DNA barcodes and new primers for nature's pest controllers: the social wasps

Ikechukwu Eugene Onah[1,2*] & Seirian Sumner[2]

[1] Department of Zoology and Environmental Biology, University of Nigeria, 410001 Nsukka, Enugu State Nigeria

[2] Centre for Biodiversity and Environment Research, University College London, London, UK, WC1E 6BT

*Corresponding author: Ikechukwu Eugene Onah, Department of Zoology and Environmental Biology, University of Nigeria, 410001 Nsukka, Enugu State Nigeria. Email: ikechukwu.onah@unn.edu.ng

Abstract

Globally, biodiversity is declining as a result of anthropogenic pressures, and this could lead to extinction of some species before they are discovered. The loss of insect taxa is of prime concern, given recent reports of significant declines in the populations of many taxa across the globe. Efforts to document biodiversity have met with several challenges, amongst which are the difficulties in using morphological features to discriminate species, especially in insects. DNA barcoding is a rapid and reliable method for species identification and discovery, but choosing appropriate primers to amplify the barcode region without coamplifying contaminants remains a key challenge. We developed and tested a set of primers for PCR amplification of the DNA barcode region of the COI gene in polistine wasps. We tested their efficacy in 36 species of vespid wasps, and the solitary wasp *Zethus miniatus* Saussure. Samples were obtained from Africa, Americas, Asia and Europe. The polistine-specific primers successfully amplified the barcode region for all polistines tested, without amplifying any *Wolbachia* present; they also worked with many species from the other Vespidae wasp subfamilies. The new primers are valuable for the discovery and accurate documentation of polistine wasps in the four continents.

Keywords: COI gene, DNA barcoding, social wasps, Vespidae

Introduction

Human activities such as intensification of agriculture, industrialization and urbanization, habitat loss, increased spread of invasive species, pollution, and climate change have caused an increased rate of biodiversity loss (Gascon et al. 2015; Pomerantz et al. 2018) and accelerated the rate of extinction of species (Ceballos et al. 2010; Pimm et al. 2014; Ceballos et al. 2017). The extinction rate is higher in the developing countries of Africa, parts of Asia and Latin America; species continue to decline as habitats are further degraded (UNEP-WCMC 2016). There is concern that many species might go extinct before they are discovered; thus, there is an urgent call to document biodiversity in response to the increased loss of biodiversity and accelerated rate of extinction of species.

Insects have generally been widely highlighted as vulnerable to anthropogenic change (Bidau 2018; Goulson 2019), with declining populations across the globe (Potts et al. 2010; Hallmann et al. 2017; Sieg et al. 2018; Powney et al. 2019; Seibold et al. 2019). The order Hymenoptera, which includes the vespid wasps (Vespidae), is an ecologically important taxon in terrestrial ecosystems whose ecosystem services will be seriously compromised by increased extinction rates (Goulson 2019).

The vespid wasps comprise over 5000 species, including both solitary and social species; these insects are thought to provide important ecosystem services as pollinators of agricultural crops (Hunt et al. 1991; Brodmann et al. 2008; Mello et al. 2011) and natural enemies and regulators of natural arthropod populations and crop pests in agroecosystems (Abd-El-Samie et al. 2018; Prezoto et al. 2019; Southon et al. 2019). However, their impact is largely unstudied, even though they are highly speciose, diverse in form, and distributed worldwide (Nguyen 2007). Given the important role of vespid wasps in ecosystems, and the lack of knowledge about them, there is an urgent need to provide rapid identification tools for vespid wasps in order to document the species and make them more accessible to scientists and agriculturalists for use.

Around 1000 species of vespid wasps are social, living in groups with a (usually) single egg-laying queen and many non-reproductive workers; social wasps may be of particular value for their contributions as natural enemies due to the large number of foraging workers, their generalist diets (Richter 2000) and their abundance in anthropogenically modified landscapes (Zanette et al. 2005). Their roles may be especially important in tropical and developing countries, where they are especially species-rich and ecologically abundant, and where natural methods of pest control are likely to be most valuable (Prezoto et al. 2019; Southon et al. 2019).

Although there are 9,888 barcodes for the family Vespidae in Barcode of Life Data (BOLD), only 578 species out of over 5000 known species in the family are represented as of September 7th, 2020. Hence, most of the polistine wasps (especially in developing countries, where they are most diverse but least studied) lack a DNA barcode in publicly available databases like the BOLD, GenBank, European Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ).

Molecular sequence data complement the use of morphological data in species identification, but sometimes molecular data remain the only reliable method for rapid identification of species as they accurately capture the differences among species (Bezeng et al. 2017). The COI gene is effective in identification of animal specimens (Hebert et al. 2003) though with some exceptions (Gibbs 2018), and has been employed extensively in species identification including vespid wasps (Neumeyer et al. 2014; Schmid-Egger et al. 2017; Abd-El-Samie et al. 2018). Constructing a barcode of life library for species is thus advocated (Hebert et al. 2010), and availability of such reference data enable rapid identification of species using the barcode sequences (Puillandre et al. 2012). Despite the many advantages of DNA barcoding, the coamplification of nuclear mitochondrial pseudogenes (numts) (Bensasson et al. 2001; Song et al. 2008; Buhay 2009; Blacket et al. 2012) and *Wolbachia* (Linares et al. 2009; Xiao et al. 2012) has been a major drawback in DNA barcoding when using universal primers (LCO1490

and HCO2198) (Folmer et al. 1994) to amplify the barcode region. For this reason, designing specific primers for each taxonomic group has been suggested as a solution (Linares et al. 2009; Stahlhut et al. 2012; Francoso and Arias 2013). To the best of our knowledge the utility of the universal barcoding primers (LCO1490/HCO2198) for DNA barcoding of social wasps has not been evaluated, especially for species from developing countries where social wasp diversity is greatest, but taxonomic information on them is most lacking. Moreover, we lack vespid-specific barcode primers which accurately capture the barcode region of COI gene, but exclude numts and *Wolbachia.*

The objectives of this study were to test the efficacies of the frequently used hymenopteran barcode primers BarbeeF/MtD9 (Francoso and Arias 2013) and the universal primers LCO1490/HCO2198 in DNA barcoding of social wasps. We then designed polistine-specific primers and tested their efficacies in DNA barcoding of polistine wasps, a subfamily of the Vespidae, in the presence of *Wolbachia* infection. We then tested these polistine-specific primers on representative species from the other social vespid subfamilies – Stenogastrinae (hover wasps) and Vespinae (hornets and yellow jackets), and a member of Zethinae, *Zethus miniatus* Saussure. Finally, this study also aimed to contribute new reference DNA barcode sequences to public DNA databases for vespid wasps.

Methods

Sample collection and morphological identification

A total of 36 species of vespid wasps were collected across four continents (Table 1) between 2015-2017. The wasps were collected from their nests using insect nets or forceps, and stored directly into 80% ethanol, RNALater or frozen at -20 ℃. We used two methods to identify the specimens using morphology prior to barcoding. Many of the species were already established as models in the labs of the authors or their close collaborators; species identification was further verified using established keys (Bequaert 1918; Richards 1982; Carpenter and Nguyen

2003; Dvořák and Roberts 2006; Kojima et al. 2010). Where the species were less familiar to us, species identities were further verified with type and voucher specimens at the Natural History Museum (NHM) UK and National Museum of Natural History (NMNH) Paris. Other samples were sent to wasp taxonomy expert, J.M. Carpenter of the American Museum of Natural History (AMNH) for morphological identification (see Table 1 for species verification method used). Initial barcoding trials, primer development and testing were conducted on 10 species of polistine wasps collected in Nigeria; the primers were then tested on the full set of vespid wasps studied (see Table 1). The polistines from Nigeria were used for primer design because of the failure of initial barcoding primers BarBee/MtD9 to amplify four samples of *Ropalidia* spp. collected, the detection of *Wolbachia* in the sequences of the four *Ropalidia* spp. with LCO1490/HCO2198 primers, and the presence of three key genera of social wasp - *Belonogaster*, *Polistes* and *Ropalidia*.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from a single leg of adult samples using DNeasy® Blood and Tissue Kit (Qiagen, Germany) according to manufacturer's instructions, with the modification of eluting the DNA in 100 µl instead of 200 µl of buffer AE to make it more concentrated. The barcode region of the COI gene from ten species of social wasps collected in Nigeria, which included three different genera of the Polistinae (*Polistes, Belonogaster* and *Ropalidia* see Table 1), was amplified and sequenced using the BarbeeF/MtD9 primer pair (Francoso and Arias 2013). The two primers were chosen because they do not coamplify numts or *Wolbachia* (Francoso and Arias 2013). All ten wasp samples were amplified and sequenced using these two primer sets; however, the primer pair failed to amplify some species in the genus *Ropalidia*. Accordingly, the universal barcoding primers LCO1490/HCO2198 (Folmer et al. 1994) were used to amplify and sequence these outstanding samples from the genus *Ropalidia.*

The PCR reaction mix for each DNA extract consisted of a 25 µl final reaction volume comprising 2 µl DNA template, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.2 µM each of forward and reverse primers, and 2U Platinum™ *Taq* DNA Polymerase (Invitrogen, USA). The BarbeeF/MtD9 primer thermal cycle programme was as described by Francoso and Arias (2013); the thermal cycle programme for LCO1490/HCO2198 primer pair was as described by Blacket et al. (2012). The PCR products were analyzed on 2% agarose gel and visualized under UV light using BioDoc-It^2® Imager, UK. All PCRs were performed using an Appendorf Mastercycler nexus GSX1, Germany. The PCR products were cleaned using Wizard® SV Gel and PCR Clean-Up System according to manufacturer's instructions. The cleaned PCR products were sequenced using Sanger sequencing methods for both the forward and reverse directions using the respective forward and reverse primers and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA). Capillary electrophoresis was done using 3730xl DNA Analyzer (Applied Biosystem, Hitachi Japan) in the Biosciences Sequencing Facility, University College London.

Sequence analysis revealed the presence of stop codons when applying the invertebrate mitochondrial genetic code in four species of *Ropalidia* amplified and sequenced with the universal barcoding primer pair LCO1490/HCO2198. The stop codons were due to the *Wolbachia* infection in the *Ropalidia* spp. which were coamplified along with COI gene by the universal barcode primers. The sequences yielded greater than 92.1% similarity with *Wolbachia* when analyzed by BLAST against the NCBI database. Consequently, it was necessary to design species-specific primers that amplify the wasp mtDNA COI gene, but not *Wolbachia.*

Primer design

Sequences of *Belonogaster juncea* Fabricius, *Polistes marginalis* Fabricius and *Ropalidia* sp._NG_05 obtained with the BarBee/MtD9 and LCO1490/HCO2198 primers and three

sequences from GenBank: *B. juncea* (accession number GU596848), *P. marginalis* (accession number EF136446) and *Ropalidia fasciata* Fabricius (accession number AB969808) were aligned using multiple alignment in Genetyx® Version 8.0 with the default settings gap insert (<=0) -12, gap extend (<=0) -4; using Primer 3 Blast in the Genetyx® software, a forward polistine specific primer POL1372F 5´ TTTGGTATATGAGCAGGAATAATTGG 3´ was designed, with the default settings: target length 1-700, product length 200-650, primer length 20-30, GC% range 20-80, Tm range 57-63, primer salt conc. 50.00 mM, primer DNA conc. 50.00 nM, and primer Max. Diff. Tm 3.00. Species in the genera *Belonogaster*, *Polistes* and *Ropalidia* were used in the primer design because they contribute a large part of the species diversity in the subfamily Polistinae and are distributed worldwide. The new primer was combined with the reverse primer HCO2198 of the universal barcoding primer to amplify and sequence all the polistine wasps collected from Nigeria. Sharma and Kobayashi (2014) recommend the design of a forward primer at different taxonomic levels since, unlike the forward primer LCO1490, the reverse primer HCO2198 is highly conserved.

Two additional primers ROP1378F 5´ TGGGCTGGAATAATCGGAACAGC 3´ and ROP1939R 5´ TCACCTCCTCCTGTAGGATCAAA 3´ were designed specifically for the genus *Ropalidia* due to the difficulties encountered with this group using BarbeeF/MtD9 and LCO 1490/HCO 2198 primer pairs. The PCR thermal cycle programme for the new primers was determined through gradient PCR with annealing temperatures from 45-55 °C using Eppendorf Mastercycler nexus GSX1, Germany. The PCR reaction mix was as described earlier and the thermal cycle programme for all the new primer combinations comprised 1 cycle of initial denaturation at 94 °C for 4 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 1.30 min, extension at 64 °C for 2 min; 1 cycle of final extension at 64 °C for 7 min, and finally holding at 4 °C until analyzed. Thereafter, the primer pair POL1372F/HCO2198 was used to amplify and sequence all the polistine wasps collected from

Nigeria and the other social wasps from Asia, Europe and Central America to determine its effectiveness in DNA barcoding of social wasps (table 2). The primer combinations POL1372F/ROP1939R and ROP1378F/ROP1939R were used in addition to amplify and sequence *Ropalidia* spp. while POL1372F/ROP1939R also worked well for *Polistes* species from Nigeria (table 1).

We evaluated the utility of the universal barcoding primers LCO1490/HCO2198 in DNA barcoding of Polistinae, Stenogastrinae and Vespinae from the four continents. In this latter case all the *Belonogaster* species were collected from Nigeria, with other polistines, stenogastrines and vespines collected from Asia, Europe and Central America (Table 1). In each case, these species were amplified and sequenced using the reaction mix and thermal cycle programme described for this primer set above.

PCR clean up and sequencing for all the primer combinations were as described earlier. Voucher specimens for all the samples were deposited in Sumner Lab, Center for Biodiversity and Environment Research, University College London and the NHM UK while the COI barcode sequences for all the species were deposited in GenBank/EMBL/DDBJ (Accession Numbers LCO510310-LC510353; LC510516-LC510547) (Table 1). Voucher DNA is also stored in the Sumner Lab, UCL.

Sequence data analysis

Raw sequence data for each species was BLAST searched in GenBank, and the percentage similarity score, the percentage query cover and alignment examined. The species with the highest percentage similarity and query cover was downloaded. The raw reverse sequence was converted to the reverse complement using Genetyx® ver. 8.0. Raw data for the forward and reverse sequences were aligned with the reference sequence from GenBank using multiple alignment in Genetyx® Version 8.0 with the default parameters, gap insert (<=0) -12, gap

extend (<=0) -4. The sequences were edited by removing the primer sequences at the extremes and crosschecking the chromatogram in Chromas software when there was a mismatch in the sequence alignment. The identity of each sequence was verified using BLAST search in GenBank. All the edited sequences for each primer pair and species were aligned using multiple alignment in Genetyx® version 8.0 and also in MEGA7 using ClustalW with gap opening penalty of 15 and gap extension penalty of 6.66 for both pairwise and multiple alignment (Kumar et al. 2016) to compare the similarities among the sequences produced with the different primer combinations. The aligned sequences were translated into protein sequences in Genetyx® version 8.0 and also in MEGA7 to check for the presence of stop codons in the sequences. We reconstructed a phylogenetic tree based on COI gene to show the relationship among the studied species (S1).

Results

DNA barcoding of social wasps

A total of 35 species of social wasps and 1 solitary wasp (*Zethus miniatus*) were barcoded in this study. The social wasps comprised 10 polistines of the genera *Belonogaster, Polistes* and *Ropalidia* from Nigeria, 3 polistines *(Polistes)* and 4 vespines from Europe, 3 stenogastrines from Malaysia, 15 polistines and 1 Zethini from Central America (Table 1). Of the 35 social wasps barcoded, 4 *Ropalidia* spp. from Nigeria, 3 *Mischocyttarus* spp. and 1 *Polistes* sp. from Trinidad were (to the best of our knowledge) undescribed species.

The primer pairs BarbeeF/MtD9 and LCO1490/HCO2198 amplified and sequenced 676 bp and 658 bp respectively in the Nigerian polistine wasps. BarbeeF/MtD9 amplified *Ropalidia* sp._NG_05 but failed to amplify the remaining four *Ropalidia* spp. where *Wolbachia* was detected in the COI gene. The failed *Ropalidia* spp. was amplified and sequenced successfully with LCO1490/HCO2198 but sequence analysis revealed that *Ropalidia* sp._NG_09,

Ropalidia sp._NG_17, *Ropalidia* sp._NG_25 and *Ropalidia guttatipennis* Saussure have *Wolbachia* in the COI gene generated.

The new primers designed in this study successfully amplified and sequenced the barcode region of all the polistine wasps tested and in addition, *Parischnogaster alternata* Sakag which is a stenogastrine wasp. The success of all the primer combinations in the DNA barcoding of the social wasps are shown in table 2. The optimum annealing temperature for all the new primer combinations was established to be 49 °C. The primer pairs POL1372F/HCO2198, POL1372F/ROP1939R, and Pol1378F/Pol1939R amplified and sequenced 616 bp, 568 bp and 562 bp respectively, in the samples where they worked well, corresponding to the regions 1372-1987 bp, 1372-1939 bp, and 1378-1939 bp respectively of the *Polistes jokahamae* Radoszkowski (KR052461) mitochondrial genome. The polistine wasp primer POL1372F/HCO2198 did not amplify the vespines from Europe or the Stenogastrinae genus *Liostenogaster* spp. from Malaysia. The primer pairs POL1372F/ROP1939R amplified and sequenced *Ropalidia* spp. and *Polistes* spp. but did not work for *Belonogaster* spp. while POL378F/ROP1939R amplified and sequenced only *Ropalidia* spp. There were no stop codons in the sequences when translated into protein sequences.

Sequence similarities among the different primer combinations

All the combinations of primer pairs for each sample generated identical sequences. The DNA sequence for the different primer combinations differed only in the number of base pairs amplified and sequenced. The identical sequences produced by the different primer combinations indicate the robustness of the primers developed in this study for DNA barcoding.

Discussion

DNA barcoding primers for social wasps

The primer pair BarbeeF/MtD9 performs excellently with polistines, capturing a long fragment of 676 bp; however, it failed to amplify the COI gene of the four *Ropalidia* spp. that have *Wolbachia* in the sequences generated with LCO1490/HCO2198 primers. In addition, the PCR thermal programme for this primer takes long time (approximately 4 hours) to successfully complete amplification of the COI gene.

The universal barcoding primers LCO1940/HCO2198 also perform well for social wasps, capturing 658 bp of the barcode region: we had 100% success rate with this primer pair for all the social wasp samples tested and COI gene amplification cycle is short (approximately 2 hours). However, this primer pair also performed poorly with the four *Ropalidia* spp. as the sequences generated have *Wolbachia.* This primer pair combination remains an excellent choice for DNA barcoding provided that contaminants are taken into consideration.

The new polistine wasp primer pair combination POL1372F/HCO2198 proved very successful for the polistine wasps but had varying degrees of success with wasps from other subfamilies (e.g. Stenogastrinae). Importantly, it amplifies the barcode region in the presence of *Wolbachia* without coamplifying the *Wolbachia*. The primer amplifies a short fragment (616 bp) of the barcode region and might therefore prove especially useful when the sample's DNA is degraded. However, the primer amplification cycle is slow (approximately 4 hours). The primer pair POL1372F/ROP1939R proved very successful for species in the genera *Ropalidia* and *Polistes.* The primer pair ROP1378F/ROP1939R proved to be specific to *Ropalidia* spp: the primers amplify shorter fragments of 568 bp and 562 bp respectively and amplify the barcode region in the presence of *Wolbachia* without coamplifying the *Wolbachia*. The short fragments targeted by these primers might also be an advantage in amplifying samples with degraded DNA. They are limited to *Ropalidia* spp. and *Polistes* spp. as they failed to amplify *Belonogaster* spp. and species in the subfamily Stenogastrinae and Vespinae; it also takes approximately 4 hours to successfully amplify the barcode region of COI gene.

Universal barcoding primers have been reported to coamplify numts (Song *et al*. 2008; Buhay 2009; Bensasson *et al*. 2001; Blacket *et al*. 2012) and *Wolbachia* (Linares *et al*. 2009; Xiao *et al*. 2012) in a number of arthropods which has been an impediment in DNA barcoding. In our study, the sequences of COI gene in the genera *Belonogaster*, *Polistes*, *Mischocyttarus*, *Liostenogaster*, *Parischnogaster*, *Apoica*, *Agelaia* and the species *Zethus miniatus*, *Vespula vulgaris* Linnaeus, *Vespula germanica* Fabricius, *Vespa crabro* Linnaeus and *Vespa velutina* Lepeletier had no detected *Wolbachia* infection using the primer pairs investigated. However, the sequences of four different species of *Ropalidia* from Nigeria have *Wolbachia*, highlighting that these infections can be common and widespread, and thus the importance of using primers that do not coamplify these bacteria.

DNA barcoding, and species discovery

DNA barcodes have been put forward as a useful tool to discover new species (Smith et al. 2005; De Salle 2006; Butcher et al. 2012). In this study DNA barcoding aided the discovery of the four *Ropalidia* spp., which to our knowledge are new to science. Species of this group are difficult to separate morphologically (Bequaert 1918), and the separation of the five species by DNA sequence data enabled detailed study of morphology of the different species and thorough comparison with museum type and voucher specimens. DNA barcoding has been effective in uncovering cryptic species and species that are difficult to separate morphologically also in other insect groups (Janzen et al. 2005; Smith et al. 2006). Many species described by morphological features and present as voucher specimens in the museums included two or more morphological groups of individuals that harbour very divergent COI sequences (Hebert et al. 2004; Campbell et al. 2008; Locke et al. 2010).

The systematic revision of the social wasps is continually developing (Pickett and Carpenter 2010; Piekarsi et al. 2018), and many new species of polistine wasps have been discovered in recent years using DNA barcoding. In Europe DNA barcoding lead to the discovery of *Polistes*

helveticus Neumeyer (Neumeyer et al. 2014), *P. austroccidentalis* van Achterberg and Neumeyer., and *P. maroccanus* Schmidt-Egger (Schmid-Egger et al. 2017). The African continent likely harbours many more undescribed polistine wasp species. DNA barcoding makes it accessible to rapidly document the biodiversity of social wasps in less-studied regions like Africa.

Conclusion

Social wasps are understudied particularly in the developing and tropical countries of Africa and Central/South America. The paucity of knowledge of social wasp diversity in these regions could be a result of taxonomic challenges in identifying the species and the dearth of expert taxonomists, molecular skills and DNA tools in these regions. This knowledge gap is holding back research on this diverse group, limiting the degree to which people can benefit from the ecosystem services of social wasps, and thus limiting our ability to manage their populations for sustainable environmental purposes. This study is a first step in addressing the limits in social wasp identification. We have presented a reliable set of DNA barcodes for a wide diversity of wasps from the largest social wasp subfamily Polistinae.

There are limitations in the use of the new primers in that they are sub-family specific and so cannot help much with the Vespinae and Stenogastrinae. However, given that Polistinae are the largest subfamily of social wasps, and the ones that are most common in tropical and developing countries, these identification resources are likely to open up opportunities for further research into these species and their potential role as biocontrol agents. Our barcoding set provides a rapid and affordable solution to documenting and categorizing the diversity of polistine wasps across the globe.

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1 Table 1: Vespid wasps barcoded in the study

Subfamily	**Species (if known)**	**Country (Locality)**	**Collector**	**Voucher**	**GenBank Accession Numbers**
Polistinae	*Polistes marginalis** Fabricius	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510537, LC510344, LC510314, LC510326
Polistinae	*Polistes africanus** de Beauvois	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510535, LC510313, LC510342, LC510324
Polistinae	*Polistes nimpha** Christ	Italy (Tuscany)	AC	n/a	LC510538, LC510345
Polistinae	*Polistes semenowi** Morawitz	Italy (Tuscany)	AC	n/a	LC510539, LC510346
Polistinae	*Polistes dominula** Christ	Italy (Tuscany)	AC	n/a	LC510536, LC510343
Polistinae	*Polistes* sp.†	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510540, LC510347
Polistinae	*Polistes versicolor*† Olivier	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510541, LC510348
Polistinae	*Polistes lanio*† Fabricius	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510533, LC510340
Polistinae	*Polistes canadensis*† Linnaeus	Panama	SS	Sumner Lab & NHM UK	LC510532, LC510339
Polistinae	*Apoica pallens*† Fabricius	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510519
Polistinae	*Apoica* sp.†	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510518
Polistinae	*Agelaia pallipes*† Olivier	Trinidad (Arima Valley)	SS	AMNH	LC510517, LC510329
Polistinae	*Agelaia cajennensis*† Fabricius	Trinidad (Arima Valley)	SS	AMNH	LC510516, LC510328
Polistinae	*Metapolybia cingulata*† Fabricius	Trinidad (Arima Valley)	SS	AMNH	LC510525, LC510333

Polistinae	*Mischocyttarus basimacula basimacula*† Cameron	Panama (Panama Canal Zone)	SS	AMNH	LC510527, LC510336
Polistinae	*Mischocyttarus alfkenii*† Ducke	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510526, LC510335
Polistinae	*Mischocyttarus* sp.2†	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510531
Polistinae	*Mischocyttarus collarellus*† Richards	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510528, LC510337
Polistinae	*Mischocyttarus* sp.1†	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510530, LC510338
Polistinae	*Mischocyttarus* sp. †	Trinidad (Arima Valley)	SS	Sumner Lab & NHM UK	LC510529, LC510334
Polistinae	*Ropalidia* sp. NG_05*	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510350, LC510315, LC510319, LC510325, LC510542
Polistinae	*Ropalidia guttatipennis**Saussure	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510349, LC510318, LC510323
Polistinae	*Ropalidia* sp. NG_25*	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510353, LC510317, LC510322
Polistinae	*Ropalidia* sp. NG_09*	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510351, LC510320, LC510327
Polistinae	*Ropalidia* sp. NG_17*	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510352, LC510316, LC510321
Polistinae	*Belonogaster macilenta**Fabricius	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510522, LC510312, LC510332
Polistinae	*Belonogaster dubia** Kohl	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510520, LC510310, LC510330
Polistinae	*Belonogaster juncea**Fabricius	Nigeria (Nsukka)	IEO	Sumner Lab & NHM UK	LC510521, LC510311, LC510331
Vespinae	*Vespula vulgaris**Linnaeus	United Kingdom (London)	SS	n/a	LC510546

Vespinae	*Vespula germanica*[*] Fabricius	United Kingdom (London)	SS	n/a	LC510545
Vespinae	*Vespa crabro*[*] Linnaeus	United Kingdom (London)	SS	n/a	LC510543
Vespinae	*Vespa velutina*[*] Lepeletier	Italy (Milan)	AC	n/a	LC510544
Stenogastrinae	*Parischnogaster alternata*[*] Sakag	Malaysia (Fraser's Hill)	SS	Sumner Lab & NHM UK	LC510534, LC510341
Stenogastrinae	*Liostenogaster vechti*[*] Turillazzi	Malaysia (Fraser's Hill)	SS	Sumner Lab & NHM UK	LC510524
Stenogastrinae	*Liostenogaster flavolineata*[*] Cameron	Malaysia (Fraser's Hill)	SS	Sumner Lab & NHM UK	LC510523
Zethinae	*Zethus miniatus*[†] Saussure	Trinidad (Arima Valley)	SS	AMNH	LC510547

Note: AC = Alessandro Cini; IEO = Ikechukwu Eugene Onah; SS = Seirian Sumner

NHM = Natural History Museum London; n/a = not applicable; AMNH = American Museum of Natural History

Species verification methods: [*]Identified using keys and type/voucher specimens by the authors; [†]Verified by J.M. Carpenter (AMNH).

12 Table 2: Performance of different primer combinations across the vespid wasps studied

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Subfamily	**Species (if known)**	**BarBee/ MtD9**	**LCO1490/ HCO2198**	**POL1372F/ HCO2198**	**POL1372F/ ROP1939R**	**ROP1378F/ ROP1939R**
Polistinae	*Polistes marginalis*	+	+	+	+	-
Polistinae	*Polistes africanus*	+	+	+	+	-
Polistinae	*Polistes nimpha*		+	+		
Polistinae	*Polistes semenowi*		+	+		
Polistinae	*Polistes dominula*		+	+		
Polistinae	*Polistes* sp.		+	+		
Polistinae	*Polistes versicolor*		+	+		
Polistinae	*Polistes lanio*		+	+		
Polistinae	*Polistes canadensis*		+	+		
Polistinae	*Apoica pallens*		+	+		
Polistinae	*Apoica* sp,		+	+		
Polistinae	*Agelaia pallipes*		+	+		
Polistinae	*Agelaia cajennensis*		+	+		
Polistinae	*Metapolybia cingulata*		+	+		
Polistinae	*Mischocyttarus basimacula basimacula*		+	+		
Polistinae	*Mischocyttarus alfkenii*		+	+		
Polistinae	*Mischocyttarus* sp.2		+	+		
Polistinae	*Mischocyttarus collarellus*		+	+		
Polistinae	*Mischocyttarus* sp.1		+	+		
Polistinae	*Mischocyttarus* sp.		+	+		
Polistinae	*Ropalidia* sp. NG_05	+	+	+	+	+
Polistinae	*Ropalidia guttatipennis*	-	**+ (Wolbachia)**	+	+	+
Polistinae	*Ropalidia* sp. NG_25	-	**+(Wolbachia)**	+	+	+
Polistinae	*Ropalidia* sp. NG_09	-	**+(Wolbachia)**	+	+	+
Polistinae	*Ropalidia* sp. NG_17	-	**+(Wolbachia)**	+	+	+
Polistinae	*Belonogaster macilenta*	+	+	+	-	-
Polistinae	*Belonogaster dubia*	+	+	+	-	-
Polistinae	*Belonogaster juncea*	+	+	+	-	-
Vespinae	*Vespula vulgaris*		+	-		

Vespinae	*Vespula germanica*		+	-		
Vespinae	*Vespa crabro*		+	-		
Vespinae	*Vespa velutina*		+	-		
Stenogastrinae	*Parischnogaster alternata*		+	+		
Stenogastrinae	*Liostenogaster vechti*		+	-		
Stenogastrinae	*Liostenogaster flavolineata*		+	-		
Eumeninae	*Zethus miniatus*		+	+		

Note: + = Success, - = failed, blank = not tested

Supplementary materials

Phylogeny

Phylogenetic trees are typically constructed for barcoding studies in order to check that they reflect expected phylogenetic relationships, and the differences in the clustering of mtDNA COI and COI with contaminants (numts or *Wolbachia*) (Blacket et al. 2012). The phylogeny was assembled automatically in the Phylogeny.fr platform (Dereeper et al. 2008). Sequences were aligned with MUSCLE v3.8.31 configured for highest accuracy using the default settings (Edgar 2004). Regions containing gaps or missing data in the multiple alignment were removed automatically using Gblocks v0.91b (Castresana 2000). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT) (Dereeper et al. 2010). The HKY85 substitution model was selected and 4 gamma-distributed rate categories to account for rate heterogeneity across sites (Guindon and Gascuel 2003). The gamma shape parameter was estimated directly from the data (gamma=0.726). Reliability of the internal branches was assessed using the aLRT test (SH-Like) (Anisimova and Gascuel 2006). The tree was drawn and edited with TreeDyn v198.3 (Chevenet et al. 2006). The analysis involved 40 nucleotide sequences.

COI Genes Reflect the Expected Phylogenetic Relationships in the Social Wasps

Although only one gene had been sequenced (mtDNA COI gene), it is useful to construct a molecular phylogeny of the species examined when developing and testing barcoding primers. The reconstructed phylogeny corresponded well to the expected phylogenetic relationships, with species clustering as separate subfamilies for the Vespinae, Stenogastrinae, and Polistinae (Piekarski et al. 2018; Figure 1). The species in the subfamily Polistinae form four separate clusters that follow the expected tribal relationships. The tribe Polistini (*Polistes* spp.) formed a clear single clade, but with separation of species by region - Italy, Nigeria and Central America each formed a separate, well supported cluster. Similarly, all species in the tribe Mischocyttarini (*Mischocyttarus* spp.) from Trinidad formed a cluster while species in the tribe Epiponini (*Apoica*, *Agelaia*, and *Metapolybia* species) from Central America formed their own cluster. All the species in the tribe Ropalidiini (*Ropalidia* spp. and *Belonogaster* spp.) from Nigeria formed different clusters each, while all the stenogastrines from Malaysia and vespines from Europe formed their own separate groups (Figure 1).

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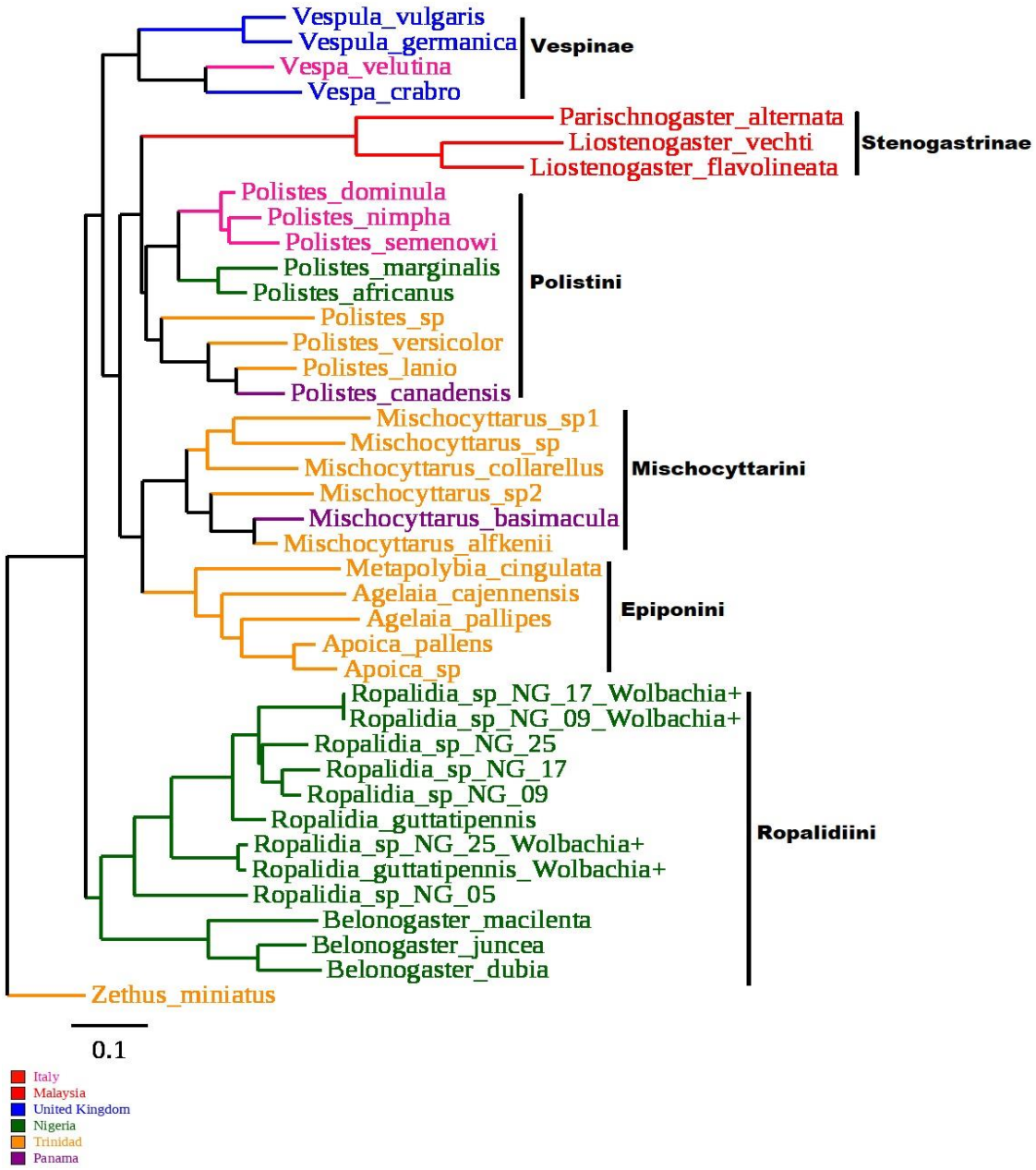

Fig.1: Molecular phylogeny of the vespid wasps based on COI gene