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Cryptic diversity of the jewel beetles *Agrilus viridis* (Coleoptera: Buprestidae) hosted on hazelnut

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Abstract
The genus *Agrilus* (Coleoptera: Buprestidae) represents a taxonomic puzzle, since the boundaries between species, subspecies and morphotypes tied to different host plants are sometimes difficult to establish on morphological characteristics alone. Some *Agrilus* species can cause severe agricultural damage; this makes correct distinctions of the taxon and knowing whether the insects switch from one host plant to another important. This study of mtDNA examined the genetic characteristics of lineages of *A. viridis*, a jewel beetle recently found causing damage to the hazelnut *Corylus avellana* in NW Italy. Three mitochondrial markers (a portion of the 12S rDNA and a DNA-fragment including partial NADH dehydrogenase subunit I gene, the tRNA Leucine gene and partial 16S rDNA, and partial Cytochrome c oxidase) were compared between individuals collected on birch *Betula* sp., beech *Fagus* sp., willow *Salix* sp., alder *Alnus* sp. and hazelnut. We found a high genetic distance between *A. viridis* sampled on different host plants, while individuals sampled on the same host plant were similar despite a considerable geographic gap between sampled areas. Our study supports the general pattern for strong ecological separation between populations living on different host plants.

Keywords: Agrilus viridis *complex, hazelnut parasite, mtDNA*

Introduction

The jewel beetles, family Buprestidae, represent a large group comprising about 15,000 species (Evans et al. 2015). Classification within the Buprestidae family is controversial (Bellamy 2003). The group includes four subfamilies: Schizopodinae, Julodinae, Buprestinae and Agrilinae (Lawrence & Newton 1995), with the latter comprising the genus *Agrilus* (Lawrence & Newton 1995). The genus *Agrilus* includes about 2880 species (Bellamy 2008), making it probably the biggest living animal genus (Curletti 2001, 2010), and represents a taxonomic puzzle with the boundaries between species, subspecies and morphotypes tied to different host plants making them difficult to be characterised on morphological traits alone. The classification of *Agrilus* is complex (Curletti 1994), and classification errors have been multiplied by the previous description of numerous races, varieties and forms within the same species.

One of the best-known examples of taxonomic difficulties in this genus is represented by the Palearctic species *A. viridis* (Linnaeus, 1758) (Jendek 2016). This jewel beetle is polyphagous, a feeding habit rarely encountered in this genus, which could enhance our ability to discover sister species with little morphological differentiation. According to the late C. L. Bellamy (pers. comm. to GC), *A. viridis* includes 24 synonyms, 15 unavailable names and only one subspecies, the *poppiusi* Obenberger 1924 from east Siberia (see also Jendek & Grebennikov 2011). Recent studies have shown the presence of different varieties or ecotypes strictly linked to geographical area, altitude or arboreal host species (Brechtel & Kostenbader 2002; Bernhard et al. 2005). These studies considered the birch *Betula* and the willow *Salix* and indicated correlation between genetic variants of *A. viridis* and tree species (Bernhard et al. 2005; Pentinsaari et al. 2014b). The hazelnut tree can host *A. viridis* as the

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primary xylophagous species (Corte et al. 2009), as well as *A. angustulus* (Illiger, 1803), *A. graminis* Laporte & Gory, 1837, and *A. olivicolor* Kiesenwetter, 1857 as secondary xylophagous hosts within the jewel beetles (Ciampolini & Ugolini 1975). Jendek and Poláková (2014) reported *A. viridis* association with 23 genera of host plants through their entire distribution range.

In recent years, the Piedmont hazelnut agriculture has had to deal with an increase in *A. viridis* attacks. This woodboring jewel beetle digs long and winding tunnels in the cambium of the hazelnut, thus causing a slow decline until desiccation. Probably a combination of factors favoured the intensification of attacks, with the main cause attributed to climatic and agricultural factors, such as the repeated superposition of dry years with production overhead. The phenomenon causes stress in hazelnuts, and the weakened trees easily become victims to the parasite. The symptoms are foliage yellowing during summertime, drying of branches and, in severe cases, the death of the plant due to lymphatic cycle breakage (Ciampolini & Ugolini 1975; Moraglio et al. 2013). During inspections, only undulating or linear cortical reliefs can be observed, without the ability to find holes with larval penetration, while it is possible to discover the emergence holes of adults. The larval dig includes a first involvement of sub-cortical tissue and then proceeds into the xylem (Pollini 2006).

Recent studies have examined the genetic relationships within this species, which appears to include several varieties or ecotypes (Brechtel & Kostenbader 2002; Bernhard et al. 2005), with possible mitochondrial introgression events (Pentinsaari et al. 2014b). In the past, some ecotypes hosted by different trees were described, such as *A.*v. *ignotus* (Schaefer, 1946) for hazelnut, *A.* v. *fagi* Ratzeburg, 1839 for beech *Fagus* sp., and *A.*v. *ribesii* (Schaefer, 1946) for blackcurrant *Ribes nigrum* (Ciampolini & Ugolini 1975). The last taxon is currently classified as a true species, *Agrilus ribesi*, while the poplar *Populus* jewel beetle, *A.*v. *populneus*, is now considered a subspecies of *A. suvorovi* Obenberger, 1935 (Curletti 2013) or a synonym of *A. suvorovi* (Jendek 2016).

In this study, we investigated the links between *A. viridis* genetic lineages and their host trees. We utilised three markers (a portion of the 12S rDNA and a DNA-fragment including partial NADH dehydrogenase subunit I gene, the tRNA Leucine gene and partial 16S rDNA and partial Cytochrome c oxidase) of the mitochondrial DNA in jewel beetles sampled on different angiosperms: birch *Betula* sp., beech *Fagus* sp., willow *Salix* sp., poplar *Populus* sp. and hazelnut *Corylus avellana*. The aim of our study was to test whether *A. viridis* hosted on different trees show a clear genetic separation. This could indicate that different lineages are not likely to switch from one host plant to another, which would help in planning future actions targeted at containing this harmful species. Interventions can be limited to plants of agricultural interest without needing to operate on wild plants present in the same area (Maloy 2005).

Methods

Sampling

Jewel beetles were collected in the field during autumn through selective cutting of branches infested by *A. viridis*, after the typical damage caused by the larvae was recognised by one of the authors (GC). The tree branches with larvae were stored during the autumn and winter seasons in a fresh cage, at ambient temperature, until the emergence of adults in spring. Individuals that emerged were identified as *A. viridis* and were subsequently transferred into test tubes containing pure ethanol and stored in a freezer at −20°C until the DNA extraction procedure. Samples and localities are listed in Table I.

Table I. List of samples of *Agrilus viridis* and *Agrilus suvorovi*. All individuals were collected at the larval stage and reared to emergence by G. Curletti.

ID	Year	Species	Host plant	Locality
Ag1	2012	*A. viridis*	Hazelnut	Bossolasco, NW Italy
Ag2	2012	*A. viridis*	Hazelnut	Bossolasco, NW Italy
Ag3	2012	*A. suvorovi*	Poplar	Caramagna, NW Italy
Ag4	2012	*A. suvorovi*	Poplar	Caramagna, NW Italy
Ag5	2012	*A. viridis*	Willow	Caramagna, NW Italy
Ag6	2012	*A. viridis*	Willow	Caramagna, NW Italy
Ag7	2012	*A. viridis* var. *fagi*	Beech	Mont Avic Park, Aosta, NW Italy
Ag12	2015	*A. viridis*	Willow	San Costantino Albanese, S Italy
Ag13	2015	*A. viridis*	Willow	San Costantino Albanese, S Italy
Ag14	2015	*A. viridis*	Willow	San Costantino Albanese, S Italy
Ag15	2015	*A. viridis*	Willow	San Costantino Albanese, S Italy
Ag16	2015	*A. viridis*	Hazelnut	Carrù, NW Italy
Ag17	2015	*A. viridis*	Hazelnut	Carrù, NW Italy
Ag18	2015	*A. viridis*	Hazelnut	Carrù, NW Italy
Ag19	2015	*A. viridis*	Hazelnut	Carrù, NW Italy
Ag20	2015	*A. viridis*	Hazelnut	Carrù, NW Italy
Ag21	2015	*A. viridis*	Hazelnut	Carrù, NW Italy
Ag22	2015	*A. viridis*	Willow	San Costantino Albanese, S Italy
Ag23	2015	*A. viridis*	Hazelnut	Carrù, NW Italy

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DNA extraction, PCR amplification and sequencing

Each adult individual was sectioned along the median sagittal plane and one half was used entirely for DNA extraction. The genomic DNA was isolated using a commercial kit for the extraction of nucleic acids to silica columns (NucleoSpin® Tissue Machery Nagel). First, thermal shocks in liquid nitrogen were carried out to facilitate the rupturing of the cell. In addition, a lysis buffer (Buffer FLB Macherey Nagel) designed for small amounts of DNA or degraded DNA was used. The DNA extracted after the final centrifugation and resuspension in standard buffer solution (Tris-EDTA pH 8–9) was stored at −20°C for subsequent molecular analysis.

We amplified three portions of the mtDNA: (i) 596 bp from the coding portion of Cytochrome c oxidase (COI) using the primers LCOI490: GGTCAACAAATCATAAAGATATTGG and HCO2198: TAAACTTCAGGGTGACCAAAAAA TCA (Folmer et al. 1994) with an annealing temperature (Ta) of 50°C; (ii) a 312-bp portion of 12S rDNA using the primers SR-J-14233: AAGA GCGACGGGCGATGTGT and SR-N-14588: AAACTAGGATTAGATACCCTATTAT (Simon et al. 1994) with Ta 50°C; and (iii) a 493-bp fragment embracing a coding portion of NADH dehydrogenase subunit 1, tRNA leucine gene, and a portion of 16S rDNA using the primers N1-J-12248: AAGCTAATCTAACTTCATAAG and LR-N-12866: ACATGATCTGAGTTCAAACC GG (Simon et al. 1994) with Ta 45°C.

The reactions were set up in a final volume of 25 µL with 10 mM polymerase chain reaction (PCR) buffer, 25 mM $MgCl_2$, 2.5 mM for each dNTP, 25 µM for each primer, 0.5 unity/µL of Taq DNA polymerase, 10% BSA, and 0.3–0.5 ng/µL of extracted DNA. The amount of DNA and TAQ polymerase was varied according to the state of degradation of the biological sample. The amplification reactions were obtained in a Bio-Rad C1000 thermal cycler using the following protocol (94°C × 5′), 29–32 cycles at (94°C × 30”) (Ta°C × 30”) (72° C × 1′), final extension 7 min at 72°C.

The DNA concentration was determined on 1.8% agarose gel (TBE 1%) using GelRedTM nucleic Acid stain gel (Biotium Inc., Hayward, California, USA) and quantified by UV-transilluminator Gel Doc XR (Bio-Rad Laboratories Inc., Hercules, California, USA) with Molecular Imager ChemiDoc XRS Systemand Quantity One (Bio-Rad) software. The amplification products intended for sequencing were purified by treatment with ExoSAP-IT ® using the following protocol – (37°C × 45′), (85°C × 15′) – and were sequenced on an ABI 3730XL (Applied Biosystems, Foster City, CA, USA) automated DNA sequencer at the BMR genomics lab (Padova, Italy).

The obtained sequences were controlled through the use of software for the visualisation of electropherograms, FINCHTV (http://www.geospiza.com/Products/finchtv.shtml; Geospiza, Seattle), then aligned utilising MEGA 6.0 (Tamura et al. 2013). The identification of haplotypes and calculations of various statistical indices was made by DNASP software 5.1 (Librado & Rozas 2009). Haplotype sequences were deposited in GenBank (COI: accession numbers MF543033–MF543037; 12S: accession numbers MF543029–MF543032; ND1-16S: accession numbers MF543040–MF543046).

Data analyses

From GenBank, we downloaded the sequences of five *A. viridis* collected in Germany on *Fagus sylvatica* and *Salix* (accession numbers 12S: AJ965440-41-42-43-45; ND1-16S: AJ937890-91-92-93-95); from the BOLD system we downloaded the sequences of two *A. viridis* collected in Finland on *Alnus* and *Betula* (accession numbers – COI: MP00061–63) (Bernhard et al. 2005; Pentinsaari et al. 2014b). The species *Trachys troglodytes* Gillenhal, 1817, of the same subfamily Agrilinae (accession numbers – 12S: AJ965469; ND1-16S: AJ937917), and *Agrilus suvorovi* (two individuals collected by GC from NW Italy, accession numbers MF543038-MF543039) were utilised as outgroups.

The genetic distances were calculated using the MEGA 6.0 software applying the Kimura 2-parameter model (Kimura 1980; Tamura & Nei 1993). Phylogenetic trees were determined through the MEGA 6.0 software applying the neighbour-joining (NJ) algorithm (Saitou & Nei 1987). The confidence levels of the tree internodes were estimated using the bootstrap procedure (Felsenstein 1985), which reported the indexes as a percentage after 1000 replications and rejected values less than 50.

The networks of the haplotypes were constructed using POPART (http://popart.otago.ac.nz) with the median-joining procedure (Bandelt et al. 1999).

Results

The COI sequences showed little variability, with only two haplotypes found in the beetles collected on hazelnut and two others found in those collected on willow plants (Table II). Beetles collected on poplar showed two different haplotypes.

The NJ tree shows a genetic distinction between individuals hosted on *Corylus avellana* and those

Table II. Haplotypes found in three portions of *Agrilus viridis* and *Agrilus suvorovi* mtDNA.

ID	12S	ND1-16S	COI	Concatenated
Ag1	Hapl 1	Hapl 1	Hapl 1	Hapl 1
Ag2	Hapl 1	Hapl 2	Hapl 1	Hapl 2
Ag3	-	-	*A. suvorovi* 1	-
Ag4	-	-	*A. suvorovi* 2	-
Ag5	Hapl 2	Hapl 3	Hapl 4	Hapl 3
Ag6	Hapl 3	Hapl 4	Hapl 4	Hapl 4
Ag7	-	-	Hapl 5	-
Ag12	-	Hapl 4	Hapl 4	-
Ag13	Hapl 4	Hapl 4	Hapl 3	Hapl 5
Ag14	Hapl 4	Hapl 4	Hapl 3	Hapl 5
Ag15	Hapl 4	Hapl 4	Hapl 4	Hapl 5
Ag16	Hapl 1	Hapl 2	Hapl 1	Hapl 2
Ag17	Hapl 1	Hapl 2	Hapl 1	Hapl 2
Ag18	Hapl 1	Hapl 5	Hapl 2	Hapl 6
Ag19	Hapl 1	Hapl 2	Hapl 1	Hapl 2
Ag20	Hapl 1	Hapl 6	Hapl 1	Hapl 7
Ag21	Hapl 1	Hapl 2	Hapl 1	Hapl 2
Ag22	Hapl 4	Hapl 4	Hapl 3	Hapl 5
Ag23	-	Hapl 7	Hapl 1	-

hosted on *Salix* (Figure 1). The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree (not shown) confirmed the division into major and minor clades which was identified from the NJ tree. The haplotype network (Figure 1) shows that the haplogroup which includes the sequences of individuals on *C. avellana* is separated by a minimum distance of 40 mutations from that which comprises the sequence on *Salix*.

The genetic distances within haplogroups found on the same host plant are smaller than those between haplogroups found on different host plants, which confirms the structure of the tree phylogenetic clades (Table III). The largest genetic distances are observed between the haplotypes of *A. viridis* hosted on hazelnut and willows (0.068–0.072) and between those of alder and willows (0.066–0.068).

The 12S sequences identified four different haplotypes with four polymorphic sites and three informative sites. The ND1-16S sequences identified seven different haplotypes with 42 polymorphic sites and 32 informative sites.

The NJ tree computed on 12S and ND1-16S concatenated sequences and rooted with one sequence of *Trachys troglodytes* shows that the sequences of hazel and willow found in the same Piedmont area cluster into two distinct groups (Figure 2). Willow samples from Piedmont were also separated from a willow sample from Germany. The UPGMA tree (not shown) confirmed the division into major and minor clades which was identified from the NJ tree. The haplotype network shows a phylogenetic structure consisting of two haplogroups comprising, respectively, *A. viridis* samples from hazelnut plants and samples taken from willow, separated by a minimum distance of 35 mutations (Figure 2).

Haplotype diversity (Hd) and nucleotidic diversity (π) values are reported in Table IV. The genetic distance between the haplotypes found on hazelnut and those found on willows in the same Piedmont area was high, assuming values between 0.044 and 0.049 (Table V).

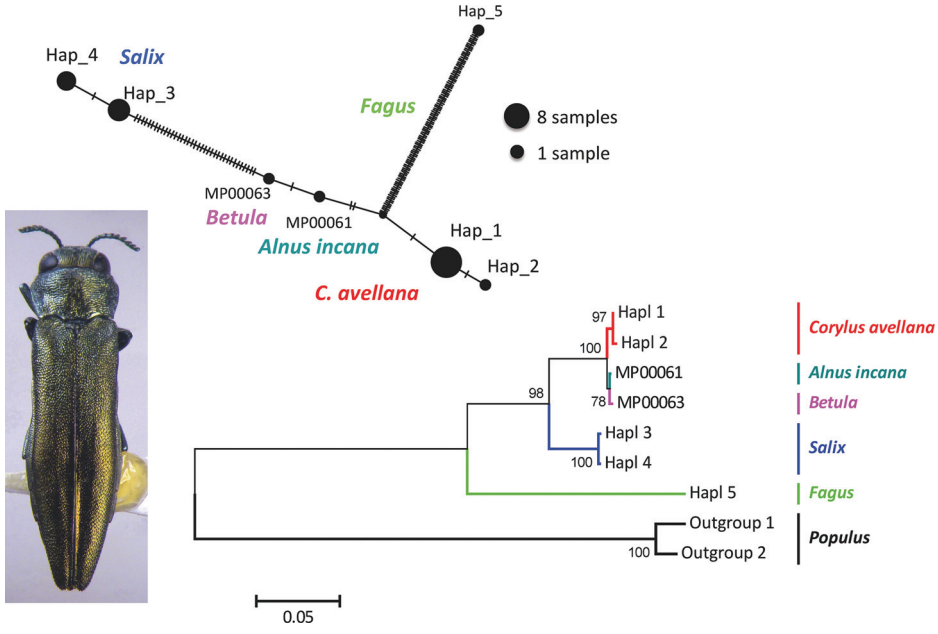

Figure 1. NJ tree estimated from COI sequences with Tamura Nei genetic distances, and median joining haplotype network. The picture shows a male of *Agrilus viridis* collected from a hazelnut.

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Table III. Matrix of Kimura 2 distances between COI haplotypes. Standard deviation values are reported above the diagonal.

Host plant	COI haplotype	*C. avellana*	*C. avellana*	*Salix*	*Salix*	*Fagus*	*Populus*	*Populus*	*Alnus incana*	*Betula*
		Hapl 1	**Hapl 2**	**Hapl 3**	**Hapl 4**	**Hapl 5**	***A. suvorovi* 1**	***A. suvorovi* 2**	**MP00061**	**MP00063**
C. avellana	**Hapl 1**		0.002	0.011	0.011	0.020	0.043	0.043	0.003	0.003
C. avellana	**Hapl 2**	0.002		0.011	0.011	0.020	0.043	0.043	0.003	0.004
Salix	**Hapl 3**	0.068	0.070		0.002	0.021	0.042	0.041	0.011	0.011
Salix	**Hapl 4**	0.070	0.072	0.002		0.021	0.041	0.041	0.011	0.011
Fagus	**Hapl 5**	0.209	0.211	0.211	0.214		0.046	0.046	0.021	0.021
Populus	***A. suvorovi* 1**	0.537	0.541	0.527	0.523	0.577		0.007	0.042	0.042
Populus	***A. suvorovi* 2**	0.534	0.538	0.517	0.513	0.573	0.029		0.042	0.042
Alnus incana	**MP00061**	0.005	0.007	0.068	0.066	0.211	0.534	0.531		0.002
Betula	**MP00063**	0.007	0.008	0.066	0.064	0.213	0.537	0.535	0.002	

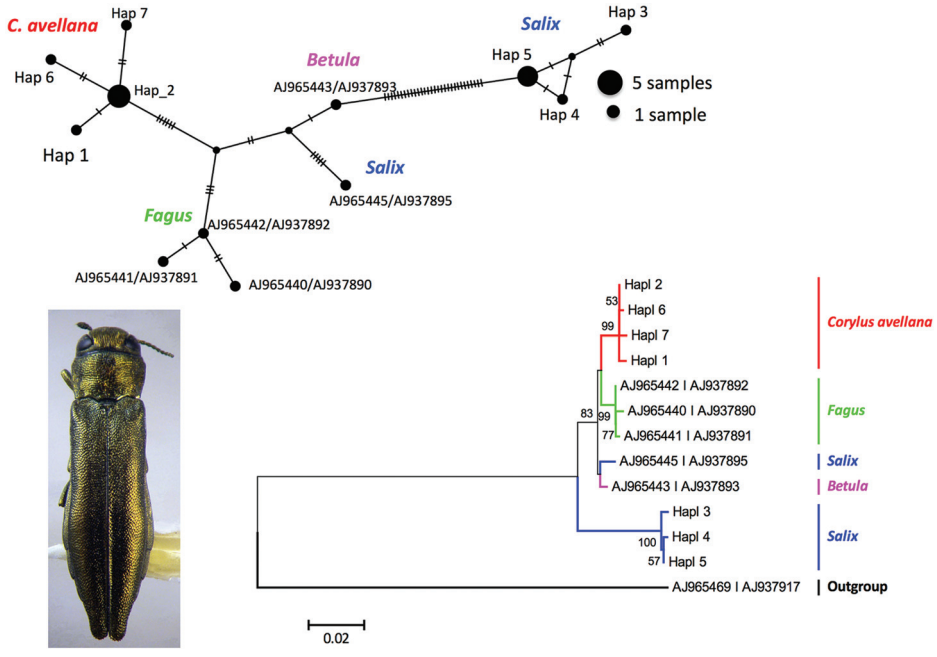

Figure 2. NJ tree estimated from 12S and ND1-16S concatenated sequences with Tamura Nei genetic distances, and haplotype median joining network. The picture shows a female of *Agrilus viridis* collected from a hazelnut.

Table IV. Genetic diversity indices for COI and concatenated 12s + ND1-16S sequences. The standard deviation values are indicated in parentheses.

	Individuals (N)	N haplotypes (h)	Haplotype diversity (Hd)	Nucleotide diversity (π)	Polymorphic sites	Informative sites	G + C	k
COI								
A. viridis	9	3	0.639	0.00119	2	1	0.382	0.722
Corylus avellana			(0.126)	(0.00031)				
Agrilus viridis	7	4	0.714	0.00219	3	0	0.405	1.333
Salix			(0.181)	(0.00065)				
Total	16	7	0.842	0.0356	44	42	0.392	21.683
			(0.059)	(0.00362)				
Concatenated 12S + ND1-16S								
A. viridis	8	4	0.643	0.00156	5	0	0.263	1.250
Corylus avellana			(0.184)	(0.00059)				
Agrilus viridis	6	3	0.600	0.00159	3	0	0.259	1.267
Salix			(0.215)	(0.00074)				
Total	14	7	0.824	0.02388	41	34	0.262	19.033
			(0.078)	(0.00274)				

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Table V. Matrix of Kimura 2 distances between 12S and ND1-16S concatenated sequences. Standard deviation values are reported above the diagonal.

Host plant		*C. avellana*	*C. avellana*	*Salix*	*Salix*	*Salix*	*C. avellana*	*C. avellana*	*Fagus*	*Fagus*	*Fagus*	*Salix*	*Betula*	*Trachys troglodytes* Outgroup
		Hapl 1	**Hapl 2**	**Hapl 3**	**Hapl 4**	**Hapl 5**	**Hapl 6**	**Hapl 7**	**AJ965442 AJ937892**	**AJ965440 AJ937890**	**AJ965441 AJ937891**	**AJ965445 AJ937895**	**AJ965443 AJ937893**	**AJ965469 AJ937917**
C. avellana	**Hapl 1**		0.002	0.008	0.008	0.008	0.002	0.002	0.004	0.005	0.005	0.006	0.005	0.019
C. avellana	**Hapl 2**	0.003		0.007	0.007	0.007	0.001	0.002	0.004	0.004	0.004	0.004	0.004	0.019
Salix	**Hapl 3**	0.049	0.046		0.002	0.002	0.007	0.008	0.007	0.008	0.007	0.007	0.007	0.020
Salix	**Hapl 4**	0.048	0.046	0.004		0.001	0.007	0.007	0.007	0.008	0.007	0.007	0.007	0.020
Salix	**Hapl 5**	0.047	0.044	0.004	0.001		0.007	0.007	0.007	0.008	0.007	0.007	0.007	0.020
C.avellana	**Hapl 6**	0.004	0.001	0.047	0.047	0.046		0.002	0.004	0.004	0.004	0.004	0.004	0.019
C.avellana	**Hapl 7**	0.005	0.003	0.048	0.048	0.047	0.004		0.004	0.004	0.004	0.005	0.004	0.019
Fagus	**AJ965442 AJ937892**	0.014	0.011	0.049	0.049	0.047	0.013	0.014		0.002	0.001	0.004	0.003	0.019
Fagus	**AJ965440 AJ937890**	0.017	0.014	0.051	0.051	0.050	0.015	0.017	0.003		0.002	0.004	0.004	0.020
Fagus	**AJ965441 AJ937891**	0.015	0.013	0.050	0.050	0.049	0.014	0.015	0.001	0.004		0.004	0.003	0.019
Salix	**AJ965445 AJ937895**	0.017	0.014	0.043	0.043	0.042	0.015	0.017	0.013	0.015	0.014		0.003	0.019
Betula	**AJ965443 AJ937893**	0.014	0.011	0.040	0.040	0.039	0.013	0.014	0.008	0.010	0.009	0.008		0.019
Trachys troglodytes Outgroup	**AJ965469 AJ937917**	0.272	0.272	0.287	0.287	0.285	0.274	0.274	0.268	0.272	0.270	0.270	0.270	

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Discussion

The results obtained in this study demonstrated the existence of a significant genetic differentiation between *A. viridis* hosted on different arboreal trees. This result is in line with previous observations that, in the absence of the host tree, these beetles are not able to switch to other plants (Heering & Biologie 1956), which is a circumstance that is likely to generate genetic diversification within the same geographical area. Our results highlight the high genetic differences between the *A. viridis* living on different arboreal types and could suggest an incipient case of sympatric speciation, a speciation model in which the ecological separation is more important than geographical isolation to influence population genetics (Filchak et al. 2000; Lechner et al. 2015).

A cut-off value higher than a 2–3% difference in the COI sequences was used in similar studies in order to separate two genetic clusters as different species or as different OTU (operational taxonomic units; Blaxter 2004; Smith et al. 2005). In the Piedmont area, the genetic distance between *A. viridis* hosted on hazelnut vs those hosted on willow was approximately 7%. This value is quite high, even in comparison with the distances found between different species of *Agrilus* or among other Buprestidae in Germany (Hendrich et al. 2015), and may suggest a future treatment of the species of these jewel beetles as distinct taxonomic entities. However, it should be noted that the distances between species of Coleoptera can be higher than that observed in other animals (Pentinsaari et al. 2014a), and that for the other mitochondrial markers (12S and ND1-16S), the genetic distance is much less. As pointed out in a previous molecular study (Bernhard et al. 2005) and in our data, the genetic distances found utilising the 12S and ND1-16S mitochondrial markers are very low between *A. viridis* hosted on different host plants. Taken together, all the data suggest that the *viridis* is probably a species complex (Bernhard et al. 2005), even when the presence of cryptic species within the complex is considered (Hendrich et al. 2015).

Besides molecular investigations, it would be worthwhile to extend the search for new biometric distinguishing features in parallel, which would allow a better discrimination of lineages hosted by the *C. avellana* trees. Indeed, morphometric differences in the male genitalia have been already reported in *A. viridis* hosted on *Betula* and *Salix* (Pentinsaari et al. 2014b). Moreover, it would be interesting to deepen the knowledge about this species to clarify whether *A. viridis* using the hazelnut as larvae have reached a complete reproductive isolation from those hosted on other trees, or if they can still interbreed.

Recognising similar but distinct taxonomic units is important for disease and pest control. Our data are of interest for applied agronomy, since the genetic separation between the lineages of *viridis* growing on different host plants suggests that any action targeted at containing this harmful species can be limited to the plants of agricultural interest without the need to operate on other wild plants present in the same area.

Future studies could examine the genetic differentiation across the whole geographical range of the species, from West Europe to East Asia, embracing samples from different altitudes, climates and host plants.

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