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Molecular insights into speciation in the *Agrilus viridis*-complex and the genus *Trachys* (Coleoptera: Buprestidae)

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Abstract. Species of the *Agrilus viridis*-complex and the genus *Trachys* are morphologically difficult to identify or even indistinguishable. However, all of them are ecologically clearly separated because their larvae develop in different host plants. Hitherto, it was unclear whether they represent varieties, ecological races or true species. In this paper the genetic variation and phylogenetic relationships within these groups are analysed using partial sequence data from mitochondrial genes (12S rDNA, and a fragment containing regions of ND1 and 16S rDNA). The phylogenetic analyses yielded largely congruent tree topologies and indicate that all species and varieties of the *Agrilus viridis*-complex belong to a monophyletic group, which is closely related to *A. cuprescens*. Compared to all other *Agrilus*-species tested, the genetic distances within the *A. viridis*-complex are very small. However, all varieties and species are clearly separated. Thus, our data support the view that both the members of the *Agrilus viridis*-complex and the species of the *Trachys*-group represent genetically separated taxa.

INTRODUCTION

The Buprestidae is a large group of polyphagous beetles containing about 15,000 species. Generally, their body is elongated or even narrow and cylindrical like the *Agrilus*-species, however some species like *Trachys* or *Habroloma* possess a short body with a rotund or drop shaped form. The larvae of Buprestidae are either xylophagous and wood-boring, or stem- and leaf-mining.

Classification within the Buprestidae differs between authors (e.g. Cobos, 1980; Bellamy, 1985, 2003). Holynski (1993) and Lawrence & Newton (1995) recognize only 4 subfamilies: Schizopodinae, Julodinae, Buprestinae and Agrilinae. The genera *Agrilus*, *Trachys* and *Habroloma*, examined in this study, are members of the latter group. However, in some classification schemes the Agrilinae are divided into different subfamilies (e.g. Bellamy, 1985) and *Trachys* and *Habroloma* are treated as members of the Trachyinae.

The Agrilinae are generally small beetles, which together with the Buprestinae include most of the buprestid species. Their imagines are mostly found on the foliage or bark of their host plants and many are very host specific.

The genus *Agrilus* has a worldwide distribution and with about 2,500 species is the largest genus of Buprestidae (Schaeffer, 1949). In Central Europe about 40 species are found, most of which have xylophagous larvae developing in broad-leafed trees or shrubs.

With about 350 and 550 species, respectively, the genera *Habroloma* and *Trachys* are clearly smaller (Obenberger, 1937). In Central Europe, only *Habroloma nana* and eight *Trachys*-species occur (Brechtel & Kostenbader, 2002). All species are small or very small (1.5–5 mm), their larvae are leaf-miniers of herbs or broad-leafed trees. Some of them are host specific, e.g. the larvae of *Habroloma nana* are only found in *Geranium sanguineum*, while others such as *Trachys minutes* develop in a wide range of broad-leafed trees (for details see Brechtel & Kostenbader, 2002).

The determination of *Agrilus*- as well as *Trachys*-species is often very difficult. Furthermore, many taxonomical ambiguities exist. Especially in the so called *Agrilus viridis*-complex, which comprises many varieties that differ in their ecology and particularly in their host plants. At the moment, it is not clear if these varieties represent host (ecological) races, variations, subspecies or "good" species (e.g. Brechtel & Kostenbader, 2002). In Central Europe the *Agrilus viridis*-complex includes *A. viridis* willow-variety, also termed *A. viridis* f. typica (larvae typically in *Salix* spec., but also recorded from *Alnus* and other trees), *A. viridis* beech-variety, also called *A. viridis* var. *fagi* (larvae only in *Fagus sylvatica*), *A. viridis* birch-variety, also termed *A. viridis* var. *fagi* (larvae in *Betula*) and *A. viridis* var. *novicus* or blue variety of *A. viridis* var. *fagi* (larvae in *Fagus sylvatica* and perhaps in other broad-leafed trees). Two further taxa of this complex, *A. populneus* and *A. ribesi*, are now generally separated as distinct species. *A. populneus* develops exclusively in *Populus*-species, while larvae of *A. ribesi* are only found in shrubs of the genus *Ribes*. *A. ribesi* was initially described as a variety of *A. viridis* (Schaefer, 1946) but later separated as a distinct species by the same author (Schaefer, 1968). The morphological differentiation of *A. ribesi* from *A. viridis* or *A. cuprescens* is very difficult without some knowledge of the habitat. The situation of *A. populneus* is more complicated. First, it

was described as "variation *populnea*" of *A. viridis*. Later, other authors treated it as *A. suvorovi* (Lompe, 1979) or as a subspecies *populneus* of *A. suvorovi* (Hellriegl, 1978). Finally, Mühle (1992) stated that *A. populneus* is a distinct species, which is not related to *A. suvorovi*. This species is also easily confused with other members of the *A. viridis*-complex.

Additional host plants, which are sometimes recorded for members of this group, must be viewed critically, because of the unclear systematic situation within the *Agrilus viridis*-complex (Brechtel & Kostenbader, 2002). Furthermore, *A. viridis* is one of the most misidentified species of the genus (Hellriegl, 1978) and many authors do not differentiate between the varieties. On the other hand, reliable determination of species or varieties without knowledge of the host plants is often very difficult, the beech- and birch-varieties of *A. viridis* var. *fagi* are morphologically indistinguishable. Hitherto, experiments on host specificity were only done by Heering (1956a, b) with the beech-variety of *A. viridis*. In his experiments the beech-varieties did not deposit their eggs on the bark of other trees. Similar to the *Agrilus viridis*-complex, the species of the *Trachys*-group within the genera *Habroloma* and *Trachys* are morphologically also very difficult to differentiate. On the other hand, they are ecologically clearly separated, because the larvae of all these species develop in different host plants (Table 1).

In this study, we undertake a phylogenetic analysis of some taxa of the *Agrilus viridis*-complex and the *Trachys*-group as well as some other *Agrilus*-species from Central Europe by sequencing a region of the 12S rDNA and a DNA-fragment including a portion of the NADH dehydrogenase subunit I (ND1) gene, the tRNA Leucine gene and partial 16S rDNA. We address the following questions: (1) do the morphologically very similar species or varieties of the *Agrilus viridis*-complex share a close phylogenetic relationship? (2) If so, what is the level of genetic divergence within this complex and also within the *Trachys*-group, and (3) do the members of these groups represent variations, host (ecological) races or recently evolved species?

MATERIAL AND METHODS

Sampling

Tissue samples were obtained from 30 individuals belonging to 20 taxa (Table 1). Imagines were captured in the field on their host plants and in some cases larvae taken from the host plants were used (see comments in Table 1). All individuals were collected directly into 70% ethanol and stored at –20°C for preservation of nucleic acids. All specimens were identified by two specialists (Werner Rose, Tübingen and Claus Wurst, Heilbronn).

DNA extraction, PCR amplification and sequencing

DNA was extracted from frozen specimens, which were pulverized in 1.5 ml microfuge tubes with a pestle following the DTAB-protocol (Gustincich et al., 1991). Two regions of the mitochondrial genome were amplified using the primers from Simon et al. (1994): (1) a region of approximately 350 bp of the 12S rDNA with the primers SR-J-14233 and SR-N-14588, (2) a fragment of approximately 600 bp spanning partial ND1, tRNA Leucine and partial 16S rDNA with the primers N1-J-12248 and LR-N-12866.

For both genes the PCR conditions were as follows: 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 50°C (12S rDNA) or 45°C (ND1-16S rDNA fragment), 1.5 min at 72°C, and a final single extension step 10 min at 72°C, using 50 µl reactions containing 0.1 µM of each primer, 200 µM dNTP, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, template DNA and 1 unit of Taq DNA polymerase (Sigma, Taufkirchen, Germany). PCR products were purified and sequenced directly with the PCR primers. Sequences were determined on an automated LI-COR DNA Sequencer 4000L (MWG-Biotech, Ebersberg, Germany) using the Thermo Sequenase fluorescent-labeled primer cycle sequencing-Kit with 7-deaza-dGTP (Amersham, Braunschweig, Germany).

Phylogenetic analyses

The program package MEGA 2.1 (Kumar et al., 1993) was used to calculate sequence statistics. Both gene fragments were combined and an alignment was carried out with ClustalX v. 1.83 (Thompson et al., 1997) with default parameters.

Phylogenies were estimated using different procedures. MODELTEST v.3.06 (Posada & Crandall, 1998) was used to find the most appropriate model of DNA substitution. Both hierarchical likelihood ratio test and Akaike information criterion selected the same best-fit model TVM, with a proportion of invariant sites (0.2375) and unequal rates (0.8000) (TVM+I+G), which then was used for maximum likelihood (ML) and Bayesian analyses.

ML analysis was calculated with PAUP* 4.0b10 (Swofford, 2002). Bayesian analyses were performed with MrBayes V3.0B4 (Huelsenbeck & Ronquist, 2001), which was used to run 1,000,000 generations, with a sampling frequency of 10 generations. From the 100,000 trees found, the first 5,000 were discarded after MrBayes reached stability.

Neighbour-joining (NJ) trees (Saitou & Nei, 1987) were constructed with MEGA 2.1 and PAUP* using the most similar models as proposed by MODELTEST, which are available in the programs. In the PAUP* analysis we chose the GTR model (Rodriguez et al., 1990), while in the analysis using MEGA the model of Tamura & Nei (1993) was performed. Both analyses were done with and without gamma correction and yielded the same tree topologies and nearly identical bootstrap values.

Maximum parsimony (MP) analyses were performed with PAUP* using the heuristic search method with 10 random stepwise additions and the TBR branch swapping option.

Bootstrap analyses (Felsenstein, 1985) were used to examine the robustness of the resulting bifurcations within the trees. MP and NJ trees were tested with 1,000 and 10,000 replicates, respectively. Because of the enormous computational time only 100 bootstrap resamplings were carried out in the ML analyses.

Two species of the genus *Anthaxia* (*A. nitidula* and *A. fulgurans*, Buprestinae) were used to root the trees.

RESULTS

For the phylogenetic analyses we isolated DNA from 30 individuals belonging to 20 buprestid taxa. We sequenced about 350 bp of 12S rDNA, about 600 bp from a fragment containing the ND1 gene (approx. 440 bp), the tRNA Leu (60 bp), and the 16S rRNA gene (approx. 100 bp), out of which 953 positions could be unambiguously aligned. Within this alignment, 565 positions were variable and 388 parsimony informative.

The A+T content in all taxa is 73.3% with only slight differences between 12S rDNA (74%) and the ND1-16S

TABLE 1. List of taxa, host plants, sampling localities and sequence accession numbers.

Taxon	Variety	Sample number	Host plants (main species)[1]	Locality[2]	Comments[3]	Accession No. 12S rDNA	Accession No. ND1-16S rDNA
Agrilus angustulus (Illiger)		1	*Quercus*, and other broad-leaved trees	Austria, Leitha		AJ965448	AJ937901
Agrilus angustulus (Illiger)		2	*Quercus*, and other broad-leaved trees	France, Chamborigaud		AJ965449	AJ937902
Agrilus biguttatus (Fabricius)			*Quercus*	Austria, Leitha		AJ965450	AJ937909
Agrilus cuprescens Ménétriés		1	*Rubus fruticosus*, and other *Rubus* and *Rosa* species	Germany, Tübingen		AJ965451	AJ937906
Agrilus cuprescens Ménétriés		2	*Rubus fruticosus*, and other *Rubus* and *Rosa* species	Germany, Rottenburg a. N.		AJ965452	AJ937907
Agrilus cuprescens Ménétriés		3	*Rubus fruticosus*, and other *Rubus* and *Rosa* species	Austria, Leitha		AJ965453	AJ937908
Agrilus derasofasciatus Lacordaire			*Vitis vinifera*	France, Chamborigaud		AJ965454	*AJ937900*
Agrilus graminis Gory & Laporte			*Quercus*	France, Chamborigaud		AJ965455	AJ937904
Agrilus laticornis (Illiger)			*Quercus*, and other broad-leaved trees	France, Chamborigaud		AJ965456	AJ937905
Agrilus populneus Schaefer		1	*Populus*	Germany, Tübingen		AJ965457	AJ937898
Agrilus populneus Schaefer		2	*Populus*	Hungary, Bugac		AJ965458	AJ937899
Agrilus pratensis Ratzeburg			*Populus*	Germany, Tübingen		AJ965459	AJ937903
Agrilus ribesi Schaefer		1	*Ribes*	Germany, Tübingen	2 imagines from same locality	AJ965460	AJ937911
Agrilus ribesi Schaefer		2	*Ribes*	Germany, Tübingen		AJ965461	AJ937912
Agrilus sinuatus (Olivier)			*Pyrus communis, Crataegus*	Germany, Tübingen		AJ965462	AJ937910
Agrilus viridis (Linné)	beech	1	*Fagus sylvatica*	Germany, Mannheim		AJ965440	AJ937890
Agrilus viridis (Linné)	beech	2	*Fagus sylvatica*	Germany, Karlsruhe	larvae	AJ965441	AJ937891
Agrilus viridis (Linné)	beech	3	*Fagus sylvatica*	Germany, Heilbronn	larvae	AJ965442	AJ937892
Agrilus viridis (Linné)	birch	1	*Betula*	Germany, Karlsruhe	1 larva and 1 imago from same locality	AJ965443	AJ937893
Agrilus viridis (Linné)	birch	2	*Betula*	Germany, Karlsruhe		AJ965444	AJ937894
Agrilus viridis (Linné)	willow	1	*Salix*	Germany, Heilbronn	2 larvae from same locality	AJ965445	AJ937895
Agrilus viridis (Linné)	willow	2	*Salix*	Germany, Heilbronn		AJ965446	AJ937896
Agrilus viridis (Linné)	willow	3	*Salix*	Germany, Leipzig		AJ965447	AJ937897
Anthaxia fulgurans (Schrank)			*Malus domestica, Prunus spinosa, P. domestica, P. avium*	Austria, Leitha		AJ965463	AJ937914
Anthaxia nitidula (Linné)			*Prunus avium, P. spinosa, P. domestica*	Austria, Leitha		AJ965464	AJ937913
Habroloma nana (Paykull)			*Geranium sanguineum*	Germany, Tübingen		AJ965465	AJ937915
Trachys fragariae Brisout			*Potentilla, Fragaria*	Germany, Tübingen		AJ965466	AJ937918
Trachys minutus (Linné)			Many broad-leaved trees	Germany, Karlsruhe		AJ965467	AJ937916
Trachys scrobiculatus Kiesenwetter			*Glechoma hederacea*	Germany, Tübingen		AJ965468	AJ937919
Trachys troglodytes Gyllenhal			*Knautia arvensis, Scabiosa columbraria, Succisa pratensis*	Germany, Tübingen		AJ965469	AJ937917

[1]Main host plants cited by Brechtel & Kostenbader (2002).
[2]Detailed locality information is available from the authors upon request.
[3]Lines without comments: 1 imago was used.

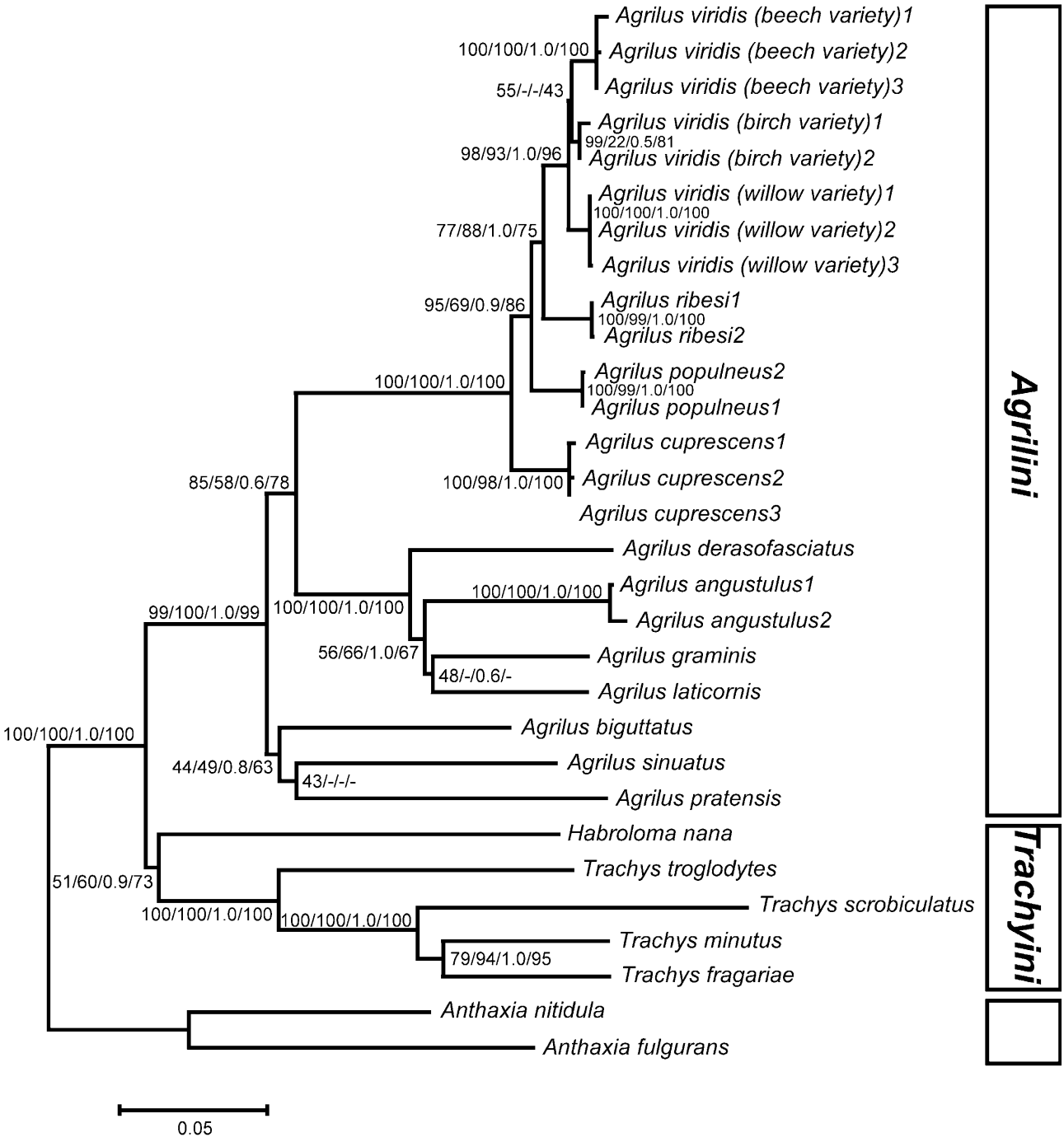

Fig. 1. Neighbor joining tree for the combined dataset (12S rDNA and ND1 sequences) for the Agrilinae species. Two *Anthaxia*-species were chosen as an outgroup. The first number at each node represents bootstrap values out of 10,000 trees in NJ analysis using the model of Tamura & Nei (1993). The second number gives bootstrap values (100 bootstrap resamplings) using the maximum likelihood (ML) method (TVM + I + G). The third number refers to posterior probabilities, which were found with Bayesian phylogenetic analysis, while the last number gives bootstrap values for 1,000 bootstrap resamplings using the maximum parsimony method.

rDNA fragment (72.8%), similar to the values in other mitochondrial genes of insects (e.g. Clary & Wolstenholme, 1985; Simon et al., 1994).

Uncorrected pairwise sequence divergence ranges from 0.1% to 0.7% between individuals of the same variety or species, and up to 30.3% between the outgroup *Anthaxia fulgurans* and *Trachys scrobiculatus*. Sequence divergences among pairwise comparisons of taxa within the *A. viridis*-complex (mean distances) yield values of 1.3–4.0% (average: 2.6%). If the close related *A. cuprescens* (Fig. 1) is included then the sequence divergence ranges up to 4.9% with an average of 3.3%.

In all trees the Agrilini as well as Trachini form monophyletic groups (Fig. 1). Within the genus *Agrilus* all

members of the *A. viridis*-complex (including *A. ribesi* and *A. populneus*) cluster together and show a close relationship with *A. cuprescens*. This group is clearly separated from the remaining *Agrilus*-species. Additionally, the genetic distances within that group are very low compared to the other branches. Within the *A. viridis*-complex the sequences of individuals from a species or variety always cluster together.

The different varieties of *A. viridis* (beech, birch, and willow) cluster closely together and also the genetic distances between them are remarkably lower than between the already recognized *A. ribesi* and *A. populneus*. In the NJ- and MP-analyses the morphologically indistinguishable beech- and birch-varieties of *A. viridis* appear as sister groups, while the willow-variety forms the sister group to them (Fig. 1). In contrast, the ML- and Bayesian trees show the willow- and birch-varieties together and the beech-variety as their sister group. However, in all analyses the branching pattern between these varieties is weakly supported.

To get a more concise picture within the *A. viridis*-complex, additional analyses were performed including only species of this group to avoid increased levels of homoplasy, which occur when increasing numbers of distantly related taxa are included in an analysis (e.g. Lecointre et al., 1994; Philippe et al., 2000). Based on our first analyses the trees were rooted with *A. cuprescens* (data not shown). However, these analyses yielded the same tree topologies as described above and there was no obvious increase in support values. Therefore, the phylogenetic relationships between the beech-, birch- and willow-varieties are not resolved unambiguously.

In addition to the *A. viridis*-complex only a few other *Agrilus*-species were included in this study, thus no other conclusions about the phylogeny within this genus can be drawn from these analyses. However, it should be mentioned that the branching pattern at the base of the genus *Agrilus* differs depending on the method used and is weakly supported in all trees (Fig. 1). Therefore, other genes need to be studied to clarify the phylogenetic relationships within this large genus.

Compared to the *A. viridis*-complex the species of the *Trachys*-group are separated from each other by large genetic distances, although their morphological similarity is similar to that of the *A. viridis*-complex. The branching pattern between the *Trachys*-species is well resolved. In all trees *Habroloma nana* branches off first, followed by *Trachys troglodytes*. *T. scrobiculatus* branches off next and *T. minutus* and *T. fragariae* cluster together.

DISCUSSION

Generally, species of the very large genus *Agrilus* (with about 2,500 species worldwide) possess only slight morphological differences. In this first molecular analysis of this group only a few species from Central Europe could be studied. Our data clearly show that all the members of the *A. viridis*-complex studied are closely related and separated from other *Agrilus*-species. Compared to the remaining *Agrilus*-species studied, the genetic distances are also clearly smaller within this complex.

Genetic variation within species or varieties is marginal (0.1–0.7% uncorrected pairwise sequence divergence). The largest difference was found between the two individuals of *Agrilus angustulus* (0.7%), which were collected from localities in Southern France and Eastern Austria, separated by approx. 1000 km. In contrast, the genetic distance between the two individuals of *A. populneus* (0.1%) is very low, although they also originate from distant localities in Southern Germany and Eastern Austria (approx. 850 km). However, for some taxa, only individuals from the same locality or from nearby habitats were available, and they show the same range of genetic variation (0.1–0.7%).

Despite the low genetic distances, all varieties or species of the *A. viridis*-complex are clearly separated from each other. The nodes linking individuals of a variety or a species are generally well supported by all the methods used, with the exception of the *A. viridis* birch-variety (Fig. 1). Therefore, despite the small number of taxa sampled the varieties and species of the *A. viridis*-complex appear genetically separated. Hence, these data support the view that host plant preference result in genetic differentiation and speciation within this complex. Within the *Trachys*-group the high genetic diversity clearly supports the view that they are all true species.

Because there are no data for their divergence from Agrilinae, external rate calibrations are required. For mitochondrial genes of insects some estimations of substitution rates are available (e.g. DeSalle et al., 1987; Brower, 1994; Juan et al., 1995, Prüser & Mossakowski, 1998), all of which range from approx. 0.5–2.3% per million years. If a rate of 1% per myr is applied to our data using the uncorrected pairwise sequence divergence, then the *A. viridis*-complex (including *A. cuprescens*) separated from other Agrilini about 18 myr ago (8–36 myr, if using 2.3 or 0.5%). Furthermore, separation of the varieties within *A. viridis* occurred in the Pleistocene (1.5 or 0.6–3 myr; using 2.3 or 0.5%), while the speciation of *A. populneus*, *A. ribesi* and *A. cuprescens* occurred 2–3 times earlier. On the other hand, estimates of diversification within the *Trachys*-group result in values about seven-fold higher than those within the whole *A. viridis*-complex.

However, these calculations should be treated with caution. First of all, the Likelihood Ratio Test indicates that neither the sequences of the *A. viridis*-complex nor those of the whole dataset evolved in a clock-like fashion. Furthermore, low numbers of substitutions, as found within the *A. viridis*-complex, can be misleading because of stochastical variation (Prüser & Mossakowki, 1998). Therefore, we think that the low genetic differences between members of the *A. viridis*-complex suggest a separation event that took place recently or is just under way.

The high diversity of beetles is explained with repeated origins of angiosperm-feeding in different beetles lineages (e.g. Farell, 1998). The relationship between food specialization and phylogeny is addressed in several

studies dealing with herbivorous beetles mainly in chrysomelid genera but also in taxa from other families such as Cerambycidae, Scolytidae, Bruchidae and Curculionidae (e.g. Funk et al., 1995; Garin et al., 1999; Kelley & Farrell, 1998; Gómez-Zurita et al., 1999; Farrell, 2001; Marvaldi et al., 2002; Kergoat et al., 2004; Swigonová & Kjer, 2004).

The respective larvae of the different *A. viridis*- or *Trachys*-taxa can be generally considered as specialists feeding on one or a few closely related plant species or genera. However, their fixation to special host plants is never absolute. Although records of host plants must be viewed critically because of potential misidentifications within these groups, there are always host plants cited in the literature in addition to the main host plants. This means that these beetles seem to possess the potential to switch to other plants, possibly in the absence of the main host. Shifts from one to another host then could lead to a rapid genetic separation within a population.

Interestingly, host plant shifts seem to have occurred within a relatively broad taxanomic range of hosts, because the host plants of the *A. viridis*-complex or *Trachys*-group are not restricted to particular plant taxa (e.g. genera or families). Instead, these shifts occurred within a relatively broad taxanomic range, e.g. *A. populneus* mainly feed on *Populus* (Salicaceae) while the closely related taxa *A. ribesi* feed on *Ribes* (Grossulariaceae) (see also Table 1).

Based on COI data Kelley & Farrell (1998) showed that within the bark beetle genus *Dendroctonus* (Scolytidae) the generalist species are ancestral and the specialized species are found at the tips of the phylogenetic tree, which provoked the question is specialization a "dead end". All the taxa of the *Agrilus viridis*-complex and the *Trachys*-group studied are clearly specialists rather than generalists, with only *T. minutus* having a wider range of host plants although it is not a basal lineage within the *Trachys*-group (Fig. 1). However, as already mentioned these highly specialized taxa seem to possess the potential for shifting between different plant taxa and therefore do not represent "dead ends".

Interpretation of the phylogenetic relationships within the rest of the genus *Agrilus* must be preliminary, because too few taxa were sampled. Interestingly, three of the four *Agrilus*-species studied (*A. angustulus*, *A. graminis*, *A. laticornis*) which feed on the same host plant (*Quercus*), cluster together, only *A. biguttatus* branches off separately. This is in contrast to the findings within the *A. viridis*-complex, where closely related species live in different host plants. However, to obtain a more detailed picture about the phylogeny and feeding preference of larvae within this large genus more species need to be studied.

Furthermore, it should be mentioned that the genetic divergences between the Trachini and Agrilini are of similar range to those between Trachini and the outgroup *Anthaxia* (Buprestinae). Therefore, the association of Agrilini and Trachyini in the subfamily Agrilinae may be questionable. Hence, further investigations using more taxa and other genes are necessary to clarify the relationships within this large beetle family.

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