Genetics, Taxonomy, and Ecology of Certain Species of *Galerucella* (Coleoptera: Chrysomelidae)

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ABSTRACT Two European chrysomelids, *Galerucella calmariensis* (L.) and *G. pusilla* (Duftschmidt), show promise as biological control agents against the weed *Lythrum salicaria* L. (purple loosestrife). *G. calmariensis* and *G. pusilla* are sympatric, share the same ecological niche on *L. salicaria*, and are separated by several morphological characters. These two *Galerucella* species were genetically compared with another sympatric species, *G. grisescens* (Joannis), found on *Lysimachia vulgaris* L. (garden loosestrife) in Europe, and to *G. nymphaeae* (L.), which occurs on water lilies and other wetland plants such as *L. salicaria* in Europe and the United States. Two additional species of *Galerucella* occur in North America: *G. stefanssoni* Brown, feeding on *Rubus chamaemorus* L. (cloudberry), and *G. quebecensis* Brown, feeding on *Potentilla palustris* (L.) Scop. (marsh-flower). Introduction and establishment of *G. calmariensis* and *G. pusilla* to combat *L. salicaria* would increase to five the number of *Galerucella* species in North America. The five species are keyed, described, and the adult and male genitalia are figured. An isozyme comparison of 33 loci among three species of *Galerucella* from Germany verified that *G. calmariensis*, *G. pusilla*, and *G. grisescens* are distinct species. *G. calmariensis* has a genetic marker represented by LDH-1. The clustering analysis based on genetic identities and some behavioral characters suggests that *G. grisescens* is more closely related to *G. nymphaeae* than to *G. calmariensis* or *G. pusilla*. The three United States samples of *G. nymphaeae* collected from two different host plants showed moderate differentiation. Mean heterozygosity ranged from 0.100 to 0.214 (mean = 0.157) across all six measured populations of *G. calmariensis*, *G. pusilla*, *G. grisescens*, and *G. nymphaeae*.

KEY WORDS *Galerucella*, systematics, isozymes

IN NORTHERN AND CENTRAL EUROPE, two chrysomelids, *Galerucella calmariensis* (L.) and *G. pusilla* (Duftschmidt), were identified as potential biological control agents for *Lythrum salicaria* L., purple loosestrife. *L. salicaria* is a European weed that has invaded wetland wildlife areas, riparian pasture and grazing lands, and agricultural areas in the United States. The plant has little or no food value for wildlife or stock animals, is highly competitive, and eliminates desirable native plants (Pfannmueller & Djupstrom 1983, Thompson et al. 1987). The plant is well established throughout the northeastern United States and into the midwestern states and adjacent Canada, and occurs in scattered locations in the northwestern states, south-central Canada, the Pacific Northwest, and California. Because of the large geographic areas involved and the type of land infested, herbicidal treatment is uneconomical, inefficient, and potentially hazardous. Biological control offers the most logical approach for controlling the weed in the most effective and least environmentally damaging way (Hight & Drea 1991).

G. calmariensis and *G. pusilla* coexist and, when in high densities, cause defoliation of whole stands of their host plant (Blossey & Schroeder 1991, Blossey 1992). It was difficult to identify morphologically 5–10% of adult field individuals using the characters developed by Palmén (1945) and Silfverberg (1974) such as the last sternite, the pronotum, and the aedeagus. The larvae were virtually indistinguishable, and preliminary findings suggested that both species share the same ecological niche on *L. salicaria*. These similarities raised the question of whether *G. calmariensis* and *G. pusilla* are genetically separate species. Before introduction of *G. calmariensis* and *G. pusilla* into North America, it is important to attain a better understanding of their systematics. Therefore, electrophoretic techniques combined with behavioral studies of mixed pairs, field data on possible competitive

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exclusion, habitat use of the two species, and detailed taxonomic evaluations were used to study this species pair. Two additional species of *Galerucella* were used as reference points in the electrophoretic study, *G. grisescens* (Joannis), occurring on *Lysimachia vulgaris* L. (garden loosestrife) in the same European habitat, and *G. nymphaeae* (L.) from North America, using *L. salicaria* as a host plant.

The objectives of this study were to compare the results of an isozyme analysis among the two European species, *G. calmariensis* and *G. pusilla*, that are planned for introduction into the United States as biological control agents of *L. salicaria*, *G. grisescens* from Europe, and three North American populations of *G. nymphaeae*, a common Holarctic species. This article presents a comparison of 22 enzyme systems and 33 enzyme loci in which some have been defined as genetic markers. This work is supported by a morphological study of adults of each species and ecological descriptions of their habitat. In addition, a key and diagnostic descriptions of *G. calmariensis* and *G. pusilla* and the three species occurring in North America, *G. nymphaeae*, *G. quebecensis* Brown, and *G. stefanssoni* Brown, are presented.

Materials and Methods

Populations Studied. Populations of four species in the genus *Galerucella* were collected, including *G. calmariensis* (50 adults), *G. pusilla* (51 adults), and *G. grisescens* (21 adults) from Germany, and three different *G. nymphaeae* populations (40–110 adults) in North America. The first two populations from Germany were collected at Bergenhusen on 23 May 1989, and the population of *G. grisescens* near Lübeck on 26 July 1989. The insects were shipped on dry ice to the Insect BioControl Laboratory at Beltsville, MD. The three populations of *G. nymphaeae* were sampled from near Ithaca, NY, on 11 August 1989, and from Maryland on 8 September 1989 in Prince George's County, one from the Beltsville Agricultural Research Center and the other from the Patuxent Wildlife Research Refuge. All adults were stored at −80°C until used in electrophoretic studies.

Electrophoretic Techniques. Electrophesis was performed on horizontal starch gels using 6% Electrostarch (lot no. "Special 89"), 6% Sigma starch (S-4501), 5% sucrose, and 350 ml gel buffer. Each individual (male or female) was ground in 30 μl of buffer, and four pieces of 4 by 10 mm sepraphore III cellulose paper (Gelman Sciences) were used as wicks to absorb the homogenate. Thus, each individual was run on four different gels simultaneously. The power used for each gel was 12.5 V/cm with the Tris·citrate buffer (TC) system of Siciliano & Shaw (1976), 13.0 V/cm and 14.7 V/cm with the morpholine buffer (Morph) system of Clayton & Tretiak (1972), and 14.5 V/cm with a modified lithium buffer (LiOH) system of Selander et al. (1969). Each gel was run 6 h, then was sliced horizontally into three to seven slices, each 1 mm thick. Each slice was stained for a specific enzyme system. Staining procedures were adapted from Harris & Hopkinson (1976), Selander et al. (1971), and Shaw & Prasad (1970). Each of 37 enzyme systems was screened on three different buffers to determine which had the best allelic resolution. Abbreviations, names, and enzyme commission numbers for the 37 enzymes assayed are listed in Table 1. After the screening of each isozyme, 22 enzyme systems were scorable. ACON, DIA, EST, GOT, HAD, LAP, LDH, MDH, ME, 6PGD, PGI, and PK were run on two Morph gels. AK, FBP, FUM, GPDH, GR, IDH, and SDH were run on TC gel. ACPH, ARGK, and PGM were run on LiOH gel. For additional details, electrophoretic procedures were described by Manguin & Hung (1991), and specific buffer systems and staining recipes are available upon request from S.M.

Data Analysis. Loci coding for the same enzyme are numbered sequentially from the most anodal to the most cathodal regions of activity. The alleles are designated by their relative mobilities, with the most common electromorph given a mobility of 100. A negative sign (−) designates an allele whose homomeric protein has a cathodal migration. Electromorph genotype frequencies were used as input for BIOSYS-1 (Swofford & Selander 1981). Analysis of each population included computation of allele frequencies, heterozygosity per locus, additional measures of genetic variability, and a test for conformance to Hardy-Weinberg equilibrium at single loci by chi-square analysis. Differentiation among the populations of *G. nymphaeae* was measured by *F* statistics. Genetic identity measures were calculated (Rogers 1972, Nei 1978). Nei's unbiased (1978) genetic identities were clustered by the unweighted pair-group method using arithmetic averaging (UPGMA) to produce the phenogram.

Taxonomic Study. Specimens studied for the taxonomic description were from the U.S. National Museum of Natural History. The number of specimens examined was as follows: 200 *G. nymphaeae*, seven *G. calmariensis*, nine *G. pusilla*, four *G. quebecensis*, and five *G. stefanssoni*.

Biological Observations. Field populations of *G. calmariensis* and *G. pusilla* at 51 different sites were examined in June 1990 in central Europe (Germany), where both *Galerucella* species occur. The frequency of each species in the total sample of sites was recorded. Only sites with populations over 10 individuals were included in the analysis. The collected beetles were identified, and the sites were grouped according to habitat characteristics into five types. Only two northern German populations of *G. grisescens*

Table 1. Enzyme strains of four species of *Galerucella*

Enzyme system	E.C. no.	Symbol	No. loci[b]	Buffer[c]
Scorable				
1. Aconitase	4.2.1.3	ACON	2	Morph
2. Acid phosphatase	3.1.3.2	ACPH	1	LiOH
3. Adenylate kinase	2.7.4.3	AK	2	TC
4. Arginine kinase	—	ARGK	1	LiOH
5. Diaphorase	1.6.2.2	DIA	1	Morph
6. Esterase	3.1.1.1	EST	4	Morph
7. Fructose biphosphatase	3.1.3.11	FBP	1	TC
8. Fumarase	4.2.1.2	FUM	1	TC
9. Glutamate oxaloacetate transaminase	2.6.1.1	GOT	2	Morph
10. ∂-Glycerophosphate dehydrogenase	1.1.1.8	GPDH	1	TC
11. Glutathione reductase	1.6.4.2	GR	1	TC
12. β-Hydroxyacid dehydrogenase	1.1.1.30	HAD	1	Morph
13. Isocitrate dehydrogenase	1.1.1.42	IDH	2	TC
14. Leucine amino peptidase	3.4.11.1	LAP	1	Morph
15. Lactate dehydrogenase	1.1.1.27	LDH	2	Morph
16. Malate dehydrogenase	1.1.1.37	MDH	2	Morph
17. Malic enzyme	1.1.1.40	ME	1	Morph
18. 6-Phosphogluconate dehydrogenase	1.1.1.44	6PGD	3	Morph
19. Phosphoglucose isomerase	5.3.1.9	PGI	1	Morph
20. Phosphoglucomutase	2.7.5.1	PGM	1	LiOH
21. Pyruvate kinase	2.7.1.40	PK	1	Morph
22. Sorbitol dehydrogenase	1.1.1.14	SDH	1	TC
Nonscorable				
23. Ethanol dehydrogenase	1.1.1.1	ADH	NA	NA
24. Aldolase	4.1.2.13	ALD	NA	NA
25. Aldehyde oxidase	1.2.3.1	AO	NA	NA
26. Alkaline phosphatase	3.1.3.1	APH	NA	NA
27. Galactose-6-phosphate dehydrogenase	—	GAL-6	NA	NA
28. Glucose dehydrogenase	1.1.1.47	GDH	NA	NA
29. Glutamate dehydrogenase	1.4.1.3	GLU	NA	NA
30. Glucose oxidase	—	GLUO	NA	NA
31. Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	G3PDH	NA	NA
32. Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH	NA	NA
33. Hexokinase	2.7.1.1	HK	NA	NA
34. Mannose-6-phosphate isomerase	5.3.1.8	MPI	NA	NA
35. Superoxide dismutase	1.15.11	SOD	NA	NA
36. Trehalase	3.2.1.28	TRE	NA	NA
37. Xanthine dehydrogenase	1.2.1.37	XDH	NA	NA

[a] Enzyme commission number.
[b] Number of scorable bands per phenotype.
[c] Refers to electrophoresis buffer.

were sampled, so only limited information is available for this species.

Results

Isozyme Analysis. Thirty-three loci were inferred from the allozyme variation observed in the 22 enzyme systems. Among the three populations of *G. nymphaeae*, 29 loci were scored for the populations from Beltsville and Ithaca and 28 for the population from Patuxent (Table 2). FBP occurred in all three *G. nymphaeae* populations, but this locus was scorable only for *G. nymphaeae* (Beltsville); its activity was very weak for the two other populations of *G. nymphaeae*. 6PGD-1 was observed only in *G. nymphaeae* (Ithaca). For the three species from Germany, 26 loci occurred for *G. calmariensis*, 25 for *G. pusilla*, and 24 for *G. grisescens* (Table 2). Five loci were species-specific: EST-2, EST-3, EST-5, and IDH-1 were observed in all three populations of *G. nymphaeae*, and LDH-1, a monomorphic locus, occurred only in *G. calmariensis*. LDH-2 was specific to *G. calmariensis* and *G. pusilla*, EST-1 was specific to the three species from Germany, and HAD and 6PGD-3 were specific to the three populations of *G. nymphaeae* and *G. grisescens*. No locus was monomorphic in all six populations, but three loci were nearly monomorphic: ARGK, FUM, and SDH (see *Appendix*).

Only seven loci (indicated with asterisks in *Appendix*) showed significant deviation from Hardy-Weinberg equilibrium: ACON-2, GR, and IDH-2 for *G. nymphaeae* (Beltsville); EST-2 for *G. nymphaeae* (Patuxent); and FBP, LDH-2, and PGM for *G. pusilla*. The resolution of ACON-2 and PGM was generally good, but rare alleles were involved and little meaning can be attached to these deviations. The allelic resolution of EST-2, GR, IDH-2, and LDH-2 was occasionally poor, and some scoring errors might be involved. This level of error had inconsequential effects on subsequent intra- and interspecific comparisons. Of the 198 comparisons made, 10

Table 2. Measures of genetic variation in four *Galerucella* species

Population	No. loci	Mean sample size per locus (SEM)	Mean no. of alleles per locus (SEM)	% polymorphic loci[a]	Mean heterozygosity	
					Direct count (SEM)	Hardy-Weinberg expected[b] (SEM)
G. nymphaeae, Beltsville	29	88.0 (2.7)	2.0 (0.1)	64.3	0.177 (0.029)	0.207 (0.034)
G. nymphaeae, Patuxent	28	35.3 (1.5)	1.9 (0.1)	60.7	0.214 (0.042)	0.209 (0.039)
G. nymphaeae, Ithaca	29	34.6 (1.6)	2.3 (0.2)	71.4	0.196 (0.033)	0.225 (0.035)
G. calmariensis	26	44.0 (1.5)	2.3 (0.2)	36.4	0.100 (0.028)	0.115 (0.032)
G. pusilla	25	45.1 (1.5)	2.0 (0.2)	50.0	0.138 (0.038)	0.165 (0.042)
G. grisescens	24	18.0 (0.7)	1.5 (0.1)	31.8	0.116 (0.043)	0.125 (0.043)

[a] A locus is considered polymorphic if more than one allele was detected.
[b] Unbiased estimate (Nei 1978).

significant deviations would be expected by chance alone, and the seven deviations observed here is unremarkable.

The mean heterozygosity (direct-count) of all loci (Table 2) ranged from 0.100 for *G. calmariensis* to 0.214 for *G. nymphaeae* (Patuxent). The average heterozygosity (direct-count) across all six populations of *Galerucella* was 0.157 (±0.046), with a mean value of 0.196 (±0.018) for the three populations of *G. nymphaeae* and 0.118 (±0.019) for the three species from Germany. The mean heterozygosity of all studied populations of *Galerucella* was close to the mean value (0.191) found for Coleoptera by Graur (1985), which confirmed the high genetic variability of these insects.

The amount of differentiation among the three populations of *G. nymphaeae* was measured with *F* statistics (Nei 1977, Wright 1978). Table 3 shows F_{st} analyses of polymorphic loci for the three populations of *G. nymphaeae*. Mean F_{st} among the three populations of *G. nymphaeae* was 0.073, which represents a negligible differentiation. However, some alleles, such as AK-1, EST-3, GOT-1, and PGM, showed higher levels of differentiation, with values above 0.1.

Allele frequency data from all six populations were used in calculating Nei's (1972) and modified Rogers's (Wright 1978) genetic distance matrices (Table 4). Nei's (1978) coefficients of genetic identity between any two populations were ≥0.95 among the three populations of *G. nymphaeae* and were <0.24 among the three populations of *G. nymphaeae* and either *G. calmariensis* or *G. pusilla*, and also among the latter two populations compared with *G. grisescens*. The values of the coefficient were intermediate for the other populations: 0.621 between *G. calmariensis* and *G. pusilla*, and from 0.507 to 0.519 between *G. nymphaeae* populations and *G. grisescens*.

Phenograms were produced by using different distance measures such as Nei's unbiased (1978), Rogers (1972), Modified Rogers (Wright 1978), Prevosti (Wright 1978), and Cavalli-Sforza & Edwards arc and chord (1967). All phenograms obtained showed identical grouping patterns. Some small variations among the methods occurred in the distances and the cophenetic correlation indices, which ranged from 0.981 to 0.998. Nei's unbiased (1978) genetic identities were clustered by UPGMA to produce the phenogram with a cophenetic correlation of 0.996 (Fig. 1). The clustering analysis suggests that *G. grisescens* is more closely related to *G. nymphaeae* from the United States than to the two species from Germany.

Taxonomic Study. If *G. calmariensis* and *G. pusilla* were to become established in North America after their introduction, there would be

Table 3. *F* statistics analysis of polymorphic loci in the three *G. nymphaeae* populations

Locus	F_{is}	F_{it}	F_{st}
ACON-1	−0.061	−0.043	0.016
ACON-2	0.172	0.224	0.063
ACPH	−0.052	−0.038	0.014
AK-1	−0.009	0.239	0.246
AK-2	0.216	0.259	0.054
DIA	−0.067	−0.021	0.043
EST-2	0.168	0.171	0.003
EST-3	0.225	0.362	0.178
EST-5	0.032	0.093	0.063
GOT-1	0.600	0.647	0.118
GOT-2	−0.011	−0.010	0.001
GPDH	0.371	0.376	0.008
GR	−0.011	−0.008	0.004
HAD	0.175	0.222	0.057
IDH-1	0.112	0.139	0.030
IDH-2	0.151	0.169	0.021
LAP	−0.032	−0.031	0.001
MDH-1	−0.058	−0.054	0.003
MDH-2	−0.065	−0.061	0.004
ME	0.053	0.056	0.003
PGD-2	0.116	0.199	0.094
PGD-3	−0.085	−0.055	0.028
PGI	0.019	0.047	0.029
PGM	0.037	0.228	0.198
PK	−0.013	−0.004	0.008
Mean	0.073	0.141	0.073

Table 4. Matrix of genetic similarity in the four species of *Galerucella*: above diagonal, Nei's unbiased (1978) genetic identity; below diagonal, Rogers (1972) genetic similarity

Population	1	2	3	4	5	6
1. *G. nymphaeae*, Beltsville	—	0.988	0.950	0.152	0.241	0.507
2. *G. nymphaeae*, Patuxent	0.934	—	0.983	0.148	0.228	0.516
3. *G. nymphaeae*, Ithaca	0.874	0.911	—	0.135	0.179	0.519
4. *G. calmariensis*	0.190	0.183	0.169	—	0.621	0.182
5. *G. pusilla*	0.263	0.248	0.214	0.587	—	0.189
6. *G. grisescens*	0.475	0.478	0.489	0.210	0.227	—

a total of five *Galerucella* species in North America. The European species would join the following three North American species (see Figs. 2–6): *G. nymphaeae* (occurs throughout the United States and Canada and feeds on *Nuphar, Polygonum, Myrica,* and occasionally on *L. salicaria*), *G. stefanssoni* Brown (found in the Northwest Territories of Canada and feeds on *Rubus chamaemorus* L. [cloudberry]); and *G. quebecensis* Brown (known from Nova Scotia and Québec to Michigan and feeds on *Potentilla palustris* (L.) Scop. [marsh-flower]).

The likelihood that two species will be added to the North American fauna of *Galerucella* necessitates a means of identification.

Key to North American Species of *Galerucella*

1. Front coxae narrowly separated by a prosternal process that nearly attains hind margin of coxae; middle coxae separated by about half coxal width; length 4.1–6.0 mm; markings as in Fig. 5 . . . . . *nymphaeae* (L.)
   Front coxae not separated by prosternum as above; middle coxae close, rarely touching; length 3.0–5.6 mm; see Figs. 2, 3, 4, 6 2
2 (1). Fourth abdominal segment pale 3
   Fourth abdominal segment partly to completely dark 4
3 (2). Antennae dark at apical ½ to ⅔; humeri often dark; aedeagus symmetrical (Fig. 4b) . . . . . *pusilla* (Duftschmidt)
   Antennae and humeri not dark; aedeagus asymmetrical (Fig. 3a) . . . . . *quebecensis* Brown
4 (2). Pronotum and elytra usually with dark markings (Fig. 2); length 3.6–5.6 mm; aedeagus slender (Fig. 2b) . . . . . *calmariensis* (L.)
   Pronotum and elytra lacking dark markings (Fig. 6); length 3.0–3.8 mm; aedeagus not slender (Fig. 6a) . . . . . *stefanssoni* Brown

Diagnostic Descriptions. The key to species plus the illustrations should facilitate identification of most specimens. In doubtful cases, the

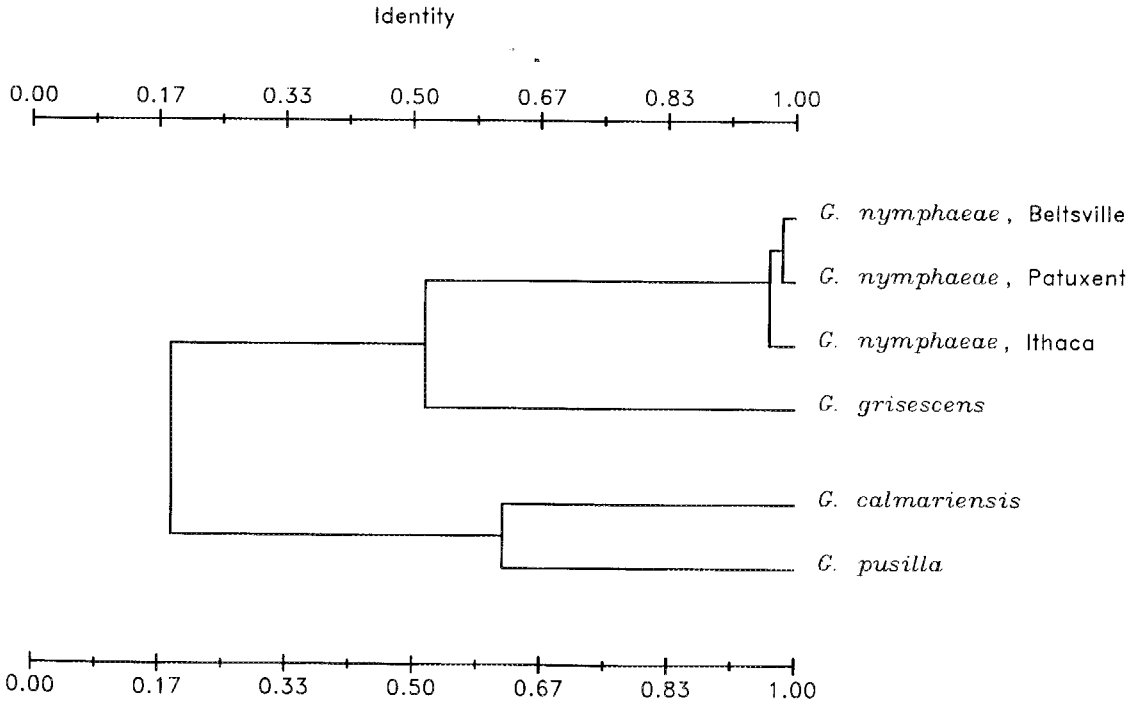
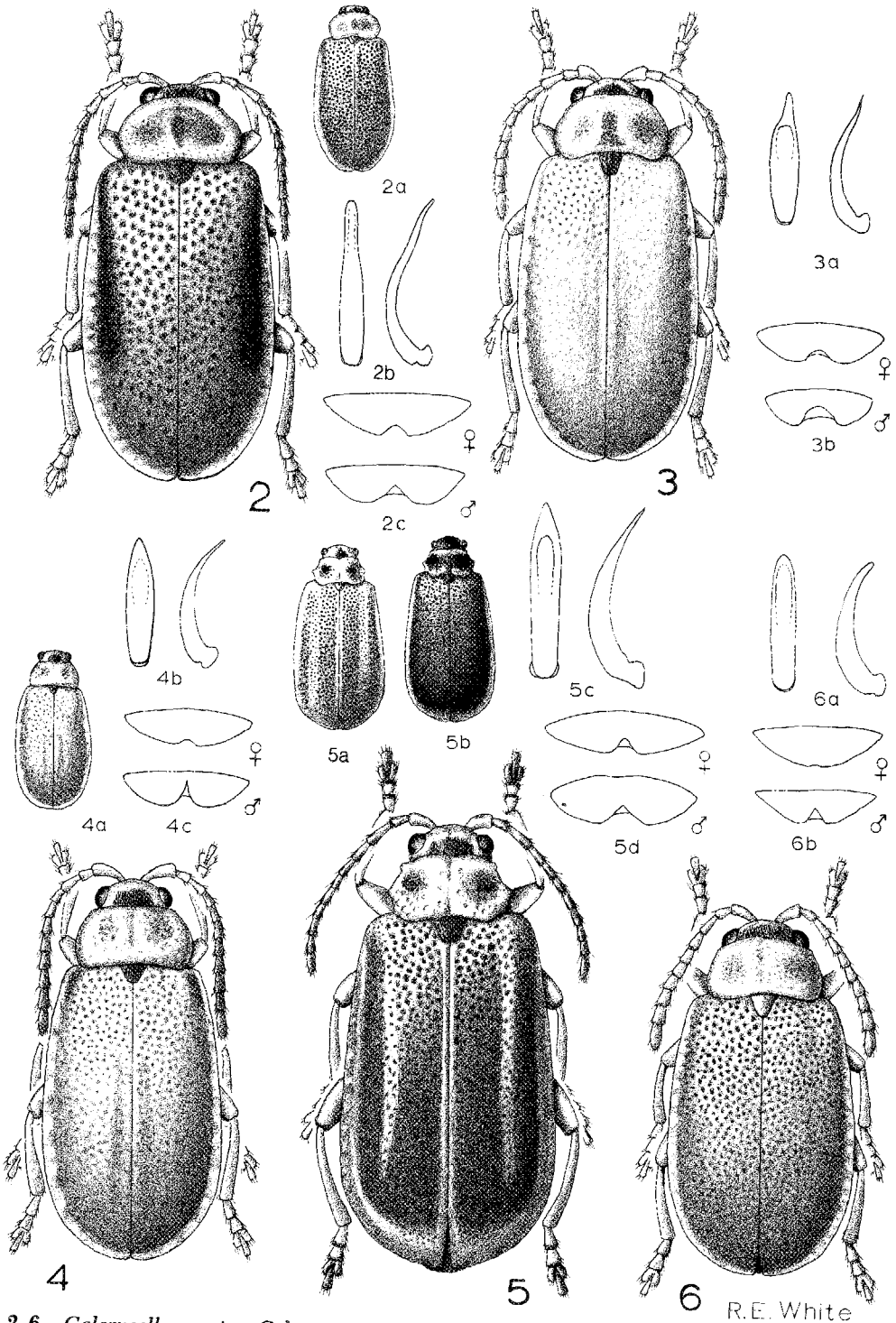


Fig. 1. UPGMA phenogram generated from Nei's unbiased (1978) genetic identity matrix for all populations and species (cophenetic correlation = 0.996).



Figs. 2–6. *Galerucella* species. Color variation (if any) shown in small figures. Aedeagus of each species shown in dorsal and lateral view. Last abdominal segment of both sexes shown for each species. (2, 2a–c) *G. calmariensis*. (3, 3a–b) *G. quebecensis*. (4, 4a–c) *G. pusilla*. (5, 5a–d) *G. nymphaeae*. (6, 6a–b) *G. stefanssoni*.

diagnostic descriptions below will provide further assistance. In the diagnostic descriptions, only those characters are presented that separate the species. Examination of the male aedeagi (Figs. 2–6) will permit definite identification to species. The sex of most specimens can be distinguished by the form of the last abdominal segment (Figs. 2–6).

G. calmariensis (L.)
(Figs. 2, 2a–c)

General. Pronotum brown, usually with black spot in middle of disk. Scutellum black. Elytra brown, humeri black and usually with elongated, dark stripe extending apically. Live specimens light brown, pronotum with a black triangle or broad black line, humeri broadly black. Body 1.9–2.0 times as long as wide. Length 3.6–5.6 mm. *Pronotum.* Widest medially, lateral margin nearly evenly arcuate, with coarsely punctate depression laterally. *Ventral Surface.* Front coxae not separated by a prosternal process. Middle coxae narrowly separated. *Aedeagus.* In dorsal view slender, symmetrical, sides subparallel at about basal ⅔, narrowed at about apical ⅓, tip blunt. Lateral view: very slender, sinuate, apex pointed.

G. quebecensis Brown
(Figs. 3, 3a–b)

General. Pronotum primarily pale brown and usually with a dark marking medially. Scutellum black. Elytra pale brown throughout. Body 2.0 times as long as wide. Length 3.5–4.0 mm. *Pronotum.* Widest medially, lateral margin weakly angulate. Surface with a shallow, punctate depression laterally. *Ventral Surface.* Front coxae not separated by a prosternal process as in *G. nymphaeae.* Middle coxae narrowly separated. *Aedeagus.* In dorsal view stout, asymmetrical, clearly narrowed at about apical ⅕, tip pointed, widest behind basal ⅓. Lateral view: slender, sinuate, tip very sharply pointed.

G. pusilla (Duftschmidt)
(Figs. 4, 4a–c)

General. Pronotum brown. Scutellum brown or black. Elytra brown, sometimes humeri black. Live specimens light brown, pronotum usually with narrow dark line, suture and humeri black. Body 2.0 times as long as wide. Length 3.5–4.6 mm. *Pronotum.* Widest medially, margin nearly evenly arcuate, with a coarsely punctate depression laterally. *Ventral Surface.* Front coxae not separated by a prosternal process. Middle coxae very narrowly separated. *Aedeagus.* In dorsal view stout, symmetrical, sides arcuate, widest medially, apex pointed. Lateral view: arcuate, curvature greatest behind apex, distinctly narrowed apically, tip pointed.

G. nymphaeae (L.)
(Figs. 5, 5a–d)

General. Pronotum varying in color from brown with a black depression laterally to nearly entirely black. Scutellum usually black. Elytra predominantly dark brown to black, lateral margin and apex usually broadly brown, suture narrowly brown, brown median stripe and more or less evident, median light stripe from humeral region to declivity; elytra varying from predominantly light to almost entirely black. Body about 2.0–2.1 times as long as wide. Length 4.1–6.0 mm. *Pronotum.* Widest medially, margin angulate from dorsal view, with a coarsely punctate depression laterally. *Ventral Surface.* Front coxae narrowly separated by a prosternal process nearly attaining hind margin of coxae. Middle coxae separated by about half a coxal width. *Aedeagus.* In dorsal view slender, symmetrical, sides nearly straight, subapical portion distinctly wider, tip pointed. Lateral view: nearly evenly arcuate and distinctly narrowing to apex, with tip sharply pointed.

G. stefanssoni Brown
(Figs. 6, 6a–b)

General. Pronotum light brown throughout with a punctate depression laterally. Scutellum brown. Elytra light to medium brown throughout. Body about 1.8 times as long as wide. Length 3.0–3.8 mm. *Pronotum.* Widest medially, margin nearly evenly arcuate. *Ventral Surface.* Front coxae not separated by a prosternal process. Middle coxae very narrowly separated. *Aedeagus.* In dorsal view with sides subparallel, symmetrical, weakly narrowed to apex, tip weakly pointed, weakly widest medially. Lateral view: nearly evently arcuate, tip blunt.

Biological Observations. The adults and larvae of the species considered here feed externally on foliage. The Holarctic *G. nymphaeae* shows the broadest host range, *G. grisescens* seems to be restricted to the genus *Lysimachia* and *Hydrocharis morsus-ranae* L., and *G. calmariensis* and *G. pusilla* feed exclusively on *L. salicaria.*

All four European species studied hibernate as adults and reappear on their host plants in May. Their oviposition periods last from May through July. A shorter oviposition period may occur when the new generation of adults oviposits before hibernating. The eggs are laid in batches of various sizes, and a single layer is attached to stems and leaves of the host plants. *G. calmariensis* and *G. pusilla* cover every egg in a batch with a thin line of frass, whereas *G. grisescens* and *G. nymphaeae* leave their eggs clean. The larvae of *G. calmariensis* and *G. pusilla* are

Table 5. Occurrence of *G. calmariensis* and *G. pusilla* in five different habitats in central Europe

Habitat	Σ Sites examined	*G. calmariensis* present		*G. pusilla* present	
	n[a]	*n*	%	*n*	%
Ditches	22	20	91	21	95
Ponds and lakes	8	6	75	8	100
Wet pastures	17	14	82	16	94
Open marshes and fens	3	2	67	3	100
Wood marsh	1	0	0	1	100
Total	51	42	82	49	96

[a] *n*, number of sites.

yellowish, whereas *G. grisescens* and *G. nymphaeae* larvae are blackish. Larvae of the latter two species pupate on the host attached to plant tissue, whereas *G. calmariensis* and *G. pusilla* pupate in the soil or the litter underneath their host plant. Thus, *G. grisescens* and *G. nymphaeae* share similar biological and morphological features, placing them apart from the two species feeding on *L. salicaria*. Biological observations on the other North American species are lacking.

The four European species co-occur at the same localities. *G. grisescens* is common in central Europe and less abundant in the north and the south (Mohr 1966). During the studies of the insects on *L. salicaria, L. vulgaris*, the host plant of *G. grisescens*, was found to be the most common plant associated with purple loosestrife in the study area (Blossey 1989). Populations of *G. grisescens* were found on plants growing along ditches and in wooded marshes in northern Germany, often alongside populations of *G. calmariensis* and *G. pusilla* feeding on *L. salicaria*. *G. calmariensis* and *G. pusilla* occur throughout Europe (except the very north) into the eastern former Soviet Union and also into Japan, coinciding with the native distribution of their host plant (Silfverberg 1974). *G. calmariensis* is found more frequently than *G. pusilla* in southern Finland and Sweden (Palmén 1945). An earlier survey for phytophagous insects in Europe showed both species to be present in all habitat types and population levels of *L. salicaria* (Blossey & Schroeder 1986). Of the 51 sites divided into five different habitat types, all except one were colonized by both *Galerucella* species (Table 5). Only *G. pusilla* inhabited the single "wood marsh" habitat. Although *G. pusilla* was more abundant at most sites than *G. calmariensis*, both species co-occurred at the majority of the sites studied.

Population fluctuations of *G. calmariensis* and *G. pusilla* occurred independently of each other at any particular site (Blossey 1991). An analysis of the relative frequency of both species in 43 mixed populations in central Europe in June 1990 showed that both species co-occur in all frequency classes (Fig. 7). Different habitat preferences or competitive superiority, or both, would lead to the dominance of either species in a mixed population. A detailed analysis of the use of individual plants on the sites showed that both species are highly aggregated and possibly attracted to each other (Blossey 1991).

Discussion

Of the four species considered for electrophoresis in this study, only the waterlily leaf

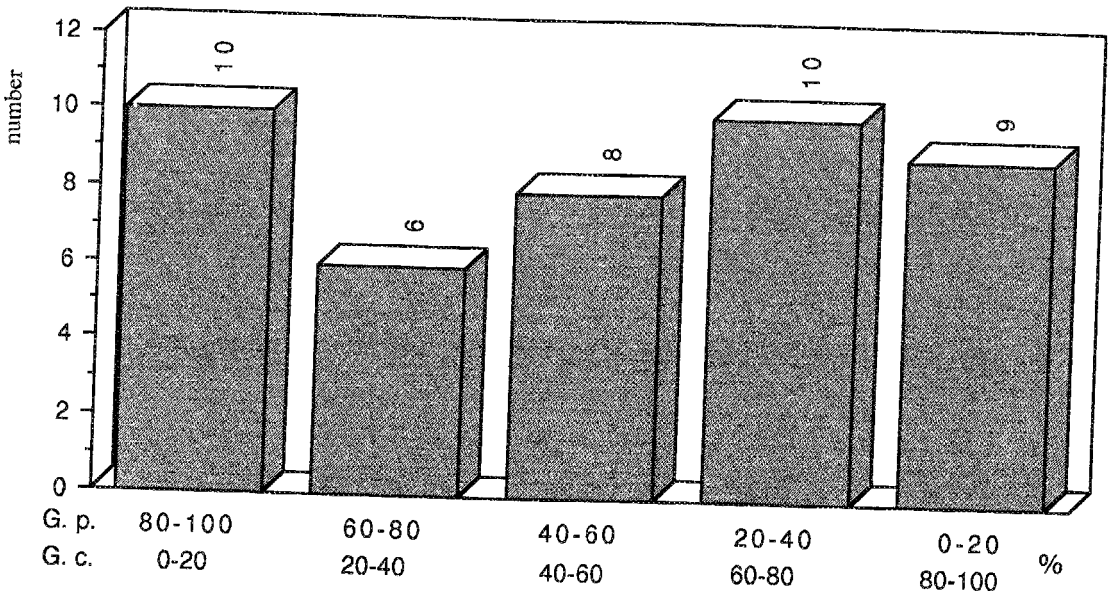


Fig. 7. Number of sites in five abundance classes shown as relative frequency (%) of *G. pusilla* (G. p.) and *G. calmariensis* (G. c.) in 43 mixed populations in Germany.

beetle, *G. nymphaeae*, is Holarctic (Lopatin 1984). There has been considerable confusion and discussion about the taxonomic status of *G. sagittariae* (Gyllenhal), a closely related species, and whether North American and European *G. nymphaeae* belong to the same species (details in Hippa & Koponen [1986], Nokkala & Nokkala [1987]). Hippa & Koponen (1986) showed reproductive isolation between beetle populations feeding on waterlilies and those on other wetland plants. They identified minor morphological differences, but the characters overlapped widely. They identified the beetle populations using *Nuphar* (waterlily) as *G. nymphaeae* and those feeding on other wetland plants as *G. sagittariae*. The host plants of *G. sagittariae* (sensu Hippa & Koponen) in Europe include a variety of wetland plants such as *Rumex* spp., *Polygonum* spp., *Comarum palustre* L., and *L. salicaria* (Hippa & Koponen 1986). Populations of *G. nymphaeae* in North America are known to feed on plants in the Nymphaceae (Wallace & O'Hop 1985).

During North American surveys of phytophagous insects associated with *L. salicaria*, *G. nymphaeae* was identified as an uncommon herbivore on the plant (Hight 1990). Populations of *G. nymphaeae* using different host plants in North America were examined for differences and evaluated for a possible sibling species pair, such as for *G. nymphaeae*/*G. sagittariae* in Europe. In addition to the Ithaca population of *G. nymphaeae* feeding on *L. salicaria*, two populations collected from *Nuphar advena* (Aiten) Ait. f., the yellow waterlily, were studied. All plants were growing in standing water. Even the *L. salicaria* plants in New York were flooded during most of the *G. nymphaeae* activity. The examination of morphological characters showed virtually no differences. Only a slightly smaller size of the specimens from Ithaca, NY, was noted. The 26 specimens from Beltsville, MD, ranged from 4.4 to 5.7 mm; the 36 specimens from Patuxent, MD, ranged from 4.4 to 5.5 mm; and the 29 specimens from Ithaca, NY, ranged from 4.1 to 5.2 mm.

When subjected to an isozyme analysis, the Maryland populations feeding on aquatic *N. advena* showed small differences from the New York population feeding on terrestrial *L. salicaria*. Four alleles, AK-1, EST-3, GOT-1, and PGM, were most variable, showing a high level of differentiation with F_{st} values >0.1. However, when all alleles are considered together, the three United States samples of *G. nymphaeae* had moderate differentiation, with F_{st} of 0.073, and a high genetic identity, with a Nei's index exceeding 0.94. One locus, 6PGD-1, showed activity only in *G. nymphaeae* from Ithaca. Perhaps "silent" alleles, which have frequently been reported for 6PGD in the literature (Parr & Fitch 1967, Blake et al. 1974), account for the inactivity observed in the other populations.

The electrophoretic analysis found minor differences between *G. nymphaeae* populations using different host plants in North America. Additional studies are required to determine whether the Ithaca population is reproductively isolated from other populations of *G. nymphaeae*, or if the differences simply reflect the three different eastern United States locations. Like the biological observations, the electrophoretic analysis placed the European *G. grisescens* closer to the North American *G. nymphaeae* than to the two European species feeding on *L. salicaria* (Fig. 1). The Holarctic distribution, the host ranges, and the similarities in their biology, as well as the electrophoretic data presented in this article, indicate that *G. nymphaeae*, *G. grisescens*, and *G. sagittariae* (sensu Hippa & Koponen [1986]) diverged from a common ancestor more recently than the separation of *G. calmariensis* and *G. pusilla* feeding on *L. salicaria*.

A further study should include more species within *Galerucella*. Of special importance would be an analysis of populations feeding on a variety of host plants in Europe and in North America, to investigate the relationship between species on the two continents. Hultén (1971) suggested that *L. salicaria* and other wetland plants were once circumpolar. As the vegetation changed and host plants became limited in their distribution, *Galerucella* populations may have become isolated. A more detailed biological and electrophoretic investigation of the other two North American species of *Galerucella* would further help to understand the evolution of this genus. Our data indicate that the separation of *G. nymphaeae*/*G. sagittariae* and *G. grisescens* from the two species feeding on *L. salicaria* occurred in a common distribution area via specialization on particular food plants. The same separation may have occurred for the two somewhat isolated North American species, *G. quebecensis* and *G. stefanssoni*.

G. calmariensis and *G. pusilla* share the same host plant, distribution, and biology, but show strong reproductive isolation. Cross-breeding experiments (Blossey 1991) and studies of chromosome numbers of different *Galerucella* species (Nokkala & Nokkala 1987) confirmed these findings. The species' use of the same ecological niche and the absence of competitive exclusion is difficult to explain with the existent theories on evolution and coexistence through niche separation. Current studies on the behavior of some European *Galerucella* species showed that the reproductive isolation is partly maintained through different courtship behavior of the males (Petersen, personal communication). However, individuals of *G. calmariensis* and *G. pusilla* can identify their mates with precision before any courtship. Males of *G. sagittariae* (sensu Hippa & Koponen [1986]), however, cannot identify females of *G. calmariensis* and *G. pusilla* and con-

tiniously try to copulate (Petersen, personal communication). This suggests that the identification of the species involves pheromones and that *G. calmariensis* and *G. pusilla* are highly specialized, not only in their host plant use, but also in their ability to identify an appropriate mate. Prerequisites for these two species' coexistence on the same host plant have yet to be determined.

In conclusion, the three populations of *G. nymphaeae* sampled in the United States show moderate differentiation, but further studies are needed to investigate their relationship with populations feeding on other wetland vegetation in North America. The electrophoretic data for *G. grisescens* show this species to be more closely related to *G. nymphaeae* than to the two species feeding on *L. salicaria*. This agrees with the observations on their biology and morphology. *G. calmariensis* and *G. pusilla* are genetically distinct species with a genetic marker represented by LDH-1 that occurs only in *G. calmariensis*. Evolution within the genus *Galerucella* has occurred mainly through specialization on various wetland plants.

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Appendix

Allele frequencies for 33 loci in four species of *Galerucella*

Locus	Allele[a]	Population					
		G. nymphaea Beltsville	*G. nymphaea* Patuxent	*G. nymphaea* Ithaca	*G. calmariensis*	*G. pusilla*	*G. grisescens*
ACON-1	*n*[b]	103	40	36	50	50	21
	186	—	—	—	0.020	—	0.976
	150	—	—	—	0.860	—	—
	123	0.005	—	0.056	0.050	—	0.024
	100	0.752	0.700	0.792	0.060	—	—
	80	0.233	0.262	0.111	0.010	0.590	—
	71	0.010	0.038	0.042	—	0.320	—
	44	—	—	—	—	0.090	—
	(H)[c]	0.330	0.500	0.417	0.260	0.480	0.048
ACON-2	*n*	93[d]	32	34	43	24	14
	−90	—	—	0.074	—	—	—
	−100	0.301	0.375	0.574	0.105	—	—
	−110	0.667	0.609	0.353	0.884	1.000	1.000
	−118	0.320	0.016	—	0.012	—	—
	(H)	0.387	0.438	0.412	0.140	0.000	0.000
ACPH	*n*	59	30	30	46	48	11
	106	—	—	—	0.065	0.188	—
	103	0.025	0.067	0.017	0.935	0.813	—
	100	0.975	0.933	0.983	—	—	1.000
	(H)	0.051	0.133	0.033	0.087	0.250	0.000
AK-1	*n*	80	40	40	39	39	21
	165	—	—	—	0.987	—	—
	117	—	—	—	0.013	1.000	—
	100	0.769	0.488	0.162	—	—	—
	88	0.231	0.512	0.837	—	—	1.000
	(H)	0.338	0.500	0.225	0.026	0.000	0.000
AK-2	*n*	60	23	20	27	38	13
	144	—	—	—	0.056	—	—
	130	—	—	—	0.796	0.066	—
	100	0.842	1.000	0.900	0.148	0.934	—
	68	0.158	—	0.100	—	—	0.500
	50	—	—	—	—	—	0.500
	(H)	0.250	0.000	0.100	0.259	0.132	0.231
ARGK	*n*	89	40	38	40	41	18
	100	1.000	1.000	1.000	—	—	1.000
	93	—	—	—	1.000	0.988	—
	84	—	—	—	—	0.012	—
	(H)	0.000	0.000	0.000	0.000	0.024	0.000
DIA	*n*	60	16	16	41	42	19

Appendix. (Continued)

Locus	Allele[a]	Population					
		G. nymphaea Beltsville	*G. nymphaea* Patuxent	*G. nymphaea* Ithaca	*G. calmariensis*	*G. pusilla*	*G. grisescens*
	240	—	—	—	0.024	0.036	—
	206	—	—	—	0.963	0.643	—
	164	—	—	—	0.012	0.321	—
	121	—	—	0.063	—	—	—
	100	1.000	1.000	0.938	—	—	0.105
	57	—	—	—	—	—	0.763
	25	—	—	—	—	—	0.132
	(H)	0.000	0.000	0.125	0.073	0.667	0.474
EST-1	*n*	110	40	40	48	46	11
	111	—	—	—	0.021	0.261	—
	105	—	—	—	0.583	0.728	—
	100	—	—	—	0.208	0.011	0.909
	96	—	—	—	0.188	—	0.091
	(H)	—	—	—	0.542	0.261	0.182
EST-2	*n*	97	37[d]	40	48	49	21
	105	0.093	0.108	0.138	—	—	—
	100	0.907	0.892	0.863	—	—	—
	(H)	0.165	0.193	0.225	—	—	—
EST-3	*n*	95	35	40	48	49	21
	123	0.384	0.600	0.883	—	—	—
	100	0.616	0.400	0.117	—	—	—
	(H)	0.389	0.480	0.167	—	—	—
EST-5	*n*	110	40	40	48	49	21
	158	0.777	0.650	0.438	—	—	—
	144	—	—	0.162	—	—	—
	100	0.223	0.350	0.250	—	—	—
	56	—	—	0.150	—	—	—
	(H)	0.300	0.500	0.650	—	—	—
FBP	*n*	44	24	24	47	49[d]	16
	171	—	—	—	1.000	—	—
	114	—	—	—	—	0.031	1.000
	100	0.727	—	—	—	0.510	—
	81	0.273	—	—	—	0.439	—
	78	—	—	—	0.020	—	
	(H)	0.322	—	—	0.000	0.367	0.000
FUM	*n*	98	40	40	48	49	18
	−81	—	—	—	0.771	1.000	0.000
	−100	1.000	1.000	1.000	0.229	—	1.000
	(H)	0.000	0.000	0.000	0.250	0.000	0.000
GOT-1	*n*	66	8	9	48	49	18
	186	—	—	—	0.990	0.990	—
	178	—	—	—	0.010	0.010	—
	115	—	—	—	—	—	0.972
	100	1.000	1.000	0.833	—	—	0.028
	73	—	—	0.167	—	—	—
	(H)	0.000	0.000	0.111	0.021	0.020	0.056
GOT-2	*n*	98	40	40	48	48	18
	−100	1.000	1.000	1.000	0.052	0.073	1.000
	−125	—	—	—	0.938	0.927	—
	−143	—	—	—	0.010	—	—
	(H)	0.000	0.000	0.000	0.125	0.146	0.000
GPDH	*n*	86	39	38	47	49	15
	114	0.058	0.077	0.066	0.043	—	0.633
	100	0.942	0.923	0.934	0.957	0.990	—
	88	—	—	—	—	0.010	0.367
	(H)	0.093	0.154	0.132	0.085	0.020	0.733
GR	*n*	75[d]	38	30	47	49	21
	100	0.153	0.105	0.083	1.000	1.000	1.000
	70	0.847	0.895	0.917	—	—	—
	(H)	0.120	0.158	0.100	0.000	0.000	0.000
HAD	*n*	101	40	40	50	51	16
	100	0.990	1.000	0.988	—	—	—
	87	—	—	—	—	—	0.406
	72	—	—	—	—	—	0.250
	55	0.010	—	0.012	—	—	—
	30	—	—	—	—	—	0.313
	10	—	—	—	—	—	0.031
	(H)	0.020	0.000	0.025	—	—	0.628
IDH-1	*n*	88	31	26	50	51	21
	120	0.091	0.145	0.308	—	—	—

Appendix. (Continued)

Locus	Allele[a]	Population					
		G. nymphaea Beltsville	*G. nymphaea* Patuxent	*G. nymphaea* Ithaca	*G. calmariensis*	*G. pusilla*	*G. grisescens*
	100	0.909	0.855	0.692	—	—	—
	(H)	0.159	0.226	0.308	—	—	—
IDH-2	*n*	95[d]	38	33	49	46	21
	240	—	—	—	0.980	0.098	—
	172	0.089	0.790	—	0.020	0.891	—
	124	—	—	—	—	0.011	—
	100	0.911	0.921	1.000	—	—	1.000
	(H)	0.116	0.158	0.000	0.041	0.174	0.000
LAP	*n*	69	37	40[d]	43	44	20
	117	—	—	0.038	—	—	0.300
	100	0.855	0.946	0.913	—	—	0.675
	83	0.145	0.054	0.050	—	—	0.025
	66	—	—	—	1.000	1.000	—
	(H)	0.203	0.108	0.125	0.000	0.000	0.450
LDH-1	*n*	49	9	9	40	32	16
	100	—	—	—	1.000	—	—
	(H)	—	—	—	0.000	—	—
LDH-2	*n*	49	9	9	45	48[d]	16
	130	—	—	—	0.011	0.052	—
	100	—	—	—	0.989	0.948	—
	(H)	—	—	—	0.022	0.063	—
MDH-1	*n*	104	40	40	50	51	21
	100	0.971	0.975	0.962	0.010	—	1.000
	77	0.029	0.025	0.038	0.990	—	—
	40	—	—	—	—	1.000	—
	(H)	0.058	0.050	0.075	0.020	0.000	0.000
MDH-2	*n*	104	40	40	48	51	21
	−100	0.644	0.650	0.587	0.021	0.020	—
	−67	0.356	0.350	0.412	0.979	0.980	1.000
	(H)	0.404	0.550	0.525	0.042	0.039	0.000
ME	*n*	88	38	40	42	50	21
	100	0.943	0.961	0.925	—	—	—
	85	0.057	0.039	0.075	—	—	0.476
	70	—	—	—	—	—	0.524
	47	—	—	—	0.012	0.870	—
	33	—	—	—	0.988	0.130	—
	(H)	0.114	0.079	0.150	0.024	0.180	0.286
6PGD-1	*n*	103	40	20	48	49	21
	100	—	—	1.000	—	—	—
	(H)	—	—	0.000	—	—	—
6PGD-2	*n*	101	40	40	48	49	21
	130	—	—	0.013	—	—	—
	100	0.743	0.750	0.700	—	—	—
	77	0.252	0.213	0.250	—	0.041	—
	58	0.005	0.038	0.038	0.021	0.949	—
	50	—	—	—	0.979	0.010	—
	(H)	0.307	0.450	0.400	0.042	0.102	—
6PGD-3	*n*	87	40	40	48	49	19
	−100	0.983	1.000	0.813	—	—	1.000
	−200	0.011	0.000	0.150	—	—	—
	−500	0.006	0.000	0.038	—	—	—
	(H)	0.034	0.000	0.275	—	—	0.000
PGI	*n*	89	28	30	48	49	21
	345	—	—	—	0.031	—	—
	318	—	—	—	0.927	—	—
	279	—	—	—	0.042	0.010	—
	250	—	—	—	—	0.939	—
	205	—	—	—	—	0.051	—
	148	—	—	—	—	—	1.000
	100	0.736	0.875	0.867	—	—	—
	67	0.264	0.125	0.133	—	—	—
	(H)	0.393	0.250	0.267	0.146	0.122	—
PGM	*n*	84	39	40	22	31[d]	18
	107	0.018	0.026	0.025	—	—	—
	100	0.673	0.551	0.762	—	—	—
	92	0.036	0.103	0.075	—	0.048	—
	80	0.274	0.321	0.014	0.023	0.226	1.000
	74	—	—	—	0.977	0.726	—
	(H)	0.429	0.615	0.375	0.045	0.161	0.000
PK	*n*	86[d]	40	40	48	49	20

Appendix. (Continued)

Locus	Allele[a]	Population					
		G. nymphaea Beltsville	*G. nymphaea* Patuxent	*G. nymphaea* Ithaca	*G. calmariensis*	*G. pusilla*	*G. grisescens*
	143	—	—	—	0.990	—	—
	134	—	—	—	0.010	—	—
	100	0.715	0.425	0.175	—	1.000	—
	91	0.285	0.575	0.825	—	—	0.050
	82	—	—	—	—	—	0.950
	(H)	0.291	0.600	0.250	0.021	0.000	0.100
SDH	*n*	99	40	40	48	49	21
	180	—	—	0.013	—	—	—
	100	1.000	1.000	0.988	1.000	1.000	—
	(H)	0.000	0.000	0.025	0.000	0.000	—

[a] Negative values indicate cathodally migrating alleles.
[b] *n*, number of specimens.
[c] (H), heterozygosity (direct count) per locus.
[d] Locus deviating from Hardy-Weinberg equilibrium.