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European and North American populations of *Galerucella nymphaeae* (Coleoptera: Chrysomelidae): Two separate species revealed by chorion polypeptide analysis

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Abstract. The *Galerucella nymphaeae* species complex is a controversial group of leaf-consuming beetles with a Holarctic distribution. It includes several closely allied species or forms living in different habitats and utilizing different food plants. In northern Europe, two species are encountered, *G. nymphaeae* (L.) living on *Nuphar*, and *G. sagittariae* (Gyllenhal) living on semiaquatic or terrestrial plants, while all North American forms have been so far considered conspecific with the European *G. nymphaeae*. In the present study we have compared chorion polypeptides of the northern European *G. nymphaeae* and *G. sagittariae* with North American *G. nymphaeae* collected from *Nuphar*. The northern European *G. nymphaeae* was found to differ from both northern European *G. sagittariae* and North American *G. nymphaeae*, which were found to be virtually identical in respect to their chorion polypeptides. The present results, coupled with earlier data concerning e.g. egg morphology, structure of larval cuticle, and comparison of several life history traits, demonstrate that northern European *G. nymphaeae* and North American *G. nymphaeae* are not conspecific, and that the North American *G. nymphaeae* may be more closely allied to the northern European *G. sagittariae*.

INTRODUCTION

The leaf-consuming chrysomelid genus *Galerucella* Crotch is a large genus widely distributed over the northern hemisphere. It is also a controversial genus presenting serious difficulties in several aspects of its classification and nomenclature (see e.g. Hippa & Koponen, 1986 and references therein). In the present study, we have adopted Silfverberg's (1974) concept of the genus, divided into two subgenera, *Galerucella* and *Neogalerucella*.

Most species included in the subgenus *Galerucella* belong to the *Galerucella nymphaeae* species complex, which is probably the most controversial group within this genus. Within the subgenus, *G. grisescens* (Joannis), *G. placida* Baly and *G. birmanica* (Jacoby) are in general considered as distinct species and separate from the *Galerucella nymphaeae* species complex (e.g. Palmén, 1945; Gressit & Kimoto, 1963; Silfverberg, 1974), although Kimoto (1969) synonymizes the two first mentioned. Up to a dozen other identified forms have been at one time or another considered conspecific with *G. nymphaeae* (L.) (see Hippa & Koponen, 1986). In northern Europe, three species or forms belonging to the complex can be identified: the typical, "aquatic" *G. nymphaeae*, which is the largest and darkest form and utilizes *Nuphar lutea* (L.) Sm. as the principal food plant, a "semiaquatic" form utilizing *Potentilla palustris* (L.) Scop. (*Comarum palustre* L.), and a "terrestrial" form living mainly on *Rubus chamaemorus* L. It has been recently established that only the "aquatic" form represents the nominal species *G. nymphaeae*, while the two last-mentioned are included under *G. sagittariae* (Gyllenhal) (Hippa & Koponen, 1986; Nokkala 1989; Nokkala & Nokkala, 1994, 1996, 1998).

In North America, five species or forms belonging to the *Galerucella nymphaeae* species complex have been identified, *G. nymphaeae* (L.), *G. femoralis* (Melsheimer), *G. marginella* (Kirby), *G. sagittariae* (Gyllenhal sensu Kirby) and *G. luctuosa* (Mannerheim), but subsequently they have all been synonymized with *G. nymphaeae* (L.) (see Wilcox, 1965, 1971; Doyen & Ulrich, 1978; Brigham, 1982; Wallace & O'Hop, 1985), and thus considered conspecific with the European counterpart (e.g. Balsbaugh & Hays, 1972). At least *Nuphar*, *Polygonum*, *Myrica* and *Potentilla* are listed as food plants of North American *G. nymphaeae*.

The size and general appearance of North American *G. nymphaeae* adults resemble more the appearance of European *G. sagittariae* than European *G. nymphaeae*, verified also by morphometric analyses by Hippa & Koponen (1986), and later by Cronin et al. (unpublished). Hippa & Koponen (1986) have also observed that the larval cuticle of American *G. nymphaeae* is similar to that of European *G. sagittariae*, both differing from that of European *G. nymphaeae*. Otto & Wallace (1989) compared several life history traits, e.g. developmental time, instantaneous growth rate, clutch size and mortality of immature stages of one northern European and one North American population of *G. nymphaeae* both inhabiting *N. lutea* and concluded that North American and northern European populations of *G. nymphaeae* represent two separate species.

Diagnostic differences between northern European *G. nymphaeae* and *G. sagittariae* were found by analyzing chorion polypeptides in these species (Nokkala & Nokkala, 1994, 1996, 1998). This encouraged us to take a similar approach to address the question of conspecificity of northern European *G. nymphaeae* and North American *G. nymphaeae*. In the present study, we report the results of comparative chorion polypeptide analysis concerning northern European *G. nymphaeae* and *G. sagittariae* and North American *G. nymphaeae*.

MATERIAL AND METHODS

Adult females of *Galerucella nymphaeae* from *Nuphar lutea*, and *G. sagittariae* from *Potentilla palustris* were collected at Vammala, Houhajärvi in Southern Finland in early June, 1996. Adult females of North American *G. nymphaeae* were collected from *Nuphar advena* (Soland.) from Painter Lake near Edwardsburg, Michigan in early June, 1996, and brought to Finland alive. They were allowed to feed on their original food plant until the preparation of the samples for electrophoresis occurring a day or two after their arrival. The present study included 14 females of Finnish *G. nymphaeae*, 12 females of Finnish *G. sagittariae* and 15 females of North American *G. nymphaeae*.

Three to five mature eggs were dissected from the ovaries of each female in insect Ringer's solution. The eggs were then dechorinated, and the chorions were washed briefly in 0.1% Nonident P-40 in Ringer's solution and dissolved in 30–40 µl of sample buffer (0.05 M Tris-HAc, pH 7.5; 10% glycerol; 2% SDS; 5% 2-β-mercaptoethanol; 0.00125% bromophenol blue). Samples were heated for 5–6 min in a bath of boiling water before submitting to SDS-PAGE electrophoresis. Parallel samples prepared from each population were run on the same gel. We used precast ultrathin gradient gels from Pharmacia (ExcelGel™ SDS, gradient 8–18), which were subsequently stained with $AgNO_3$, according to the instructions given by the manufacturer.

RESULTS

Chorion polypeptide analysis is based on differential migration of molecules according to their molecular weights in the electric field. Each polypeptide is a gene product, and

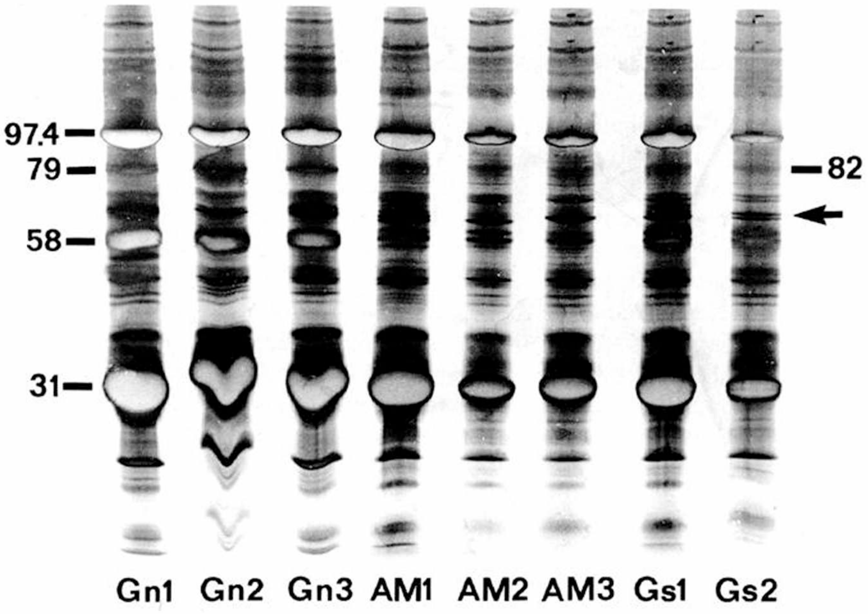

Fig. 1. Chorion polypeptide patterns in Finnish *G. nymphaeae* (Gn), American *G. nymphaeae* (AM) and Finnish *G. sagittariae* (Gs). Major bands of 97.4 kD, 82 kD in American *G. nymphaeae* and *G. sagittariae*, 79 kD in Finnish *G. nymphaeae*, 58 kD and 31 kD are indicated. The conspicously polymorphic region around 62 kD is also indicated by an arrow.

mutations in the gene, if reflected in the molecular weight of the product, can be observed as a shift in band position on the gel. A band may consist of a single polypeptide, i.e. the product of a single gene, or it may be composed of several comigrating polypeptides, sharing similar molecular weight but representing different gene loci. A locus is revealed if both heterozygotes and homozygotes can be detected.

The general pattern of polypeptide distribution is similar in the two Finnish and one American populations, molecules falling in the range of ca. 15–150 kD (Fig. 1). However, the American samples give virtually identical chorion polypeptide patterns to the Finnish *Galerucella sagittariae*, whereas the Finnish *G. nymphaeae* shows unique characteristics (see also Nokkala & Nokkala, 1994, 1996, 1998). The single most important feature is the presence of a prominently stained yellow band in the 79 kD region in Finnish *G. nymphaeae*, not found in *G. sagittariae* or North American *G. nymphaeae*, while in these species there is a quite distinct brown band in the 82 kD region. The 79 kD band was found in all 14 individuals of Finnish *G. nymphaeae* analyzed in the present study, and also in an additional 320 individuals of *G. nymphaeae* studied in parallel studies originating from Vammala and three other locations in Finland. The band is totally absent both from North

American *G. nymphaeae* (15 individuals studied) and Finnish *G. sagittariae* (12 individuals studied in the present study, and 328 individuals from Vammala and five other locations studied in parallel studies). It is evident that the prominent band in the 79 kD region is composed of several co-migrating polypeptides. The region is nearly monomorphic in *G. nymphaeae*, but occasionally heterozygotes may be found, as demonstrated by sample Gn1 in Fig. 1. The doublet band does not consist of two equal bands, but the band with higher molecular weight is thinner than the standard band indicating heterozygosity of only one polypeptide from several. In some individuals of North American *G. nymphaeae* and Finnish *G. sagittariae*, one may observe a much more faintly-stained band in the 79 kD region, but its intensity of staining is so different from the prominent band of Finnish *G. nymphaeae* that the bands obviously represent co-migrating but not identical gene products. The 82 kD band in *G. sagittariae* and North American *G. nymphaeae* is also a composite band, and the region is highly polymorphic. It is evident that in the 79 and 82 kD regions several mutations have occurred, which have concentrated polypeptides in the 79 kD region in *G. nymphaeae* and to a somewhat lesser extent in the 82 kD region in *G. sagittariae* and North American *G. nymphaeae*. There are also clear quantitative differences; the 58 kD region is much more strongly stained and the 31 kD region somewhat more strongly stained in Finnish *G. nymphaeae* than in the other two.

As might be expected, there are a number of polymorphic loci within each sample population – an example of these may be detected in Fig. 1. Sample AM1 represents a heterozygote in the 62 kD region, sample AM2 represents a homozygote with alleles coding for the lighter variant of the polypeptide, and sample AM3 a homozygote with allelles coding for the heavier variant. Sample Gs1 is a similar homozygote as AM3, but sample Gs2 is heterozygous, yet not similar to AM1 but sharing one allele with AM3 and Gs1, while the other allele codes for a still heavier variant of the polypeptide. The Finnish *G. nymphaeae* seems to differ from the other two, since corresponding bands tend to concentrate in the 65 kD region.

Despite several polymorphic loci in all sample populations, the characteristics described above always separate northern European *G. nymphaeae* from *G. sagittariae* and North American *G. nymphaeae*.

DISCUSSION

The results of the present study confirm earlier suggestions (Hippa & Koponen, 1986; Otto & Wallace, 1989; Cronin et al., unpubl.) that the *Galerucella nymphaeae* from North America is not conspecific with the *G. nymphaeae* from northern Europe, despite similar food plants and habitat utilization. In morphometric analyses of adults (Hippa & Koponen, 1986; Cronin et al., unpubl.), North American populations originating either from *Nuphar* or from various semiaquatic or terrestrial plants give measurements indistinguishable from each other and from European *G. sagittariae*. European *G. nymphaeae* adults are larger than European *G. sagittariae* or American *G. nymphaeae*, and their pronotum is relatively shorter than in the other two.

The larvae of each instar of northern European *G. nymphaeae* are slightly larger and have a relatively wider head than those of *G. sagittariae* (Hippa & Koponen, 1986) and American *G. nymphaeae* (Otto & Wallace, 1989). The larvae of northern European *G. nymphaeae* and *G. sagittariae* differ also in the surface structure of their cuticle (Hippa &

Koponen, 1986). The dorsal cuticle of *G. nymphaeae* has small conical papillae, which makes the larvae seem dull to the naked eye, while the dorsal surface of *G. sagittariae* lacks conical papillae, and larvae appear shiny. Hippa & Koponen (1986) observed also that the larval cuticle structure of North American *G. nymphaeae* was similar to that of European *G. sagittariae*, not *G. nymphaeae*.

Hippa & Koponen (1986) have studied egg morphology of northern European *G. nymphaeae* and *G. sagittariae*. They found that eggs of *G. nymhaeae* are larger than eggs of *G. sagittariae*, and that egg surface structure is different in the two species. Unfortunately, there are no studies of egg surface structure in North American *G. nymphaeae*, but Otto & Wallace (1989) found the egg dry mass of European *G. nymphaeae* to be larger than that of North American, indicating the similarity of egg size in American *G. nymphaeae* and European *G. sagittariae*.

The observations concerning egg morphology are consistent with the results of the present study. Egg chorion polypeptides are components of structural proteins, linked directly to egg surface structure, where they are exposed to selective pressures. The great similarity in chorion polypeptide patterns in *G. sagittariae* and North American *G. nymphaeae* coupled with morphological data indicate that the latter may be more closely allied to *G. sagittariae* than to the European *G. nymphaeae*.

The close alliance of American *Nuphar*-consuming *Galerucella* beetles with the northern European *G. sagittariae* is very interesting, since we know that European *G. sagittariae* cannot survive on *Nuphar lutea* in Europe (Hippa & Koponen, 1986; Nokkala & Nokkala, 1998). American beetles transferred as pupae to Finland and reared in the laboratory prefered *Rubus* and *Potentilla* over *Nuphar lutea*, which seemed to be impossible for them to gnaw on (personal observation), while Finnish *G. nymphaeae* allways prefers *Nuphar* (Nokkala & Nokkala, 1998). Otto & Wallace (1989) made a consistent observation that American larvae collected as eggs from American *Nuphar lutea* and transferred to Sweden found Swedish *N. lutea* unpalatable and starved to death. It seems that the quality of North American *Nuphar* species differs markedly from that of northern European *N. lutea*.

There is a gap in the distribution of all species of *Nuphar* between eastern Asia and western North America, but *Rubus* and *Potentilla* are circumpolar plants (Hultén & Fries, 1986). Thus it seems possible that populations belonging to the *Galerucella nymphaeae* complex have distributed over the northern hemisphere utilizing *Rubus*, *Potentilla*, or some other terrestrial or semiaquatic plant occupying corresponding habitats. It is somewhat puzzling what made the beetles change to an aquatic habitat and food plant to *Nuphar* in North America. It is clear that we need to have detailed knowledge of European and Asian, as well as North American, populations belonging to the *Galerucella nymphaeae* species complex, their distributions, and the distributions of their food plants to unravel the biological and taxonomic relations, the direction of evolution, and the modes of speciation in this controversial complex.

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