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Molecular approaches for blood meal analysis and species identification of mosquitoes (Insecta: Diptera: Culicidae) in rural locations in southern England, United Kingdom

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Abstract

Thirty-four species of Culicidae are present in the UK, of which 15 have been implicated as potential vectors of arthropod-borne viruses such as West Nile virus. Identification of mosquito feeding preferences is paramount to the understanding of vector-host-pathogen interactions which, in turn, would assist in the control of disease outbreaks. Results are presented on the application of DNA barcoding for vertebrate species identification in blood-fed female mosquitoes in rural locations. Blood-fed females (n = 134) were collected in southern England from rural sites and identified based on morphological criteria. Blood meals from 59 specimens (44%) were identified as feeding on eight hosts: European rabbit, cow, human, barn swallow, dog, great tit, magpie and blackbird. Analysis of the cytochrome *c* oxidase subunit I mtDNA barcoding region and the internal transcribed spacer 2 rDNA region of the specimens morphologically identified as *Anopheles maculipennis s.l.* revealed the presence of *An. atroparvus* and *An. messeae*. A similar analysis of specimens morphologically identified as *Culex pipiens/Cx. torrentium* showed all specimens to be *Cx. pipiens* (typical form). This study demonstrates the importance of using molecular techniques to support species-level identification in blood-fed mosquitoes to maximize the information obtained in studies investigating host feeding patterns.

Key words. Blood meals, mosquitoes, cytochrome *c* oxidase I, internal transcribed spacer 2, DNA barcoding, southern England, United Kingdom

Introduction

Mosquitoes transmit numerous pathogens which are responsible for numerous zoonotic diseases and a driver of numerous emerging infectious diseases around the world (Schaffner *et al.*, 2013; Petersen *et al*., 2016). Correct identification of mosquito vectors is critical to defining pathogen transmission pathways and is the first step in preventing pathogen transmission. Studies focused on determining the host selection and host feeding preferences of mosquitoes and other haematophagous arthropods, via the identification of their blood meals, have become a vital component in understanding the dynamics of vector-host-pathogen interactions (Gariepy *et al.*, 2002; Mukabana *et al*., 2002; Kent & Norris 2005; Kent, 2009; Schönenberger *et al*., 2016). Identifying the host-feeding preferences of vector species reveals the vertebrate species at risk of arthropod-borne pathogen transmission and

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the mechanism of spread of disease within an environment (Alcaidae *et al.*, 2009; Chaves *et al.*, 2010; Brugman *et al*., 2015). Systematic characterisation of bird and mammalian host genetics, in particular, has increased the specificity of studies driven by the use of molecular techniques. These techniques have largely replaced serological methods for blood-meal identification, such as the precipitin test and enzyme-linked immunosorbent assays (ELISA) (Kent, 2009). The high copy number and conserved nature of mitochondrial genes such as cytochrome *b* and cytochrome *c* oxidase subunit I (COI) have made them popular targets for species identification applied to host-feeding preference studies (Muñoz *et al*., 2012). However, not all mosquito species groups can be differentiated using these genes alone (Laurito *et al*., 2013) and other DNA regions are used to provide greater resolution between closely related species.

One of the main obstacles in host-preference studies is the accurate identification of vector species. This is for several reasons: the morphological homogeneity in certain life stages among many species; the increasing lack of taxonomic expertise; the presence of species complexes; and because of the damage of important diagnostic characteristics upon which identification keys are based (Cranston *et al*., 1987; Hernández-Triana *et al.*, 2012; 2014; Murugan *et al.*, 2015). To overcome these impediments, sequencing of the COI mtDNA barcode region of organisms, particularly mosquitoes, is now widely used (Hebert *et al*., 2003a,b; Cywinska *et al.*, 2006; Kumar *et al.*, 2007; Gunay *et al.*, 2015; Versteirt *et al.*, 2015). In addition, ribosomal genes such as the internal transcribed spacer (ITS2) sequence of ribosomal DNA is also used in identification of mosquito species, either independently or in combination with COI (Linton *et al.*, 2001, 2005; Danabalan *et al*., 2012; Khoshdel-Nezamiha *et al.*, 2016).

In the United Kingdom (UK), early identifications of mosquito blood meals were achieved using the precipitin assay (Service, 1971). More recently, Danabalan *et al.* (2014) used a multiplex primer assay to identify host species at three sites and identified seven species (human, deer, goat, horse, cow and dog) fed on by *Anopheles maculipennis s.l*. A key disadvantage of using these targeted approaches was the introduction of a selection bias towards particular species and, therefore, the inability to detect non-target hosts. For example, Danabalan *et al.* (2014) were only able to identify the vertebrate host in 138/242 (57%) of blood-fed specimens. Whilst some of these unknown feeds can likely be attributed to non-viable DNA sequences due to digestion, as well as assay failure at either the PCR or sequencing stages, it is probable that several of the specimens contained viable blood from non-target hosts. In recognition of these challenges, subsequent UK studies have used a different approach based on a cocktail of universal primers followed by sequencing, originally developed by Ivanova *et al.* (2007). This approach enabled the un-biased identification of the blood meal hosts of members of the *Anopheles maculipennis* complex (Brugman *et al.*, 2015) and *Culiseta annulata* (Fernández de Marco *et al.*, 2016). Identification of hosts in turn facilitated targeted screening for epidemiologically relevant pathogens (xenosurveillance), resulting in the detection of myxoma virus in rabbit-fed and *Theileria orientalis* in cattle-fed mosquitoes, respectively.

The aims of the present study were twofold. The first was to identify vertebrate blood-feeding hosts of mosquitoes collected from a wider range of field sites than previously trialled. The second was to ascertain whether the parallel sequencing of two gene regions, COI and ITS2, was sufficient to support the morphological identification of the collected mosquitoes to species level. Collections were conducted in five field sites in southern England considered to be at risk of the introduction of exotic viruses of human and veterinary importance (Bessell *et al.*, 2014).

Material and methods

Collection and morphological identification of blood-fed mosquitoes. Five rural sites in southern England were selected for mosquito collections: four farms and one residential village. These were: Church Farm, Oxfordshire (51.715807, -1.38081); Northney Farm, Hampshire (50.828166, -0.962151); Elmley Nature Reserve, Kent (51.377587, 0.783954); White Lodge, Surrey (51.322255, -0.637692), and Frimley, Surrey (51.316221; -0.743290). Mosquitoes were collected between June and September 2013 using mouth aspirators (John W. Hock, Gainesville, Florida, USA) from buildings found at the various locations to target the resting, blood-fed females in a 10–20 m radius around presumed larval habitats. Sweep netting of vegetation was used in some locations to capture outdoor-resting females. Blood-fed mosquitoes were placed in a vessel containing dry ice for transportation to the laboratory, at which point they were stored at –20°C until processing. All specimens were identified to

species level based on morphological features using the keys of Cranston *et al.* (1987) and Snow (1990). *Culex pipiens* has two ecological forms, *Cx. pipiens* typical form and *Cx. pipiens* form *molestus*, which are morphologically indistinguishable, sympatric and can hybridize along their distribution range. Both forms and hybrids are present in the UK (Manley *et al*., 2015).

DNA extraction from mosquito abdomens. DNA was extracted from engorged mosquito abdomens following the protocols of Brugman *et al.* (2015). In brief, abdomens were separated from the rest of the body on a chilled plate using forceps and placed into individual 1.5 mL Eppendorf tubes containing 200 µl of phosphate buffered saline (PBS). The abdomens were then pressed against the wall of the tube using the forceps to release the blood meal. The remainder of the body of each mosquito was stored at –20°C for morphological reference. Forceps were cleaned between specimens handling using a three-stage wash to avoid cross-contamination. Washing consisted of a first wash of 5% Decon (Decon Laboratories Ltd, Hove, UK), a second of 100% ethanol and a third of sterile water, at which point all excess liquid was wiped off with task wipes (Kimtech Science, Georgia, USA). Each sample was incubated with 20 µl proteinase K and 200 µl buffer AL (QIAgen, Manchester, UK) for 30 min at 56°C in a water bath. DNA extraction was carried out using the DNeasy Blood and Tissue Kit (QIAgen, Manchester, UK), following the manufacturer's spin column-protocol. All DNA extractions were stored at 4°C until processing.

Identification of blood meal host. Vertebrate host species were identified in the blood meals using a vertebrate-specific, M13-tailed, triple primer cocktail (VF1_t1 + VF1d_t1 + VF1i_t1 / VR1_t1 +VR1d_t1 + VD1i_t1) targeting a 685 base pairs (bp) sequence of the COI gene (Ivanova *et al.*, 2007; Brugman *et al.*, 2015). This primer combination was expected to amplify DNA of all vertebrate species. Reaction contents were obtained from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK) unless otherwise stated. The final PCR reaction mix of 50 µL consisted of: 31 µL H_2O, 5 µl GeneAmp 10X PCR buffer I (Applied Biosystems, Life Technologies Ltd, Paisley, UK), 1 µL dNTPs (at 0.2 pmol/µL), 1 µL of each set of primers (three forward and three reverse at 10 pmol/µL, total of primers 6 µL), 0.3 µL AmpliTaq Gold DNA Polymerase (10 units/ µL) (Applied Biosystems, Life Technologies Ltd, Paisley, UK), 0.7 µL dimethyl sulfoxide (DMSO), 1 µ*L* tetramethylammonium chloride (TMAC), and 5 µL of extracted DNA. The thermal profile consisted of an initial denaturation step at 94°C for 10 min, followed by 40 cycles of 94°C for 30 s, 535C for 30 secsonds, 72°C for 1 min, followed by a final elongation step of 72°C for 10 min. PCR products were separated on a 1.5% agarose gel, and samples producing an amplicon of the correct size were sequenced. Sequencing was performed using M13 primers at 1pmol/µL as in Brugman *et al.* (2015). Amplification products were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All sequences were edited using Lasergene version 12.1 (DNASTAR, Inc., Madison, Wisconsin, USA) and assigned to a particular vertebrate species when agreement was ≥98 % with sequences of named species in GenBank.

Molecular identification of mosquito species. Due to of the morphological similarity of several mosquito species, we used the COI barcoding approach to supplement and support morphological identification. In this case, the same DNA extraction from the fully engorged abdomen was used for the molecular identification of each specimen. We used the COI barcoding approach as in Hebert *et al.* (2003a,b) and followed the Canadian Centre for DNA Barcoding (CCDB) protocols (www.dnabarcoding.ca). In brief, PCR primers were those developed by Folmer *et al.* (1994) (LCO1498 and HCO2190), which are considered the standard for the amplification of the 658 bp region located at the 5' end of the COI gene (Hebert *et al.*, 2003a,b). PCR products were obtained using a QIAgen PCR system using the following reaction mix, final volume 48 µL: 25 µL H_2O, 5 µL NH_4, 5 µL of dNTPs (2 pmol/µL), 2.5 µL of $MgCl_2$ (25 pmol), 0.1 µL Bioline Taq Polymerase (Bioline Reagents Ltd, London, UK), 5 µL of each forward and reverse primers (each at 10 pmol/µL), and 0.38 µL of BSA (bovine serum albumin) (20 mg/ml), to which 2 µL of DNA templates was added. The thermal profile consisted of an initial denaturation step at 94°C for 1 min, 5 cycles of pre-amplification of 94°C for 1 min, 45°C for 1.5 min, 72°C for 1.5 min, followed by 35 cycles of amplification: 94°C for 1 min, 57°C for 1.5 min and 72°C for 1 min, followed by a final elongation step of 72°C for 5 min.

For species-level identification of *Anopheles maculipennis s.l.* and *Culex pipiens*, we employed primers 5.8SF and 28SR (Collins & Paskewitz, 1995) to amplify a 435 bp region of ITS2 (Linton *et al.*, 2001, 2005; Danabalan *et al.*, 2012, 2014) using SYBRGreen JumpStart ReadyMix for a real time PCR system. The reaction mix contained 2 µL of DNA template, 14 µL H_2O, 20 µL SYBRGreen JumpStart *Taq*-ReadyMix (Sigma-Aldrich) and 2 µL of each forward and revised primers (each at 10 pmol/ µL) in a final volume of 40 µL. The thermal profile consisted of an

initial denaturation step at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, followed by a final elongation step of 72°C for 10 min. Reactions were amplified in a Mx3000P real-time PCR system (Stratagene, Agilent Technologies, Cheshire, UK). All PCR products were visualized on a 1.5% agarose gel, and samples showing bands of the correct size were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All sequences were assigned to a particular mosquito species when agreement was ≥98% with sequences of named species in GenBank.

Detailed specimen records and sequence information (including traces files) were uploaded to the Barcode of Life Data Systems (BOLD, www.boldsystems.org) and can be found within the Working Group 1.4 Initiative "Human Pathogens and Zoonoses": "Barcoding mosquitoes blood meals, UK-2013 [DS-MBM13]". The Digital Object Identifier (DOI) for the publically available data set is dx.doi.org/10.5883/DS-MBM13. All sequences have been submitted to GenBank (accession numbers: KU876944 to KU877023). All voucher specimens are housed at the Animal and Plant Health Agency, United Kingdom.

Sequence analysis. Paired bi-directional sequence traces for COI or ITS2 were combined to produce a single consensus sequence. All sequences were analysed in MEGA v.6 (Tamura *et al.*, 2013). Neighbour Joining (NJ) analysis was carried out using the K2P distance metric to represent their clustering pattern; bootstrap values were calculated to test the robustness of the phenogram and were obtained by conducting 1000 pseudoreplicates (Hernández-Triana *et al.*, 2014).

Results

In total, 134 blood-fed specimens were collected at Church Farm, Northney Farm, Elmley, White Lodge and Frimley (see Table 1). Most of the specimens were identified as belonging to *An. maculipennis s.l.* (n = 95, 49%), although representatives from eight additional species were also collected (*n* = 39).

TABLE 1. Mosquito species (identified by morphology or COI sequence analysis) and number of blood-fed specimens collected in southern England in 2013.

Mosquito species	No. of blood-fed specimens (*n*)	Specimen identifications based on COI sequence
Anopheles maculipennis s.l. [1, 2]	95	39[1]/6[2]
Culex modestus	1	1
Culex pipiens/Culex torrentium	8	6[3]
Culiseta annulata	6	4
Ochlerotatus cantans/Oc. annulipes	2	2[4]
Ochlerotatus detritus	13	13
Ochlerotatus nr. *flavescens*	2	2
Ochlerotatus punctor	1[5]	0
Coquillettidia richiardii	6	6
Total	134	79

[1]*An. atroparvus*; [2]*An. messeae*; [3]*Cx. pipiens* typical form; [4]*Oc. cantans*; [5]Specimen subsequently identified as *Oc. rusticus* by COI sequence (see also Table 2 for species identification).

For the analysis of the blood meals, we obtained sequences from 59 specimens (44% sequencing success). These results revealed that most mosquito species fed on mammalian species, primarily the European rabbit (*Oryctolagus cuniculus*), cow (*Bos taurus*) and human (*Homo sapiens*) (Table 2). The exception was *Cx. pipiens* that fed on birds including barn swallow (*Hirundu rustica*), great tit (*Parus major*), blackbird (*Turdus merula*) and magpie (*Pica pica*).

TABLE 2. Mosquito species, vertebrate hosts and numbers of specimens identified after molecular analysis of the same DNA extract were applied using the COI barcoding region and ITS2 in blood-fed specimens collected in southern England, 2013.

Mosquito species	Vertebrate hosts of blood meal	Number of specimens (*n*)
*Anopheles atroparvus****	European rabbit	33
	Cow	3
*Anopheles messeae****	Cow	6
	Dog	1
Culex pipiens typical form***	Barn Swallow	3*
	Great Tit	1
	Blackbird	1
	Magpie	1
Culex modestus	Human	1
Coquillettidia richiardii	Human	1
Culiseta annulata	Cow	1
Ochlerotatus nr. *cantans*	Cow	1
	Human	1
Ochlerotatus detritus	Human	4
	Cow	1
Ochlerotatus nr. *flavescens*	[PCR failed]	_
*Ochlerotatus rusticus***	[PCR failed]	_
Total		59

*One specimen was previously mis-identified as *An. maculipennis s.l.*; **recorded previously as *Oc. punctor* (see Table 1); ***species identification also confirmed by ITS2 (see also Table 1).

We obtained 79 COI barcodes for mosquitoes, representing 59% of the collected specimens. The remaining samples failed to amplify in spite of several re-amplification attempts. NJ analysis of the 82 barcode sequences separated them into 10 discrete clusters (Fig. 1), and revealed some misidentifications based on morphological characters. For example, one specimen morphologically identified as *Ochlerotatus punctor* (Table 1) matched *Oc. rusticus* (Table 2). This methodology was also able to identify specimens that were initially grouped together due to similar morphology such as *Oc. cantans/Oc. annulipes*, which were later confirmed to be *Oc. cantans* (see Table 2). In addition, all specimens morphologically identified as *Cx. pipiens / Cx. torrentium* were grouped (with 100% bootstrapped values) to *Cx. pipiens*. Further molecular analysis carried out by Brugman (2015) using the duplex PCR assay to separate the two ecoforms of *Culex pipiens*, based on the CQ11 microsatellite locus (Bahnck & Fonseca, 2006; Fonseca *et al*., 1998), ruled out the presence of *Cx. pipiens* form *molestus* in the samples we processed. Specimens of *An. maculipennis s.l.* were separated into two discrete species, *An. atroparvus* and *An. messeae*, both with 99–100% confidence. COI BLAST searches for specimens identified as *An. messeae* did not retrieve matches with *An. daciae* in our data set. To date, *An. atroparvus* is regarded as being primarily mammalophilic (Danabalan *et al.*, 2014); thus, we re-examined one damaged specimen identified as this species that had fed on a barn swallow (Table 2), which had grouped together with specimens of *Cx. pipiens* (100% GenBank match). Based on its morphology, and molecular data, it proved to be *Cx. pipiens*. In general, levels of sequence divergence were variable across the taxa; conspecific individuals exhibited zero or very low divergence values. Mean intraspecific divergence ranged from 0–0.8%, while mean interspecific divergence ranged from 2.4–18.9%.

In a parallel analysis, DNA samples were also processed using ITS2 sequencing of taxa where the presence of species complexes are known, in this case *An. maculipennis s.l.* Analysis of the ITS2 sequences confirmed that 33 specimens feeding on European rabbit and three feeding on cow were *An. atroparvus* (38% of identification success) (GenBank accession number AY365007) (Table 2). In addition, six specimens feeding on cow and one feeding on dog (*Canis familiaris*) were *An. messeae* (Genbank accession number AY238412). ITS2 BLAST searches for specimens identified as *An. messeae* did not retrieve matches with *An. daciae* in our data set.

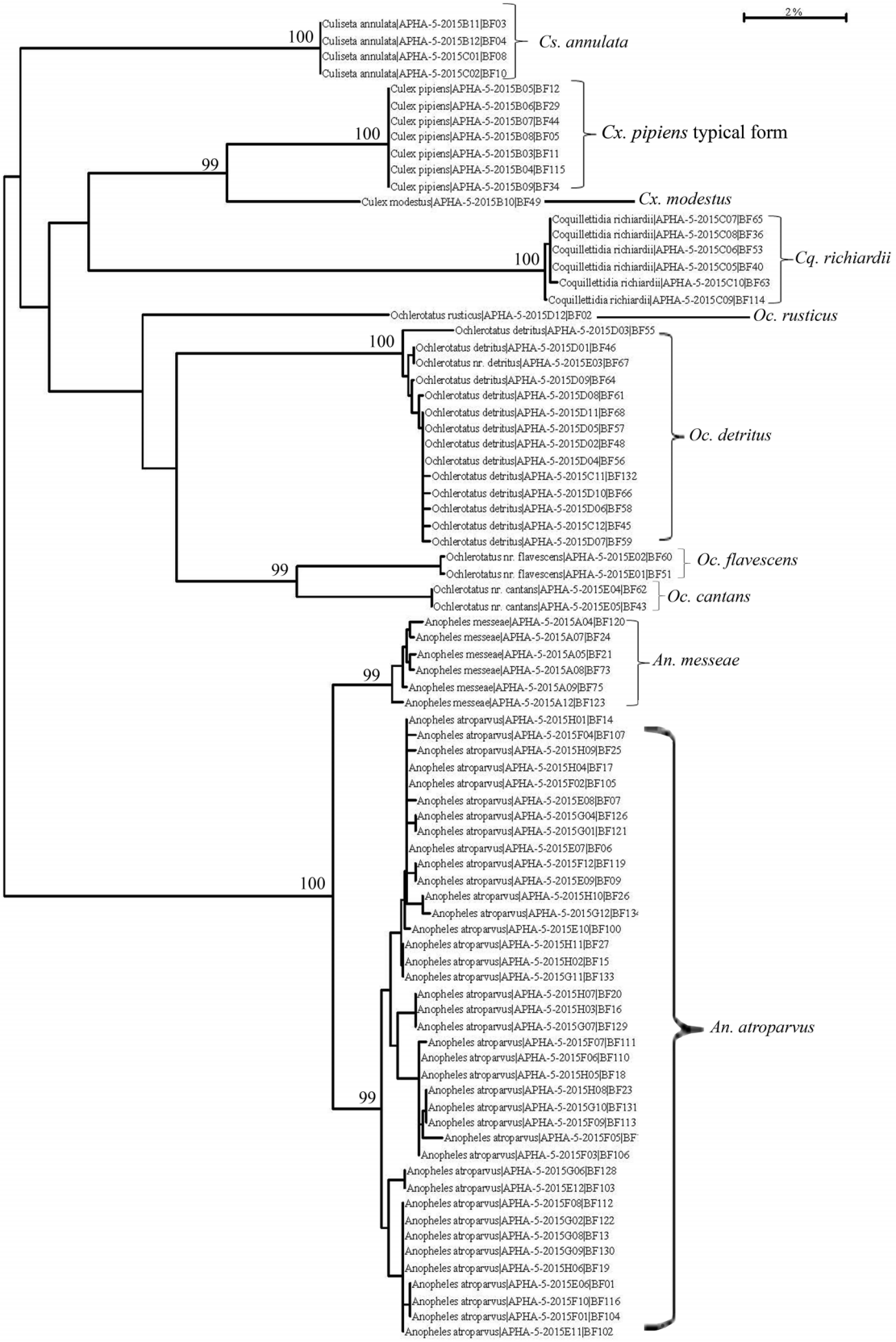

FIGURE 1. Neighbor Joining (NJ) tree of full-length barcodes (658 bp) for species of mosquitoes. A divergence of >2% is indicative of separate operational taxonomic units. Each specimen is labelled with a species name based on its molecular identification. BF refers to the alphanumeric number given to the blood-fed samples. Bootstrap values higher than 80% are only shown in the tree on each node.

BLAST searches on samples of *Cx. pipiens* feeding on birds (see Table 2) confirmed that all specimens shared greater than 98% identity with published sequences of typical *Cx. pipiens* (GenBank accession number HQ881674).

Discussion

This study further confirms that a single DNA extract from the abdomen of a blood-fed mosquito can be successfully used for different purposes in a sequential workflow as proposed by Brugman (2015) and Brugman *et al.* (2015): (1) identification of the vertebrate origin of a blood meal by sequencing the COI barcode region and (2) the molecular identification of mosquito species by sequencing the COI barcode region and ITS2. Our success in determining the blood meal source was much lower than that of Brugman *et al.* (2015) and other large-scale studies in Germany (Börstler *et al.*, 2016), and the reasons for these results are difficult to explain. It may be due to poor performance of the blood meal identification assay we employed at the PCR stage, which could still require further optimization with continuous attempts to amplify those samples that failed to amplify. In addition, the quality of the DNA extracted from the blood meal could have been low due to the degradation of the blood within the specimens prior to collection. Nevertheless, this approach can refine or correct identifications based on morphological criteria. This study also affirms the need to maximise data obtained from a single blood-fed specimen, because collecting large numbers of blood-fed mosquitoes is challenging using currently available methods. Ideally, future studies will focus on collecting larger numbers of blood-fed individuals to compensate for the relatively low PCR success rate, when amplifying degraded host DNA. In contrast with similar studies in tropical areas (Tchouassi *et al.*, 2016), the relatively low numbers of blood-fed specimens obtained in this study reflects the low probability of catching engorged females in one field season in southern England.

The successful identification of blood meal hosts decreases rapidly with time as digestion within the insect takes place (Tuten *et al*., 2012; Martínez de la Puente *et al.*, 2013). Whilst every effort was made to keep blood-fed mosquitoes chilled immediately after capture, the time between the mosquito taking the blood meal and capture was impossible to control and is a likely contributor to the low success rate. The difficulties in amplifying highly degraded DNA can perhaps be overcome by employing high-throughput sequencing, which is often more sensitive for this purpose compared to conventional Sanger-based sequencing (Prosser *et al.*, 2016). However, even though this can now be outsourced to private companies or other institutes at competitive rates (see Lee *et al.*, 2015), it is still expensive and requires novel and costly computing programmes for data analyses. The use of DNA metabarcoding (the simultaneous sequencing of the barcode region from multiple samples) is also proving useful in overcoming the aforementioned issue by allowing the identification of multiple species from a single bulk sample, for example Kocher *et al.* (2016). Nonetheless, the approach described here could pave the way for the development of larger studies that integrate morphological and molecular approaches, and which would facilitate the detection of pathogens in mosquito blood meals (xenosurveillance) that are of veterinary/medical importance in the UK. Similar host preference studies in Germany and Switzerland have proved useful in identifying feeding patterns of mosquitoes and highlighted potential bridge vector species (Börstler *et al*., 2016; Schönenberger *et al*., 2016). The concomitant identification of both the mosquito species and its blood meal host also allows for the targeted selection of samples for further analysis of potentially cryptic species. In this case, the standard COI barcoding approach was used in combination with the ITS2 nuclear marker to increase taxonomic resolution in cases where COI alone was insufficient. Alternative molecular assays could also be performed on the same DNA extract to further delineate species in cases with extreme phylogenetic complexity, for example to separate hybrid forms of the *Culex pipiens* complex (*e.g.* Rudolf *et al.*, 2007; Shaikevich, 2007; Smith & Fonseca, 2004).

DNA barcoding is widely used in the identification of mosquito species (Cywinska *et al.*, 2006; Kumar *et al.*, 2007; Gunay *et al.*, 2014; Versteirt *et al.*, 2015), as well as biting flies and other organisms, including plants (Hebert *et al.*, 2003a,b). The approach presented here proved very useful in supporting the morphological identifications of the blood-fed females across collection sites. Indeed, this molecular support is often necessary, as mosquitoes collected in traps frequently become damaged, resulting in the loss of key diagnostic features (Cranston *et al.*, 1987; Murugan *et al.*, 2015). Moreover, the large numbers of specimens of different species that are found in traps can make the identification of all specimens a difficult exercise (Murugan *et al.*, 2015), particularly in the absence of appropriate taxonomic expertise. The advantage of adopting an integrative approach for mosquito

species identification is evidenced in this study by the detection of morphology-based errors in some cases and the verification of morphological identifications in other cases (see Table 1). The values for intraspecific and interspecific genetic divergence within and between species were expected, as most of the specimens originated from a single locality, and reflect the ranges obtained in other studies, for example Cywinska *et al.* (2006) and Linton *et al.* (2005).

Mosquitoes living within the same rural ecosystem in the United Kingdom feed on different vertebrate hosts despite the presence of the same range of hosts. For example, while *An. atroparvus* fed largely on rabbits, *An. messeae*, *Cs. annulata* and *Oc. detritus* fed mainly on cattle in Kent. Furthermore, the typical form of *Cx. pipiens* from the same site only fed on birds, in agreement with earlier literature on mosquito feeding preferences in England (Cranston *et al.*, 1987), and other regions such as Europe (Börstler, 2016) and the USA (Tuten *et al*., 2012). These differences in feeding behaviour likely result from both variation in host availability within and between locations as well as reflecting a degree of intrinsic host preference. However, it is these differences that will influence the ability of a particular mosquito species to serve as vectors of pathogens of livestock and other animals.

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