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Molecular and morphological identification of *Cinara juniperi* and *Cinara mordvilkoi*

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Abstract

Cinara juniperi (De Geer) and *Cinara mordvilkoi* (Pasek) (Rhynchota Aphidoidea), the species infesting *Juniperus* sp., are morphologically very similar to each other and difficult to distinguish. A COI analysis of mitochondrial DNA proved 9% distinction between them. A phylogenetic analysis based on the distance neighbour-joining method proved the highest similarity between *Cinara juniperivora* (Wilson) and *C. juniperi*, amounting to 95%. It was also proven that the body length, tibia length, the length of antennae segment III, the length of the rostrum and the total length of the antennae are the optimal statistically significant morphological features that enable to distinguish those species.

Key words: *Cinara* sp., *Juniperus* sp., cytochrome oxidase I, morphology.

Introduction

Aphids of the *Cinara* genus (Rhynchota Aphidoidea) infest lignified parts, branches, trunks, roots and leaves of coniferous Pinaceae and Cupressaceae trees. This genus includes about 200 species, out of which about 150 are native of N. America, 30 of Europe and 20 of the Far East (Blackman and Eastop, 1994). Approximately 26 species of *Cinara* occur in Poland and Central Europe, mostly infesting *Pinus*, *Abies* and *Picea*. The number of species is still increasing due to expansion of the warm climate species i.e. *Cinara tujafilina* (Del Guercio) (Durak *et al*., 2006). The species of this genus do not require a change of host plant in their life cycle. *Cinara* belongs to Eulachnini tribes and Lachninae subfamily, which in the light of the latest molecular studies have turned out to be the basal group for the other aphids (Ortiz-Rivas and Martinez-Torres, 2010).

Cinara differs from other aphids, as many species may infest the same host plant. Numerous *Cinara* species infest *Pinus* trees, e.g. as many as 14 species are associated with *Pinus edulis* Engelmann (Voegtlin and Bridges, 1988). Many *Cinara* species are related to the Cupressaceae plants. According to Blackman and Eastop (1994) the whole *Juniperus* genus is associated with 17 aphid species, with some of them known only from authors' original descriptions. All the *Cinara* species are yellow-brown to dark brown, more rarely green or black, up to 8 mm long and difficult to determine (Szelęgiewicz, 1978).

Cinara species associated with *Juniperus* are poorly known and very difficult to distinguish. The most frequently recorded species is *Cinara juniperi* (De Geer), which occurs in Europe, Middle East, Australia, New Zealand and USA (Blackman and Eastop, 1994).

Cinara mordvilkoi (Pasek) is a very rare species recorded in the Czech Republic, Poland, Lithuania, Sweden and Italy, known only from a few localities (Szelegiewicz, 1962; Heie, 1995; Herczek *et al*., 1977; Danielsson and Carter, 1992; Binazzi, 1996). Those species may occur in mixed colonies, which add to the difficulty of their distinction and determination. Some authors consider *C. mordvilkoi* to be a synonym of *C. juniperi* (Carter and Maseln, 1982; Barbagallo *et al*., 1995). Due to its rare occurrence, some data on the species are based on a single specimen of an aphid (Binazzi, 1996). The rarity of its occurrence and unspecific morphological characteristics render it even more difficult to distinguish the species and analyse their mutual phylogenetic relations (Foottit and Mackauer, 1990; Foottit, 1992; Watson *et al*., 1999).

The aim of the study was to prove genetic and morphological distinction between the *Cinara* species infesting *Juniperus*. In molecular determination of both species cytochrome oxidase (COI) of mitochondrial DNA was used. Cytochrome oxidase is commonly used to identify insects belonging to various genera, also aphids (Milankov *et al*., 2005; Foottit *et al*., 2008). It was also used in genetic and phylogenetic research within the *Cinara* genus (Favret and Voegtlin, 2004; Durak *et al*., 2008; Mujtar *et al.*, 2009).

Materials and methods

Specimens of *C. juniperi* and *C. mordvilkoi* species were collected in summer 2008 and 2009 from *Juniperus communis* L. in Brenna (Poland). The aphids were preserved in 95% ethanol.

The DNA was extracted from single aphids with a standard phenol procedure and DNA from 10 aphids of each species has been obtained. Then the DNA fragments were PCR-amplified with LCO1490/HCO2198 primers (Folmer *et al*., 1994), which give about 650 bp of the COI gene from the mitochondrial genome. PCR reactions were carried out in 50 µl reaction aliquots containing 1 µl DNA, 1.5 µl of each primer (10 pM), 0.5 µl of Taq DNA polymerase (5U/µl), 5 µl of buffer 3 (Expand Long Template PCR System, Roche), 1 µl of 10 mM dNTPs and ultra-pure water. The temperature profile for the amplification of the COI gene fragment included an initial denaturation step of 95 °C for 2 min

followed by 3 cycles of 95 °C for 30 sec, 47 °C for 30 sec, 72 °C for 1 min 10 sec and 32 cycles of 95 °C for 30 sec, 53 °C for 30 sec, 72 °C for 1 min 10 sec and a final extension period of 72 °C for 10 min. Amplification products were resolved by electrophoresis in 2% agarose gels. PCR products were cleaned with High Pure PCR Product Purification Kit (Roche) and then sequenced in Genomed service (www.genomed.pl).

In order to verify the difference and similarity between the species infesting *Juniperus* the analyses also included *Cinara juniperivora* (Wilson) gathered from the branches of *Juniperus virginiana* L.. The COI sequence of *C. juniperivora* is available from GenBank (AY300221). The sequences were assembled and aligned with Lasergene software package (DNASTAR Inc., USA). A phylogenetic tree based on neighbour-joining method and divergence/similarity matrix was drawn using the same Lasergene software.

Microscope slides were prepared for morphological identification of 30 aphids of each species. The following aphid body parts were measured: body length, tibia length, the length of antennae segments III, IV, V and VI, rostrum length. The microscope slides are deposited at the Department of Invertebrate Zoology, University of Rzeszow (Poland).

In order to determine statistical significance of morphological differences between the species the ANOVA variance analysis as well as Mann-Whitney U (Z) and T tests (T) were conducted with STATISTICA for Windows.

Results

With COI primers 628 bp nucleotide parts were amplified for the studied species. A sequence analysis for 628 bp lengths of mitochondrial COI-coding DNA proved an abundance of A-T nucleotides. The proportion of A+T in *C. juniperi* was 73.57%, while for *C. mordvilkoi* it amounted to 71.79%. The share of particular nucleotides was as follows: for *C. juniperi* A 35.19%, T 38.38%, C 16.24%, G 10.19%, while for *C. mordvilkoi* A 34.95%, T 36.83%, C 16.61%, G 11.6% (figure 1). Both presented sequences were the first and only ones deposited at GenBank for those species (JN190924, JN190923). No differences were observed between specimens of the same species both in *C. juniperi* and in *C. mordvilkoi*; the sequences were identical within a species.

55 nucleotides differentiating COI of *C. juniperi* from the one of *C. mordvilkoi* were observed. The total similarity between the species was 91%. The 9% threshold constituting the difference clearly states the difference between the studied species (figure 1).

C. juniperivora was also covered by this study for comparative purpose. It was proven that the percentage of similarity between *C. juniperivora* and *C. juniperi* is the highest and reaches 95%. The similarity between *C. mordvilkoi* and the remaining species was 89% (figure 2).

An analysis of morphological traits showed significant statistical differences between the parameters of the two species: body length ($Z = -2.63$; $P = 0.0084$), tibia length ($Z = -2.63$; $P = 0.0084$), the length of antennae segment IV ($Z = -2.46$; $P = 0.0137$), the length of antennae segment VI ($Z = -2.123$; $P = 0.0337$). Significant differences were also found between the lengths of antennae segments III ($T = -3.216$; $P = 0.0092$) and the lengths of antennae segment V ($T = -2.7628$; $P = 0.02$). The lengths of rostrum and antennae differed significantly between the species ($Z = -2.63$; $P = 0.0084$ and $T = -3.2$; $P = 0.0016$ respectively). Those species also differ in the relation of rostrum length to body length (table 1). On the basis of measurements it was found that the segments of *C. mordvilkoi* antennae vary and each subsequent one is longer than the previous one (table 1).

Discussion

C. juniperi and *C. mordvilkoi* infest the same host plant and only slightly differ in morphology, most of which concerns secondary features such as body colour and sheen. This creates serious identification problems for both species, particularly when they occur in mixed colonies and are often wrongly classified. Klimaszewski *et al*. (1977) proved that both species differ in the composition of hemolymph proteins. Anatomical analysis of the male reproductive system indicates a number of similarities between the species. They both have 4 tracts in testicles and their deferent ducts in their proximal part are widened and connected, however, they do not differ in terms of histology and size (Wojciechowski, 1977).

Table 1. Morphological features of the apterous viviparous females *C. juniperi* (n = 30) and *C. mordvilkoi* (n = 30). Minimum - maximum (mean). Statistical significance: * = $P < 0.05$; ** = $P < 0.01$.

Characters	Lenght measurements (mm)		
	C. juniperi	*C. mordvilkoi*	
body length	2.1 - 2.45 (2.33)	2.75 - 3.12 (2.94)	**
tibia lenght	0.9 - 1.12 (1.04)	1.45 - 1.55 (1.5)	**
antennae segment III	0.22 - 0.35 (0.28)	0.32 - 0.4 (0.37)	**
antennae segment IV	0.12 - 0.15 (0.128)	0.15 - 0.17 (0.162)	*
antennae segment V	0.15 - 0.21 (0.18)	0.2 - 0.25 (0.21)	*
antennae segment VI	0.22 - 0.26 (0.23)	0.25 - 0.27 (0.26)	*
antennae lenght	0.82 - 1.1 (0.96)	1.17 - 1.22 (1.19)	**
rostrum lenght	0.85 - 1.0 (0.9)	1.32 - 1.72 (1.43)	**
rostrum/body	38.7%	48.6%	

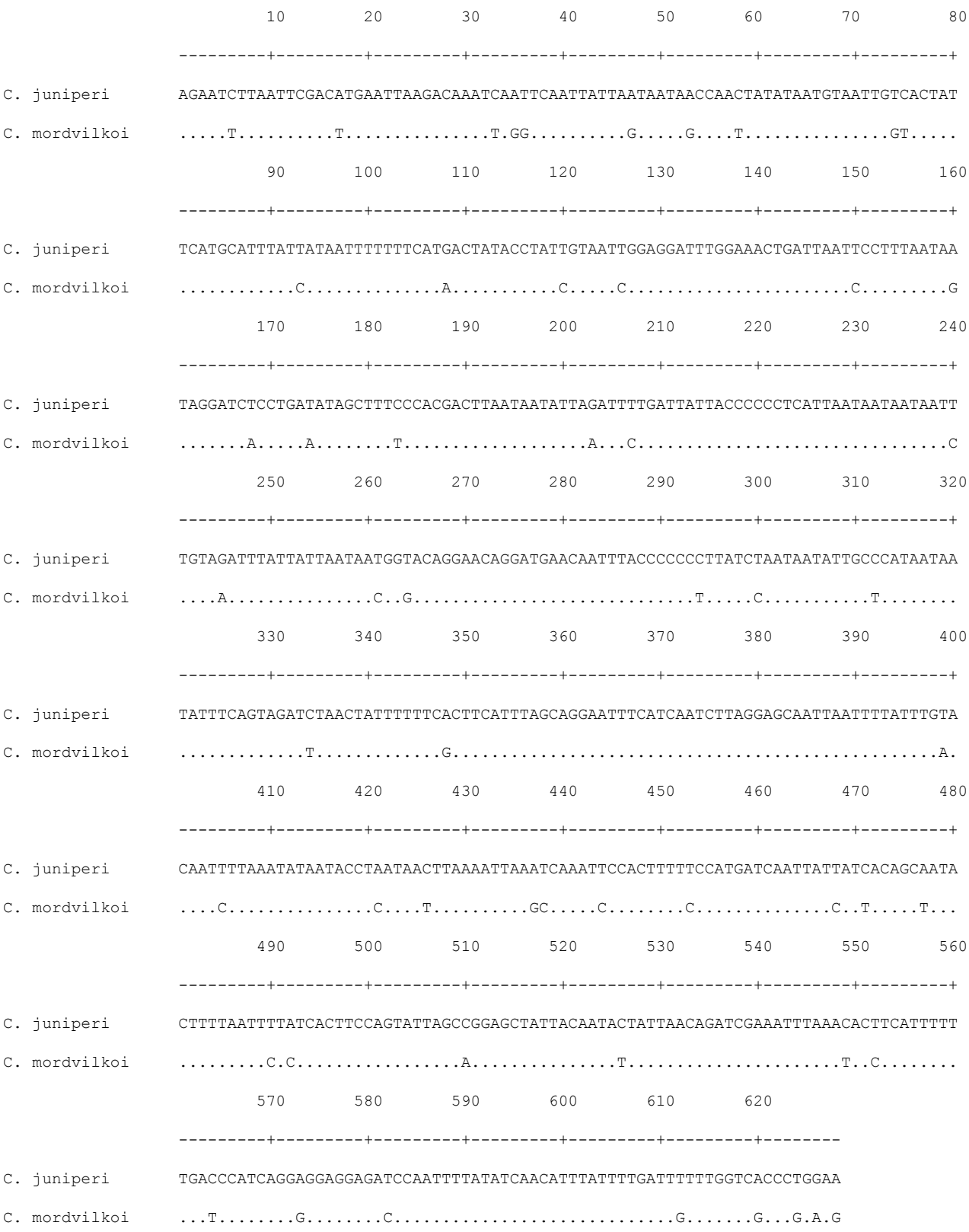

Figure 1. Alignment of COI sequences for *C. juniperi* and *C. mordvilkoi*. Dots indicate identical nucleotides as the topmost sequence.

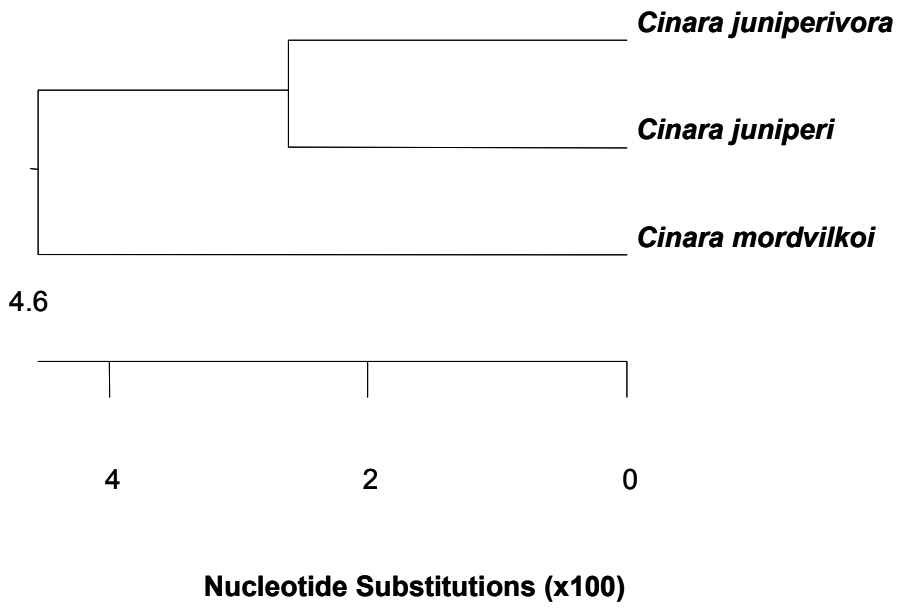

Figure 2. Phylogenetic clustering of three aphids species in relation to partial COI mitochondrial gene, based on neighbour-joining method.

Despite many similarities an analysis of mitochondrial DNA clearly indicates genetic distinction of the species. A high, reaching 9% difference between COI coding sequences indicates the species distinction on the one hand and enables their precise identification on the other. A differentiation of mtDNA sequences on a level of 2% allows to classify the aphids into species (Hebert *et al*., 2003; Stern *et al*., 1997). Previous analysis of COI sequence was used to classify morphologically undeterminable species of *Cinara contortae* Hottes and *Cinara ponderosae* (Williams), infesting the *Pinus* trees (Favret and Voegtlin, 2004). These points at a possibility of effective application of the method used to identify this difficult group.

An analysis of similarity between the three *Cinara* species infesting *Juniperus* sp. indicated a high (5-11%) difference between them. It seems interesting that the level of difference between *C. juniperi* and *C. juniperivora* is lower than between those two and *C. mordvilkoi*. This might corroborate the theory that within *Cinara* particular species are more closely related if they infest similar microhabitats (feeding on different plants) than species infesting the same host plant. In such case there was less difference between *C. juniperi* and *C. juniperivora*, feeding on branches and twigs of different host plants (*J. communis* and *J. scopulorum*). This might also corroborate the possibility of *C. mordvilkoi* infesting not only branches but also roots, which has not been confirmed as yet, however, it has been indicated as possible by biological observations. This theory could be further corroborated by a morphological adaptation in the form of rostrum length in this species and its high relation to body length. The aphids infesting roots, trunks and branches have longer stylets than those feeding on twigs (Bradley, 1961; Favret and Voegtlin, 2004). The presented study showed relationships between the feeding spot of the species and the length of rostrum as well as percentage relation between its length and body length. Both the rostrum length and its relation to body length were considerably higher for *C. mordvilkoi.* Morphological adaptations are supplemented with the COI-based analyses.

Significant statistical differences between morphological traits of both species presented herein facilitate their identification. The presented study clarified doubts as to the length of antennae segments IV, V and VI. The measurements confirm that for *C. mordvilkoi* segment V is longer than IV and shorter than VI, as previously indicated by Pašek (1954). This feature has been problematic so far and some authors, e.g. Mamontova (1972), or Danielson and Carter (1992) indicated that segments V and VI are of the same length. The presented lengths of antennae segments as well as the lack of sclerites on the first and second tergit of the abdomen are characteristic of *C. mordvilkoi*, and the absence of those features may confirm the doubts put forth by Zhuravlev (2003) on the absence of this species in Ukraine.

However, it must be stressed that general identification of *Cinara* specimens is very difficult. This results from their significant morphological similarity. Most *Cinara* are considerably large, brown, grey or grey-black. A slight morphological variety, according to Heie and Wegierek (2009), results from comparatively slow evolution of Sternorrhyncha in comparison with other groups, e.g. mammals. Aphids evolve more in terms of adaptation than morphology. The body structure of *Cinara* formed in Miocene has not significantly changed. Evolutionary changes in this group concern rather infestation of new host plant species (observed since mid-Tertiary) or changes in their life cycles. Molecular identification of species belonging to *Cinara* will certainly enable to learn and understand their phylogenetic relations.

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