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A new aphid subspecies on the endemic Cyprus cedar *Cedrus brevifolia*: *Cinara cedri brevifoliae* ssp.n. (Aphididae Lachninae)

Francesco BINAZZI[1], Agostino STRANGI[1], Francesco PAOLI[1], Giuseppino SABBATINI PEVERIERI[1], Pio Federico ROVERSI[1], Andrea BINAZZI[2]
[1]*Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA), Research Center for Agrobiology and Pedology, Cascine del Riccio, Florence, Italy*
[2]*Bagno a Ripoli, Florence, Italy*

Abstract

A subspecies of *Cinara cedri* Mimeur, *C. cedri brevifoliae* ssp.n., is described from apterous viviparous females. The authors report on the discovery of this new taxon in Cyprus on the endemic cedar *Cedrus brevifolia* (Hooker fil.) Henry, suggesting that the aphid is co-endemic with its host conifer. Morphological evaluation is provided in order to support this conclusion and a key to separate the Cedar *Cinara* species is given. Moreover, new insights on the distribution of *C. cedri* in the Mediterranean area were gained by molecular analysis. Gene sequences were deposited in Genbank and the type specimens located in the collection (A.B.) of CREA-Research Center for Agrobiology and Pedology, Florence, Italy.

Key words: Aphididae, Lachninae, *Cinara* new subspecies, *Cedrus brevifolia*, Cyprus, Cedar aphids.

Introduction

The discovery in Cyprus of *Cinara cedri* Mimeur on the endemic *Cedrus brevifolia* (Hooker fil.) Henry was recently reported and discussed by Binazzi *et al.* (2015). It is worth noting that *C. cedri*, though widespread in the Mediterranean basin, had never been previously recorded on *C. brevifolia* in its natural environment. So far, in Europe, this aphid had been reported to feed on *C. atlantica* and *C. deodara* wheresas in Turkey it was observed on *C. libani* (Tuatay and Remaudière, 1964; Covassi and Binazzi, 1974; Notario *et al*., 1984).

However, preliminary morphological evaluation of the Cyprus cedar aphids based on mature specimens mounted on slides, highlighted significant differences between them and other individuals of the nominotypical species. This outcome led Binazzi *et al.* (2015) to suggest that the Cyprus cedar aphid is native to the island, thus representing an endemism together with its host cedar. In the present work, further morphological evaluation allowed the last A. to define this lacnid as a new subspecific taxon while the molecular analysis shed light on *C. cedri* distribution in the Mediterranean basin.

Materials and methods

In September 2015, *C. cedri* individuals were detected on a young *C. brevifolia* tree located in the Troodos mountains (1700 m a.s.l.) (34°56'N, 32°50'E) near Prodromos (Cyprus). The sample, composed of 12 apterous viviparous females plus some immature specimens, was collected by shaking cedar branches over a white cotton towel. Specimens were then stored in 100% ethanol (Binazzi *et al*., 2015).

Moreover, living specimens of *C. cedri* were collected on a *C. atlantica* ornamental tree (Bagno a Ripoli, close to Florence, Italy, 15.X.2015, 300 m a.s.l., 43°45'N, 11°21'E) and then stored, as the Cyprus samples, in order to perform molecular analysis. In addition, the following specimens (slides in "A. Binazzi" = A.B. collection, CREA, Firenze, Italy) were employed for morphological comparison: 3 from *C. atlantica*, same data as above (sl. C10/931); 2 from *C. atlantica*, Florence, 28.V.1974 (sl. C2/102); 2 from *C. deodara*, Aci S. Antonio (Catania-Sicily), 30.X.1974 (sl. C2/110-109); 1 from *C. atlantica*, Piano Zucchi (Palermo-Sicily), 24.VI.1983 (sl. C2/122); 2 from *Cedrus* sp., Madrid (Spain), 28.VII.1981 and 02.X.1981 (sl. C2/117 and sl. C2/119).

Additional morphological information was obtained from the original description by Mimeur (1935).

As regards the morphological analysis specimens were mounted on slides and identified using the keys from Binazzi (1984), Blackman and Eastop (1994; 2015) and Nieto Nafria *et al.* (2002).

For the molecular analysis Genomic DNA was extracted from the whole body of three single individuals using the ZR Tissue & Insect DNA MicroPrep (Zimo Research) kit according to the manufacturer's instructions, while final elution step was performed in 30.0 µl of DNA Elution Buffer. Quality of DNA preparations was evaluated with both spectrophotometer (QIAxpert, QIAGEN) and agarose gel electrophoresis. Amplification and sequencing of the barcode gene region belonging to mitochondrial Cytochrome Oxidase Complex I gene (COI) were performed according to Folmer *et al*. (1994).

Cytocrome b (*CytB*) and Elongation Factor 1 alpha (*EF1α*) DNA fragments comprised between primers CP1 - CP2 (amplicon length: 798 bp) and Ef3 - Ef6

(amplicon length: 1070 bp) respectively, were amplified according to Jousselin *et al.* (2013). PCR products were sequenced at the Centro di Servizi per le Biotecnologie di Interesse Agrario Chimico ed Industriale (CIBIACI), University of Florence, Italy, and the resulting sequences were submitted to GenBank.

Among all the species of the genus *Cinara* recorded on *Cedrus* spp., only those whose both COI and *CytB* sequences were available in GenBank or in the specific barcode / taxonomy database for European aphids (Coeur D'Acier *et al.*, 2014) were considered for phylogenetic reconstruction. Single gene phylogenetic trees were calculated independently both for COI and *CytB loci* using Maximum Likelihood (ML) and Bayesian inference (BI) methods.

The starting alignment of 12 sequences belonging to a region of COI [mean nucleotide frequencies were 34.80% (A), 40.41% (T), 14.55% (C), and 10.24% (G)] resulted in a matrix of 13 informative sites on 630 positions. The transition/transversion rate ratios were k_1 = 47.761 (purines) and k_2 = 16.28 (pyrimidines) while the overall transition/transversion bias was R = 10.742.

The alignment of sequences belonging to *CytB* locus from the same specimens had 5 informative sites on 692 positions with the following mean nucleotide frequencies 35.06% (A), 43.43% (T), 12.60% (C), and 8.91% (G). The transition/transversion rate ratios were k_1 = 1000 (purines) and k_2 = 18.845 (pyrimidines) while the overall transition/transversion bias was R = 131.025.

Phylogenetic distance estimations in ML trees were obtained adopting the Tamura and Nei (1993) substitution model, and trees were tested performing 5000 bootstrap replicates.

BI trees were calculated assuming the same substitution model used for ML analysis under a relaxed log normal molecular clock hypothesis. A phylogenetic tree based on COI sequences was obtained under coalescent constant population approximation while *CytB* better fitted with Yule model. BI analyses were run for 10 million generations, sampling every 1,000 generations (first million discarded as burn-in). Five independent Markov chain Monte Carlo analyses were performed starting from a randomly chosen tree. Maximum clade credibility tree was summarized.

Multilocus phylogenesis was performed starting from an alignment of 1322 positions with 18 informative sites and the following mean nucleotide frequencies 34.94% (A), 41.99% (T), 13.53% (C), and 9.55% (G). The transition/transversion rate ratios were k_1 = 57.581 (purines) and k_2 = 19.804 (pyrimidines) while the overall transition/transversion bias was R = 12.331.

Tamura-Nei substitution matrix was adopted in both phylogenetic tree reconstructions methods. The optimal ML tree was obtained using moderate branch swapping algorithm and it was tested with 5000 bootstrap replicates. BI tree adopted a relaxed log-normal molecular clock and tree reconstruction under Yule speciation model. BI analyses were run for 10 million generations, sampling every 1,000 generations (first million discarded as burn-in). Five independent Markov chain Monte Carlo analyses were performed starting from a randomly chosen tree. Maximum clade credibility tree was summarized.

All ML trees showed in this work were computed using MEGA 6 software (Tamura *et al*., 2013), while BI analyses were obtained with BEAST 2 ver. 2.4.1 (Bouckaert *et al.*, 2014).

Cinara cedri brevifoliae Binazzi A., ssp.n.

Morphological description

Apterous viviparous female (from 9 specimens) (figure 1, 1-2)

Small- to medium-sized body, 2.21-2.57 mm long, light bronzy in color when alive, with four rows of roundish intersegmental sclerites on dorsal abdomen. Siphuncular cones brown and medium sized with marked apical flange. Head, prothorax and mesothorax rather lightly sclerotized. Metathorax without sclerotizations and the same absence in abdominal tergites I to VI. Tergite VII with a reduced spinal lightly sclerotic area or with several fragmented sclerites whereas the VIII bears a well sclerified solid bar, occasionally interrupted in the middle. Cauda dark brown. Genital plate small and light colored. Antennae rather pale with only the segment I dark brown while light brown are the segment II, the distal part of III and IV, the distal half of V and the whole VI. Rostrum reaches the V abdominal segment with dark brown apical segments. Legs rather uniformly dark brown except for a paler basal part of femora and tibiae; the latter are darker on hind legs. Tarsi brown, a little paler than the distal end of tibiae.

Length of the antennal segments, III, 0.372-0.480, IV, 0.150-0.186, V, 0.186-0.222, VI, 0.120-0.144 mm long (p.t., 0.042-0.051 with 4 subapical setae); secondary rhinaria, 0-1 on III, 1-2 on IV, 1 on V; ratio antennal segments V to VI, 1.07-1.15; ratio antennal segment III to diameter of siphuncular cones, 1.01-1.5; rostral segments IV and V, 0.174-0.204 and 0.090-0.102 mm long, their ratio 1.82-2.13; hind tibiae, 1.16-1.68 mm long; hind tarsal segment (I), basal length, 0.036-0.042, dorsal length, 0.060-0.072, ventral length, 0.108-0.144 with ratio dorsal to basal, 1.43-1.71; hind tarsal segment (II), 0.258-0.312 mm long, its ratio to the IV rostral segment, 1.29-1.62.

Antennal hairs rather hyaline, those on segment III up to 0.130 mm long (0.114-0.132), sometimes sinuous, the longest 3.3-3.5 times the diameter of that segment at the insertion point. Abdominal tergites I to VII with numerous pale brown and somewhat translucent long hairs, those on tergites V and VII, 0.150-0.198 and 0.162-0.192 mm long, respectively; tergite VIII and genital plate bearing 18-26 and 6-9 hairs, respectively; fourth rostral segment with 6-8 accessory hairs; siphuncular cones with 0.132-0.198 mm long hairs; hind tibiae with numerous pale brown translucent hairs, 0.156-0.204 mm long, the longest, 2.5-3 times as long as the tibial diameter at their insertion point.

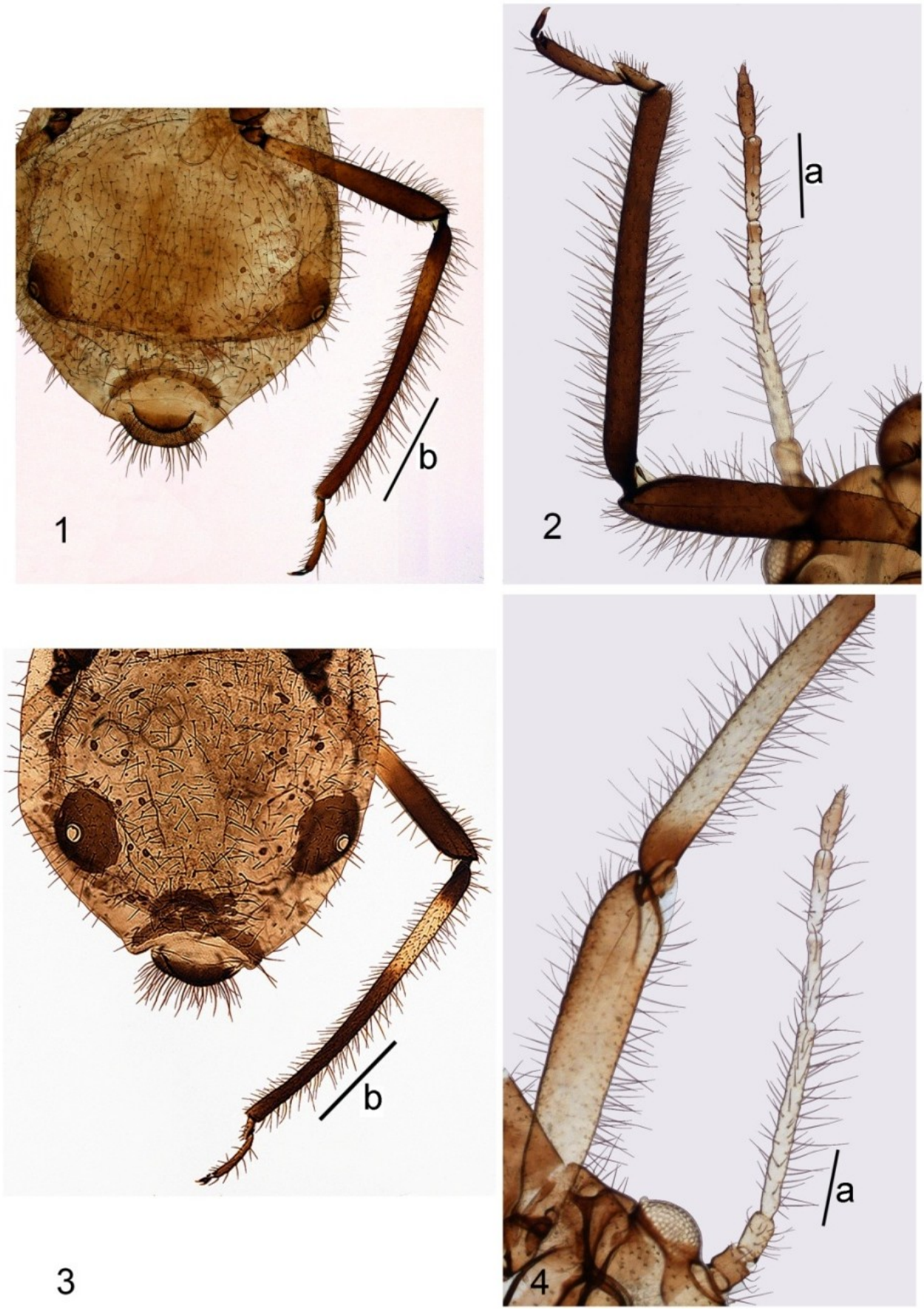

Figure 1. Morphological details: **1-2)** *C. cedri brevifoliae*; **3-4)** *C. cedri cedri*. Scales: a = 0.2 mm; b = 0.5 mm.

Table 1. Main discriminant features (mm) between *C. cedri brevifoliae* and *C. cedri cedri*. Specimens are apterous viviparous females (see keys of Binazzi, 1984; Blackman and Eastop, 1994; 2015; Nieto Nafria *et al.*, 2002). Collection data in the text.

	C. cedri brevifoliae	*C. cedri cedri*	*C. cedri* orig. descr. by Mimeur, 1935
Number of specimens	9	10	"plusieurs"
Body length	2.21-2.57	2.86-3.55	2.50-3.00
Hind tibia length	1.16-1.68	1.54-2.11	1.87
Diameter of siphuncular cones	0.258-0.378	0.354-0.450	-
Length of antennal segment III	0.372-0.480	0.400-0.576	0.450
Length of antennal segment IV	150-.186	0.168-0.270	0.200
Length of antennal segment V	0.186-0.222	0.192-0.252	0.220
Length of antennal segment VI base	120-.144	0.108-0.138	0.130
Length of antennal segment VI p.t.	0.042-0.051	0.042-0.060	0.024
Ratio antennal segment V to VI	1.07-1.18	1.17-1.40	1.43
Length of rostral segment IV	174-.204	0.180-0.222	0.190
Length of rostral segment V	0.090-0.102	0.078-0.096	0.080-0.090
Ratio rostral segment IV to V	1.82-2.13	2.06-2.50	2.23
Length of the II segment of hind tarsus	0.258-0.312	0.270-0.350	0.340
Length of the longest hair on:			
abdominal tergite VII	0.162-0.192	0.156-0.240	-
antennal segment III	0.114-0.132	0.120-0.170	-
hind tibia	0.156-0.204	0.156-0.240	-
Number of hairs on the genital plate	6-9	9-12	-

H o l o t y p e

Aptera vivipara, *Cedrus brevifolia* (Hooker fil.) Henry, Troodos Mountains near Prodromos, Cyprus, 1700 m a.s.l., 05.ix.2015, leg. F. Binazzi (slide C10/928).

P a r a t y p e s

8 apterae, same data as holotype (slides C10/929-930).

Preservation of material at the Research Center for Agrobiology and Pedology, Florence, Italy, included in the A.B. collection.

D e r i v a t i o n o m i n i s

From the specific name of the host plant of the new subspecies.

Taxonomy

C. cedri brevifoliae ssp.n. differs from the nominotypical species (figure 1, table 1) in having smaller body size (2.21-2.57 vs. 2.86-3.55), shorter hind tibiae (1.16-1.68 vs. 1.54-2.11 mm) and shorter antennae (whole length, .882-1.080 vs. .966-1.236 mm). Furthermore the ratio antennal segment V to VI is lower, i.e., 1.07-1.18 vs. 1.17-1.40, as well the ratio fourth rostral segment to fifth, which is 1.82-2.13 vs. 2.06-2.50. Other differences in the new subspecies, although somewhat lower, are the minor length of the second hind tarsal segment (.258-.312 vs. .270-.350) and the shorter hairs on antennae and on the dorsal abdomen and tibiae. Moreover, in *C. cedri brevifoliae*, the legs and particularly the hind tibiae are more dark-brown pigmented except for a slightly paler short basal part before the knees. At last, the dorsal tergite VII bears a smaller spinal sclerotic area, or fragmented sclerotizations, and a smaller genital plate with only 6-9 hairs (vs. 9-12).

A d d i t i o n a l n o t e s a n d k e y t o c e d a r *C i n a r a* s p e c i e s

To our knowledge, only three other species of *Cinara* are reported to feed on Cedar trees, i.e., *C.* (*Cedrobium*) *laportei* Remaudiere, *C. indica* Verma and *C. deodarae* Seo. The only known *Cedrobium* species seems to be restricted to the Mediterranean Basin with the exception for records in the Middle East (Blackman and Eastop, 1994; 2015) and in South Africa (Millar, 1990). It is thought to have originated from the Atlas Mountains of North Africa (Morocco and Algeria) (Remaudière, 1954). *C. indica* is native to the Himalayan region (Verma, 1970) but it was recently recorded in Turkey (Şenol *et al*., 2015) where it was probably introduced by human activities. Conversely, the latter species, *C. deodarae*, was reported only in Korea by Seo (1994) and never recorded again in other parts of the world. Two other congeneric species, generally feeding on *Abies*, have been observed also on *Cedrus*, i.e., the Holarctic *C. confinis* (Koch) and the Nearctic *C. curvipes* (Patch). The former was found (sub *abieticola*) on *C. deodara* in Italy by Covassi (1971) and in the UK and India by Eastop (1972), the latter was recorded on *C. deodara* in India by Agarwala and Ghosh (1984) and by Ghosh and Singh (2004).

The apterous viviparous females of the *Cinara* species and subspecies recorded worldwide on *Cedrus* can be differentiated as follows:

1	-	5-segmented antennae. [Small, roundish body, 1.6-2.0 mm long. Abdominal tergites II to V with sclerotized plates each bearing a typical rising protuberance backwards. IV and V rostral segments 0.120 e 0.060 mm long, the IV with two accessory hairs. Ratio dorsal to basal length of the I segment of hind tarsus, 0.5-0.6. Dorsal hairs short, pale and knobbed with apical spikes. VIII tergite bearing eight hairs on its posterior margin, up to 0.100 mm long and distally ramified. On *Cedrus*, on shoots and twigs] . . . . . ***Cinara* (*Cedrobium*) *laportei***
	-	6-segmented antennae. **2**
2	-	Small- to medium-sized aphids, 2.2-3.8 mm long **3**
	-	Large-sized aphids, 4.0-7.8 mm long **6**
3	-	Abdominal tergites I-VI without sclerotizations **4**
	-	Abdominal tergites I-VI with an extensive pattern of dark sclerotization. [Aptera with c. 5 secondary rhinaria on antennal segment III. On *Cedrus deodara*] . . . . . ***Cinara deodarae***
4	-	Diameter of siphuncular cones up to 0.450 mm. Hairs on dorsal abdomen, tibiae and antennae, long and strong, variously brown pigmented, up to 0.240 (abdomen and tibiae) and 170 (antennae) mm long. Legs more or less uniformly pigmented **5**
	-	Diameter of siphuncular cones more than 0.450 mm. Hairs on dorsal abdomen, antennae and tibiae, shorter and fine, variously paler pigmented, 0.100, 0.060-0.150 and 0.030-0.110 mm long respectively. Legs pale save the distal portion of femora and bases and apices of tibiae. On twigs of *Cedrus deodara* . . . . . ***Cinara indica**** * not seen by the AA.
5	-	Hind tibiae, 1.5-2.1 mm long. Ratio apical rostral segments IV to V, 2.0-2.5. Ratio antennal segments V to VI, 1.2-1.4. Long and strong hairs, those on antennal segment III and tergite VII up to 0.170 e 0.240 mm long, respectively. On *Cedrus* spp., on two- to three-years-old twigs (table 1) . . . . . ***Cinara cedri cedri***
	-	Hind tibiae, 1.1-1.6 mm long. Ratio apical rostral segments IV to V, 1.8-2.1. Ratio antennal segments V to VI, 1.0-1.2. Less long and strong hairs, those on antennal segment III and on tergite VII up to 0.130 e 0.190 mm long, respectively. On *Cedrus brevifolia* (table 1). . . . . ***Cinara cedri brevifoliae* ssp.n.**
6	-	Hairs on dorsal abdomen (tergite V) 0.012 mm long at most. Ratio dorsal to basal length of the I segment of hind tarsus about 1.3. Greyish aphids. Native to North America and feeding there on nearctic *Abies*. On trunks and branches of *Abies* and, occasionally, of *Cedrus* . . . . . ***Cinara curvipes***
	-	Very long hairs, the ones on dorsum (tergite V) up to 0.240 mm. Ratio dorsal to basal length of the I segment of hind tarsus < 1.1. Dark brown aphids. On branches and trunks of *Abies* and, occasionally, of *Cedrus* . . . . . ***Cinara confinis***

Molecular analysis

Molecular results evidenced that the aphid samples belong to *C. cedri*. Identical COI sequences were obtained from specimens collected in the same geographic locations (GenBank Accession Number: KU321598). DNA-based species attribution was supported by all phylogenetic reconstruction methods used (ML and BI).

However, two different clades were identified on the basis of COI and *CytB* gene trees (figure 2). Clade I includes specimens from Languedoc-Roussillon (France), Sicily and Washington (USA); Clade II includes specimens from Cyprus, Florence (Italian mainland) and China (from unknown geographical coordinates). The subdivision into two clades was found well supported in both ML and BI trees based on COI sequences, while only ML tree reconstruction on *CytB* sequences fully supported this subdivision. The same situation was obtained combining data from the two loci figure 2 (2-3).

The EF1alpha sequences of specimens collected in Cyprus differed from the Italian FB102015 for only one base. Unfortunately, the polymorphism occurred in a region that shows many base ambiguities in the sequences available in GenBank. For this reason, sequences were submitted in the public database but no further analyses were performed on this locus.

Discussion

Foottit *et al*. (2008; 2009) proposed DNA barcoding as a standardized approach to identify aphid species and explore their diversity. In the present study, Cyprus cedar aphids were initially identified as *C. cedri* by either morphology or molecular analysis. However, on the basis of further morphological evaluation this aphid was accorded subspecific rank, assuming that the new taxon, *C. cedri brevifoliae*, is native to the island where it is endemic together with its host cedar. In fact, the differences in the main taxonomic characters between the specimens collected in Cyprus and those sampled in the rest of the Mediterranean basin are too relevant to be simply interpreted as the consequence of a different feeding regime of *C. cedri* on the endemic *C. brevifolia*. Hence, the new taxon was established.

Moreover, a phylogenetic tree obtained from the multilocus analysis evidenced the presence of two distinct groups within *C. cedri s.l.* Such result was independently obtained in either the reconstructions based on a fragment of COI gene or in those based on *CytB*, individually considered. It is worth mentioning that the low posterior probability value of the node indicated with asterisk (*) in figure 2 (3) of multilocus reconstruction could be due more to *CytB* contribution rather than COI.

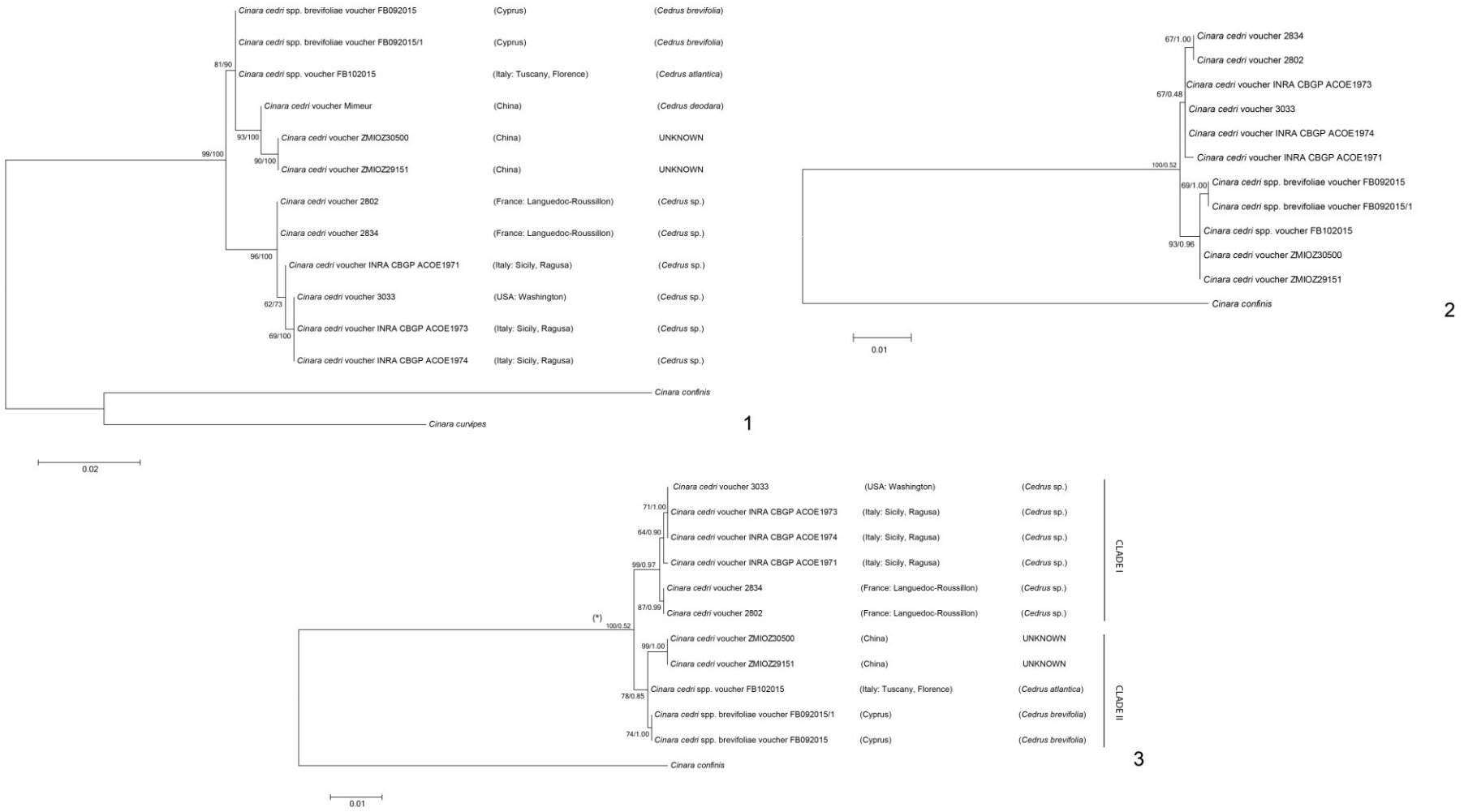

Figure 2. Phylogenetic analyses of *C. cedri* populations. Trees were annotated with bootstrap values of ML and Posterior Probability of BI analyses respectively. **1)** Molecular relationships between *C. cedri brevifoliae* and *C. cedri cedri* based on COI gene; **2)** Molecular relationships between *C. cedri brevifoliae* and *C. cedri cedri*, based on *CytB* gene; **3)** Multilocus phylogeny based on COI-*CytB* mitochondrial genes showing Clade I and Clade II subdivision. (*) Node fully supported in ML analyses but not in Bayesian approach.

In fact, the presence of a lower number of informative sites in *CytB* (5 on 692 positions) compared with COI (13 on 630 positions) led to hypothesize that *CytB* is a less useful target compared with COI for an intraspecific analysis of variability within this species.

Though further investigations are required, the presence in *C. cedri* of either the two groups identified by molecular analysis or the new subspecies defined by morphological evaluation, might be part of a dynamic scenario determined by ongoing speciation processes.

Therefore, the current evolutionary context could be the consequence not only of the intense import/export routes that since ancient times affected Cedar species in the Mediterranean basin, but also of the peculiar adaptation of the aphids to specific hosts and environments such as those experienced in Cyprus. Moreover, similar findings may be also supported by recent studies showing that within the genus *Cinara*, climatic events and landscape history are probably as relevant as ecology in shaping diversity and driving speciation (Jousselin *et al*., 2013).

In the phylogenetic tree all the available data on *C. cedri* were considered including those recorded outside its natural range. Records from China or USA are probably the consequence of recent Cedar trade outside the Mediterranean basin. Conversely, in western and north Europe, the first introduction of *Cedrus* spp. in parks and gardens, for ornamental purposes, is relatively old, dating back to the 19th century. In fact, *C. deodara* and *C. atlantica* were imported for the first time in 1822 and 1842 respectively, while *C. libani* in 1863 (Covassi and Binazzi, 1974; Binazzi *et al*., 2015). Since then, all cedar species have been spread all over the world due to the beauty of their foliage and their capacity to adapt to various climatic conditions. As a consequence of trade of nursery material, all cedar aphids have been dispersed outside their original range determining the current distribution.

In the light of the above, because of the complexity, the wide scale and the long time span of such a phenomenon, additional studies, both molecular and morphological, are necessary to understand the main routes of invasion of the lacnids associated with Cedars.

In Binazzi *et al*. (2015) the question was discussed whether this new taxon could represent a threat for *C. brevifolia* and why it had never been previously detected. Whatever the answer, we believe that *C. cedri brevifoliae* does not endanger its native host as long as severe climate changes or other disruptive events do not occur (Gokcekus and Gucel, 2010; Binazzi *et al*., 2015). Nevertheless, additional research is required in order to fully clarify its cycle, behaviour and ecology.

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Authors' addresses: Andrea BINAZZI (corresponding author, e-mail: andrea.binazzi@gmail.com), via di Vernalese 41, 50012 Bagno a Ripoli, Florence, Italy; Francesco BINAZZI (francesco.binazzi@crea.gov.it), Agostino STRANGI (agostino.strangi@crea.gov.it), Francesco PAOLI (francesco .paoli@crea.gov.it), Giuseppino SABBATINI PEVERIERI (giuseppino.sabbatini@crea.gov.it), Pio Federico ROVERSI (piofederico.roversi@crea.gov.it), Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, via Lanciola 12/A, Cascine del Riccio, 50125 Florence, Italy.

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