Comparison between molecular and morphological-based phylogenies of galerucine/alticine leaf beetles (Coleoptera: Chrysomelidae)

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Insect Syst.Evol.

Kim, S. J., Kjer, K. M. & Duckett, C. N.: Three conflicting hypotheses describing the relationship between the Chrysomelid lineages of the Flea Beetles and the galerucines exist. *Insect Syst. Evol.* 34: 53-64. Copenhagen, March 2003. ISSN 1399-560X.

Phylogenies of representative genera based on 3 molecular markers are presented. These data are combined with previously published morphological data (Lingafelter & Konstantinov 2000). The results of separate 28S-D2 rRNA, combined analysis of molecular data and molecular data combined with morphological characters for 27 genera indicate monophyly of Galerucinae *s.s.* and paraphyly of Flea Beetles. These results also mean that our molecular data do not support the Flea Beetles as a tribal ranking (Alticini) within the Galerucinae, nor is it's status as a separate subfamily (Alticinae) as has been proposed by other researchers. We suggest that addition of more independent characters is still needed to resolve the question of the relationships between the Flea Beetles and the galerucines.

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Introduction

The Flea Beetles and the galerucine chrysomelids constitute a highly diverse monophyletic group (Jacoby 1908, Farrell 1998) and are arguably the most economically important group of insects for which no basic phylogenetic consensus exists (see Konstantinov 1998a for review). The Flea Beetles, most of which are tiny and jump like fleas, are composed of over 560 genera and 8000 described species (Seeno & Wilcox 1982). The galerucine leaf beetles (Galerucinae) are similarly diverse, with over 480 genera and 5,800 species (Wilcox 1975), including the rootworms (Luperini), with over 3500 species world wide (Wilcox 1972a, b). The Flea Beetles and the galerucines have been classified as separate subfamilies, Galerucinae and Alticinae, by some scientists (e.g. Seeno & Wilcox 1982), however, the Flea Beetles have often been subordinated as a tribe, Alticini within the Galerucinae (see Lingafelter & Konstantinov 1999 for review). Both groups contain species being used for biological control of weeds (White 1996), as well as important pest species. The luperine rootworms (Galerucinae) are especially notable for their multibillion-dollar damages to corn and bean crops (Spike & Tollefson 1991). Both Flea Beetles and galerucines are used in basic scientific studies, for example, the evolution of host choice (Futuyma & McCafferty 1990, Farrell & Mitter 1990), chemical ecology (Metcalf 1994, Pasteels & Rowell-Rahier 1991), evolution of pharmacophagy (Tallamy et al. 1999, 2000), and diversity generating evolutionary mechanisms (Farrell et al. 1992, Mitter & Farrell 1991). The lack of a reliable phylogenetic framework for these beetles is a significant barrier to research on basic evolutionary processes as well as to pest management studies.

Taxonomic History. – The taxonomy and systematic position of the Flea Beetles has been greatly rearranged since Latreille (1802) first proposed Galerucinae *sensu lato* (*s.l.*) as the tribe 'Galerucites', including Flea Beetles and galerucine genera (reviewed in Lingafelter & Konstan-

tinov, 2000). Since the Flea Beetles were first generally recognized as a subfamily by Jacoby (1908), many researchers have suggested that Galerucinae plus the Flea Beetles constitute a clade, Galerucinae *s.l.* (Latreille 1802, Chapuis 1875, Jacoby 1908, Böving & Craighead 1931, Chen 1940, Gressitt 1942, Jolivet 1959, Furth & Suzuki 1994, Reid 1995, Crowson & Crowson 1996, Schmitt 1996, Lingafelter & Konstantinov 1999). Thirty-eight supra-generic groupings of Flea Beetles are currently recognized, only 5 of which are widely accepted as monophyletic (Scherer 1983, Virkki 1989, Virkki & Santiago-Blay 1996, Konstantinov 1998b, Duckett 1999, Lingafelter & Konstantinov 1999). Galerucine tribal names were stabilized early with Weise's (1924) revision, and remained unchanged until Seeno and Wilcox (1982) restricted the Galerucinae to the following 5 tribes: the Oidini, the Galerucini, the Metacyclini, the Sermylini, (correctly Hylaspini Chapuis 1875 [Silferberg 1990]) and the Luperini.

However, there is no agreement about the taxonomic rank or monophyly of the Flea Beetles, nor about the status of named sections within them, nor about the relationships among the different named tribes and sections of the Galerucinae *sensu stricto* (*s.s.*) to each other or to the Flea Beetles (Seeno & Wilcox 1982, Reid 1995, Lingafelter & Konstantinov 1999). Recent cladistic studies with regard to the Flea Beetle's relationship to galerucines *s.s.* using either molecular or morphological data or both have resulted in the three conflicting hypotheses (Figs 1a-c and Figs 2 a, b).

Farrell (1998) combined molecular data from 18S rRNA with morphological data from Reid (1995), including 6 Galerucinae *s.s.* genera and 11 Flea Beetle genera, and obtained a phylogeny in which Flea Beetles and galerucines are monophyletic sister taxa (MGA = monophyletic Galerucinae & Alticinae Hypothesis; see Fig. 1c and Fig. 2a for reanalysis of his molecular data). Although Farrell's (1998) primary objective was to document larger patterns of generation of biological diversity, and not to hypothesize the relationships between the subfamilies of the Chrysomelidae, the tree presented weakly supported the monophyly of both Alticinae and Gallerucinae. Suzuki & Furth (1992) and Furth & Suzuki (1994) examined only the 'problematic' or 'transitional' genera (genera without characters which definitively place them in the alticines or the galerucines); and concluded that no taxonomic decisions should favor the subordination of the Flea Beetles to tribal status without a subfamily wide revision.

A paraphyletic Flea Beetles hypothesis with a monophyletic Galerucinae (= MG) (see Fig. 1a) has been proposed by Reid (1995) and Crowson & Crowson (1996) using adult and some larval characters. Reid (1995) explicitly stated that the Flea Beetles are poly- or paraphyletic, basing his assertion on the ease of loss of the jumping mechanism, and therefore included only one taxon, the Galerucinae *s.l.* as part of his analysis of the relationships between the subfamilies of Chrysomelidae. Crowson & Crowson (1996) examined a comprehensive series of morphological characters and found no larval characters to distinguish Flea Beetles from galerucines. Crowson & Crowson (1996) rejected the monophyly of the Flea Beetles. They strongly agree with Böving and Craighead (1931) that study of the larvae of Flea Beetles and Galerucinae *s.s.* is essential to understanding their phylogenetic relationships.

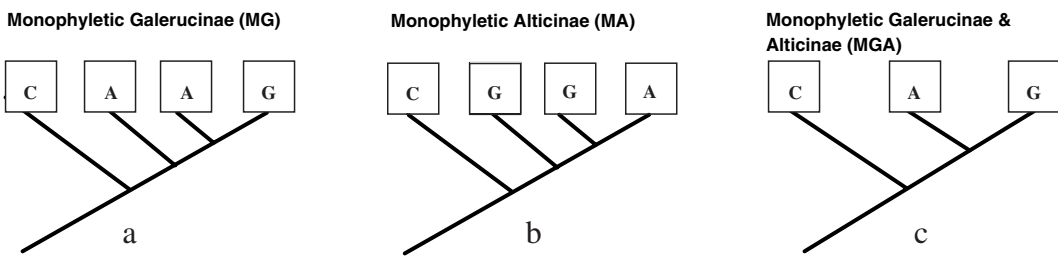

Figure 1. Three conflicting hypotheses of relationships among galerucines and Flea Beetles. C: Chrysomelinae, G: Galerucinae, A: Alticinae. (a) MG = monophyletic Galerucinae Hypothesis, implies paraphyletic alticines; (b) MA = monophyletic Alticinae Hypothesis, implies paraphyletic galerucines; (c) MGA = Monophyletic Galerucinae & Alticinae Hypothesis, implies monophyly of both Galerucinae and Alticinae.

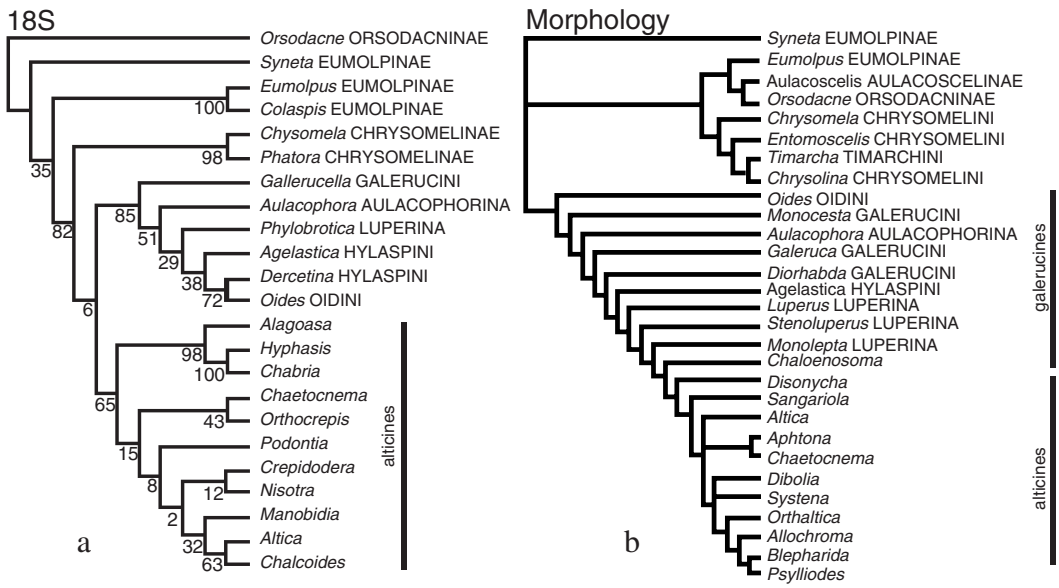

Figure 2. Current hypotheses of relationships among galerucines and Flea Beetles. (a) 18S data from Farrell (1998), realigned according to secondary structure (Kjer 1995), and analyzed with Likelihood under an HKY model with a gamma correction for among site rate variation. Numerals below the internodes are bootstrap values from 100 heuristic likelihood replicates. (b) Morphological proposal of Lingafelter and Konstantinov (1999), redrawn from their analysis.

A paraphyletic Galerucinae giving rise to a monophyletic Alticinae hypothesis (= MA) (see Fig. 1b) was proposed based on analysis of an adult morphological character matrix (Lingafelter & Konstantinov 2000). Lingafelter & Konstantinov (1999) explicitly sought to test the monophyly of the Flea Beetles and their relationship to the Galerucinae *s.s.* In their study, 50 morphological characters of 12 major Flea Beetle tribes and lineages, as well as four of the five galerucine tribes and a comprehensive set of outgroups, were subjected to cladistic analysis. Although monophyly of the alticines was supported, this taxon was nested within the galerucine subtribe Luperina. However, it should be noted that this data set required four iterations of successive weighting to support this hypothesis (Lingafelter & Konstantinov 1999). Their placement of the Flea Beetles within the Luperini is intriguing because the Luperini are morphologically specialized and have root-feeding larvae; however, the Luperini and Metacyclini were not extensively sampled (see Fig. 2b, diabroticine luperines & Metacyclini are not represented). The tribe Galerucini *s.s.* were also found to be polyphyletic (Lingafelter & Konstantinov 1999). The authors, however, acknowledged that the taxon sampling was too poor to address taxonomic or phylogenetic questions in the Galerucinae, but proposed that the tribal level taxon Alticini be accepted for the Flea Beetles.

Our understanding of evolutionary trends in these beetles is dependant on their phylogeny; if either one of the paraphyletic hypotheses is true, this would have profound implications for this economically and biologically important group of herbivores. Both of these paraphyletic hypotheses imply significant taxonomic changes to the presently accepted system.

In summary, recent cladistic analysis of the Flea Beetle/galerucine relationships using both molecular and morphological data have resulted in 3 different hypotheses for the relationship between the two "taxa". The fact that 3 respected groups of systematists, although using different sets of taxa and characters, get 3 different hypotheses indicates that the relationships between these taxa will not be trivial to uncover. These differing results indicate that additional independent characters may help in resolving this difficult question. Here, we test the hypotheses of relationships between

Table 1. Taxon Sampling.

Subfamily	Tribe	Species	Locality	28S-D2	COI	EF-1α
Orsodacnae	Orsodacni	*Orsodacne atra* Ahrens	Tony Grove. UT, USA	*	*	-
Eumolpinae	Synetini	*Syneta adamsi* Baly	Kangwon, Korea	*	*	*
Chrysomelinae	Timarchini	*Timarcha tenebricosa* Fabricius	Vosges, France	*	*	*
	Chrysomelini	Chrysomela tremulae Fabricius	Orleans, France	*	*	*
		Chrysolina coerulans (Scriba)	L.Biabout, Belgium	*	*	*
		Zygogramma piceicollis Stål	AZ, USA	*	*	*
		Paropsis porosa Erichson	Creekotow, Tasmania	*	*	*
Galerucinae	Oidini	*Oides decempunctata* (Billberg)	Chonbuk, Korea	*	*	*
	Hylaspini	*Agelastica caerulae* Baly	Seoul, Korea	*	*	*
	Galerucini	*Monocesta depressa* Clark	USA	*	*	*
		Galeruca rudis Leconte	UT, USA	*	*	*
		Diorhabda elongata (Brulle)	USA	*	*	*
		Schematiza flavofasciata (Klug)	Coxilhia do Fogo, Canguçu, RS Brazil	*	*	*
	Luperini	*Aulacophora indica* (Gmelin)	Taipei, Taiwan	*	*	*
		Diabrotica undecimpunctata howardi Mannerheim	Newark, DE, USA	*	*	–
		Phyllobrotica sp.	Newcastle Co, Delaware,USA	*	*	–
		Monolepta nigrotibialis Jacoby	Umtumvuna, South Africa	*	*	*
Alticinae: Flea Beetles		*Allochroma sp.*	Areia Branca PA, Brazil	*	*	*
		Systena bifasciata Jacoby	Coxilhia do Fogo, Canguçu, RS Brasil	*	*	*
		Disonycha conjuncta	Rio Grande do Sul, Brazil	*	*	*
		Altica sp.	Montpelier, France	*	*	*
		Blepharida rhois Förester	NJ, USA	*	*	*
		Aphthona strigosa Baly	Hongdo, Korea	–	–	*
		[a]*Aphthona nioniscutis* Foudras	Krasnodar, Russia	*	–	–
		Chaetocnema costulata	Chejudo, Korea (Motschulsky)	*	*	*
		Chaetocnema sp.	Mexico	*	*	*
		Sangariola fortunei (Baly)	Kyongbuk, Korea	*	*	*
		Dibolia borealis Chevrolat	TN,USA	*	*	*
		Orthaltica copalina Fabricius	NC, USA	*	*	*

[a]Note that different species for *Aphthona* were used in 28S-D2, * corresponding data available.

the Flea Beetles and the Galerucinae using 3 disparate gene fragments from the mitochonrial cytochrome oxidase subunit 1 (COI), the nuclear elongation factor 1 alpha (EF1-a), and the nuclear large ribosomal subunit (28S) expansion segment 2 (D2). We chose taxa in order to make our work comparable with the Lingafelter & Konstantinov (1999) study so that molecular and morphological data could be analyzed together. Below, we present the results of our molecular study and its combination with the Lingafelter & Konstantinov (1999) data set.

Materials and methods

Sampling. – The species sampled in this study are listed in Table 1, including genera used by Lingafelter & Konstantinov (1999). All tribes of Chrysomelinae and Galerucinae were sampled except the galerucine tribeMetacyclini. Although classification of the Flea Beetles has not been well established, the 10 genera included in this study represent a broad taxonomic sampling based on current classification.

Gene Choice. – We collected sequence data from three independent gene fragments: the second variable region (D2) of nuclear rRNA large subunit (28S; 450nt), elongation factor-one α (EF-1α; 480nt collected from the middle 1/3rd of the gene), and mitochondrial cytochrome oxidase one (COI; 470nt, collected from the middle 1/3rd of the gene). Sequences have been deposited in Genbank under the following accession numbers- AF479420, AF466311, AY171396-AY171471.

DNA Extraction, PCR, and Sequencing. – DNA extractions and purifications from single individuals were performed from one or two legs or thoracic muscles following standard techniques (Hillis and Davis, 1986). For most samples, DNA was extracted from alcohol-preserved adults, while a few dried specimens were used for some species.

The primer pairs for the PCR amplification of the three gene fragments were; GAA ACC GWT CAG GGG TAA ACC TG paired with CCT TGG TCC GTG TTT CAA GAC for the 28S-D2; TAA TTG GAG GAT TTG GWA AYT G paired with CCY GGT AAA ATT AAA ATA TAA ACT TC for the COI; and GGC CCA TGA AAT GGG NAA RGG YTC paired with AAC ATR TTR TCN CCR TGC CAT CC for the EF-1α. The same primers were used for PCR and cycle sequencing.

PCR conditions were as follows; 4 min at 96°C followed by 40 cycles of 95°C for 30s, 50°C for 45s, and 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR products were purified with a "Gene Clean" kit (Bio101). Cycle sequencing was done using a Perkin Elmer/ABI Dye Terminator Cycle Sequencing Kit and run on a thermocycler using the profiles recommended by the manufacturers, except that we used 1/8 the recommended amount of the enzyme. Cycle sequencing products were purified by ethanol precipitation and sequenced using an Applied Biosystems 377 automated sequencer (Forster City, CA) following manufacturer's instructions.

Alignment. – Sequence data of COI and EF-1α were aligned with the ClustalX and default parameters (Vers. 1.81; Thompson & al., 1999) and Mac Clade (Vers. 4.0; Maddison & Maddison, 2000) was used to check for stop codons and proofread the edited sequences. The 28S-D2 rDNA alignment was constructed manually based on secondary structural information (Kjer 1995) designed from the secondary structure models of Gutell & al. (1994), downloaded from the website http://www.rna.icmb.utexas.edu (Gutell 2000). Regions in which alignment was ambiguous were defined according to Kjer (1997). The nucleotides from these regions were eliminated, but the sequences within them were coded as single multistate characters as described in Lutzoni & al. (2000). Indels of uniform length were coded as in Kjer & al. (2001). A Nexus file with the aligned data is posted on Kjer's website. Parsimony-sequences were analyzed with PAUP*, version 4.0b10 (Swofford 1999). We initially carried out parsimony analyses with characters equally weighted and unordered, using 500 random addition heuristic searches with "TBR" branch swapping. Support of individual nodes was assessed with 500 nonparametric bootstrap replicates (Felsenstein 1985), each comprising 100 heuristic random addition searches. In this study we arbitrarily refer to branches with <50%, between 50% and 90%, and >90% bootstrap support as poorly, moderately, and highly supported. Decay indices (or Bremer support; Bremer 1988) were calculated using AutoDecay (Vers. 4.0.2; Erikson 2000) with 100 random-addition searches evaluating individual nodes. For each data set and the combined data sets, the most-parsimonious trees and the strict/50% majority rule consensus trees were saved for further analyses.

Likelihood. – Maximum likelihood (ML) analyses were also conducted on the molecular data sets. We used the program MODELTEST (Vers. 3.04; Posada 1998) to select an optimal model. To reduce computation time, parameters such as the R-matrix, alpha, and the proportion of invariant sites were estimated from a starting tree, and preset for the analysis. We conducted ML analyses with 100 random addition full heuristic searches. Bootstrap values were calculated with 100 replicates by fast step-wise addition. Alternatively, Bayesian inferences were used to estimate phylogeny and branch support under likelihood with MrBAYES (Vers.2.01; Heulsenbeck 2000). We ran two sets of 480,000 iterations, sampling and saving every 400th tree to a file, with the first 200 trees discarded. A majority-rule consensus of 2000 trees was constructed to examine estimates of posterior probabilities, interpreted as nodal support. For the combined molecular data, we used a site-specific rate model that reweights characters according to their best rescaled consistency index (base of 5) for each character on 1000 trees obtained from a fast heuristic bootstrap analysis (Kjer & al. 2001). To test different tree topologies based on current conflicting hypotheses, we used filter tree options implemented in PAUP* to estimate the Bayesian posterior probabilities.

Data combination. – The majority of genera (Table 1) were considered for combined analyses, although several genera lacking either of the three gene sequences were coded as missing in the

molecular combined data (*Orsodacne* Latreille 1802, *Aphthona* Chevrolat 1837, *Diabrotica* Chevrolat 1837 and *Phyllobrotica* Chevrolat 1837*)*. In the case of *Aphthona*, sequences that we obtained for the EF-1α and 28S-D2 rRNA genes were sampled from different species of the same genus and then combined as a single taxon for genus-level phylogenetic analysis; (given that *Apthona* has recently been revised and found to be monophyletic (Konstantinov 1998a) we felt this approach was justified). When the morphological data from Lingafelter & Konstantinov (1999) were combined to our molecular data sets, several genera were removed because these genera were not represented in our molecular data sets (see Table 1). Therefore, the 23 morphological genera from Lingafelter & Konstantinov (1999) were added to the molecular combined data. In most cases, sequences used in molecular analyses were sampled from different species of the same genus that were sampled by Lingafelter & Konstantinov (1999).

Results and discussion

Analysis of 28S-D2 rRNA. – A χ^2 test for base frequency heterogeneity among taxa showed that proportions of base composition were not significantly different among taxa (χ^2 = 84.35, df = 78, P = 0.30). When *Orsodacne*, the most distant outgroup was deleted from the analysis (which has a base composition of 15%A, 34%C, 34%G, 17%T), the P value rose to 0.94.

Analysis of COI. – Mean base frequencies for COI show the typical AT bias of insect mitochondrial DNA and cytochrome oxidase genes in particular (Simon & al. 1994). A χ^2 test for base composition showed no significant deviation from these proportions among taxa (χ^2 = 31.36, df = 78, P = 0.99). However, some taxa that we expected to be divergent, but were recovered together in the equally weighted parsimony analysis share similar bias in base composition: for example, *Timarcha* Latreille 1829 (Chrysomelinae) and *Chaetocnema* Stephens 1831 (a Flea Beetle) share an AT (70%) bias over the mean values (65%), and *Chrysomela* Motschulsky 1860 (Chrysomelinae) and *Blepharida* Chevrolat 1837 (a Flea Beetle) share a composition of 22% C compared to the mean value of 19%. We note that of all partitions, COI does not show a significant left skew in the tree distributions (Table 2; g1 statistic), and according to Hillis & Heulsenbeck (1992) we should not expect the "true tree" to be among the shortest trees, using equally weighted parsimony with these characteristically randomized data. Lack of significant signal is seldom reported, but in this study, the COI is retained because it is useful in resolving more apical nodes.

Analysis of EF-1α. – Mean base frequencies for EF-1α (Table 2) show relatively unbiased nucleotide composition (χ^2 = 77.24, df = 78, P = 0.50). There was an elevated percentage of C and G observed in *Zygogramma* Chevrolat 1837 (46%, 33% respectively), which was much above the

Table 2. Nucleotide composition, and phylogenetic informative sites. Asterisks in the g_1 column indicate significance at p = 0.01.

Gene	Total	Inform.	A/C/G/T (P value)	g_1	Ti/Tv	alpha	invarian
28S-D2	448	138	19/26/30/25 (0.30)	-0.51*	2.55	0.52	0.22
COI							
all	462	198	29/19/15/37 (0.99)	-0.07	2.22	1.64	0.00
1st	154	46	28/19/25/28 (1.00)	-0.14*	2.50	0.57	0.49
2nd	154	14	15/27/17/41 (1.00)	-0.70*	2.40	0.64	0.68
3rd	154	138	43/10/4/43 (0.09)	-0.05	2.10	0.48	0.00
EF-1α							
all	420	137	30/22/22/26 (0.50)	-0.52*	2.06	0.93	0.59
1st	140	9	31/15/37/17 (1.00)	-0.98*	3.75	1.67	0.70
2nd	140	1	32/26/13/29 (1.00)	-2.27*	0.01	∝	0.00
3rd	140	127	26/27/14/33 (0.01)	-0.47*	2.17	1.43	0.02
Molecular combined							
all	1624	506	26/21/23/30 (0.01)	-0.51*	2.40	0.78	0.47

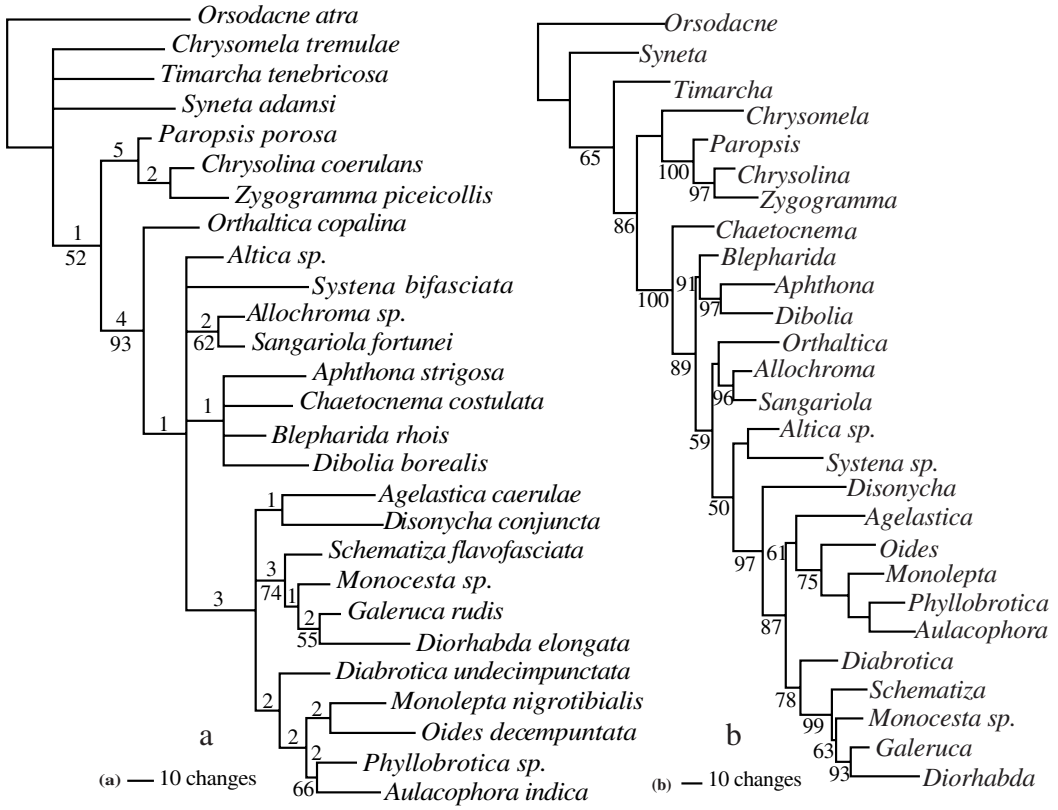

Figure 3. Phylogenetic analysis of 28S-D2 rRNA data. (a) A strict consensus tree of equally weighted parsimony (865 tree steps). Numbers below the internodes represent bootstrap values, above the internodes represent decay indices. (b) A majority-rule consensus tree of Bayesian maximum likelihood. Numbers below the internodes represent posterior probabilities of the final 2000 trees.

means for other taxa. Excluding this genus from the data equalized proportions across taxa (χ^2 = 47.95, df = 75, P = 0.99). A χ^2 test with uninformative sites excluded showed highly heterogeneous base composition among taxa (χ^2 = 240.79, df = 78, P = 0.00). Third-codon position base composition (27%, 26%, 12%, 35%) was significantly heterogeneous among taxa for EF-1α (χ^2 = 83.62, df = 60, P = 0.02), while the first and second codon base compositions were homogenous (both P = 1.00). As expected, the third codon dominates sequence divergence, constituting up to 88% of the variable sites among taxa.

Phylogenetic analysis of molecular data. – The unconstrained, equally weighted parsimony analysis (EP) of the 28S-D2 rRNA data resulted in 11 most-parsimonious trees (MPTs) of a length of 865 steps (Fig. 3a) and the resulting topology is compared with that of the Bayesian likelihood in Fig. 3b. The strict consensus EP tree shows strong bootstrap support for the monophyly of the Galerucinae *s.l.* but failed to support a clade of Galerucinae *s.s*. (note the placement of *Disonycha* Chevrolat 1837 within the Galerucinae *s.s*.). Also one of the outgroups, *Syneta* Dejean 1835, was placed within the Chrysomelinae, while the strict consensus tree did not resolve the relationship among *Chrysomela* Latreille 1858, *Timarcha* Latreille 1829 and *Syneta*. The monophyly of both Galerucinae *s.l*. and Galerucinae *s.s.* were highly supported (MG) under the Bayesian likelihood with different tree topology from the EP tree. The tribe Galerucini (*Monocesta* Clark 1865, *Diorhabda* Weise 1883, *Schematiza* Chevrolat 1837,

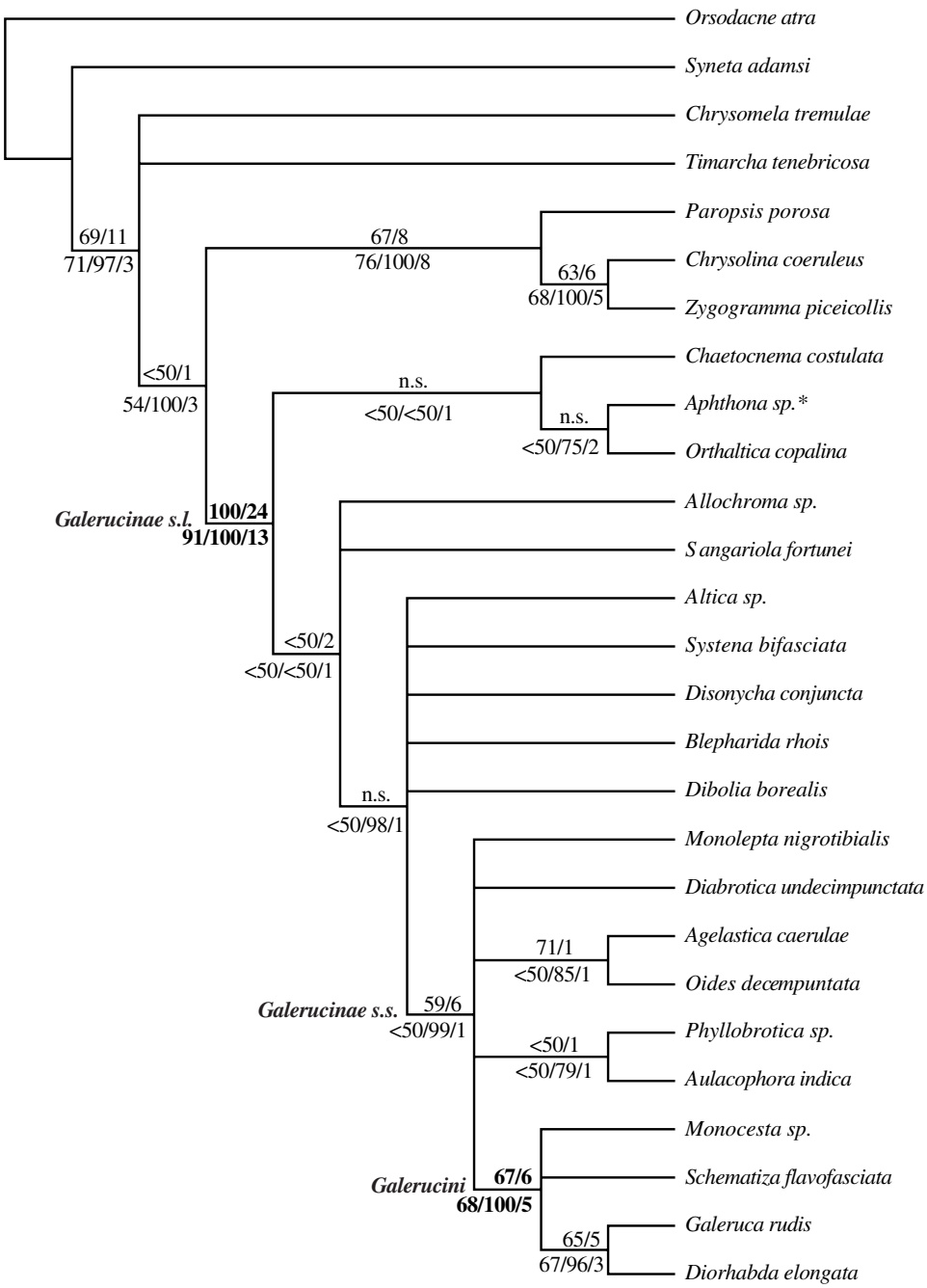

Figure 4. Phylogenetic analysis of combined molecular data. Numerals below the internodes represent bootstrap values/posterior probability estimates/decay indices for the combined molecular data. The consensus of the combined molecular and morphological data can be reconstructed from this tree by collapsiing the nodes marked "n.s." and resolving *Timarcha* as described in the text. Support for the combined morhological and molecular data is indicated above the internodes, with numerals representing bootstrap values/decay indices. *For 28S-D2 rRNA *Aphthona strigosa*, for COI *Aphthona nioniscutis.*

and *Galeruca* Geoffroy 1762) was robustly supported in both MP and Bayesian likelihood analyses, while the "Luperini" appeared paraphyletic in both analyses. Members of the subfamily Chrysomelinae were the sister group to the Galerucinae *s.l.,* and this subfamily appears to be paraphyletic in all analyses. However, MPTs constrained to a monophyletic Chrysomelinae were only two steps longer. ML was conducted under GTR+G+I model. All parameters evaluated with MODELTEST (Table 2) were preset before the analyses. ML also recovered monophyletic Galerucinae *s.l.* and *s.s.* and suggested a paraphyletic Chrysomelinae and Flea Beetles (i.e. MG, see Fig. 1a, trees not shown).

Equally weighted MP analysis (EP) of the molecular combined data sets gave 9 minimum-length trees (tree length = 3208). Parsimony bootstrap and Bayesian posterior probabilities are shown in Fig. 4. Both parsimony and Bayesian likelihood trees resolve the Galeucinae *s.l.* as monophyletic with high bootstrap values (91) and Bayesian scores (100). Bayesian maximum likelihood highly supports the monophyly of the Galerucinae *s.s.*, and also indicates paraphyly of the Flea Beetles. The support for a monophyletic Galerucini *s.s.* appeared low in the parsimony bootstrap analysis because the effect of EF-1α data sets weakened the node to less than 50% support (not shown). Both results are topologically concordant in classifying the Galerucini *s.s.* as monophyletic and the Luperini as a polyphyletic group. ML also resolved a paraphyletic Flea Beetles with a monophyletic Galerucinae *s.s.* (not shown).

Analysis of combined molecular and morphological data. – The combined molecular and morphological data set, analyzed with equally weighted parsimony resulted in 10 trees of 3385 steps, a strict consensus of which can be recovered from Fig. 4. The combined data tree was identical to the combined molecular tree except for the nodes marked "n.s." on Fig. 4, and except that *Timarcha* was resolved as the sister taxon to Galerucinae *s.l.* plus *Paropsis*, *Chrysolina* and *Zygogramma*, supported with a decay index of 1. The similarity of the combined tree to the molecular tree implies a strong influence of molecular data on the analysis (Fig. 1, MG). As in all molecular analyses, the monophyly of the Galerucinae *sensu lato,* and *sensu stricto* and the Galerucini were recovered.

Systematics of Flea Beetle/galerucine Leaf beetles. – The separate 28S-D2 rRNA (Fig. 3), the combined analysis of our molecular data, and molecular data combined with 50 morphological characters from Lingafelter & Konstantinov (2000) (Fig. 4) for these genera indicate monophyly of Galerucinae *s.s.* and paraphyly of the Flea Beetles. This is similar to Reid's (1995) hypothesis (See Fig. 1a). The monophyly of the galerucines in this study is strongly supported (Bayesian posterior probabilities 99%) while the monophyly of the Flea Beetles was recovered in only one of 2000 trees, resulting in a posterior probability estimate of 0.05%. Although our combined-data supports the monophyly of Galerucinae *s.s.,* only two steps are needed to support the monophyly of Flea Beetles as well as the monophyly of Galerucinae *s.s.* (Table 3). The major conclusion of Lingafelter and Konstantinov's study, as discussed above, was the proposal that the Flea Beetles are a monophyletic clade, and the tribal rank, the Alticini, proposed. Our data do not support this hypothesis.

The molecular phylogeny shown in Figs 3 and 4 supports the synonymy of the Flea Beetles within the Galerucinae *s.l.*, but not as one tribe, and the need for further study of the potential formation of new taxonomic sections in the Flea Beetles and possibly the Galerucinae *s.s.*, specifically the

Table 3. Testing different tree topologies based on current conflicting hypotheses.

	28S-D2		Molecular combined		All combined	
Hypotheses	[a]MG	[b]MA	MG	MA	MG	MA
Tree length of [c]EP	865	875	3208	3216	3385	3387
Bayesian posterior probabilities (%)	86%	0.05%	99%	<0.05%	NA	NA

[a]MG: monophyletic Galerucinae hypothesis, [b]MA: monophyletic Alticinae hypothesis, [c]EP: equally weighted parsimony, NA: not available

Luperini. Our DNA data supports the taxonomic hypotheses made by two morphologists (Reid 1992, 1995, Crowson & Crowson 1996). Reid hypothesized the paraphyly of the Flea Beetles (Reid 1992, 1995) and a monophyletic core of Galerucini *s.s.* (Reid 1992) as did Crowson & Crowson (1996). Crowson & Crowson also hypothesized a non-monophyletic Luperini (1996), which we find paraphyletic (Fig. 3).

Intriguingly, although the data presented here represent only 3 gene fragments, the fact that this study shows a cladogram similar to the Lingafelter & Konstantinov hypothesis if it were re-rooted within the Flea Beetles (see Fig. 3), gives us confidence that our molecular data is going to have a significant impact on controversies about character polarization in Chrysomelidae based on morphological data (Reid 1995). In fact, our 28S-D2 rRNA data, (Fig. 3) strongly supports (97% posterior probability), *Disonycha*, as the sister taxon to the Galerucinae *s.s.* Lingafelter & Konstantinov's tree (Fig. 2b) shows *Disonycha* as the 2nd most basal Flea Beetle. Also, if the Lingafelter & Konstantinov hypothesis is re-rooted with *Orsodacne,* the Chrysomelinae become the sister group to the galerucine/Flea Beetle clade as both Reid (1995) and Farrell (1998) show.

It is also interesting and relevant to note the positions of the "problematic" genera in our analysis. "Problematic genera" are defined by Furth & Suzuki (1994) and Lingafelter & Konstantinov (2000) as Flea Beetles without the characteristic large hind femora of other Flea Beetles, and galerucines that appear to have hind femora that are specialized for jumping. *Orthaltica*, which Furth & Suzuki (1994) suggest should be removed from the Flea Beetles because it lacks the jumping apparatus, groups basally within the Flea Beetles (See Fig. 3). This placement suggests that not all Flea Beetles jump.

In summary, our data do not support the Flea Beetles as a subfamily, Alticinae, nor as Alticini, a tribal ranking within the Galerucinae. Our data also suggest that the root worms, Luperini, may also be a paraphyletic assemblage (Gillespie 2001). These findings indicate that addition of more independent characters, and probably the study of more taxa is needed to resolve the question of the relationships between the Flea Beetles and the galerucines. There are works in progress by Duckett & al. (invited book chapter), and Gillespie & al. (invited book chapter) that have added both characters and taxa to this question, and preliminary results from these works do not strongly contradict our findings.

Acknowledgements

We thank Joe Gillespie, Zuzana Swigonova and John Lapolla for aid in sequencing. We are profoundly grateful to the following individuals for collecting, identifying or helping to procure specimens for this study: Dr. Seung Lak An, Shawn Clark, Edward G. Riley, David G. Furth, Joe Gillespie, Luciano Moura, Alexander Konstantinov. We also thank Joe Gillespie, and C.A.M. Reid for discussion and comments. Alex Konstantinov also provided valuable discussion and good humored remarks on our lack of appropriate character polarization. We thank an anonymous reviewer, Joe Gillespie and Dan P. Duran for comments on the manuscript. We gratefully acknowledge NSF grants DEB-9972036 and DEB-0137624 to C.N.D. and K.M.K. and DEB-96-32879 to K.M.K. and DEB 9707544 to C.N.D. We also thank the New Jersey Agricultural Experiment Station for support.

References

Böving, A.G. & F. C. Craighead. (1931) An illustrated synopsis of the principal larval forms of the order Coleoptera. *Entomol. Am. (N.S.)* 11(1): 351 pp.

Bremer, K. (1988) The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* 42: 795-803.

Chapuis, F. (1875) Famille des Phytophages. Pp. 124-134 *in* Lacordaire,T. & Chapuis, F. *Histoire naturelle des insectes. Genera des Coléoptères. Vol. 11*, 420pp. Paris.

Chen, S. H. (1940) Attempt at a new classification of the leaf beetles. *Sinesia* 11 (5-6); 451-481, illus.

Crowson, R.A. & E. A. Crowson. (1996) The phylogenetic relations of Galerucinae-Alticinae. Pp. 97-118 *in* P.H. Jolivet and M. L. Cox, (Eds): *Chrysomelidae Biology, vol. 1: The classification, Phylogeny and Genetics*. SPB Publishing, Amsterdam, The Netherlands.

Duckett, C.N. (1999) A preliminary cladistic analysis of the subtribe Disonychina with special emphasis on the series *Paralactica* (Chrysomelidae: Galerucinae: Alticini). Pp. 105-136 in Advances *in Chrysomelidae Biology.* M. L. Cox, (Ed): Backhyus Publishers, Leiden, The Netherlands.

Duckett, C.N., J.J. Gillespie, & K.M. Kjer (in press) Relationships among the subfamilies of Chrysomelidae inferred from small subunit ribosomal DNA and morphology, with special emphasis on the relationship between the Flea Beetles and the Galerucinae. *in* P. H. Jolivet, J.A. Santiago-Blay & M. Schmitt, (Eds.) *New Contributions to the Biology of Chrysomelidae.* SPB Academic Publishers, The Hague, Netherlands.

Eriksson, T., (2000) AutoDecay 4.0. Bergius foundation, Royal Swedish academy of science. Stockholm.

Farrell, B. D.(1998) "Inordinate Fondness" Explained: Why are there so many beetles? *Science* 281: 555-559.

Farrell, B. D., and C. Mitter (1990) Phylogeny of host-affiliation: have *Phyllobrotica* (Coleoptera: Chrysomelidae) and the Lamiales diversified in parallel? *Evolution* 44:1389.

Farrell, B. D., C. Mitter, and D. J. Futuyma (1992) Diversification at the insect-plant interface. *BioScience* 42:34-42.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

Furth, D. G. & K. Suzuki (1994) Character correlation studies of problematic genera of Alticinae in relation to Galerucinae (Coleoptera: Chrysomelidae). Pp 150 *in* Furth, D. G. (Ed.) *Proceedings of the third International Symposium on the Chrysomelidae*, Leiden, 1992, Beijing.

Futuyma, D. J. and S. S. McCafferty (1990) Phylogeny and the evolution of host plant association in the leaf beetle genus *Ophraella* (Coleoptera, Chrysomelidae). *Evolution* 44 (8):1885-1913.

Gillespie, J. J. (2001) Inferring phylogenetic relationships among basal taxa of the leaf beetle tribe Luperini (Chrysomelidae: Galerucinae) through the analysis of mitochondrial and nuclear DNA sequences. Masters Thesis. University of Delaware.

Gillespie, J.J., K.M. Kjer, & D.W. Tallamy (in press) The evolution of cucurbitacin pharmacophagy in rootworms: insight from Luperini paraphyly. *in* P. H. Jolivet, J.A. Santiago-Blay & M. Schmitt, (Eds.). *New Contributions to the Biology of Chrysomelidae*. SPB Academic Publishers, The Hague, Netherlands.

Gressit, J.L. (1942) Plant-beetles from south and west China. I. Sagrinae, Donaciinae, Orsodacninae and Megalopodinaea (Coleoptera). *Lingnan Sci. Jorn*. 20 (2-4): 271

Gutell, R. R., N. Larsen & C. R. Woese. (1994) Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev*. 58: 10-26.

Hillis, D. M., & S. K. Davis. (1986) Evolution of ribosomal DNA: fifty million years of recorded history in the frog genus *Rana*. Evolution 40: 1275-1288.

Hillis, D. M., & J. Huelsenbeck. (1992) Signal, noise and reliability in molecular phylogenetic analysis. *J. Hered*. 83:189-195.

Huelsenbeck, J. P. (2000) MrBayes: Bayesian inference of phylogeny. Distributed by author, Department of Biology, University of Rochester, N.Y.

Jacoby, M. (1908) Coleoptera: Chrysomelidae. Pp 534, vol. 1 *in* Bingham (Ed.): *Fauna of British india including Ceylon and Burma*, London.

Jolivet, P., and T. J. Hawkeswood. (1995) Host-plants of Chrysomelidae of the world. Backhuys Publishers, Leiden, Netherlands.

Kjer, K. M. (1995) Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. *Mol. Phylogenet. Evol*. 4: 314-330.

Kjer, K. M. (1997) An alignment template for amphibian 12S rRNA, domain III: Conserved primary and secondary structural motifs. *J. Herpetol*. 31:599-604

Kjer, K. M., Blahnik, R. J, & Holzenthal, R. (2001) Phylogeny of Trichoptera (Caddisflies): Characterization of Signal and Noise Within Multiple Datasets. *Syst. Biol*. 50(6): 781-816

Konstantinov, A. S. (1998a) Revision of the Palearctic species of *Aphthona* Cheevrolat and a cladistic classification of the Aphthonini (Coleoptra: Chrysomelide: Alticinae). *Memoirs on Entomology, International*. Vol. 11. 429 pp. Associated Publishers Gainesville, Florida.

Konstantinov, A. S. (1998b) On the structure and function of female genitalia in Alticinae (Coleoptera: Chrysomelidae). *Proc. Ent. Soc. Wash*. 100(2): 353-360.

Latreille, P. A. (1802) *Histoire naturelle, générale et particulière, des crustacés et des insectes. Tome troisième. Familles naturelles des generes.* xii + 468 pp. 'An X', Paris.

Lingafelter, S. W. & A. S. Konstantinov. (1999) The monophyly and relative rank of alticine and galerucine leaf beetles: A cladistic analysis using adult morphological characters (Coleoptera: Chrysomelidae). *Ent. Scand*. 30: 397-416.

Lockhart, P. J., M. A. Steel, M. D. Hendy, & D. Penny. (1994) Recoding evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol*. 11: 605-612.

Lutzoni F., P. Wagener, V. Reev, & S. Zoller. (2000) Integrating ambiguously aligned regions of DNA sequence in phylogenetic analyses without violating positional homology. *Sys. Biol*. 49: 628-651

Maddison, D. R. & W. P. Maddison. (2000) MacClade 4: Analysis of Phylogeny and Character Evolution. Version 3.0. Sinauer, Sunderland, MA.

Mitter, C. & B. D. Farrell. 1991. Macroevolutionary aspects of insect-plant relationships. *in* E. A. Bernays [ed.]. *Insect-Plant interactions*. pp. 35-78. CRC Press, Boca Raton.

Metcalf, R. L. (1994) Chemical ecology of Diabroticits. p. 153-169 *in* P. H. Jolivet, M. L. Cox and E. Petitpierre, eds. *Novel aspects of the biology of Chrysomelidae.* Kluwer Academic Publ. Boston.

Pasteels, J. M. & M. Rowell-Rahier (1991) Proximate and ultimate causes of host plant influence on chemical defense of leaf beetles (Coleoptera: Chrysomelidae). *Entomoligia generalis* 15: 227-235.

Posada, D., & K. A. Crandall (1998) Modeltest: Testing the model of DNA substitution. *Bioinformatics.* 14: 817-818.

Reid, C.A.M. (1992) The leaf-beetle genus *Microdonacia* Blackburn (Coleoptera: Chrysomelidae: Galerucinae): revision and systematic placement. *Syst. Ent*. 17: 359-387.

Reid, C.A.M. (1995) A cladistic analysis of subfamilial relationships in the Chrysomelidae *sensu lato* (Chrysomeloidea). *In* J. Pakaluk & S.A. Slipinski (Eds.): *Biology, Phylogeny, and Classification of Coleoptera: Papers Celebrating the 80th Birthday of Roy A. Crowson*. Muzeum i Instytut Zoologii PAN, Warszawa.

Scherer, G. (1983) Diagnostic Key for the Neotropical Alticine Genera, (Coleoptera: Chrysomelidae: Alticinae). *Ent. Arb. Mus. Frey*,31/32:1-89.

Schmitt, M. (1996) The phylogenetic system of the chrysomelidae-History of ideas and present state of knowledge. Pp. 57-96 *in* P.H. Jolivet & M. L. Cox, [Eds], *Chrysomelidae Biology, vol. 1: The classification, Phylogeny and Genetics*, SPB Publishing, Amsterdam, The Netherlands.

Seeno, T. N. & Wilcox, J. A. (1982) Leaf Beetle Genera (Coleoptera: Chrysomelidae). *Entomography* 1:1-222.

Silferberg, H. (1990) The nomenclaturally correct names of some family-groups in Coleoptera. *Entomol. Fenn*. 1: 119-121.

Spike, B. P. & J. J. Tollefson (1991) Yield response of corn subjected to western corn rootworm (Coleoptera: Chrysomelidae) infestation and lodging. *J. Econ. Entomol.* 84:1585-1590.

Swofford, D. L. (1999) PAUP*: Phylogenetic Analysis Using Parsimony, Version 4. Computer program distributed by Sinauer.

Suzuki, K. & D. G. Furth (1992) What is classification? A case of study in insects systematics: potential confusion before order. *Zool. Sci*. 9: 1113-1126.

Tallamy, D. W., J. Stull, N. Erhesman & C. E. Mason (1997) Cucurbitacins as feeding and oviposition deterrents in nonadapted insects. *Environ. Entomol*. 26:678-688.

Tallamy, D. W., C. A. Mullin & J. L. Frazier (1999) An alternative route to insect pharmacophagy: the loose receptor hypothesis. *J. Chem. Ecol*. 25:1987-1997.

Tallamy, D.W., P. M. Gorski & J. K. Burzon (2000) Fate of male-derived cucurbitacins in spotted cucumber beetle females. *J. Chemical Ecology*. 26:413-427.

Thompson, J. D., F. Plewniak & O. Poch. (1999) A comprehensive comparison of multiple sequence alignment programs. *Nucleic Acids Res*. 27:2682-2690.

Virkki, N. (1989) On the cytological justification of the Flea Beetle subtribes Oedionychina and Disonychina (Bechyné & Springlová de Bechyné 1966). *Entomography* 6: 545-550.

Virkki, N. & J. A. Santiago-Blay. (1996) Atypical cytology in some neotropical Flea Beetles (Coleoptera: Chrysomelidae: Alticinae: Oedionychina) from one of the most intense natural radiation sites known, Morro do Ferro (Brazil). *Cytobios* 85: 167-184.

Weise, J. (1924) *Coleopterum Catologus, Chrysomelidae: Galerucinae*, pars 78(13): 1-225. [vol. 25].

White, R.E. (1996). Leaf beetles as biological control agents against injurious plants in North America. Pp 373-399. *in* P.H. Jolivet and M. L. Cox, (Eds): *Chrysomelidae Biology, vol. 2: The classification, Phylogeny and Genetics*. SPB Publishing, Amsterdam, The Netherlands.

Wilcox, J.A. (1971-1975) *Chrysomelidae: Galerucinae. Coleopterum Catalogus* (Schenkling, S. ed) pars 78(1-4) 1-770. W. Junk, Berlin.

Wilcox, J. A. (1972a) *Coleopterum Catalogus Supplementa (Chrysomelidae: Galerucinae, Luperini: Diabroticina and Aulacophorina), Pars 78, Fasc. 2. 2nd ed.* Dr. W. Junk, Gravenhage, Netherlands.

Wilcox, J. A. (1972b) *Coleopterum Catalogus Supplementa (Chrysomelidae: Galerucinae, Luperini: Luperina), Pars 78, Fasc. 3. 2nd ed.* Dr. W. Junk, Gravenhage, Netherlands.

Revised manuscript accepted December 2002