Genus	Vol. 23(1): 17-24	Wrocław, 30 IV 2012

Molecular identification of atypical morph of *Paracletus cimiciformis* von Heyden, 1837 described as *Forda rotunda* Theobald, 1914 (Hemiptera: Aphidoidea: Eriosomatidae)

Ewa Mróz[1] & Łukasz Depa[2]
Zoology Department, Faculty of Biology and Environmental Protection, University of Silesia, Bankowa 9, PL 40-007 Katowice; [1]ewa.mroz@us.edu.pl, [2]lukasz.depa@us.edu.pl

Abstract. The paper presents the results of molecular analysis of aphids morphologically identical with *Forda rotunda*, a species hitherto not recorded in Polish fauna. The molecular analysis indicates that DNA sequences of "*F. rotunda*" (mitochondrial gene *cox1* and nuclear *ef1α*) were identical with sequences of Mediterranean populations of *Paracletus cimiciformis*. The analysis thus suggests presence of a morphologically different form of *P. cimiciformis* in Poland. The applied molecular analysis proved its usefulness for solving taxonomic problems in a highly polymorphic group like aphids.

Key words: entomology, taxonomy, molecular markers, aphids, *Paracletus*, *cox1*, *ef1α*.

INTRODUCTION

Paracletus cimiciformis von Heyden, 1837, a representative of tribe Fordini of family Eriosomatidae, is a species of European range. In the Mediterranean zone, on the territory of its primary host – *Pistacia terebinthus* – it is holocyclic and diecious (Nieto-Nafria et al. 2003). Bisexual generation reproduces and feeds on pistacia, while exules feed on different species of grasses, where they reproduce exclusively parthenogenetically. In Europe north of the Mediterranean, only such a permanently anholocyclic populations, feeding on grass roots, occur (Szelegiewicz 1968).

In these conditions these aphids are strictly myrmecophilous – they occur only in nests of *Tetramorium caespitum* (L.) and in Spain also in nests of the ant genus *Tapinoma* (Nieto-Nafria et al. 2003). The morphs inhabiting ant nests are easily distinguished, with very characteristic 6-segmented antennae and white color of body, which is also

strongly flattened. Research suggests that this morph does not need to feed on plants as it can obtain food from ants on the way of trophallaxis (Zwölfer 1958, Heie 1980). All hitherto reported observations of this species in Poland concern the presence of this morph (Szelegiewicz 1968, Płachta et al. 1996, Osiadacz & Hałaj 2009).

1. The morphology of the studied form ('A') of the *Paracletus cimiciformis*

During the authors' research on the anholocyclic populations of the tribe Fordini, we noticed that despite extensive field studies including a few dozens of sites with xerothermic vegetation, we found no specimens of *P. cimiciformis* accordant to the above description of its anholocyclic morph.

Instead, the specimens with morphological features of the genus *Forda* were recorded, which should be determined as *Forda rotunda* Theobald, 1914, an aphid species never recorded in Poland. Those aphids were already reported in different parts of Europe, inhabiting nests of *T. caespitum* and feeding on the grass roots. Collected apterous viviparous females were round and shining green, with 5-segmented antennae (Ortiz-Rivas et al. 2009) (Fig. 1.).

However, Roberti (1983, 1993) and later Ortiz-Rivas et al. (2009), basing on the molecular studies of Iberian representatives of the genus *Paracletus*, suggested that *F. rotunda* may be a morph (so called form "A") of *P. cimiciformis*. Because the factor triggering the development of the green morph is unknown, the molecular analysis was necessary to resolve the taxonomic identity of the studied aphids. For this purpose, two molecular markers were sequenced: mitochondrial *cox1* and nuclear *ef1α*. These sequences were then compared to sequences of the taxon of interest deposited in GenBank by other authors.

Mitochondrial genes have been widely used in the molecular systematics of insects (Simon et al. 1994). Due to their maternally inherited characteristics (Hoy, 1994), mitochondrial sequences are suitable for reconstructing phylogenetic relationships of parthenogenetic organisms like aphids (von Dohlen & Moran 2000). Mitochondrial genes encoding cytochrome oxidase subunit 1 (*cox1*) and subunit 2 (*cox2*) have been

Table 1. Collection data and accession numbers of used sequences.

Species	Form	Voucher number	Collection site	Collection date	Host plant	Ant species associated with aphids	GenBank Accession No.	
							ef1α	*cox1*
Paracletus cimiciformis	AA	D1a	Lubaczów	23.09.2010	*Poa sp.*	*Tetramorium caespitum*	JQ241785	JQ241782
	AA	D1b	Lubaczów	23.09.2010	*Poa sp.*	*Tetramorium caespitum*	JQ241786	JQ241783
	AA	D3	Sandomierz	22.09.2010	*Poa sp.*	*Tetramorium caespitum*	JQ241787	JQ241784
Species	*Paracletus cimiciformis*						FM163597.1	AY227089.1
	Forda formicaria						DQ499608.1	AY227086.1
	Forda marginata						FM163596.1 DQ499609.1	EU701668.1
	Forda riccobonii							EU701669.1 AY227076.1
	Pemphigus bursarius						DQ779154.1	EU701834.1

used in phylogenetic studies of the three aphid subfamilies, Hormaphidinae (Stern 1994; 1998; Stern et al. 1997), Lachninae (Normark 1999; 2000) and Aphidinae (von Dohlen et al. 2006). These genes appear to be very useful for clarifying phylogenetic relationships also at the tribal and generic levels in aphids (Normark 2000; Stern 1994). Among nuclear markers, also the nuclear gene encoding elongation factor 1-alfa (*ef1α*) has often been used for species identification and phylogenetic reconstruction (Ortiz-Rivas et al. 2009).

The aim of the study was to check the species identity of collected specimens of *F. rotunda* using molecular markers.

MATERIAL AND METHODS

The material comprised the three specimens of *F. rotunda* (which were the supposed form "A" of *P. cimiciformis*), collected from two different sites located in southeastern Poland (Table 1).

MOLECULAR RESEARCH

Aphids were collected in 95% - 100% ethanol and stored at 4°C. We extracted total genomic DNA using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Prior to extraction, we removed the entire abdominal tagma to avoid possible contamination from gut content.

Voucher specimens were collected in 80% ethanol and deposited in Zoology Department of the University of Silesia. Samples for extraction consisted of individuals from the same colony and were probably members of the same clone as the voucher specimens.

Polymerase chain reaction (PCR) products were generated from a mitochondrial gene: cytochrome oxidase I (*cox1)*, and nuclear gene: elongation factor 1-alfa (*ef1α)*. The primers used for the PCR amplifications were applied following published sequences (listed in Table 2), for *cox1* using primers LCO1490 and HCO2198. The mitochondrial gene fragment was amplified as follows: initial denaturation at 94°C for 1 min, followed

Table 2. The sequences of primers applied in the studied species

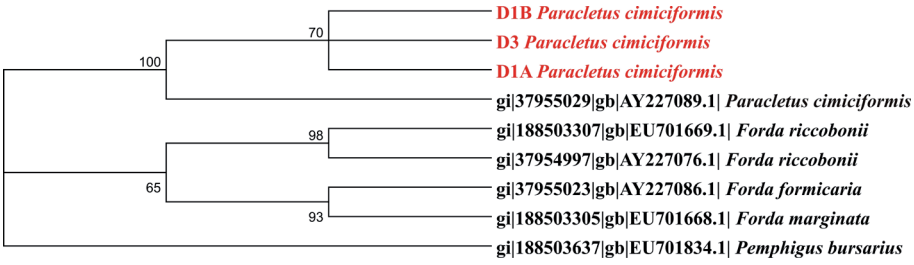
Region	Gene location	Primers name	Primer sequence (5′ - 3′)	Reference
Mitochondria	*cox1*	LCO1490 HCO2198	5′-GGTCAACAAATCATAAAGATATTGG-3′ 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer *et al*. 1994
Nuclear DNA	*ef1α*	Efs175 efr1	5′GGAAATGGGAAAAGGCTCCTTYAAGTAYGCYTGGG-3′ 5′-GTGTGGCAATSCAANACNGGAGT-3′	Moran *et al*. (1999) Ortiz Rivas *et al*. (2009)
Nuclear DNA	*ef1α* nested	Efs1 nested Efr3 nested	5′-TGGACAAAYTKAAGGCTGAACG-3′ 5′-GTRTASCCRTTGGAAATTTGACC-3′	Ortiz Rivas *et al*. (2009)

by 35 cycles of 94°C for 30s; annealing temperatures 47 for 45s; extension at 72°C for 1min; final extension at 72°C for 3 min.

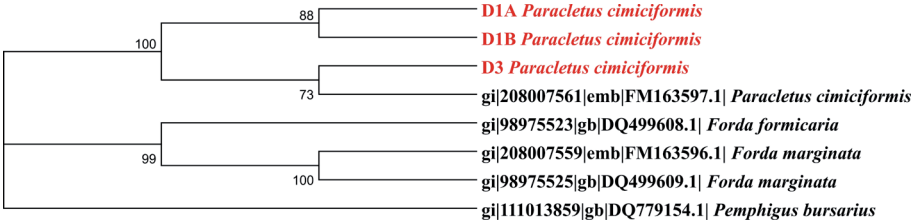
Two sets of nested primers were used for *ef1α* amplification. The first PCR was made using primers Efs175 and Efr1 with the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30s; annealing temperatures 52 for 1 min; extension at 72°C for 2 min; final extension at 72°C for 7 min. Second, nested PCR was necessary, using primers Efs1, Efr3 on 0,5µl taken from the first PCR. Conditions were identical except that the annealing temperature was 56°C.

Products were sequenced at the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (IBB PAN).

Amplified DNA was visualized using 1.5 % agarose gel electrophoresis with ethidium bromide staining. PCR products were purified using a QIAquick® PCR purification Kit (QIAGEN) and sequenced directly with an automated sequencer (Genome Sequencer GS FLX Roche) at IBB PAN (Laboratory of DNA Sequencing and Oligonucleotide Synthesis). Complete sequences were deposited in GenBank under accession numbers given in Table 1, together with the accession numbers of sequences used for data comparison.



2. Maximum parsimony (MP) tree from the analysis of the cox1 gene sequences from species in the genera Forda and Paracletus. Bootstrap values (as a percentage) are indicated when higher than 50. There were a total of 425 positions in the final dataset, out of which 49 were parsimony informative. Phylogenetic analyses were conducted in MEGA4



3. Maximum parsimony (MP) tree from the analysis of the ef1α gene sequences from species in the genera Forda and Paracletus. Bootstrap values (as a percentage) are indicated when higher than 50. There were a total of 907 positions in the final dataset, out of which 64 were parsimony informative. Phylogenetic analyses were conducted in MEGA4

Computer analysis of DNA sequences

The target sequences were 425 bp from the mitochondrial gene encoding cytochrome oxidase subunit (*cox1*), and 907 bp of the nuclear gene encoding elongation factor 1-alfa (*ef1α*) including both exons and introns.

Chromatograms were analysed and assembled using the CHROMAS V2.3 (Technelysium Pty Ltd. 2004). Multiple alignments were done with Clustal X v1.81 (Thompson et al. 1997) with gap opening and gap extension penalties of 10.0 and 0.2, respectively, and subsequently manually revised.

Molecular phylogenetic analysis

The phylogenetic trees were constructed using MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al. 2007).

Aligned sequences were tested using bootstrap method and to construct the tree the maximum parsimony (MP) method was applied. The MP tree was obtained using the close-neighbor-interchange algorithm (Nei & Kumar 2000) with search level 3 (Felsenstein 1985) in which the initial trees were obtained with the random addition of sequences (100 replicates). The codon positions included were 1st+2nd+3rd+Noncoding.

RESULTS

The results of molecular analysis obtained with both applied markers indicated that sequences of studied specimens create a clade with those deposited in the data bank belonging to *P. cimiciformis*, which was supported with high bootstrap values. The results are presented on generated phylogenetic trees (Figs. 2 and 3). The clade consisting of a *P. cimiciformis* sequences is a sister clade to the one comprising the representatives of the genus *Forda*. The sequence of *Pemphigus bursarius* was applied as an outgroup as a representative of the sister subfamily Pemphiginae.

DISCUSSION

The sequences of analyzed specimens of the *P. cimiciformis* form a clade in case of both applied markers, together with the sequences obtained from GenBank. This evidence seems sufficient to be sure that the form 'A' is a morph of the *P. cimiciformis*, and not a different species of the genus *Forda*. The *Forda* species constitute a separate clade, a sister group to the clade with *Paracletus*. Thus, the analysis of Central-European representatives of the tribe Fordini confirms the idea of synonymy of *Forda rotunda* with *P. cimiciformis* proposed by Ortiz-Rivas et al. (2009) on the basis of molecular research of Iberian populations.

This is an interesting case of usefulness of molecular analysis for taxonomical purposes in aphids. The taxonomic problems of the species identity usually concern very closely related species, which are often morphologically very similar and thus, making it difficult to unambiguously determine the species (Wang & Qiao 2009, Rakauskas et al. 2011). In the studied case there are two forms which may easily be determined as belonging to two different (although closely related) genera. In such case, when the

polymorphism of a single species reaches the genus-level, only molecular analysis may correctly confirm the species identity.

The results also clearly indicate that the bionomics of this species is not well recognized yet. The factors determining the origin of both forms are still unknown and their ecological function is still a matter for further research.

REFERENCES

Felsenstein, J., 1985 Confidence limits on phylogenies: An approach using the bootstrap. Evolution, **39**: 783-791.

Heie, O.E.,1980. The Aphidoidea (Hemiptera) of Fennoscandia and Denmark. I. General Part. The families: Mindaridae, Hormaphididae, Thelaxidae, Anoeciidae and Pemphigidae. Fauna Entomologica Scandinavica, **9**: 1-236.

Hoy, M.A., 1994. Chapter13. Insect molecular systematics and evolution. In Insect Molecular Genetics (San Diego, USA: Academic Press Inc.), pp. 337–379.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York. 333 pp.

Nieto Nafría, J.M., Mier-Durante, M.P., Binazzi, A., Pérez–Hidalgo, N., 2003. Fauna Ibérica. Hemiptera Aphididae II. Museo Nacional de Ciencias Naturales, CSIC, Madrid.

Normark, B.B., 1999. Evolution in a putatively ancient asexual aphid lineage: Recombination and rapid karyotype change. Evolution, **53**: 1458–1469.

—, 2000. Molecular systematics and evolution of the aphid family Lachnidae. Molecular Phylogenetics and Evolution, **14**: 131–140.

Ortiz-Rivas, B., Martínez-Torres, D., Pérez-Hidalgo, N., 2009. Molecular phylogeny of Iberian Fordini (Aphididae: Eriosomatinae): implications for the taxonomy of genera *Forda* and *Paracletus*. Systematic Entomol., **34**: 293-306.

Osiadacz, B., Hałaj, R., 2009. The aphids (Hemiptera: Sternorrhyncha: Aphidinea) of Poland. A distributional checklist. Polish Entomological Monographs, **6**: 1-96.

Płachta, J., Klimaszewski, S.M., Wojciechowski, W., 1996. Aphid groupings (Homoptera, Aphidodea) in *Dicrano-Pinion* coniferous forests of the Pomorze Lake District. Acta Biologica Silesiana 29(46): 66-82.

Rakauskas, R., Turčinavičienė, J., Bašilova, J. 2011. How many species are there in the subgenus Bursaphis (Hemiptera: Sternorrhyncha: Aphididae)? Co-I evidence. European Journ. Entomol., **108**: 469-479.

Roberti, D., 1983. Note su alcune specie di Fordinae (Homoptera – Aphidoidea Eriosomatidae). Entomologica, **28**: 141–205.

Roberti, D., 1993. Gli Afidi d'Italia (Homoptera – Aphidoidea). Entomologica, **25**: 3-387.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. Ann. Entomol. Soc. Am., **87**: 651–701.

Stern, D.L., 1994. A Phylogenetic analysis of soldier evolution in the aphid family Hormaphididae. Proc. R. Soc. Lond. B. Biol. Sci., **256**: 203-209.

—, 1998. Phylogeny of the tribe Cerataphidini (Homoptera) and the evolution of the horned soldier aphids. Evolution, **52**: 155-165.

Stern, D.L., Aoki, S., Kurosu, U., 1997. Determining aphid taxonomic affinities and life cycles with molecular data: a case study of the tribe Cerataphidini (Hormaphididae: Aphidoidea: Hemiptera). Systematic Entomol., **22**: 81-96.

Szelegiewicz, H., 1968. Mszyce – *Aphidodea*. w: Katalog fauny Polski. Warszawa. T.XXI, 4:1-316.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biol. Evol., **24**:1596-1599.

Technelysium Pty Ltd., "Chromas," http://www.technelysium. com.au/chromas.html, 2004.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., **24**: 4876-4882.

von Dohlen, C.D., Moran, N.A., 2000. Molecular data support a rapid radiation of aphids in the Cretaceous and multiple origins of host alternation. Biol. J. Linn. Soc., **71**: 689-717.

von Dohlen, C.D., Rowe, C.A., Heie, O.E., 2006. A test of morphological hypotheses for tribal and subtribal relationships of Aphidinae (Insecta: Hemiptera: Aphididae) using DNA sequences. Molecular Phyl. Evol., **38**: 316-329.

Wang, J., Qiao, G. 2009. DNA barcoding of genus *Toxoptera* Koch (Hemiptera: Aphididae): identification and molecular phylogeny inferred from mitochondrial COI sequences. Insect Science, **2009**: 475–484.

Zwölfer, H., 1958. Zur Systematik, Biologie und Ökologie unterirdischer lebender Aphiden (Homoptera, Aphididae). Teil III. Zeitschr. Angew. Entomol., 42(2).