



RESEARCH ARTICLE

Molecular phylogeny of *Monomorium pharaonis* (Hymenoptera: Formicidae) based on rRNA sequences of mitochondrial gene

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Abstract. Many studies on forensic entomology have focussed on Diptera and lack the Hymenopteras population. Nonetheless, hymenopterans are part of the entomofaunal colonization of a corpse. Morphologically, it is difficult to identify and distinguish between them. In this study, using mitochondrial DNA knowledge, the molecular analysis was performed to classify the recovered species of hymenoptera collected from rabbit carcass, quickly and accurately. A molecular identification method with a 251-bp fragment of the 16S ribosomal gene RNA (16S rRNA) from a single ant species was evaluated. The maximum likelihood method analysis has recovered a generally well-supported phylogeny, with most taxa and species groups currently being recognized as monophyletic. The aculeate consists of some Hymenoptera's best known. Their sister group has traditionally been considered in Ichneumonoidea. In addition, Trigonaloidea was found as the aculeates' sister group and Crabronidae in Apoidea forming the Formicidae's sister group. These results will play an important role in the implementation of the Saudi database forensically relevant ants.

Keywords. forensic entomology; ant phylogeny; formacidae; mitochondrial genes.

Introduction

Hymenoptera (sawflies, wasps, ants and bees) is one of four mega-diverse insect orders (Aguilar *et al.* 2013; Peters *et al.* 2017). Ants are one of the most successful groups of eusocial insects (Hölldobler and Wilson 1990; Lach *et al.* 2009; Chen *et al.* 2013). Currently, there are more than 13,000 species of ant recognized worldwide (Bolton 2014). The most frequently used species to examine a crime scene are flies and beetles (Biavati *et al.* 2010). Although less studied, ants and other insects are also part of the corpse fauna, actively participating in the fractionation and decomposition of vertebrate carcass (Moretti and Ribeiro 2006). Since some of the ant species are necrophagous, they can help to solve crime in forensic entomology studies (Amendt *et al.* 2000, 2010; Cameron and Williams 2003; Grassberger and Frank 2003; Tabor *et al.* 2005; Byrd and Castner 2010; Prado e Castro *et al.* 2014; Paula *et al.* 2016; Maciel *et al.* 2016; Mashaly *et al.* 2018).

All the species of ant are grouped into a single family, the Formicidae, divided into 17 subfamilies based on a morphological and behavioural cladistic analysis (Baroni Urbani *et al.* 1992; Derr *et al.* 1992; Ohnishi *et al.* 2003; Brady *et al.* 2014), but four of these subfamilies, Dolichoderinae, Formicinae, Myrmicinae and Ponerinae are made up almost 90% of all the known species (Bolton 2014). The Myrmicinae subfamily is the most biologically complex and prolific of these 'big four'. With ~6475 species described, the myrmicines are a hyperdiverse clade that inhabits most of the earth's surface and includes a wide variety of lifestyles including generalist and specialist predators, scavengers, omnivores, granivores and herbivores (Kugler 1979; Brown 2000). *Monomorium* Mayr 1855 is one of the most important genera of myrmicine ants, comprising a total of 359 species (Heterick 2006).

In many respects, the higher phylogeny and ants classification remains controversial (Astruc *et al.* 2004). Within the Formicidae family, sequences of mitochondrial or

ribosomal DNA genes were often used to define phylogenetic relationships at the generic or tribal or subfamily level (Baur *et al.* 1993; Crozier *et al.* 1995; Ayala *et al.* 1996; Wetterer *et al.* 1998; Brandão *et al.* 1999; Chiotis *et al.* 2000; Feldhaar *et al.* 2003; Astruc *et al.* 2004; Ward and Downie 2005; Moreau *et al.* 2006; Ouellette *et al.* 2006; Rabeling *et al.* 2008; Ward *et al.* 2010).

To date, in Saudi Arabia, the molecular phylogenetic analysis has not been performed on Myrmicinae subfamily. Therefore, the objective of this study was to document the presence of ants in the ecological habitat of the carcass experiment in Saudi Arabia. In addition, the phylogenetic position of *Monomorium pharaonic* within Formicidae belonging to the Hymenoptera was determined and this was done on the basis of the sequences of the mitochondrial 16S rRNA gene.

Materials and methods

The ant specimens were collected from rabbit carcasses in Riyadh, Saudi Arabia, and fixed in 70% ethanol until they were used for molecular analysis. DNA was extracted from whole ants using QIAamp DNA mini kit (Qiagen, Germany), following the manufacturer's protocol. Polymerase chain reaction (PCR) amplifications were performed with a total volume of 20 μ L using Thermo Cycler (Perkin Elmer) with an amplification profile consisting of an initial denaturation step at 95°C for 3 min followed by 35 cycles each consisting of denaturation at 95°C for 15 s, annealing at 48°C for 15 s, extension at 72°C for 1 min and an extension at 72°C for 5 min. The 16S rRNA gene region was amplified with 16S rRNA forward universal primers 5'-CGC TGT TAT CCC TAA GGT AA-3' and 16SRNA reverse 5'-CTG GTA TGA AAG GTT TGA CG-3', as mentioned by Li *et al.* (2010). The amplified PCR products were purified and suspended in a 25 μ L TE buffer with a DNA-purification kit (Qiagen). All PCR products were sequenced in both directions using Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) on 3130x1 Genetic Analyzer (factory refurbished) (Biosystems 3130, Thermo Fisher Scientific, USA). All procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals and have been approved and authorized by the Institutional Animal Care and Use Committee (IACUC) at King Saud University, Riyadh, Saudi Arabia.

To identify the related sequences in the NCBI database, a BLAST search was performed. CLUSTAL-X v1.83 aligned the obtained sequences (Thompson *et al.* 1997). The alignment was manually adjusted using the BioEdit 4.8.9 software (Hall 1999). The data were analysed in MEGA 7.0 with maximum parsimony (neighbour-interchange (CNI) level 3, random addition trees 100) (Tamura *et al.* 2007). Bootstrap analyses (1000 replications) were used on distance trees to obtain estimates of nodal

support. Tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer phylogenetic tree.

Results

A total of 251 bp with a GC content of 19.12% was analysed and the resulting sequences were deposited in GenBank under the accession number MN588189.1, for the mt16S rRNA gene region of the present hymenoptera species. Phylogenetic analysis was carried out on the basis of a comparison with 27 related species using the maximum likelihood method based on the Jukes–Cantor model representing the Hymenoptera order (figure 1).

Comparison of nucleotide sequences and divergence showed that the 16S rRNA of this species revealed sequence identities with taxa belonged to Hymenoptera as 83.64–76.23% with Formicidae, 81.48% with Trigonalidae, 76.86% with Crabronidae, 77.19% with Braconidae, 76.62–79.26% with Bethylidae, and 76.71–78.22% with Chrysididae. While, Formicidae had sequence identities with taxa as 76.23–83.64% with Myrmicinae, and 77.68–78.38% with Formicinae. The maximum identity with lowest divergent values were reported with formicidal species, *M. pharaonis* (83.64%, gb| DQ023051.1), *Aphaenogaster cardenai* (82.70%, gb| LT623127.1), *Cardiocondyla bulgarica* (79.11%, gb| DQ023040.1), *C. sahlbergi* (78.85%, gb| DQ023056.1), *C. ulianini* (78.83%, gb| DQ023059.1), *Pheidole gagates* (78.74%, gb| HM015951.1), *Myrmica scabrinodis* (78.40%, gb| LN607806.1), *Solenopsis invicta* (78.38%, gb| HQ215538.1), *S. geminata* (78.07%, gb| HQ215537.1), *P. cramptoni* (77.58%, gb| HM015943.1), *Protomognathus americanus* (76.38%, gb| DQ023055.1), and *Pristomyrmex punctatus* (76.23%, gb| AB556947.1).

The constructed dendrogram is divided into two clades, the first clustered some formicoids representing the subfamily Formicinae and only two genera *Aphaenogaster* and *Pheidole* within Myrmicinae, in addition to species belonging to Bethylidae within Ichneumonoidea, Chrysididae within Chrysidoidae, and Braconidae within Ichneumonoidea. Nevertheless, the second clade consisted mainly of species belonging to the subfamily Myrmicinae, Crabronidae and Trigonalidae within Trigonaloidae, with strong nodal support. Formicidae forming a sister group for Bethylidae + Chrysididae + Braconidae with low support values. Crabronidae formed a sister group to Formicidae with strong nodal support. In addition, Trigonalidae formed a sister group to Formicidae by low support value. The ME tree showed a well-resolved distinct clade with other hymenopteran members of the Formicidae family and deeply embedded in the *Monomorium* genus for the present formicid species, closely related to the previously recorded *M. pharaonis* (gb| DQ023051.1) in the same taxon with a moderate support value (44).

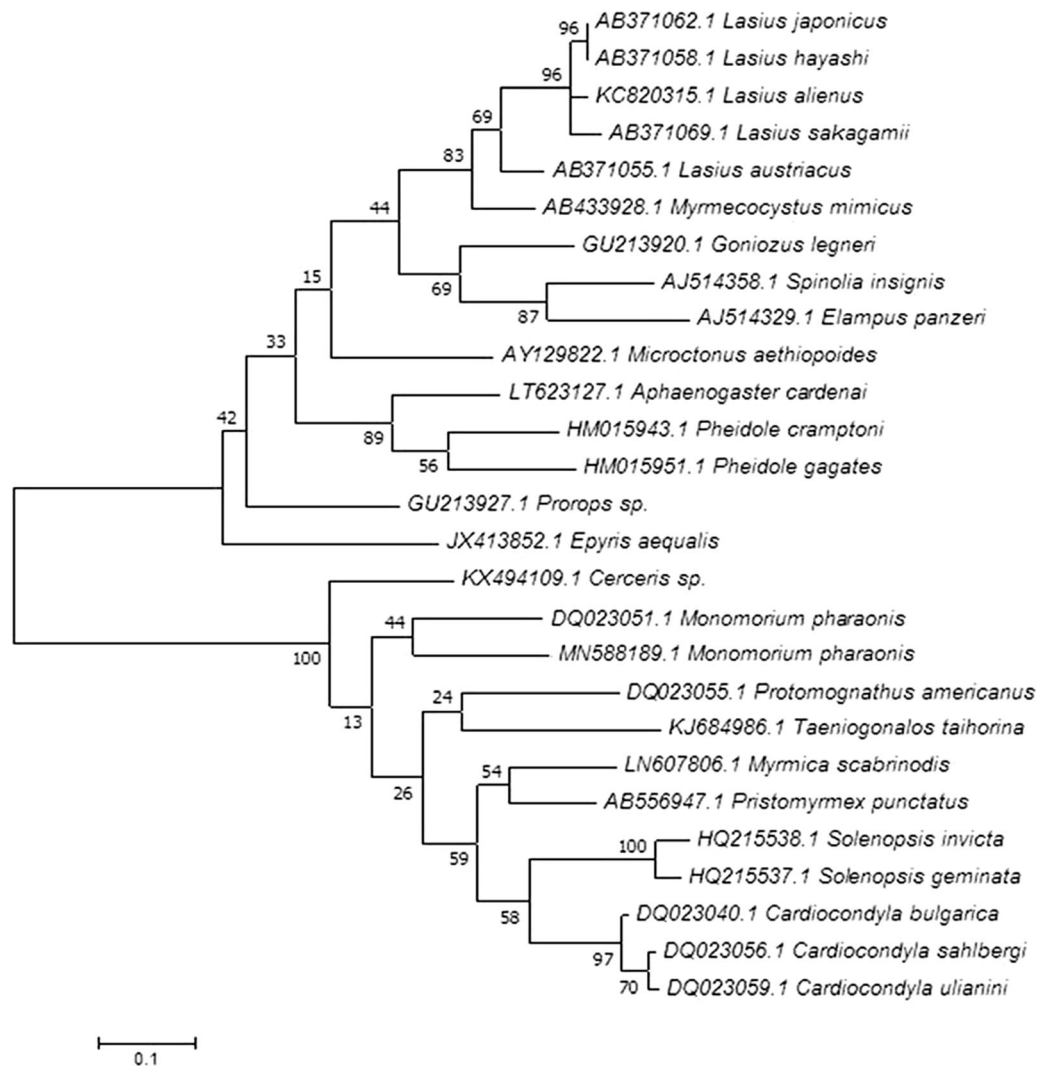


Figure 1. Molecular phylogenetic analysis by maximum likelihood method based on the Jukes–Cantor model. The tree with the highest log likelihood (−4101.45) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Discussion

Carrion insects help to clarify the cause of death and neglect cases (Catts and Goff 1992; Benecke 2001). Ants can both inhibit carrion colonization by necrophagous flies and predate large amounts of immatures (eggs and larvae) on animal carcasses and human corpses. This behaviour is particularly important because it may interfere with the calculation of the post-mortem interval (PMI), as demonstrated by Wells and Greenberg (1994), Lindgren *et al.* (2011) and Moretti *et al.* (2013).

There are currently few studies dealing with morphological identification of different ant species because of high morphological characters conflict (Agosti 1991; Baroni Urbani *et al.* 1994; Lorite *et al.* 2004; Schneider *et al.* 2018; Ma *et al.* 2019). Molecular phylogenetic studies can provide better resolution of ant phylogeny than morphology

studies only and can show an accurate relationship between subfamilies (Dowton and Austin 1994; Gimeno *et al.* 1997; Sullander 1998; Ohnishi *et al.* 2003). Previous molecular studies of hymenopteran phylogeny used mitochondrial 16S and COI and small fragments of 18S and 28S ribosomal (D2–D3). Such experiments are usually either based on Symphyta (Schulmeister 2003, Schulmeister *et al.* 2002; Kuo *et al.* 2003; Zheng *et al.* 2018) or on Apocrita (Castro and Dowton 2006; Dowton and Austin 1994, 2001; Dowton *et al.* 1997). In the present study, the 16S rRNA mitochondrial gene is used strongly to support the recovered hymenoptera species, which was in agreement with previous studies by Avise (1986, 1991, 1994) and Ballard and Whitlock (2004), who reported that the mtDNA genomes generally have several properties that are ideally suited for phylogenetic studies of closely related ant species.

Many relationships were found in this study, such as the relationship between Parasitica (parasitic wasps) represented by Bethyridae within Ichneumonoidea and Aculeata (aculeate wasps) represented by Chrysididae within Chrysoidea and Formicidae within Vespoidea, agreed with observations by Rasplus *et al.* (2010). Similarly, Ronquist *et al.* (1999) and Sharkey (2007) reported that Hymenoptera was divided into the Symphyta (sawflies) and Apocrita, the latter further divided into Parasitica (parasitic wasps) and Aculeata (aculeate wasps) and supported Apocrita and Aculeata monophyletic classification. Brothers and Carpenter (1993) have claimed that the relationship between Formicidae and Chrysididae might be due to the maxilla involving the relative sizes of the galea and lacinia. Similarly, Carpenter and Wheeler (1999), Dowton and Austin (1994), Dowton *et al.* (1997), Whiting (2002), Davis *et al.* (2010), Vilhelmsen *et al.* (2010), and Cockx *et al.* (2016) reported that Bethyridae and Chrysididae within Chrysoidea are a fairly well-supported sister pairing due to the synapomorphic state of the reduced metapostnotum, which has also been shown to be sister to alternative families. Dowton *et al.* (1998) and Grimaldi and Engel (2005) stated that the Ichneumonoidea (Ichneumonidae + Braconidae) is often considered to be the sister group of Aculeata.

Johnson *et al.* (2013) followed by Branstetter *et al.* (2017) considered ants to be Apoidea's sister, which agreed with our Crabronidae findings in Apoidea that Formicidae's sister group was formed with strong nodal support. In addition, in the molecular analyses by Heraty *et al.* (2011) and Klopstein *et al.* (2013), Trigonaloidea was retrieved as the sister group of the aculeates, this was accepted with our findings as Trigonidae within Trigonaloidea forming sister group to Formicidae by weak support value. Sharkey *et al.* (2012) and Zimmermann and Vilhelmsen (2016) confirmed that the presence of a secondary tentorial bridge and a subforaminal cup in Evanioidea and Aculeata and a bent cibarium in Trigonidae, Evanioidea and Aculeata add morphological evidence to the molecularly validated hypothesis of Trigonidae + (Aculeata + Evanioidea) sister group relationship. Peters *et al.* (2011) also noted that Trigonaloidea was the sister group of Aculeata in paraphyletic Evaniomorpha through a combination of morphology and molecular analysis with low branch support.

In addition, the present study focussed on the generic relationship within subfamily-level groups of ants such as Myrmicinae and Formicinae within Formicidae, two subfamilies which traditionally regarded as belonging to separate groups but closely related in this analysis, as agreed with previous reports by Hölldobler and Wilson (1990), Schmitz and Moritz (1998), Ohnishi *et al.* (2003), Krieger and Ross (2003), Fisher and Cover (2007), Ward (2007), and Brady *et al.* (2014) claimed that Formicidae was divided into 21 subfamilies, including Myrmicinae, the largest Formicidae subfamily with 138 genera, followed by Formicinae with 39 genera and Ponerinae with 25 genera. Several species-rich genera of myrmicine in this study, including

Aphaenogaster Mayr 1853, *Pheidole* Westwood 1839, *Monomorium* Mayr 1855, *Protomognathus* Wheeler 1905, *Myrmica* Linnaeus 1758, *Pristomyrmex* Mayr 1866, *Solenopsis* Westwood 1840, and *Cardiocondyla* Emery 1869, with a strong support values; this was in line with data from Ward *et al.* (2015), where, the recovered hymenopteran species belongs to the genus *Monomorium*. The species studied here, *M. pharaonis*, as previously defined by morphological taxonomic study, was reported to be distinct species with a very close relationship to the *M. pharaonis* previously described (gb|DQ023051.1).

In conclusion, the mitochondrial 16S rRNA gene is useful in distinguishing between the forensic ant species in our study. Further, experiments are recommended to collect more specimens of Hymenoptera in a wider area of Saudi Arabia and then improve the molecular method by using different and more genes to classify appropriate forensic ants.

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