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Phylogenetic analyses of the leaf beetle genus *Galerucella*: Evidence for host switching at speciation?

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ABSTRACT

It is still the subject of lively debate whether sympatric speciation is a general mode of speciation as opposed to allopatric speciation. In herbivorous insects, host switching, i.e. colonization of, and adaptation to, a new host by a herbivore, has been proposed as one of the driving mechanisms of sympatric speciation. Evidence for host switching as a speciation driving mechanism can be inferred from phylogenies of herbivores and host plants: if the host plant phylogeny is randomly distributed over the herbivore phylogeny, this indicates host switching. The Chrysomelid beetle genus *Galerucella* is a good taxon to study for evidence of host switching, because several closely related *Galerucella* species form sympatric species complexes associated with various unrelated plant species. Here we present the phylogenetic relationships of 10 species in the genus *Galerucella*, based on the mitochondrial gene fragments of the NADH-2 (410 bp) and CO-I (659 bp) genes, and analyzed with Bayesian, Maximum Likelihood and Maximum Parsimony methods. The resulting molecular phylogenetic tree proved to be largely congruent with morphologically based taxonomy. The host-associated taxa of the *Galerucella nymphaeae* species complex are not defined as distinct gene pools under the phylogenetic species concept (PSC), however, the species complex as a whole is. Two results indicate the contribution of host switching to the speciation of *Galerucella*: (1) the host-associated taxa of the *G. nymphaeae* species complex have diverged very recently and (2) constrained ML analyses showed that host use constraints led to a significantly different *Galerucella* tree compared to unconstrained analyses. This evidence for host switching, together with the observation that several sister taxa using unrelated host plants live in sympatry, suggests that sympatric speciation by host race formation can be an important mode of speciation in this genus.

1. Introduction

The study of speciation is one of the active areas of biological sciences. Many researchers favor the allopatric model of speciation, worked out by Mayr (1942, 1963), whereas others favor the sympatric model worked out later by Bush (1975). Bush proposed a mechanism for the formation of a new species by host switching (Bush, 1975), which is the colonization of and adaptation to a new host by a herbivore or parasite while gene flow is continuing. In his model several steps are required to complete the process of sympatric ecological speciation. Host races are the first step of the diverging populations toward genetic isolation. If host fidelity then develops, host races can eventually evolve into reproductively isolated species even when there is gene flow. Bush's model is supported by several theoretical and simulation studies (Rice, 1984; Kondrashov and Mina, 1986; Diehl and Bush, 1989; Kondrashov and Kondrashov, 1999; Dieckmann and Doebeli, 1999; Doebeli and Dieckmann, 2000). The experimental work on *Rhagoletis* fruit flies (e.g. Feder et al., 2003) shows, however, that empirical evidence for sympatric speciation by host race formation is difficult to obtain. Although empirical evidence for sympatric ecological speciation in general has been provided by recent molecular studies on palm trees (Savolainen et al., 2006) and on fishes that have recently colonized lakes (Barluenga et al., 2006), it remains unclear whether sympatric speciation is the exception or the rule (Berlocher and Feder, 2002; Jiggins, 2006).

Herbivorous beetles are among the most diverse animal groups. While this enormous diversity has partly been explained by the rise of the angiosperms (Farrell, 1998; Barraclough et al., 1998) it remains unclear whether herbivorous beetles have speciated in a sympatric or an allopatric mode. Insights into the mode of speciation in herbivorous beetles will contribute to the debate on the generality of sympatric speciation. One of the leaf beetle genera that is the subject of research into sympatric speciation by host race formation is the genus *Galerucella*.

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Galerucella belongs to the family Chrysomelidae, one of the very species-rich families of herbivorous beetles. Many species in the genus *Galerucella* have a very large geographical range and many exhibit broad range overlap (Silfverberg, 1974). They occur in moist habitats, but feed on many different hosts belonging to at least seven distantly related host plant families (see Appendix).

The wide variety of distantly related but commonly co-occurring host plants and the observation of closely related host-associated *Galerucella* taxa occurring in the same habitat make *Galerucella* an interesting genus for studying host switching, with possible consequences for sympatric ecological speciation (Hippa and Koponen, 1986; Nokkala and Nokkala, 1998; Pappers et al., 2001, 2002a; Ikonen et al., 2003). Whether sympatric speciation by host switching has contributed to the current diversity of *Galerucella* can only be decided after the phylogenetic relationships between the members of the genus have been resolved (Barraclough and Nee, 2001). We can then assess whether taxa associated with the same host are indeed most closely related to each other and which host switches have taken place.

The systematics of species in the genus *Galerucella* has traditionally been based on morphological characteristics. However, the large geographical ranges of the species allow much local variation, which has resulted in different species descriptions in different parts of their range, yielding many synonyms for a single species (see for example Reid (2001)). Moreover, several authors believe that leaf beetles adapt and specialize to a particular host plant (monophagy) or several related host plants (oligophagy) (e.g. Novotny et al., 2002) and treat the host-associated taxa as monophagous or oligophagous species (Lohse, 1989; Kangas, 1991). However, since taxa associated with different hosts are able to produce viable and fertile offspring (Hippa and Koponen, 1986; Pappers et al., 2002b), both Ikonen et al. (2003) and Pappers et al. (2002b) favored a subdivision into host races rather than species, based on the biological species concept (Mayr, 1942).

This study presents a molecular taxonomy of the genus *Galerucella*, including Palearctic, Nearctic, African and Australian species. The mtDNA characteristics allowed us to construct a phylogenetic tree of *Galerucella* and test whether typological species are monophyletic. In addition, the phylogenetic tree allowed us to infer the evolutionary relationships between the host-associated taxa within the *Galerucella nymphaeae* species complex. Finally, we looked at the distribution of host plant use over the phylogenetic tree of *Galerucella* to find indications for evolutionary relationships.

2. Materials and methods

2.1. Sampling, taxa and host plant association

The taxonomy of *Galerucella* taxonomy has been revised several times (see Silfverberg, 1974). Most *Galerucella* species that have been described are from the Palearctic region. Wilcox (1971) recognizes 3 North-American *Galerucella* species, 2 of which were included in our study, along with 8 of the 9 West-Palearctic *Galerucella* species recognized by Silfverberg (1974) and 1 *Galerucella* species from Africa. Of the total of 10 *Galerucella* species included in this study, 5 belong to the subgenus *Galerucella* and 5 to the subgenus *Neogalerucella*, (Silfverberg, 1974). Each species was represented by 1 or more individuals. The host-associated groups of *G. nymphaeae* were treated as *G. nymphaeae* species complex.

Tricholochmaea decora and *Lochmaea suturalis* are two species closely related to the genus *Galerucella* (as reported in Borghuis et al. (unpublished manuscript), based on a partial CO-I sequence from 45 species of the Galerucini subtribe). We therefore used these species to root our phylogeny of *Galerucella*.

The species in the genus *Galerucella* feed on host plants belonging to several families. Table 1 shows the *Galerucella* taxa and outgroup species we studied, their host plant associations and their geographical origins.

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted by grinding up the heads and thoraxes of individual dried, fresh, frozen or alcohol-preserved specimens. Abdomens were kept as voucher material. The alcohol in alcohol-preserved specimens was allowed to evaporate at room temperature before grinding. 500 μl 5% CHELEX (Biorad) solution and 10 μl 20 mg/ml Proteinase-K were added to the ground-up individuals and incubated at 65 °C for 12 h. Samples were subsequently heated to 95 °C for 10 min, to inactivate Proteinase-K.

Mitochondrial gene fragments were obtained using the following PCR primers: a modified Tm-J-206 (5′-GCTAADYAAGCTAT TGGGTTCAT-3′) and a newly developed primer N2-N-670 (5′-TTGGGGCTAATTTTTGTCA-3′) following the nomenclature by Simon et al. (1994) were used to amplify approximately 465 bp of the NADH-2 gene, while the primers C1-J-2164 and TL2-N-3014 (Simon et al., 1994) were used to amplify approximately 850 bp of the CO-I gene for a subset of the taxa, as listed in Table 1. PCRs were carried out in 25 μl with 100 μM dNTPs, 3 mM $MgCl_2$, 0.4 μM primer and 1 U ABgene Taq polymerase, using the following PCR profile: 4 min at 95 °C followed by 20 cycles of denaturation for 30 s at 95 °C, annealing at 60 °C dropping 0.5 °C/cycle for 1 min and extension at 72 °C for 1.5 min, with a final extension step at 72 °C for 7 min. PCR products were visually checked using agarose gel electrophoresis, and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to the manufacturer's instructions, and eluted in 20 μl ddH_2O. Purified PCR products were used for direct sequencing with the original PCR primers and with internal primers. Internal primers for the NADH-2 gene were N2-J-231 (5′-CCCACTTATAAAGATTACTC-3′) and N2-N-534 (5′-CAGTAATCTGAATTGTTGG-3′); internal primers for the CO-I gene were C1-J-2577 (5′-ATGTTCTTTCAATAGGRG CAG-3′) and C1-J-2626 (5′-ATGATTCCCTTTATTTACAG-3′) C1-N-2757 (5′-AAATGTTGNGGRAAAAATGTTA-3′), according to the nomenclature by Simon et al. (1994). Sequence PCRs were performed using the CEQ 2000 DTCS sequencing kit (Beckman Coulter) following the manufacturer's instructions. Sequences were run on a Beckman 8000 sequence analyzer.

2.3. Sequence analysis

Two to four sequence chromatograms from each gene fragment were used to construct a consensus sequence for each individual. Both gene regions were aligned using CLUSTAL_X (Thompson et al., 1997) using the default options. Neither of the mitochondrial gene fragments had insertions or deletions in the coding region, making alignment unambiguous. Polymorphisms were double-checked on the chromatograms. Sequence data were submitted to GenBank. GenBank accession numbers of the amplified NADH-2 and CO-I fragments are shown in Table 1.

2.4. Phylogenetic analysis

Bayesian and maximum likelihood (ML) phylogenetic analyses were performed with MRBAYES v3.1.2 (Ronquist and Huelsenbeck, 2003) and Maximum Parsimony (MP) phylogenetic analyses with PAUP v.4.0b10 (Swofford, 2002). Model settings for the Bayesian and maximum likelihood analyses were determined using MODELTEST v.3.7 (Posada and Crandall, 1998). This program compares 56 possible models of sequence evolution and selects the model identified as optimal by the Akaike Information Criterion (Akaike,