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Barcoding Turkish *Culex* mosquitoes to facilitate arbovirus vector incrimination studies reveals hidden diversity and new potential vectors

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ABSTRACT

As a precursor to planned arboviral vector incrimination studies, an integrated systematics approach was adopted using morphology and DNA barcoding to examine the *Culex* fauna present in Turkey. The mitochondrial *COI* gene (658 bp) were sequenced from 185 specimens collected across 11 Turkish provinces, as well as from colony material.

Although by morphology only 9 species were recognised, DNA barcoding recovered 13 distinct species including: *Cx.* (*Barraudius*) *modestus*, *Cx.* (*Culex*) *laticinctus*, *Cx.* (*Cux.*) *mimeticus*, *Cx.* (*Cux.*) *perexiguus*, *Cx.* (*Cux.*) *pipiens*, *Cx.* (*Cux.*) *pipiens* form *molestus*, *Cx.* (*Cux.*) *quinquefasciatus*, *Cx.* (*Cux.*) *theileri*, *Cx.* (*Cux.*) *torrentium*, *Cx.* (*Cux.*) *tritaeniorhynchus* and *Cx.* (*Maillotia*) *hortensis*. The taxon formerly identified as *Cx.* (*Neoculex*) *territans* was shown to comprise two distinct species, neither of which correspond to *Cx. territans* s.s. These include *Cx.* (*Neo.*) *impudicus* and another uncertain species, which may be *Cx.* (*Neo.*) *europaeus* or *Cx.* (*Neo.*) *martinii* (herein = *Cx.* (*Neo.*) sp. 1). Detailed examination of the Pipiens Group revealed *Cx. pipiens*, *Cx. pipiens* f. *molestus* and the widespread presence of the highly efficient West Nile virus vector *Cx. quinquefasciatus* for the first time. Four new country records are reported, increasing the *Culex* of Turkey to 15 recognised species and *Cx. pipiens* f. *molestus*. A new taxonomic checklist is provided, annotated with respective vector competencies for transmission of arboviruses.

1. Introduction

Culex mosquitoes are increasingly being implicated in the transmission of arboviral and parasitic diseases worldwide. A prerequisite to successful control of these diseases is the accurate identification of the mosquito vector species involved. *Culex* mosquitoes reported from Turkey to date comprise thirteen species in four subgenera: *Cx.* (*Barraudius*) *modestus*, *Cx.* (*Bar.*) *pusillus*, *Cx.* (*Culex*) *laticinctus*, *Cx.* (*Cux.*) *mimeticus* Noè, *Cx.* (*Cux.*) *perexiguus* (previously reported as *Cx. univittatus* in Harbach, 1999), *Cx.* (*Cux.*) *pipiens*, *Cx.* (*Cux.*) *theileri*, *Cx.* (*Cux.*) *torrentium*, *Cx.* (*Cux.*) *tritaeniorhynchus*, *Cx.* (*Maillotia*) *deserticola*, *Cx.* (*Mai.*) *hortensis*, *Cx.* (*Neoculex*) *martinii* and *Cx.* (*Neo.*) *territans* (Irdem, 1939; Süyev, 1953; Erel, 1967; Parrish, 1959; Harbach, 1999; Alten et al., 2000; Ramsdale et al., 2001).

Early reports documented the presence of *Cx.* (*Cux.*) *quinquefasciatus* (Parrish, 1959), and *Cx.* (*Lasiosiphon*) *adairi* [as *Cx. pluvialis* by Süyev, 1953] in Turkey, However as these one-off reports fell outside the normal distribution of these species, Ramsdale et al. (2001) regarded these as erroneous and deleted these species from the Turkish faunal list. Ramsdale et al. (2001) also reported that following Kirkpatrick's (1925) mistaken identification of *Cx. theileri* as *Cx. tipuliformis* (=synonym of *Cx. vagans*), this error was inadvertently perpetuated in later derivations of the Turkish faunal lists (Irdem, 1939; Süyev, 1953; Erel, 1967; Alten et al., 2000).

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The taxonomic status of *Cx. territans* in Europe has long been under scrutiny, with specimens initially misidentified as *Cx. apicalis* (Parrish, 1959), which is now regarded as restricted to the Nearctic (Knight and Stone, 1977). Originally described from the USA, *Cx. territans* has been widely reported in the European and Mediterranean subregions of the palaearctic. However, a morphological re-examination of topotypic material (Charleston, South Carolina) and comparison with specimens collected in Avo, northern Portugal revealed a new closely related species, *Cx. europaeus*, described in da Cunha Ramos et al. (2003). This paper detailed morphological characters based on the male and female genitalia to differentiate these two species. Since then it has been widely accepted that the European mosquitoes previously referred to as *Cx. territans* were most likely to be *Cx. europaeus*. However, which species the previous Turkish records of *Cx. territans* refer to remains unclear.

The application of genetic markers has proven extremely useful for resolving cryptic and hidden species of mosquitoes. In particular, the increasingly availability of DNA barcode data, based on linked taxonomic voucher specimens and mitochondrial cytochrome c oxidase I (*COI*) gene sequences, has helped to confirm the identity of invasive or previously undocumented species that local entomologists are unfamiliar with and are most likely to misidentify (e.g. Ruiz-Lopez et al., 2012; Danabalan et al., 2012; Linton et al., 2013; Oter et al., 2013). We employed an integrated morphological and molecular approach to better understand the taxonomic status of Turkish *Culex* mosquitoes. DNA barcode sequences were generated from morphologically identified samples to assess their utility for robust identification and incrimination of mosquito vectors in future arboviral surveys in Turkey.

2. Materials and methods

2.1. Specimen data

Specimens of *Culex* were field-collected in 11 provinces across Turkey between 2005–2011. Mosquitoes were mostly collected as immatures and link-reared; others were collected in light traps or as resting adults. Morphologically identified representatives of 10 species totalling 185 specimens were gathered for this study as follow: *Cx. hortensis* ($n = 13$), *Cx. laticinctus* ($n = 9$), *Cx. modestus* ($n = 1$), *Cx. mimeticus* ($n = 6$), *Cx. perexiguus* ($n = 7$), *Cx. pipiens s.l.* ($n = 64$), *Cx. territans* ($n = 8$), *Cx. theileri* ($n = 61$), *Cx. torrentium* ($n = 8$) and *Cx. tritaeniorhynchus* ($n = 8$). Specimens of *Cx. pipiens* f. *molestus* ($n = 13$) were obtained from the autogenous colony maintained at Hacettepe University, Ankara. This colony was established in 2004 from wild-caught specimens from around Ankara and from sites in Hatay and Şanlıurfa provinces. Despite our extensive collection efforts and reaching out to other Turkish mosquito workers, no specimens of *Cx. pusillus*, *Cx. deserticola* or *Cx. martinii* were available for inclusion, although all have previously been reported from Turkey (Alten et al., 2000; Ramsdale et al., 2001).

Mosquitoes were identified using available morphological keys (Harbach, 1988; Samanidou-Voyadjoglou and Harbach, 2001; Schaffner et al., 2001; Samanidou and Harbach, 2003; Becker et al., 2010), individually labelled, and stored in 95% ethanol prior to DNA extraction. DNA was extracted from mosquito abdomens or legs only, with the remainder of each specimen stored at the Natural History Museum, London (BMNH) to serve as voucher specimens for the molecular study. DNA extracts are deposited in the frozen repository of the National Museum of Natural History (NMNH), Smithsonian Institution, USA or in the Molecular Collections Facility of the Natural History Museum (BMNH), UK.

Bi-directional, edited *COI* trace files and specimen details (including exact localities with georeferences and specimen identifiers) are freely available in the "*Culex* of Turkey" project (CXTUR) on the Mosquitoes of the World section of the Barcode of Life Data Systems database (BOLD: http://www.boldsystems.org) (Ratnasingham and Hebert, 2007). Sequences appear in GenBank as "barcode red flag" data indicating their high quality and voucher standards under accession numbers KJ012066–KJ012250.

2.2. Molecular methods

DNA was extracted using the QIAgen® BioSprint 96 DNA Tissue Kit (QIAgen®, Crawley, England, UK) on the QIAgen® automated DNA extraction platform, with all solutions at half the manufacturers recommended volumes. The universal LCO and HCO barcoding primers of Folmer et al. (1994) were used to amplify the barcode region of the mtDNA *COI* gene (658-bp after primer removal). The PCR reactions comprised 1 μl template DNA, 1 μl 10× NH_4 buffer, 0.5 μl dNTPs at 2.5 mM, 0.3 μl each primer at 10 μM, 0.4 μl $MgCl_2$ at 50 mM and 0.2 μl of Taq polymerase (BioLine, London, England) made up to 10 μl with ddH_2O. Reactions comprised initial denaturing at 95 °C for 5 min, then 34 cycles of 95 °C for 30 s, 48 °C for 30 min and 72 °C for 45 s, followed by a 5-min extension at 72 °C and a 10 °C hold.

PCR products were visualised on 2% agarose gels stained with ethidium bromide. Products were purified using the Millipore® vacuum manifold system, following the manufacturers instructions. Bidirectional DNA sequences were generated using the Big Dye® Terminator Kit (PE Applied BioSystems, Warrington, England) and run on an ABI 3730 automated sequencer (PE Applied BioSystems®). Sequences were edited using Sequencher[a] version 4.8 (Genes Codes Corporation, Ann Arbor, MI) and alignments verified in CLUSTAL X (Jeanmougin et al., 1998). Nucleotide sequences were translated to amino acid sequences using the invertebrate mitochondrial code (Clary and Wolstenholme, 1985). The second base of the 658-bp barcode sequence is equal to the first position of the amino acid codon. Alignment of the *COI* fragments was unambiguous and no evidence of pseudogenes was noted.

Sequences generated in this study were directly compared with those publicly available in GenBank using Blast (http://blast.ncbi.nlm.nih.gov/) and as yet unreleased sequence data held in the BOLD database, including sequences generated by the Mosquito Barcoding Initiative (MBI). Sequence statistics, calculation of pairwise distance parameters using Kimura's 2-parameter algorithm (Kimura, 1980) and the bootstrapped neighbor-joining tree (Saitou and Nei, 1987) was constructed in MEGA v. 5.2.2 (Tamura et al., 2011).

The optimal neighbor-joining tree with the sum of branch length = 0.54578624 is shown in Fig. 2. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale. The distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 185 nucleotide sequences. Codon positions included 1st + 2nd + 3rd + noncoding. There were no ambiguous data and a total of 658 positions in the final dataset (Fig. 2).

3. Results

Full-length DNA barcodes (658-bp) were generated from 172 wild-caught *Culex* specimens, collected in 11 provinces of Turkey (Fig. 1). Morphologically, the specimens were identified as belonging to nine species: *Cx. hortensis*, *Cx. laticinctus*, *Cx. mimeticus*, *Cx. modestus*, *Cx. pipiens*, *Cx. territans*, *Cx. theileri*, *Cx. tritaeniorhynchus* and *Cx. torrentium*. In addition, 13 specimens of *Culex pipiens* f.

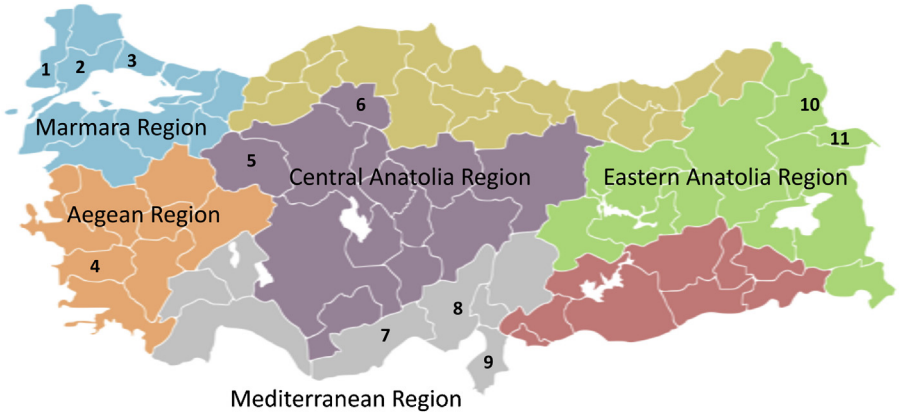

Fig. 1. Map of Turkey indicating the 11 provinces where *Culex* specimens used in this study were obtained. (Map outline source: www.fr.academic.ru). Species confirmed are indicated by region and province. Marmara Region (blue): 1. Edirne (*Cx. pipiens*, *n* = 7), 2. Tekirdağ (*Cx. pipiens*, *n* = 13), 3. Istanbul (*Cx. pipiens*, *n* = 1); Aegean Region (orange): 4. Aydın (*Cx. perexiguus*, *n* = 1 & *Cx. quinquefasciatus*, *n* = 1); Central Anatolia Region (mauve): 5. Eskişehir (*Cx. pipiens*, *n* = 1), 6. Çankiri (*Cx. pipiens* f. *modestus*, *n* = 1, *Cx. pipiens*, *n* = 1 & *Cx. theileri*, *n* = 1); Mediterranean Region (grey): 7. Içel (*Cx. laticinctus*, *n* = 9, *Cx. perexiguus*, *n* = 5, *Cx. pipiens*, *n* = 1 and *Cx. quinquefasciatus*, *n* = 6), 8. Adana (*Cx.* (*Neo.*) sp. 1, *n* = 2, *Cx. hortensis*, *n* = 7, *Cx. impudicus*, *n* = 6, *Cx. mimeticus*, *n* = 2, *Cx. perexiguus*, *n* = 1, *Cx. pipiens*, *n* = 3, *Cx. quinquefasciatus*, *n* = 4 and *Cx. tritaeniorhynchus*, *n* = 8), 9. Hatay (*Cx. pipiens*, *n* = 1 and *Cx. mimeticus*, *n* = 4); Eastern Anatolia Region (green): 10. Kars (*Cx. hortensis*, *n* = 6, *Cx. pipiens*, *n* = 8, *Cx. quinquefasciatus*, *n* = 1, *Cx. theileri*, *n* = 45 and *Cx. torrentium*, *n* = 8), 11. Iğdır (*Cx. pipiens*, *n* = 3 and *Cx. theileri*, *n* = 15) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

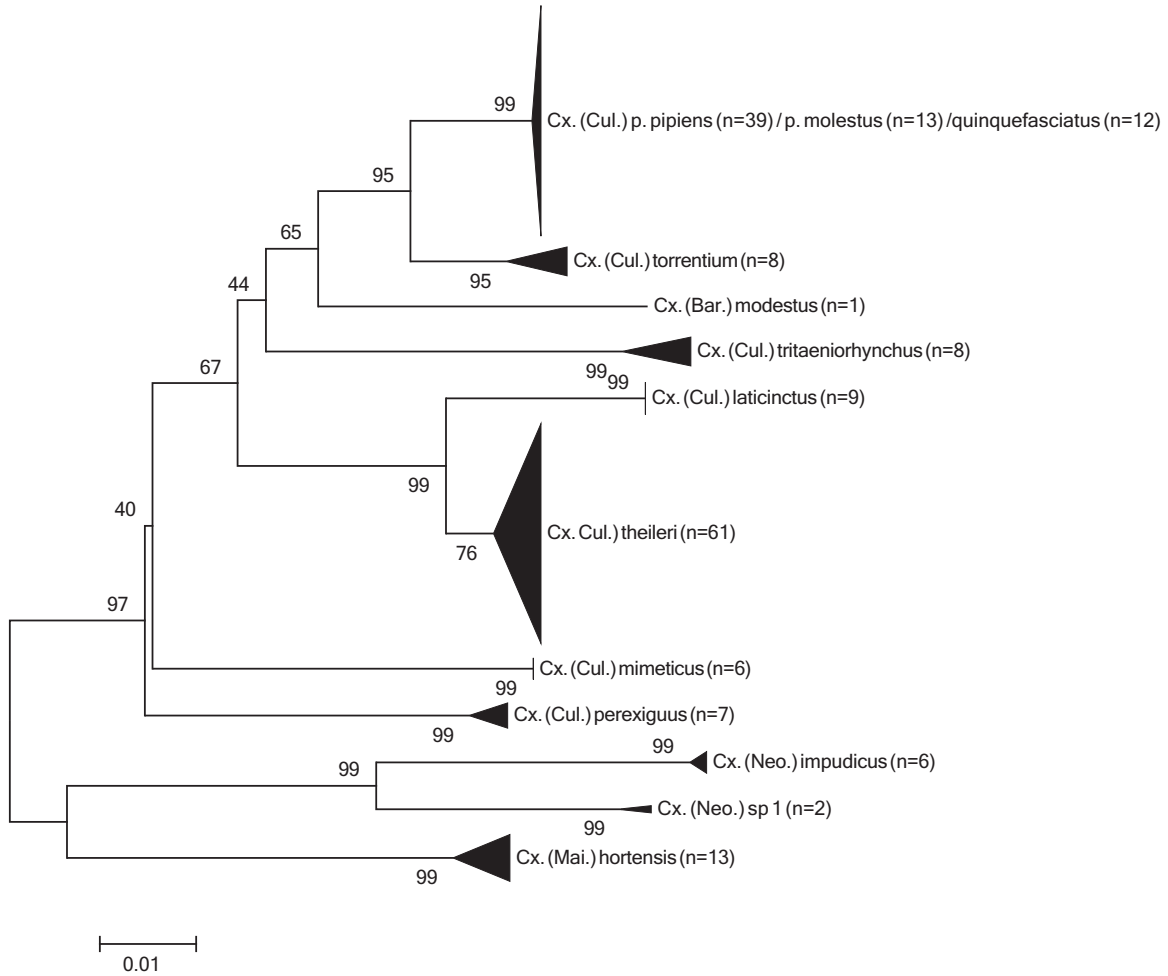

Fig. 2. Relationships between *Culex* sequenced showing robust bootstrap values for terminal clades. Pairwise genetic distances between 185 nucleotide *COI* sequences were computed using the Kimura 2-parameter method (Kimura, 1980) and the relationships were inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and in MEGA v.5.2.2 (Tamura et al., 2011). The optimal tree with the sum of branch length = 0.54578624 is shown.

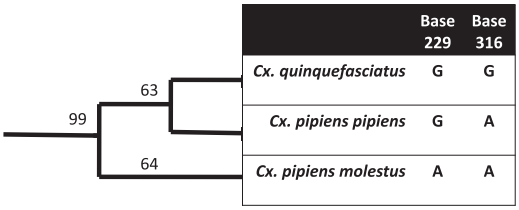

Fig. 3. Alignment showing the three combinations of two variable bases on the *COI* barcode fragment which represent fixed differences between *Cx. pipiens* (*n* = 39), *Cx. pipiens* f. *molestus* (*n* = 13) and *Cx. quinquefasciatus* (*n* = 12). The tree figure and bootstrap support values were from the neighbour-joining analysis using the whole 185 sequence dataset for all 658 bases of mtDNA *COI* gene conducted in MEGA v.5.2.2 (Tamura et al., 2011), as in Fig. 2. The percentages of replicate trees in which the associated species clustered together in the bootstrap test (5000 replicates) are shown next to the branches (Felsenstein, 1985).

molestus were sequenced from a colony maintained at Hacettepe University, Ankara.

DNA sequence analysis using the 2% sequence threshold of the MBI revealed 11 distinct genetic entities, with nine supported with 99% bootstrap values in the neighbor-joining phylogram (Fig. 2). Eight of these corresponded to the morphologically identified species *Cx. hortensis* (*n* = 13, haplotypes (*H*) = 12 from Adana and Kars), *Cx. laticinctus* (*n* = 9, *H* = 1 from Içel), *Cx. modestus* (*n* = 1, *H* = 1 from Çankiri), *Cx. mimeticus* (*n* = 6, *H* = 1 from Adana and Hatay), *Cx. perexiguus* (*n* = 7, *H* = 4 from Adana, Aydın and Içel), *Cx. theileri* (*n* = 61, *H* = 24 from Çankiri, Kars and Iğdır), *Cx. torrentium* (*n* = 8, *H* = 5 from Kars) and *Cx. tritaeniorhynchus* (*n* = 8, *H* = 7 from Adana) (Table 1, Fig. 1). However morphologically identified specimens of *Cx. territans* and *Cx. pipiens* revealed hidden species.

Genetic differentiation of the *COI* barcode region of species within the Pipiens Group has previously been reported as extremely low but comprising fixed differences in the members of the group worldwide (Shaikevich, 2007; Danabalan et al., 2012). According to our neighbour-joining tree, drawn based on Kimura's 2-parameter distance algorithm, all 64 sequences of *Cx. pipiens* s.l. fall together, supported by 99% bootstrap value (Fig. 2), indicative of a single species. However, close examination of the sequences revealed three fixed haplotypes (Fig. 3), which exactly matched multiple publically available *COI* sequences for *Cx. pipiens* (*n* = 39), *Cx. pipiens* f. *molestus* (*n* = 13) and *Cx. quinquefasciatus* (*n* = 12) (Table 1). This level of variation is much lower than most intraspecific variation reported for all the other species (Table 2), but the variable bases appear fixed in these specimens and other MBI sequences available the BOLD and GenBank databases (Fig. 3).

The *Cx. pipiens* f. *molestus* sequenced herein were from Hacettepe University colony, which originated in 2004 from specimens collected in Ankara and the provinces of Hatay and Sanlıurfa. *Culex pipiens* identified in this study were widely collected from the provinces of Çankırı, Edirne Eskişehir, Hatay, Igdır, İstanbul and Tekirdağ; *Culex quinquefasciatus* was collected in Aydın, with both species present in İçel, Adana and Kars (Fig. 1). Discounting the single unverified record of *Cx. quinquefasciatus* in Parrish (1959), this is the first confirmed record of *Cx. quinquefasciatus* in Turkey.

Specimens morphologically identified as *Cx. territans* were found to comprise two genetic entities. A BLAST search confirmed that neither species shares more than 96% sequence similarity with *Cx. territans* from the USA [e.g. JX259927, JX259923] (Table 1). One of these species was shown to match an archive specimen of *Cx. impudicus* housed at the Natural History Museum (BMNH), London (Labels read: Sardinia, Geremeas, 26.ix.1952, THG Aitken, Serial No 42) (BOLD process ID: NHMCX573-11; GenBank accession KP037055) sequenced through the efforts of the Mosquito Barcoding Initiative. This retrospective identification indicates the presence of *Cx. impudicus* in Turkey for the first time. Tempting as it is to assume that the other species is *Cx. europaeus*, we have no DNA sequences or males to verify this identification, and thus instead, we carefully refer to this species as *Cx.* (*Neo.*) sp. 1 (Fig. 2, Table 1). Both species were collected in Adana (Fig. 1).

Overall examination of these 185 DNA barcodes revealed 168 variable bases (25.5%), 153 of which are parsimony informative and only 17 represent singleton mutations. Nucleotide variation was heavily skewed to the third position of the codon, accounting for 86.9% of all variation (146 variable bases; 14 singletons). First position changes (22 variable bases; 1 singleton) accounted for the remainder of the variation, as no changes were noted in the second codon position.

Mean intra-specific genetic distance ranged from 0 in *Cx. laticinctus* (*n* = 9), *Cx. mimeticus* (*n* = 6), *Cx. pipiens* (*n* = 39), *Cx. pipiens* f. *molestus* (*n* = 13) and *Cx. quinquefasciatus* to 0.010 in *Cx. tritaeniorhynchus* (*n* = 8). One specimen of *Cx. tritaeniorhynchus* [GenBank accession KJ012244] was significantly different from the others (max difference 0.023), elevating the overall mean genetic distance between these specimens (Table 1).

Table 1
Intraspecific COI sequence diversity statistics (658-bp) for Turkish *Culex* specimens sequenced in this study (*n* = 185). Statistics include specimens per species (*n*), number of unique *COI* haplotypes (*H*) and genetic distances (mean and range calculated using Kimura's 2-parameter distance algorithm (Kimura, 1980). GenBank accessions for sequences reported herein are given by species, along with the closest available published sequence matches. *Indicates first barcode records for these species.

Species	*n*=	Il=	Mean distance (range)	GenBank accessions	Closest available public sequence (>96% sequence match)
*Cx. hortensis**	13	12	0.007 (0–0.014)	KJ012068–080	None
*Cx. laticinctus**	9	1	0	KJ012087–095	None
Cx. mimeticus	6	1	0	KJ012096–101	99% *Cx. mimeticus* AB738235 99% *Cx. mimeticus* AB738226 Japan
Cx. modestus	1	1	n/c	KJ012102	99% *Cx. modestus* JN592748 France 99% *Cx. modestus* JN592723–729 UK
*Cx. perexiguus**	7	4	0.005	KJ012103–109	None
Cx. pipiens	39	1	0	KJ012110–148	100% *Cx. pipiens* JN592737 UK 100% *Cx. pipiens* AM403476 Russia
Cx. pipiens f. *molestus*	13	1	0	KJ012149–161	100% *Cx. pipiens* f. *molestus* AM403492 Russia 100% *Cx. pipiens* f. *molestus* FN395171 Russia
Cx. quinquefasciatus	12	1	0	KJ012162–173	100% *Cx. quinquefasciatus* AY729977 India 100% *Cx. quinquefasciatus* CQ16576 Uganda
Cx. (*Neo.*) sp. 1*	2	2	0.006	KJ012066-067	96% *Cx. territans* JX259923 USA
*Cx. impudicus**	6	5	0.003 (0–0.005)	KJ012080–086	96% *Cx. territans* JX259927 USA
Cx. theileri	61	24	0.003 (0–0.015)	KJ012174–234	99% *Cx. theileri* FJ210898–900 Iran 99% *Cx. theileri* HE610457–459 Portugal
Cx. torrentium	8	5	0.007 (0–0.015)	KJ012235–242	100% *Cx. torrentium* JQ253809 UK 100% *Cx. torrentium* HM008672 Germany
Cx. tritaeniorhynchus	8	7	0.010 (0–0.023)	KJ012243–250	99% *Cx. tritaeniorhynchus* AB738194 Japan 99% *Cx. tritaeniorhynchus* AB738269 Japan

Table 2
Mean intra- and interspecific genetic distances (calculated using K-2P algorithm (Kimura, 1980). Highest mean interspecific genetic distances are underlined and in bold. Mean genetic distances of less than 2% are highlighted in bold.

SPECIES	*n*=	SP1												
Cx. (*Neo.*) sp. 1	**2**	**0.006**	**HOR**											
Cx. hortensis	**13**	0.108	**0.007**	**IMP**										
Cx. impudicus	**6**	0.063	0.108	**0.003**	**LAT**									
Cx, laticinctus	**9**	0.120	0.110	0.126	**0.000**	**MIM**								
Cx, mimeticus	**6**	0.120	0.110	0.124	0.099	**0.000**	**MOD**							
Cx. modestus	**1**	0.126	0.117	0.133	0.084	0.082	**n/c**	**PER**						
Cx. perexiguus	**7**	0.132	0.097	0.123	0.081	0.077	0.083	**0.005**	**PIP**					
Cx. pipiens	**39**	0.126	0.110	0.137	0.074	0.075	0.057	0.086	**0.000**	**PMO**				
Cx. pipiens f. *molestus*	**13**	0.125	0.109	0.135	0.075	0.073	0.058	0.084	**0.002**	**0.000**	**QUI**			
Cx. quinquefasciatus	**12**	0.125	0.112	**0.139**	0.075	0.077	0.058	0.088	**0.002**	**0.003**	**0.000**	**THE**		
Cx. theileri	**61**	0.115	0.103	0.119	0.027	0.084	0.071	0.071	0.064	0.062	0.065	**0.003**	**TOR**	
Cx. torrentium	**8**	0.126	0.107	0.132	0.066	0.069	0.067	0.085	0.030	0.029	0.032	0.058	**0.007**	**TRI**
Cx. tritaeniorhynchus	**8**	0.138	0.125	**0.139**	0.096	0.089	0.089	0.096	0.073	0.072	0.075	0.083	0.080	**0.008**

Discounting the exceptionally low mean interspecific distances in *Cx. pipiens*, *Cx. pipiens* f. *molestus* and *Cx. quinquefasciatus* (0.002–0.003) discussed above, mean interspecific distances ranged from 2.9% (*Cx. torrentium*–*Cx. pipiens* f. *molestus*) through to 13.9% (*Cx. impudicus* with both *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus*) (Table 2).

Translation of nucleotide data to amino acids resulted in an alignment of 219 amino acids, five (2.3%) of which were found to be variable overall. Non-synonymous amino acid changes were *V*/*I*, *L*/*I*, *M*/*L*, *V*/*I* and *I*/*V* at AA bases 13, 19, 101, 135 and 153, respectively. Protein sequences were fixed within species, and sometimes even between species; all specimens of *Cx.* (*Neo.*) sp. 1, *Cx. hortensis*, *Cx. laticinctus* and *Cx. theileri* share identical AA sequences, as do *Cx. pipiens*, *Cx. pipiens* f. *molestus* and *Cx. quinquefasciatus*. DNA barcode sequences are presented for *Cx. hortensis*, *Cx. impudicus*, *Cx. laticinctus*, *Cx. perexiguus* and *Cx.* (*Neo.*) sp. 1 for the first time (Table 1).

4. Discussion

It is evident from our studies that the application of DNA to baseline faunal surveys prior to undertaking large-scale arbovirus screening programmes is a fruitful and prudent first step. Only by knowing the threat can its magnitude be fully understood and appropriate vector control actions taken. Mosquito research in Turkey is highly active and the available species list was believed to be comprehensive (Alten et al., 2000; Ramsdale et al., 2001), yet we determined the presence of three more species (*Cx. quinquefasciatus*, *Cx. impudicus*, *Cx.* (*Neo.*) sp. 1), and confirmed the urban variant of *Cx. pipiens*, i.e. form *molestus* (see Table 3).

Accurate identification of members of the Pipiens Group has long been problematic, and the gold standard to identify *Cx. pipiens*, *Cx. pipiens* f. *molestus* and *Cx. quinquefasciatus* in North America is based on a microsatellite assay that exploits the length variation of the TG di-nucleotide repeat of the CQ11 locus (Bahnck and Fonseca, 2006). However comparing of CQ11 results with *COI* sequences, Danabalan et al. (2012) showed that the often sympatric occurrence of the closely related *Cx. torrentium* renders this assay unusable in Europe, as *Cx. torrentium* samples return the same purported species-diagnostic size bands as *Cx. pipiens*, *Cx. pipiens* f. *molestus* and *pipiens x* f. *molestus* hybrids. They reported the same fixed differentiation bases between *Cx. pipiens* and *Cx. pipiens* f. *molestus* as reported here, and that used in the RFLP assay of Shaikevich (2007). Genetic differences (0.2–0.3% sequence differences) between *Cx. quinquefasciatus*, *Cx. pipiens* and its recognised form *molestus* are almost 10% lower than most would accept as defining separate species, however, they maintain genetic integrity despite geographic distance. Whether these taxa truly represent different species is beyond the scope of this paper, but these entities have differing ecologies and vector competencies, which affect their respective vector capacity, making their identification highly important in epidemiological terms.

That *Cx. quinquefasciatus* is present in Turkey is highly significant in terms of arboviral disease transmission, as this species feeds on both birds and mammals and has been widely incriminated as an extremely effective vector of West Nile virus across its distribution. In other parts of its range, *Cx. quinquefasciatus* also acts as a major vector of Japanese encephalitis virus in SE Asia (Kumari et al., 2013), St Louis encephalitis virus in the USA (Rios et al., 2006), and is capable of experimental transmission of *Plasmodium relictum* and *Wuchereria bancrofti* (Calherios et al., 1998; LaPointe et al., 2005). The wide geographical distribution of *Cx. quinquefasciatus* revealed in this study (from Aydın in the West and in İçel, Adana and Kars in the south and east of the country) suggests that it is probably present across much of southern Turkey, where its presence is likely to be masked by that of the closely related and widespread *Cx. pipiens*. Dehghan et al. (2013) reported northerly records of *Cx. quinquefasciatus* in central areas of Iran in sympatry with *Cx. pipiens* in the central regions. Hybrids between *Cx. quinquefasciatus* and *Cx. pipiens* were detected on the Greek island of Kos (Shaikevich and Vinogradova, 2014). All together, these reports may indicate a northerly expansion of the range of *Cx. quinquefasciatus* in the Palaeartic Region.

In Turkey, routine surveys for vector-borne viruses began on human blood donor and veterinary specimens at the beginning of the 1970s. In one of the earliest published studies, 763 sera from southeastern Anatolia were analyzed and 41.80% were found positive for West Nile (WN) virus antibodies (Meço, 1977). During an extensive survey across Anatolia, WN neutralizing antibodies were detected from a wide range of mammals (dog 37.7%, horse 13.5%, mule 2.5%, cattle 4%, sheep 1%), including humans (20.4%) (Özkul et al., 2005). Although most of these are not recognised as natural hosts for WN, cumulatively they represent a high percentage of all mammals in Turkey and, if non-viremic transmission is a significant factor in mosquito-borne virus transmission, Özkul et al. (2005) argued that common farmyard animals could contribute to virus dispersal. Attempts to isolate the virus from wild-caught mosquitoes in SE Anatolia were negative ($n = 6547$), whereas 29 of 181 (16%) serum samples collected in the same region were found to be WN positive (Özer et al., 2007).

Increasing reports of human WN cases clearly indicates the heightening public health significance of the disease in Turkey (Ergunay et al., 2011; Özkul et al., 2013). Recent studies by our group reported WN positive pools in two regions of Turkey: Thrace in the west and Içel Region in the south of the country. In Thrace, Ergunay et al. (2013) reported *Ochlerotatus caspius* and *Culex pipiens* as WN positive. Seven specimens collected concurrently with the virus-tested mosquitoes were retrospectively identified against reference DNA barcodes from this study and were identified as *Cx. pipiens* [GenBank accessions: KJ012112, KJ012122, KJ012124–25,

Table 3
Annotated checklist of *Culex* species reported from Turkey detailing reported arbovirus vector capacity for each species.

Subgenus	Species	Virus	Experimental transmission	Field incrimination	Vertical transmission
Barraudius Edwards	*Cx. modestus*	Lednice		Lundström (1994)	
		Tahyna		Lundström (1994)	
		West Nile	Balenghien et al. (2007)		
	Cx. pusillus	None reported			
Culex Linnaeus	*Cx. laticinctus*	None reported			
	Cx. mimeticus Noè	None reported			
	Cx. perexiguus	Barkedji		Kolodziejek et al. (2013)	
		Rift valley fever	Turell et al. (1996)		
		Sindbis		Samina et al. (1986)	
		Usutu		Vazquez et al. (2011)	
		West Nile		Orshan et al. (2008), Samina et al. (1986)	
	Cx. pipiens	Japanese Encephalitis		Johansen et al. (1986)	
		Ockelbo		Francy et al. (1989)	
		Rift valley fever	Turell et al. (1996), Gad *et al.* (1989)		
		Sindbis		Samina et al. (1986)	
		Tahyna		Lundström (1994)	
		West Nile		Orshan et al. (2008), Samina et al. (1986)	
		Usutu		Busquets et al. (2008)	
	Cx. pipiens f. molestus	None reported			
	Cx. quinquefasciatus	Japanese Encephalitis		Do et al. (1994)	
		St. Louis Encephalitis			Flores et al. (2010)
		West Nile			Goddard *et al.* (2003)
	Cx. theileri	Ockelbo		Francy et al. (1989)	
		Rift Valley		Ribeiro et al. (1988)	
		Sindbis	Jupp (1985)	McIntosh et al. (1967)	
		West Nile		McIntosh et al. (1967)	
	Cx. torrentium	Ockelbo	Lundström (1994)		
	Cx. tritaeniorhynchus	Japanese Encephalitis			Rosen et al. (1980)
		Getah		Takashima and Hashimoto (1985)	
		Rift Valley Fever		Jupp et al. (2002)	
		Sindbis		Wills et al. (1985)	
		Tembusu		Pandey et al. (1999)	
Maillotia Theobald	*Cx. deserticola*	None reported			
	Cx. hortensis	None reported			
Neoculex Dyar	*Cx. impudicus*	None reported			
	Cx. martinii	None reported			
	Cx. sp. 1	Unknown			

KJ012145–47]. Although this was useful information, it still did not address the issue of which exact taxa was involved in the transmission of WN in the Thrace Region.

Learning from the previous study (Ergunay et al., 2013), when samples were later collected for West Nile screening in the Içel Region, mosquito legs were removed and stored in ethanol prior to viral screening (Ergunay et al., 2014). In this later study, West Nile virus was detected in one pool of morphologically identified as *Culex pipiens*. DNA barcoding was carried out on seven samples from the WN-positive pool: six were identified as *Cx. quinquefasciatus* [GenBank accessions KJ012162–63, KJ012168–71]. Thus, not only do we now know that *Cx. quinquefasciatus* is in Turkey, we also know it is involved with the transmission of West Nile virus in the country. One of the seven specimens barcoded was misidentified, identified instead as *Cx. perexiguus* [GenBank accession KJ012103], which also plays an important role as a bridge vector of WN (Muñoz et al., 2012). Misidentifications are not unexpected as mosquito identification is carried out rapidly to preserve viral RNA, but can lead to misinformation on vector identification and mismanagement of vector control efforts. This study demonstrates the value of retrospective DNA barcoding on voucher tissue from individuals pooled for viral screening and we advocate the utility of DNA barcoding for accurate vector incrimination in future. Further intensive arboviral studies are planned for the 2014 season in southern Turkey, and this methodology will be adopted to optimise vector identification and incrimination.

With the exception of *Cx.* (*Bar.*) *modestus*, all potential arbovirus vectors in Turkey belong to *Culex* subgenus *Culex* (Table 3). Along with *Cx. pipiens*, *Culex modestus* is widely accepted as a most effective vector of West Nile virus in Europe (Hannoun et al., 1964; Mouchet et al., 1970; Balenghien et al., 2006; Balenghien et al., 2007), due in no small part to its abundance and opportunistic feeding habits on both birds and mammals (Hubálek and Halouzka, 1999). Despite reports of the perceived spread of *Cx. modestus* in central Europe (Votýpka et al., 2008) and its high density in the Czech Republic (Votýpka et al., 2008), France (Balenghien et al., 2006) and in neighbouring Greece (Chaskopoulou et al., 2013), *Cx. modestus* seems quite rare in Turkey, with only one verified specimen collected in Çankiri, despite significant collection efforts spanning several years and many provinces. Thus, unless locally abundant in regions as yet unsampled, it seems highly unlikely that *Cx. modestus* will play a significant role in arbovirus transmission in Turkey. It also seems unlikely that *Culex hortensis* will be involved in arboviral transmission, due to its feeding preferences on reptiles (e.g. lizards) and amphibians (Snow, 1990; Roiz et al., 2012).

Integration of DNA sequence data into incrimination studies is extremely helpful. For instance, through correlation of our DNA sequences, we can be sure that the Turkish *Cx. theileri* is the same as that incriminated as a vector of *Dirofilaria immitis* (dog heartworm) in Iran [GenBank FJ210898-900] (Azari-Hamidian et al., 2009), and from four pools of which an new insect-specific flavivirus was isolated in southern Portugal [GenBank HE610457–459] (Parreira et al., 2012). As well as *Cx. theileri*, *Cx. modestus*, *Cx. pipiens* and *Cx. tritaeniorhynchus* are all reportedly involved in the transmission of dirofilariasis in other countries (Ludham et al., 1970; Gutsevich et al., 1974; Rossi et al., 1999; Santa-Ana et al., 2006).

Culex tritaeniorhynchus is a competent vector of a wide variety of arboviruses especially in SE Asia (Table 3), and has also been found naturally infected with *Wuchereria bancrofti* and *Brugia malayi* (Takashima et al., 1983a,b). One COI sequence from one of the *Cx. tritaeniorhynchus* in our dataset showed high genetic differentiation (2.3%) from all others (Table 2). This was somewhat

surprising given that all *Cx. tritaeniorhynchus* were taken from the same collection in Doğankent, Adana. Deep genetic divergences like this at the subspecific level can reflect incipient speciation, and can impact the vector competences of given strains (McKeon et al., 2010). The specific status of *Cx. tritaeniorhynchus* warrants further investigation across its extensive range, stretching from Greece in the west to Sri Lanka in the south and Vietnam in the east. There are several misidentified *COI* sequences in GenBank. For example AY917215, presented in GenBank as *Cx. tritaeniorhynchus* (Pradeep Kumar et al., 2007) is actually *Cx. pseudovishnui*, sharing 100% identity with link-reared vouchers in the MBI database on BOLD, and 99% and 98% sequence identity with *Cx. pseudovishnui* HM769283 and HM769284 (Pradeep Kumar et al., direct submissions 2010), and HM769282 (Pradeep Kumar et al., direct submission 2010) and AY834248 (Pradeep Kumar et al., 2007), respectively. It would appear that GenBank entry JQ728350 (labelled as *Cx. tritaeniorhynchus*) is also misidentified, sharing 98% identity with *Cx. vishnui* [GenBank AB738303, AB738092, AB738094 and AB738246]. GenBank entry AB69084 (labelled as *Cx. vishnui*, Japan) is also misidentified, showing closest homology (98%) with *Cx. pseudovishnui* [GenBank AB738303, AB690844, AB738092, AB738094 and AB738246].

Using DNA barcodes, we were able to categorically determine that the species formerly identified as *Cx. territans* in Turkey comprises two separate species, neither of which are *Cx. territans* (96% sequence similarity). *Culex territans* has been removed from the species list of Turkey (Table 3). It seems likely that, as with *Cx. apicalis* (Knight and Stone, 1977), the distribution of *Cx. territans* is restricted to the Nearctic and that species referred to as *Cx. territans* in Europe should more correctly be referred to as the Territans Group (see Table 3). da Cunha Ramos et al. (2003) determined that specimens in southern Portugal comprised a new species (*Cx. europaeus*) and suggested that the European species previously identified as *Cx. territans* is most likely to correspond to *Cx. europaeus*, but warned that several other species, e.g. *Cx. impudicus*, *Cx. judaicus*, *Cx. martinii* and *Cx. rubensis*, could easily be confused. Whereas one of the two species has here been confirmed as *Cx. impudicus* by direct comparison to an archive specimen (held in the BMNH, London), the identity of the remaining species (*Cx.* (*Neo.*) sp. 1) remains unclear. Although this could still be *Cx. europaeus*, all of the *Cx.* (*Neo.*) tested in the present study were collected in Adana, which is the type locality of *Cx. martinii*. Characteristics of the male genitalia are considered the most reliable method for identifying species of *Neoculex* (Bohart, 1948; Bickley and Harrison, 1989). Only when comparable DNA barcodes are generated from samples that have been verified by male genitalia preps can the true identity of *Cx.* (*Neo.*) sp. 1 be resolved.

Herein we show that including DNA barcoding in baseline faunal surveys reveals more species than by morphology alone. Our limited study on the *Culex* of Turkey, clarified the identities of *Cx. pipiens* and *Cx. territans*, adding four species to the Turkish faunal list. This included the previously undetected presence of *Cx. quinquefasciatus*, a highly efficient arboviral vector. Given that we now have quality reference barcode sequences, retrospective vector incrimination by DNA will be much more accurate, even in the absence of voucher specimens. We advocate the use of integrated faunal baseline surveys as precursors to establishing successful mosquito and arbovirus surveillance programmes in future.

Authors' contributions

The study was designed by YML, FG, FS, AA and BA. Specimens were collected by FG, BA, FS, AA and YML and sequences generated by FG, YML, BA and FS. The data were analysed and figures produced by YML and FG. The manuscript was drafted by YML and FG, and improved by BA and FS. All authors agree to the findings in this publication and hereby declare no conflict of interest. Funders of this study had no role in the design, collection, analysis or interpretation of the results, nor in the writing of this report or the decision to publish it.

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