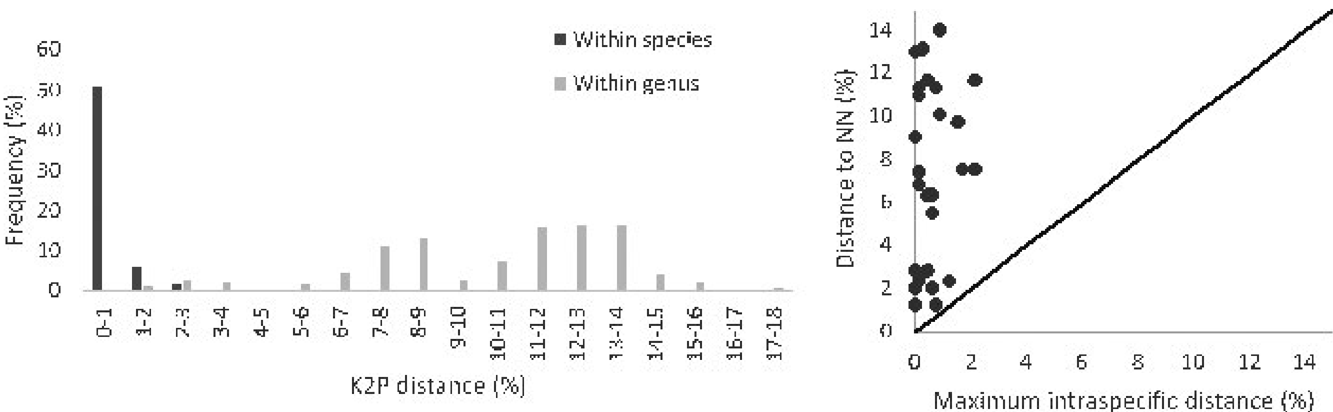

FIGURE 1 Evaluation of the existence of a barcode gap. (a) Frequency histogram of COI sequence divergence (K2P) within species (dark grey) and among congeneric species (light grey). (b) Scatter plot of maximum intraspecific distance and distance to NN; black line with a 1:1 slope representing the point at which the difference between both is zero. Each dot represents a species. All dots fall above the diagonal line meaning that for all species distance to the closest non-conspecific (i.e., nearest neighbour (NN)) was higher than the maximum intraspecific distance

2.4 | Ethical approval statement

Ethical approval was not required because this study does not use vertebrates or higher order invertebrates, and no personal data were collected.

3 | RESULTS

One hundred and three samples (*N* = 103), representing 26 species and six genera [*Aedes* (*N* = 7 species), *Anopheles* (*N* = 5 species), *Coquillettidea* (*N* = 1 species), *Culex* (*N* = 10 species), *Culiseta* (*N* = 2 species), *Uranotaenia* (*N* = 1 species)] were selected for amplification and sequencing. Of these, five sequences had low quality and were not used for the analysis.

Ninety-eight specimens (*N* = 98) were successfully amplified, with average nucleotide frequencies of C (15.8%), T (39.4%), A (29.0%) and G (15.8%) similar to those previously reported in other studies. Cytochrome Oxidase I DNA sequence length varied between 641 and 658 bp, average 657 bp. Full barcode sequences were obtained from 84.7% of samples (*N* = 83) and 88.8% (*N* = 87) sequences were classified as 'Barcode compliant', by the BOLD platform as a guarantee of their quality.

On average, 3.8 sequences were analysed per species (range 1–13), with 92% (*N* = 24) of the species represented by more than one specimen.

Genetic distances increased from lower to higher taxonomical levels. Average confamilial distance was 14.87% whilst the average congeneric distance was 10.48% (range 1.23%–17.22%), based on 1,082 comparisons. The mean intraspecific divergence was 0.28% (range 0.00%–2.17%). The average K2P genetic distance within conspecific specimens was 37-fold lower than the average value found in congeneric species (Table 2).

Twenty-four species (92.3%) had intraspecific diversity <2%. Maximum intraspecific divergence (2.17%) was observed in two species, *Aedes aegypti* and *Aedes detritus*. Three species revealed no intraspecific variability (Intraspecific divergence = 0%), and five species showed very low levels of divergence (average intraspecific divergence between 0.04% and 0.1%): *An. maculipennis*, *Cx. mimeticus*, *Cx. univittatus*, *Culiseta annulata* and *Cs. longiareolata* (Table 3).

K2P distances within species and K2P distances within genus show a clear separation except for a few comparisons in the [1%–3%] range, in which an overlap can be observed (between 1.23%–2.17%) (Figure 1).

Congeneric distances <2% occur exclusively for *Cx. territans-Cx. impudicus* comparisons (range 1.23%–1.85%, average 1.39%). Distances between 2% and 3% happen between *Cx. pipiens-Cx. torrentium* (range 2.796%–2.979%, average 2.89%), *Cx. theileri-Cx. laticinctus* and *An. atroparvus-An. maculipennis*.

Ninety-eight per cent of intraspecific comparisons are below the 2% divergence, whilst 99.8% of congeneric comparisons are greater than 2%, with 97% being above 6%.

All specimens were above the line that represents the existence of a barcoding gap, meaning that for each individual the difference between the distance to the NN and the distance to the furthest conspecific is above zero (Figure 1).

Genetic distances of each species to their nearest neighbour (NN) (i.e. non-conspecific specimen with the lowest interspecific distance) were always higher than the maximum intraspecific genetic distance, for all species (Table 3). Distance to NN was always above 2%, except for *Cx. territans* and *Cx. impudicus*. *Cx. laticinctus* was considered *Cx. theileri* NN (and vice-versa), with a distance very close to the 2% threshold.

The NJ tree grouped sequences of the same taxonomically identified species in distinct non-overlapping clusters supported with high bootstrap values (≥99%), for the 26 species. All biotypes of *Cx. pipiens* clustered together. *Anopheles maculipennis* and *An. atroparvus*

that belong to the *An. maculipennis* complex, clustered separately in the NJ tree, with high bootstrap values of 99%. A sub-branch inside the *An. atroparvus* branch can be observed, although having a medium bootstrap support (94%). Deep splits were observed for *Aedes aegypti* and *Ae. detritus* species (Figure 2).

Overall, the species delimitation algorithms had a strong performance as both identified 25 MOTUs out of the 26 taxonomically identified species, corresponding to a 96.1% success rate.

The ABDG method identified 25 MOTUs at the prior maximal distance of 4.64 e-3. Results are consistent with the NJ tree clustering and taxonomic classification, except for *Cx. laticinctus* and *Cx. theileri* that were grouped within the same MOTU.

BIN assignment tool allowed the distinction of 25 BINs, with most delimited MOTUs consistent with the clustering pattern observed in the NJ tree and concordant with morphological identification. The only exception is for *Cx. pipiens* and *Cx. torrentium* that were placed together in the same BIN (BOLD:AAA4751). This corresponds to the single discordant BIN, (where two different nominal species share the same BIN), found by the BIN discordance analysis. A total of 21 concordant BINs, basically indicating BINs containing records from only one species, were found. Furthermore, 2 BINs that include only one single sequence were also detected (*Aedes vittatus* and *Cx. theileri)*. A new unique BIN, meaning that any existent BIN was considered similar enough, was also created - BOLD: AEE2449, corresponding to the six *Cx. territans* specimens identified.

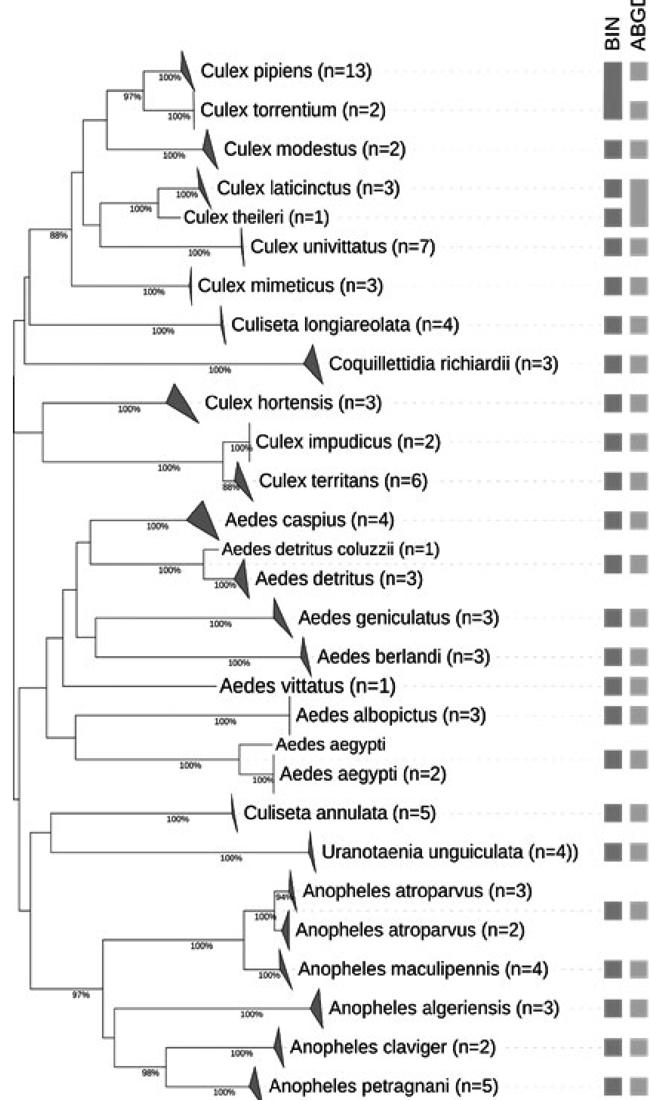

FIGURE 2 Neighbour joining tree based on K2P distances among 98 COI sequences of 26 Portuguese mosquito species. The number of specimens inside each species is shown in brackets. Bootstrap values (1,000 replicates) above 85% are shown below the branches. The MOTUs created by each delimitation algorithm - Barcode index number (BIN) and Automatic Barcode Gap Discovery (ABGD) - are represented as squares on the right, with rectangles depicting species that were considered as a single MOTU. The node for each species with multiple specimens is collapsed to a vertical line or triangle, with the horizontal depth indicating the level of intraspecific divergence

4 | DISCUSSION

The present work represents the first comprehensive study combining morphological and molecular identification of mosquito species commonly found in Portugal. COI sequences of 26 mosquito species were obtained and used to assemble a reference library of DNA barcodes to be used for identification of mosquitoes, including the major disease vectors. Species delimitation algorithms were used to assess the validity and reliability of the generated library. The analysis of the results obtained by distinct clustering methods offer an additional level of confidence in the inferred MOTUs in Portuguese mosquitoes.

Mosquito barcoding was first used to identify 37 mosquito species in Canada (Cywinska et al., 2006) and since then several other countries have successfully used this method for mosquito identification (Ashfaq et al., 2014; Hernández-Triana et al., 2019; Kumar et al., 2007; Versteirt et al., 2015; Wang et al., 2012). The results of this study prove that DNA barcoding is an effective tool for the identification of Portuguese mosquitoes. Most authors refer that the successful use of DNA barcodes for specimen identification and species delimitation, depends on the existence of a barcoding gap. This can be defined as the difference or distance between the mean intraspecific sequence variability and interspecific variability for congeneric COI sequences (Meyer & Paulay, 2005).

Although an overlap between intra- and interspecific K2P divergence values occurred in our study, (Table 2; Figure 1), this should not be interpreted as the inexistence of a barcode gap. These distances were calculated for all pooled species analysed in the study, so it can be expected that intraspecific distances for one species exceed interspecific distances for other species in the analysis. This can be interpreted as a failure to define an universal cut-off value, a threshold applicable for all species, or a 'global' barcode-gap, but not a 'local' barcode gap, that can be used for each particular data set (Collins & Cruickshank, 2013). A best option to infer about the

successful use of barcodes for specimen identification is a dot-plot graphic, in which, for each individual data point, the distance to the furthest conspecific (maximum intraspecific distance) is plotted against the distance to the NN, with a 1:1 slope representing the point at which the difference between the two is zero (i.e. no local barcoding gap) (Collins & Cruickshank, 2013). In our study, the distance to the NN is higher than the maximum intraspecific distance, for all species, meaning there is a clear local barcoding gap, which enables the successful use of DNA barcoding for specimen identification of common Portuguese mosquito fauna. Furthermore, we show that average distance amongst congeneric mosquito species was 37 times higher than the average differences within species. Nevertheless, the overlap between intra- and interspecific K2P distances observed in our data may be related to the presence of closely related species, such as cryptic species or species complexes.

Other of the aims of DNA barcoding is 'species discrimination' or 'species discovery' (Collins & Cruickshank, 2013). Several studies using DNA barcoding were able to reveal cryptic species (Batovska et al., 2016; Chan-Chable et al., 2019; Delgado-Serra et al., 2020; Wang et al., 2012), although some report the inability of barcoding to separate closely related species, namely in *Culex* and *Anopheles* genus (Cywinska et al., 2006; Kumar et al., 2007; Laurito et al., 2013; Wang et al., 2012). The species discrimination power of DNA barcoding in mosquitoes, reflected by congruence with the morphological identifications, was usually high in previous studies, ranging from 82% to 98% (Ashfaq et al., 2014; Chan et al., 2014; Rozo-Lopez & Mengual, 2015; Versteirt et al., 2015; Wang et al., 2012). The overall DNA barcoding success for species discrimination in our data set was also high. If we consider the use of each species delimitation method alone, the success rate is 96,1% (25 out of the 26 species). When considering the use of the combination of all clustering methods, all species were distinguishable.

Species delimitation algorithms were mostly congruent amongst them and with NJ tree clustering, with the definition of 25 MOTUs for all methods. Overall, the NJ tree shows that all species are clearly distinguishable through COI, forming non-overlapping monophyletic clusters. In some cases, the clustering pattern was slightly different for each algorithm. This usually occurred for species that are part of species complexes or were cryptic diversity can be expected. Moreover, our results show that, COI could also differentiate closely related species that are usually not distinguished by morphology and need the use of different markers such as ITS2 or Ace2, as found by Chan et al. (2014).

Although *Culex laticinctus* and *Culex theileri* are phylogenetically close as both belong to the Theileri subgroup (Sirivanakarn, 1976) inside the Culex Linnaeus subgenus (Harbach, 2018), they are clearly distinguishable by morphology and have different ecology. The average genetic distance between specimens from each species averaged 2.2% and they form two distinct branches in the NJ tree, with high bootstrap values. A different BIN was attributed to each species but the ABGD method considered the two species in the same MOTU. This is probably due to the low number of sequences analysed, as *Cx. theileri* was a singleton and the ABGD method requires 3–5 specimens of each species for ideal performance (Puillandre et al., 2012). In contrast, BIN was able to differentiate the two species as it uses all sequences available in BOLD database. In this way, the small differences amongst sequences of the two species, when compared with their conspecifics in the database, enabled their separation.

Culex impudicus and *Culex territans* were considered as two separated species by all methods. Although, interspecific distance between the two species was the lowest amongst all species (average 1.38%, min 1.23%) and below the 2% threshold. The two specimens of *Cx. impudicus* showed no intraspecific divergence whilst *Cx. territans* had a low intraspecific divergence value, contrary to findings from Belgium (Versteirt et al., 2015). Some authors refer that *Cx. territans* distribution is likely restricted to the Neartic region and that the only representatives of subgenus Neoculex in Portugal are *Cx. impudicus* and, what was previously identified as *Cx. territans* that should now be referred to as the Territans Group (Sirivanakarn, 1971) (Gunay et al., 2015; Ramos et al., 2003). Territans Group includes several species that are morphologically similar and can easily be confused such as *Cx. impudicus*, *Cx. judaicus*, *Cx. martinii*, *Cx. rubensis* or *Cx. europaeus* (Ramos et al., 2003). Specimens from Turkey previously identified as *Cx. territans* were barcoded and authors found that some specimens had low sequence similarity with *Cx. territans*. These specimens were putatively identified as *Cx. europaeus* (GenBank accession numbers KJ012066-067) (Gunay et al., 2015). *Culex territans* sequences obtained in this study were compared through Blast with these sequences and showed low similarity values (approx. 94%). A new unique BIN was created for all the six *Cx. territans* sequences obtained in this study, apart from all the previously existent sequences on the BOLD platform. Most sequences from this species available in BOLD are from EUA and Canada, with a few specimens from Belgium, but our specimens were considered more related to *Cx. impudicus* specimens (distance to NN=1.12%) than to *Cx. territans* from other countries. This, together with the fact that *Cx. europaeus* was firstly described based on the examination of specimens from Portugal, makes us suspect that our specimens can in fact be *Cx. europaeus*. In order to resolve the true identity of Portuguese and Turkish samples, and to fully understand Territans group diversity we need newly generated DNA barcodes of specimens identified by male genitalia, which is considered the most reliable method for Neoculex species identification (Gunay et al., 2015).

Culex pipiens and *Culex torrentium* are sibling species and are mostly not distinguishable morphologically (only male genitalia have distinct morphological characters), but several PCR based-methods using different molecular markers such as Ace2 or ITS2, are available for their identification (Rudolf et al., 2013; Smith & Fonseca, 2004; Vinogradova & Shaikevich, 2007; Werblow et al., 2014). In our study, the two species clustered in two highly supported branches in the NJ tree and were considered as two independent taxa by the ABGD method. Instead, BIN approach grouped the two species in the same BIN (BOLD:AAA4751). Average K2P distance between the two species was approximately 3%. Other authors (Hernández-Triana et al., 2019; Versteirt et al., 2015) also found delimitation of

these two species controversial, as other methods (ABGD and BIN, respectively) were not able to distinguish them, but NJ tree results showed a clear separation between the two species. The discordance between the two delimitation methods—BIN and ABGD—might be due to the different threshold values used for separating species, 2.2% by default for the BIN system method, and the threshold calculated by ABGD. In this way we found that the COI marker has a strong performance in distinguishing the two species present in our study, as also reported by other authors (Engdahl et al., 2014; Shaikevich, 2007; Werblow et al., 2014). *Culex pipiens* biotypes *Cx. p. pipiens* and *Cx. p. molestus*, and their hybrids had been previously identified using CQ11 essay. None of the algorithms was able to differentiate these biotypes based on DNA barcoding, in agreement with the majority of previous works, although some authors found fixed differences in the COI sequence of the members of the *Culex pipiens* group worldwide (Danabalan et al., 2012; Gunay et al., 2015; Hernández-Triana et al., 2019; Shaikevich, 2007).

An. maculipennis complex is a species complex that includes *An. maculipennis* and *An. atroparvus*, the only two species reported in Portugal in the last 40 years, as well as other species. For the differentiation of the species in this complex, the amplification of the ITS2 marker is recommended as morphologically, they are only distinguishable in the egg stage (Proft et al., 1999). COI amplification showed to be also a competent tool for the differentiation of *An. maculipennis s.s.* and *An. atroparvus*, as specimens of each species clustered separately in the NJ tree, with high bootstrap values of 99%. Additionally, also BIN and ABGD were also able to effectively separate the two species in two independent MOTUs, in agreement with (Hernández-Triana et al., 2017). This is contrary to results of Kronefeld et al., 2014 and Linton et al., 2002, that found that members of *An. maculipennis* complex are phylogenetically related and cannot be readily identified using only the COI genetic marker. This can be explained by the fact that these studies occurred in geographic locations where species variability inside *An. maculipennis* complex is higher, as they report occurrence of *An. messeae* and *An. daciae*.

In the NJ tree a sub-branch with high bootstrap support inside the *An. atroparvus* branch can also be observed. Although the intraspecific divergence was below the 2% threshold, we can suspect of some diversity amongst this species.

Another case in which COI barcoding seems to be successful is in the distinction between species of the *Anopheles claviger* complex: *Anopheles claviger s.s.* and *Anopheles petragnani*. Molecular identification using ITS2, was able to identify 5 of our specimens to species level as *An. petragnani* (results not presented). For two other specimens, that cluster together and apart from the previous five specimens, a definite ITS2-based identification was not possible, as sequencing was not successful. This occurred also in a recent work, in which the molecular differentiation of *An. claviger s.s.* and *An. petragnani* using ITS2 was not possible, although the two species had been previously reported for the area (Ruiz-Arrondo et al., 2019). The two species delimitation algorithms separated the two species in two different MOTUs and the NJ tree shows 2 separated branches, although with bootstrap values below 90%. These results contrast with previous studies, in which *An. claviger s.l.* clustered together with no deep splits (Hernández-Triana et al., 2019; Versteirt et al., 2015). This may be due to the inexistence of *An. petragnani* in the surveyed regions.

High intraspecific divergence values, which also reflected in deep splits in the NJ tree, occurred for the species *Aedes aegypti* and *Ae. detritus*. In the case of *Ae. aegypti*, the high genetic diversity may be explained by the occurrence of at least two different introductions in the Madeira Island (Seixas et al., 2019). *Ae. detritus* is also part of a complex, constituted by *Ae. colluzzi* and *Ae. detritus*. After ITS2 amplification we confirmed that the specimen placed farther from the other two was in fact *Ae. colluzzii*, which explains the high intraspecific divergence value.

Some of the Portuguese mosquito species have just a few COI sequences publicly available, such as *Aedes berlandi*, *Aedes vittatus* (only 32 sequences available, mostly from Africa and Asia, only one from Europe, in Spain), or *An. petragnani*. Our study contributed to international genetic databases with extra sequences of these and other species, making them publicly available and enabling the use of DNA barcoding for the identification of mosquito species. As future perspectives, we will complement the generated database with more specimens of the species already present, and also add other species according to availability, such as *Ae. eatoni*.

5 | CONCLUSIONS

The current study generated a validated national reference DNA barcode library for mosquitoes commonly found in Portugal. We contribute to the global mosquito barcoding initiative, with the submission of 98 sequences, including sequences of less represented species. A reference library with validated sequences will help future studies as it will facilitate the association of conspecific specimens and the detection of identification errors. It is a commonly referred DNA barcode limitation the existence of misidentified sequences in public databases. It will also contribute to the use of DNA barcoding as an additional identification tool in mosquito surveillance programmes.

DNA barcoding proved to be an effective tool for the identification and species delimitation of Portuguese mosquitoes. It showed to be able to discriminate some closely related species such as *Cx. pipiens* and *Cx. torrentium*, or species complexes such as *An. maculipennis* and *An. atroparvus* or *An. claviger* and *An. petragnani*. Although, COI cannot discriminate *Cx. pipiens* biotypes. *Cx. territans* needs further research to resolve its taxonomic controversy. The results of this work should be complemented and confirmed with the amplification of other molecular markers, particularly in the case of cryptic species, and sample size should also be increased.

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CONFLICT OF INTEREST

The authors declare that there is no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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