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Short communication

Molecular phylogeny and DNA barcoding in the meadow-spittlebug *Philaenus spumarius* (Hemiptera, Cercopidae) and its related species

S.G. Seabra [a,*], F. Pina-Martins [a], E. Marabuto [a], S. Yurtsever [b], O. Halkka [c,†], J.A. Quartau [a,1], O.S. Paulo [a,1]

[a] Centro de Biologia Ambiental/Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Edifício C2, Campo Grande, 1749-016 Lisboa, Portugal
[b] Biology Department, Faculty of the Arts and Science, Trakya University, 22030 Edirne, Turkey
[c] Tvärminne Zoological Station, University of Helsinki, Finland

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ABSTRACT

Philaenus spumarius, widely studied for its colour/pattern polymorphism, is a widespread species across the Holartic. The patterns of haplotype divergence at the mitochondrial gene cytochrome oxidase I (COI) found in this study suggest a postglacial western Europe (Iberian and Italian peninsulas to Britain) and a eastern (from Near East to Finland) south-to-north colonization. The haplotypes found in North America are most likely derived from the British haplotypes. The barcode fragment used here allowed the distinction of the species within genus *Philaenus* and questioned some taxonomic identifications of sequences present in Genbank.

1. Introduction

The meadow-spittlebug *Philaenus spumarius* (Hemiptera, Cercopidae) is an insect species that has been widely studied for its balanced polymorphism in adult dorsal colour/pattern (Halkka and Halkka, 1990; Stewart and Lees, 1996; Drosopoulos, 2003). This pigmentation variation is determined mainly by a single autosomal locus (Halkka et al., 1973; Yurtsever, 1999) and several selective factors have been suggested to explain the maintenance of this polymorphism (Halkka and Halkka, 1990; Stewart and Lees, 1996; Quartau and Borges, 1997). Population genetic studies of *P. spumarius* have been based on the frequency evaluations of colour phenotypes and have shown differentiation among populations (Halkka and Halkka, 1990; Stewart and Lees, 1996; Yurtsever, 2000) in spite of a low genetic variability when using allozymes (Saura et al., 1973; Loukas and Drosopoulos, 1992). This is a widespread species mainly distributed across the Holartic Region and one crucial factor for its survival is the presence of enough humidity, since the nymph stage is very vulnerable to desiccation (Halkka and Halkka, 1990; Drosopoulos, 2003).

Taxonomists have long accepted the existence of at least another species belonging to this genus, *Philaenus signatus* Melichar, 1896, which differs in body size, form of the pronotum and male genitalia (Halkka and Lallukka, 1969). More recently, other species were described from the Mediterranean region, namely *Philaenus loukasi* from Greece (Drosopoulos and Asche, 1991), *Philaenus arslani* from Lebanon (Abdul-Nour and Lahoud, 1996), *Philaenus maghresinus* from north west Africa and southern Spain, *Philaenus italosignus* from the Italian Peninsula and Sicily (Drosopoulos and Remane, 2000) and *Philaenus tarifa* from southern Spain (Remane and Drosopoulos, 2001). Finally, *Philaenus tesselatus*, found in southern Iberian Peninsula and northwest Africa, was the last species to be considered as valid (Drosopoulos and Remane, 2000; Drosopoulos and Quartau, 2002), even if described more than a century ago, since it was for a long time considered a subspecies of *P. spumarius* (Halkka and Lallukka, 1969). Current taxonomy within the genus, based on morphological similarities in the male anal tube considers these eight species as divided into the *P. spumarius* species group (*P. spumarius*, *P. tesselatus*, *P. loukasi* and *P. arslani*) and the *P. signatus* species group (*P. signatus*, *P. italosignus*, *P. maghresinus* and *P. tarifa*) (Drosopoulos, 2003). Some of these species show identical variation in dorsal colour, with the same colour morphs occurring in different species (Halkka and

* Corresponding author. Fax: +351 217500028.
E-mail address: sgseabra@fc.ul.pt (S.G. Seabra).
[1] Equal contribution.
[†] Deceased author.

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Lallukka, 1969; Drosopoulos, 2003). At a molecular level, it was found that some allozyme loci are diagnostic in the identification of *P. spumarius*, *P. signatus* and *P. loukasi* from Greece (Loukas and Drosopoulos, 1992). Chromosome variation also distinguishes the species, with four species groups: one includes *P. spumarius* and *P. tesselatus*; another includes *P. loukasi* and *P. arslani* with a reduction of the number of autosomes and the appearance of neo-XY type of sex chromosomes; another includes *P. signatus*, *P. maghresignus* and *P. tarifa*; and the last one includes only *P. italosignus* with a peculiar type of sex determination system (Maryanska-Nadachowska et al., 2008a, 2008b). A preliminary phylogenetic study on the relationships among the eight species found in the Mediterranean region also found four separate groups: *P. loukasi* and *P. arslani* as independent groups; a third group including *P. signatus*, *P. maghresignus*, *P. tarifa* and *P. italosignus*; and a forth group including *P. spumarius* and *P. tesselatus* (Maryanska-Nadachowska et al., 2008c). However, this last study did not provide statistical support values for these relationships.

In the present study, the molecular genetic variation within *P. spumarius*, as well as within the genus, is analyzed by using the cytochrome oxidase I (COI) mitochondrial gene, which has been widely used in the study of evolutionary relationships among closely related insect taxa (Caterino et al., 2000). A 648 bp region of COI has been suggested by the Barcode Initiative as a barcode sequence for identification and discovery of animal species (Hebert et al., 2003; Ratnasingham and Hebert, 2007) and has been successfully applied in the identification of many insect species, including hemipterans (Foottit et al., 2009). Its application will also be discussed for the genus *Philaenus*.

2. Materials and methods

2.1. Specimen collection

Philaenus specimens were collected or sent by collaborators between 2007 and 2009 from the Iberian Peninsula, Italian Peninsula, Sicily, Anatolian Peninsula, Great Britain, Archipelago of the Gulf of Finland in Europe and from British Columbia in North America (Table 1). A total of 39 individuals of *P. spumarius*, 3 of *P. tesselatus*, 2 of *P. tarifa* and 1 of *P. italosignus*, together with 4 outgroups within Cercopidae (3 of *Neophilaenus* and 1 of *Cercopis intermedia*) were used. Adult insects were captured using a sweep net and were taken to the laboratory dry in silica gel, in absolute ethanol or alive. The larval stages are not identifiable to species level in this group but a nymph from Oxfordshire (UK) was used in this study since no adults were available from this location, which also provided a test for the feasibility of identification of immature stages through molecular techniques.

2.2. Mitochondrial DNA analysis

The wings and the abdomen of each specimen were removed and the remaining of the body was used for DNA extraction using the E.Z.N.A.® Tissue DNA Isolation kit (Omega Bio-Tek). A fragment of about 800 bp of the 3'-end of cytochrome oxidase I (COI) mitochondrial gene was amplified by polymerase chain reaction (PCR) using the primers C1-J-2195 and TL2-N-3014 (Simon et al., 1994). PCR was done in a 12.5 μl reaction volume containing 1× buffer (Promega), 1.8 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μM of each primer and 0.02 U of Taq DNA polymerase (Promega). The PCR conditions were: 94 °C for 5 min, 35 cycles of 94 °C for 20 s, 50 °C for 30 s and 72 °C for 50 s, with a final extension period of 7 min at 72 °C.

Another fragment of about 650 bp near the 5'-end of the COI gene, corresponding to the DNA barcode sequence, was amplified using the primers LEPF and LEPR developed for butterflies and moths (Hajibabaei et al., 2006). PCR reaction volume contained 1× buffer (Promega), 2 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μM of each primer and 0.02 U of Taq DNA polymerase (Promega). The PCR conditions were: 94 °C for 1 min, 5 cycles of 94 °C for 30 s, 45 °C for 1 min and 72 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1.5 min and 72 °C for 1 min, and a final extension of 5 min at 72 °C. This fragment was amplified in a subset of samples – nine individuals of *P. spumarius*, one *P. tesselatus*, one *P. tarifa* and one *Neophilaenus* sp. (Table 1). These were aligned against nine other sequences of a fragment of 399 bp of COI available in Genbank from several *Philaenus* species (Accession numbers FJ516388–FJ516395), from which resulted an overlapped fragment of 313 bp. This fragment was used to evaluate its utility in the discrimination of species.

After visualization in a 0.8% agarose gel stained with ethidium bromide, PCR products were purified with Sureclean (Bioline) following the manufacturer's protocol and sequenced using the forward and the reverse primers with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Fragments were separated on a 310 Genetic Analyzer (Applied Biosystems) and the base composition of the DNA sequences were obtained with Sequencing Analysis Software 5.2 (Applied Biosystems).

2.3. Data analysis

Forward and reverse sequences were aligned and edited using Sequencher 4.05 (Gene Codes Corporation) and the consensus were aligned in BioEdit Sequence Alignment Editor 7.0.5.1 (Hall, 1999). The complete mitochondrial genome sequence of *P. spumarius* is available in Genbank (Acession number AY630340) and belongs to a Canadian specimen (Stewart and Beckenbach, 2005; Stewart, personal communication). It was included in the analysis and was also used to position the reading frame. The outgroups used in this study were sequenced by us in the laboratory and consisted of other species within Cercopidae: *Neophilaenus* sp., the sister genus of *Philaenus*, and *Cercopis intermedia.*

FASTA to Nexus conversions were handled using Concatenator 1.1.0 (Pina-Martins and Paulo, 2008) and phylogenetic analyses were carried out using Maximum Parsimony (MP) and Maximum Likelihood (ML) methods with PAUP* 4.0b10 (Swofford, 2002) and Bayesian inference (BI) with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fit substitution model (HKY+G) was selected using the Akaike Information Criterion (AIC) available in Modeltest 3.7 (Posada and Crandall, 1998) and in MrModeltest 2.3 (Nylander, 2004), to use in ML and in BI analyses, respectively. MP and ML analyses were done using the heuristic search with tree-bisection-reconnection (TBR) branch swapping with 100 random stepwise taxon addition. Node support was obtained with 10,000 bootstrap replicates. In the Bayesian analysis, the posterior probabilities for each node were obtained by carrying out four MCMCMC chains for 1.5×10^6 generations, sampled every 100 generations. The burn-in was set to the first 1000 trees and the remaining trees were used to obtain a consensus tree by the 50% majority rule.

Mega 4.0.2 (Kumar et al., 2008) was used to obtain base composition, number and type of nucleotide substitutions, number of degenerate sites and translation into aminoacids using the invertebrate genetic code. It was also used to calculate pairwise uncorrected genetic distances. Maximum likelihood distances were calculated in PAUP*. Homogeneity of base frequencies among taxa was also calculated in PAUP* with the chi-square test. A median-joining network was constructed for the haplotypes of *P. spumarius* using Network 4.5.1.0 (Bandelt et al., 1999; fluxus-engineering.com).