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Molecular phylogeny of *Diabrotica* beetles (Coleoptera: Chrysomelidae) inferred from analysis of combined mitochondrial and nuclear DNA sequences

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Abstract

The phylogenetic relationships of thirteen *Diabrotica* (representing *virgifera* and *fucata* species groups) and two outgroup Acalymma beetle species (Coleoptera: Chrysomelidae) were inferred from the phylogenetic analysis of a combined data set of 1323 bp of mitochondrial DNA (mtDNA) cytochrome oxidase subunit 1 (COI) and the entire second internal transcribed spacer region (ITS-2) of nuclear ribosomal DNA of 362 characters. Species investigated were *D. adelpha, D. balteata, D. barberi, D. cristata, D. lemniscata, D. longicornis, D. porracea, D. speciosa, D. undecimpunctata howardi, D. u. undecimpunctata, D. virgifera virgifera, D. v. zeae, D. viridula,* and outgroup *A. blandulum* and *A. vittatum*. Maximum parsimony (MP), minimum evolution (ME), and maximum likelihood (ML) analyses of combined COI and ITS-2 sequences clearly place species into their traditional morphological species groups with MP and ME analyses resulting in identical topologies. Results generally confer with a prior work based on allozyme data, but within the *virgifera* species group, *D. barberi* and *D. longicornis* strongly resolve as sister taxa as well as monophyletic with the neotropical species, *D. viridula*, *D. cristata* and *D. lemniscata* also resolve as sister taxa. Both relationships are not in congruence with the prior allozyme-based hypothesis. Within the *fucata* species group, *D. speciosa* and *D. balteata* resolve as sister taxa. Results also strongly supported the D. *virgifera* and *D. undecimpunctata* subspecies complexes. Our proposed phylogeny provides some insight into current hypotheses regarding distribution status and evolution of various life history traits for Diabrotica.

Keywords: Diabrotica, rootworms, mtDNA, cytochrome oxidase subunit I, ITS-2, phylogeny

Introduction

The genus *Diabrotica* represent a large group of phytophagous beetles (Coleoptera: Chrysomelidae: Galerucinae: Luperini: Diabroticites) composed of 338 valid species (Wilcox, 1972). The genus is historically neotropical, although *D. virgifera virgifera* LeConte has been recently introduced as a pest of maize, *Zea mays* L., in Europe (Camprag & Baca, 1996), with the greatest diversity of species in tropical areas (Smith, 1966; Krysan, 1986; Krysan & Smith, 1987). *Diabrotica* is generally separated into three species groups: *signifera*, *fucata* and *virgifera*, with the latter two containing identified pest species, which may explain why these groups have been disproportionately studied (Krysan, 1986). The *virgifera* species group is composed of twenty-one known species with the remainder in the *fucata* (305 species) and *signifera* (eleven species) species groups (Wilcox, 1972; Krysan & Smith, 1987).

Their pest status (fifteen species and subspecies are reported as pests of sixty-one different crops (Krysan, 1986)), has meant that *Diabrotica* species have been the focus of numerous studies on life history (Metcalf, 1979; Branson & Krysan, 1981; Krysan, 1982), species distribution (Smith, 1966; Krysan, 1986; Krysan & Smith, 1987), reproductive isolation (Krysan & Guss, 1978; Krysan et al., 1983, 1986; Guss et al., 1982, 1983a, 1983b, 1984, 1985; Giordano et al., 1997) and host-plant relationships (Metcalf, 1979; Branson & Krysan, 1981; Yaro & Krysan, 1986). For example, Branson & Krysan (1981) and Krysan (1982) suggested that species in the *fucata* species group are multivoltine and polyphagous while species in the *virgifera* species group are univoltine and oligophagous (or monophagous) with species group specific differences being attributed to host plant availability and climate. From a evolutionary and chemical ecology perspective many *Diabrotica* and related Diabroticites such as Acalymma have been the subject of many studies. Metcalf (1979, 1986) reviewed the evolution and chemical ecology of Diabroticite affinity toward triterpene cucurbitacins found in the plant family Cucurbitaceae. Various aspects of this research was embraced as it was discovered that insecticides could be mixed with selected cucurbitacins for control of pest *Diabrotica* and *Acalymma* (Metcalf et al., 1987; Weissling et al., 1989; Weissling & Meinke, 1991). Beyond cucurbitacins, Branson & Krysan (1981) presented evidence for an evolutionary relationship between D. *virgifera* and *Z. mays* as well as anecdotal evidence for associations of other *Diabrotica*, particularly Nearctic species, with native grasses.

Krysan et al. (1989) inferred evolutionary relationships of selected *Diabrotica* using allozyme data and resolved nine species into their respective clades. These authors provided evidence that closely related sympatric *Diabrotica* in the *virgifera* species group have stronger reproductive barriers in terms of reduced pheromone cross attractance than allopatric species within the same group. While this work provided an essential foundation in the study of phylogenetic relationships within Diabrotica, the authors also noted that it may have lacked resolution in the deepest nodes of the resulting phenogram because *D. cristata* (Harris) and *D. longicornis* (Say) were grouped as sister taxa in a monophyletic group with *D. barberi*, rather than the morphological sibling species *D. longicornis* and *D. barberi* Smith and Lawrence.

There is a high degree of similarity within *Diabrotica* even beyond the sibling and subspecies levels. For example, Krysan et al. (1989) observed that while they were able distinguish *D. cristata* and *D. lemniscata* LeConte neither species has phylogenetically informative characters. The analysis of DNA sequences may help solve this problem by providing additional phylogenetically informative characters. This is especially true in *Diabrotica* because this genus of beetles is characterized by morphological similarity, a problem that is common within this genus as well as other genera in the subfamily Galerucinae (Wilcox, 1965).

The use of mitochondrial DNA (mtDNA) sequence data has become a proven standard for many phylogenetic studies (Caterino et al., 2000). Among the many mitochondrial genes that have been studied, subunit I of the cytochrome oxidase gene (COI) has become a standard for phylogenetic inference of many insect groups (Lunt et al., 1996). Examples include but are not limited to Lepidoptera at the species level (Caterino & Sperling, 1999), Hymenoptera at the subgeneric level (Koulianos, 1999), and Orthoptera at the intraspecific level (Lunt et al., 1998) to name a few. Given the proven utility of COI to resolve phylogenetic relationships at many taxonomic levels, we believed the use of this gene was appropriate given there were five classification categories represented in this study (genus, species within a genus, species within a clade, sibling species, and subspecies).

The internal transcribed spacers of nuclear ribosomal DNA have been used extensively for intra- and interspecific relationships in closely related and cryptic species of insects and arthropods (Porter & Collins, 1991; Paskewitz et al., 1993; Navajas et al., 1998). While lack of interspecific variation (Kuperus & Chapco, 1994) or high intraspecific variation may limit the use of ITS regions in phylogenetic studies (Vogler & DeSalle, 1994),

other studies have shown that these regions do indeed have phylogenetic utility (Miller et al., 1996; Schilthuizen et al., 1998; Depaquit et al., 2000). Because some of the taxa selected in this study are closely related with some cryptic morphological features, we also explored the phylogenetic resolving utility of the ITS-2 region.

In this study, we begin the process of constructing a phylogeny of the genus *Diabrotica* by considering nine of the ten species that are known to occur in the United States as well as selected species that inhabit Central and South America. Presented is the first phylogenetic hypothesis of *Diabrotica* based upon DNA sequence data from mitochondrial (COI) and nuclear sources (ITS-2). The purpose of this study was to provide an initial step in understanding the evolutionary history of *Diabrotica* based on DNA sequences of this closely related, widespread and economically important group of beetles for which little phylogenetic information exists.

Results

COI sequence variation

The length of the sequenced COI fragment was 1323 bp for all *Diabrotica* evaluated and 1320 bp for both *Acalymma* species. The difference between the genera was the deletion of a codon between positions 1312 and 1314 of the *Diabrotica* fragment, which codes for a serine residue. The adjoining portion of the t-RNA-Leu gene that was 21 bp for all species represents the 3′ priming region for the COI fragment and was not subject to further analysis. Of the 1323 COI characters 434 were variable between taxa, of which 303 were parsimony informative. PTP tests indicated significant phylogenetic structure (P = 0.0002). Table 1 lists base composition for each COI codon position where nucleotide frequencies were biased toward A + T, averaging 69.78%. This bias was also apparent at the respective codon positions, with third position codons having the greatest bias at 89.48% A + T. No significant differences were detected between taxa in terms of base composition (χ^2 = 6.62 (d.f. = 42), P = 1.00). The transition/transversion ratio (estimated by maximum likelihood on a NJ tree) for COI considering all codon positions is 2.06.

ITS-2 sequence variation

The entire ITS-2 with portions of flanking 5.8S and 28S rRNA genes were successfully sequenced for all taxa. The boundaries of ITS-2 were determined by these highly conserved flanking regions (Navajas et al.,

1998). The 3′ portion 5.8S was 155 bp while the 5′ portion of 28S was 50 bp for all taxa. Not a single bp change (transition, transversion, or indel) for either flanking region was observed. Thus, these conserved regions were excluded from further consideration. The length of the entire ITS-2 varied from 323 bp (*D. adelpha*) to 352 bp (*D. v. virgifera* and *D. v. zeae*). However, alignment of all sequences yielded 362 characters when considering insertion/deletion (indel) events of which eighty-nine were variable with fifty-five of these characters being parsimony informative. PTP tests indicated significant phylogenetic structure (P = 0.0002). Indels ranged from single bp events to 10 bp deletions, in total forty-seven sites out of 362 had at least one taxa with an indel (Table 1). ITS-2 nucleotide frequencies were also biased toward A + T averaging 66.9% (Table 1). No significant differences were detected between taxa in terms of base composition (χ^2 = 12.83 (d.f. = 42), P = 1.00). The transition/transversion ratio (estimated by maximum likelihood on a NJ tree) for ITS-2 considering all sites is 1.40.

Nucleotide distances

Nucleotide distances corrected using Tamura and Nei's (1993) method among all taxa range from 0.3% to 23.7% for the ITS-2 and 0.5% to 18.4% for COI (Table 2). Inter-generic differences ranged from 13.1% to 23.7% for ITS-2 and 13.5% to 18.4% for COI (Table 2). Distances between the morphologically assigned species groups range from 5.5% to 8.5% for ITS-2 and 12.4% to 15.7% for COI (Table 2). Within the *virgifera* species group distances range from 0.3% to 3.2% for ITS-2 and 1.1% to 10.7% for COI (Table 2). Between the *fucata* species distances ranged from 0.6% to 3.7% for ITS-2 and 0.5% to 13.6% for COI (Table 2).

Combining COI and ITS-2

The partition homogeneity test between COI and ITS-2 sequences did not reject the null hypothesis that either data set was significantly different from a random partition of pooled data (P = 0.32). Thus, we combined both data sets for the phylogenetic analyses shown in this manuscript. The combined data set is comprised of 1685 total characters of which 524 were variable and 359 were parsimony informative. The PTP test detected significant phylogenetic structure in the combined data set (P = 0.0002).

MODELTEST selected the general time reversible model including the proportion of invariable sites and gamma distribution for rate variation among sites (GTR + I + G) (Lanave et al., 1984; Yang, 1994; Gu et al., 1995) as the best fit for ME and ML phylogenetic analyses of the combined data.

Rate matrix parameters estimated on the neighbour-joining tree are: R(a) [A-C] = 3.20, R(b) [A-G] = 8.21, R(c) [A-T] = 3.73, R(d) [C-G] = 1.26, R(e) [C-T] = 25.19, R(f) [G-T] = 1.00. The proportion of invariable sites (I) and the gamma shape distribution parameter (G) are approximated at 0.48 and 0.54, respectively.

Phylogenetic analysis

While describing trees that were inferred from different analyses, we considered bootstrap values of 70% or greater as strong, between 50 and 70% as moderate, and below 50% as weak (Hillis & Bull, 1993). Unweighted parsimony analysis of the combined data set results in a single tree of 1154 steps (consistency index (C.I.) = 0.609, retention index (R.I.) = 0.584) (Fig. 1). Results from the separate analyses of COI and ITS-2 data are not in conflict and are not presented here for simplicity. The same tree was recovered when transitions at third codon positions of the COI gene partition of the combined data set were down-weighted by a factor of two. Likewise, an identical tree topology was obtained under ME analysis using the GTR + I + G model described previously (Fig. 2). The topology of both analyses received moderate to strong support at most nodes, as suggested by bootstrap values with a couple exceptions. A subclade with *D. porracea* being monophyletic with the *D. virgifera* subspecies is relatively weak for both ME and MP analyses (Figs 1 and 2). ML analysis results in a similar topology as MP and ME but differs in that the moderately supported *virgifera* species group subclade (*D. lemniscata*, *D. cristata*; *D. porracea*; *D. v. virgifera*, *D. v. zeae*), was absent (Fig. 3). All analyses (Figs 1–3) clearly differentiate both morphological (*virgifera* and *fucata*) species groups, although bootstrapping of the ME analysis indicates only moderate bootstrap support (56%) for the *fucata* species group (Fig. 2). The topology of the *fucata* species group was identically supported by all analyses. Additionally, all sister taxa and subspecies groupings as well as the monophyletic placement of *D. viridula* with the sister taxa, *D. barberi* and *D. longicornis*, received strong bootstrap support for all analyses (Figs 1–3).

Discussion

Data analysis

Both COI and ITS-2 sequences display similar divergence patterns between the taxa and have significant phylogenetic structure. It is interesting that the data partitions are not conflicting based on the partition homogeneity

test considering that they come from different sources, evolve at different rates, and have quite different properties. For example, Navajas et al., 1998 reported that ITS-2 sequences appear to evolve 2.5 times faster than COI sequences in five Tetranychus species. Despite potential differences, we consistently recovered compatible trees among the data partitions, which also suggests that the models used to analyze the data were adequate in recovering the correct phylogenetic signal for the taxa examined (Miyamoto & Fitch, 1995). While we chose to combine the data sets, the issue of combining data for phylogenetic analysis is debatable without a current consensus (Bull et al., 1993; de Queiroz et al., 1995; Miyamoto & Fitch, 1995; Huelsenbeck et al., 1996). Considering that partition homogeneity tests were not significant, it is most likely that the combined data set maximizes the amount of information obtained while yielding the correct trees (Vogler & Welsh, 1997; Chippindale et al., 1999).

Two different approaches to parsimony analysis; equal weighting and differential weighting of transitions at third codon positions of the COI gene, were applied to the combined data. Differential weighting was done with the intention of down-weighting the potential misleading effect of transitions that may accumulate at high frequencies in third codon positions (Meyer, 1994; Huang et al., 2000). Both approaches resulted in no topological differences. ME analysis based on the GTR + I + G model, which allows for differing substitution ratios and among site variability proportion, also identically supports the topology acquired in the different parsimony analyses. ML based on the same model (GTR + I + G) results in some minor topological differences in the *virgifera* species group from MP and ME that are not well supported in those analyses as well. Despite these minor differences, it is our opinion that we have entered a zone of high confidence estimation because we are beyond 1000 + sequence characters, which increases the chances of recovering the correct phylogenetic tree (Hillis et al., 1994a, 1994b). However, stronger resolution will most likely not be achieved until additional *Diabrotica* species are added to future studies.

Comparison with a prior phylogenetic study and fit with information-based hypotheses

All phylogenetic estimates presented in this study support traditional morphologically based assignments of the *fucata* and *virgifera* species groups, with the latter receiving the strongest support. This was not surprising because the *virgifera* species group has received the most taxonomic study, with Krysan & Smith (1987) separating it from the rest of *Diabrotica* by examining sclerite shape on male genitalia as well as five

external characters. Smith & Lawrence (1967) and Wilcox (1972) have made important contributions in assigning other *Diabrotica* to the *fucata* species group but additional work is necessary to clarify morphological relationships.

Our results generally concur with Krysan et al.'s (1989) phenogram based on allozymes, especially considering the placement of species into their respective species groups. However we are not in congruence regarding their proposal of a monophyletic Nearctic species complex (*D. cristata*, *D. longicornis* and *D. barberi*) with *D. cristata* and *D. longicornis* regarded as sister taxa. Analyses of the combined sequences in our study place the morphological sibling species *D. barberi* and *D. longicornis* as sister taxa These results were not unexpected as other studies have reported a high degree of similarity between *D. barberi* and *D. longicornis*. For example, Krysan et al. (1983) reported that a discriminant analysis of fourteen morphometric characters failed to completely discriminate between *D. barberi* and *D. longicornis*. Moreover, examination of *D. barberi* and *D. longicornis* genitalia and spermathecae, characters which have proven reliable for distinguishing most Diabrotica, revealed no discriminating features between these two species (Krysan et al., 1983; Krysan & Smith, 1987). Meanwhile, our analyses consistently placed *D. cristata* as sister with *D. lemniscata*, albeit a longer distance sister relationship. Reproductive isolation due to a lack of sex pheromone cross-attractance is one mechanism that closely related sympatric *Diabrotica* may use as a hybridization barrier (Krysan et al., 1989). We are in congruence with Krysan et al. (1989) regarding this aspect, because both sister taxa relationships, *D. barberi–D. longicornis* and *D. cristata–D. lemniscata*, proposed in our analyses are not pheromonally cross-attractive (Guss et al., 1984, 1985; Krysan et al., 1986).

Considering distribution status, *D. barberi*, *D. longicornis,* and *D. cristata* are the only *Diabrotica* species that are entirely Nearctic (Krysan & Smith, 1987), yet both *D. longicornis* and *D. cristata* are geographically sympatric with *D. lemniscata* in Northern and central Mexico and the south-western United States (Krysan & Smith, 1987). Likewise, *D. porracea*, *D. v. virgifera* and *D. v. zeae* are distributed within Nearctic and Neotropical regions and are geographically sympatric with the Nearctic species at several localities (Krysan & Smith, 1987). The results presented in this study show no clear geographical pattern regarding phylogenetic placement of species. For example, all analyses support a monophyletic grouping of *D. viridula*, a Neotropical species, with Nearctic *D. barberi* and *D. longicornis*. Thus,

geographical range may not be a good characteristic of support for an entirely Nearctic subclade as was presented by Krysan et al. (1989).

It is also difficult to make assumptions about phylogenetic placement of *Diabrotica* species in terms of habitat especially when considering relationships with adult host plants. For example, one argument for a common adult habitat is collection of beetles on the same host plant. This becomes questionable on cucurbits given the vestigial condition of *Diabrotica* attraction toward plants containing cucurbitacins (Metcalf et al., 1980), because this condition allows for the possibility to collect many *Diabrotica* on the same plant (even the same bloom) at a given point in time. The authors and their colleagues have collected all five species of *Diabrotica* (*D. barberi*, *D. longicornis*, *D. cristata*, *D. v. virgifera* and *D. u. howardi*) that occur in Nebraska on a single *C. foetidissima* plant (Golden, 1990; T. L. Clark and L. J. Meinke, unpublished data). Beyond curburbit association, species such as *D. cristata*, *D. barberi*, *D. longicornis* and *D. v. virgifera* have been collected as adults on a variety of prairie forbs such as sunflower, *Helianthus* spp.; smartweed, *Polygonum* spp.; and amaranth, *Amaranthus* spp. This may occur when preferred pollen sources are depleted. For example, *D. barberi* adults feed on pollen and succulent reproductive tissues of *Z. mays* during their ovipositional period (Krysan, 1999), however Cinereski & Chiang (1968) observed that these beetles switch to alternate pollen sources outside the maize habitat as their ovipositional period exceeds the availability of maize pollen. Thus, the choice of adult host may actually be more of an opportunistic survival strategy rather than an indicator for an evolutionary relationship or a phylogenetic support character. In defense of habitat choice, *D. longicornis*, *D. cristata*, *D. lemniscata,* and *D. barberi* (historically, before the switch to maize) are all found as adults in ecosystems where perennial grasses are a permanent feature (Branson & Krysan, 1981; Krysan & Smith, 1987). It has also been hypothesized that *virgifera* clade ancestor evolved as a specialist on the roots of certain grasses (Branson & Krysan, 1981; Krysan & Smith, 1987). However, little is known about which grass species are actually utilized. Until more information is gained on larval host preferences or female oviposition choice, as suggested by *D. cristata* emergence on the perennial grass Andropogon gerardii Vitman (Yaro & Krysan, 1986), treatment of *Diabrotica* habitat (especially for adults) must be taken with caution when considering it as a support character for phylogenetic resolution.

Related to larval food availability, the adaptation of egg diapause during unfavorable conditions such as dry seasons or seasonal cold periods when

specific grass roots are unavailable is hypothesized to have evolved in a *virgifera* clade ancestor (Krysan, 1982). Because this life history trait has been exclusively observed in the *virgifera* species group, *D. barberi*, *D. cristata*, *D. lemniscata*, *D. longicornis*, *D. v. virgifera* and *D. v. zeae* (Krysan, 1982), it may indeed have evolved from a univoltine ancestor. Interestingly, three reported *virgifera* species group taxa, including two examined in this study (*D. viridula* and *D. porracea*), may have a multivoltine life cycle (Krysan & Smith, 1987; Eben & Barbercheck, 1996; Eben, 1999). This suggests that multivoltinism may have evolved independently from a univoltine progenitor species or that the progenitor was multivoltine with egg diapause evolving as given *virgifera* species group taxa colonized regions with alternating wet and dry seasons and/or temperate climates. The answer to this question most likely will not be known until more taxa are added to a future phylogenetic study complemented with voltinism information, which is currently lacking for most *virgifera* group species particularly those from Neotropical regions.

Within the *fucata* species group all analyses strongly supported the undecimpunctata subspecies. This was expected, as both subspecies respond identically to the sex pheromone 10-methyl-2-tridecanone (Guss et al., 1983b), and have similar life histories albeit in different geographical regions (Smith, 1966; Smith & Michelbacher, 1949). These subspecies are part of a subspecies complex that also includes *D. u. tenella* LeConte and *D. u. duodecimpunctata* F., all of which occur in different geographical areas within North America separated by a series of mountain ranges and ecological zones (Krysan, 1986). The analysis of these subspecies with a biogeographical component would be a worthwhile and interesting study considering the pest status of this complex. The monophyly of *D. speciosa* and *D. balteata* was interesting. *D. balteata* is a cosmopolitan pest that ranges throughout Central America and Mexico into the Southern United States where its range is limited by an inability to survive subfreezing temperatures (Pitre & Kantack, 1962; Saba, 1970; Krysan, 1986). *D. speciosa* apparently fills the same pest niche as *D. balteata* but in South America (Krysan, 1986). Chuman et al. (1987) isolated the sex pheromone, 6,12-dimethylpentadcan-2-one, for *D. balteata* but to-date the *D. speciosa* pheromone has not been characterized. It would be of interest to examine if these species are isolated by sex pheromones or if they have a similar pheromonal structure. For example, some species of *Diabrotica* that have recently become sympatric due to agricultural practices, such as *D. barberi* and *D. v. virgifera* on maize in the North Central United States (Krysan &

Smith, 1987), display certain degrees of sex pheromone cross attractance (Guss et al., 1985).

Possible insight into an evolutionary hypothesis

It has been hypothesized that Diabroticites such as *Diabrotica* and *Acalymma* and their Old World galerucine counterpart *Aulacophora* evolved with Cucurbitaceae as evidenced by a similar affinity toward cucurbit hosts (Metcalf, 1979). Possible factors selecting for this association include protection against predators (Ferguson & Metcalf, 1985; Nishida & Fukami, 1990), entomopathogenic nematodes (Eben & Barbercheck, 1997), and fungi (Brust & Barbercheck, 1990; Tallamy et al., 1998) as well as reproductive success via sequestration of bitter tasting cucurbitacins to eggs (Ferguson et al., 1985). While these factors most certainly promote an evolutionary relationship, the extent to which various Diabroticite species groups and species within groups utilize cucurbits is intriguing and poses possibilities for co-evolutionary study. For example, between the Diabroticite genera, *Acalymma* and *Diabrotica*, an interesting hierarchy exists when considering utilization of cucurbit hosts. *Acalymma* spp. are dependent upon Cucurbitaceae as adult and larval hosts (Smith, 1966); *fucata* species group taxa can utilize various cucurbits as a larval host but also utilize several other plant families (Metcalf, 1979; Branson & Krysan, 1981) and are more responsive to cucurbitacins than *virgifera* species group taxa (Metcalf et al., 1980; Tallamy et al., 1997). Meanwhile, *virgifera* group species while attracted to cucurbits show a wide range of variability, with taxa occurring outside the native range of Cucurbitaceae being less sensitive to cucurbitacins (Metcalf et al., 1980). Additionally, *virgifera* species group taxa are most likely unable to utilize cucurbits as larval hosts (Metcalf, 1986). This evidence indicates a possible evolutionary pattern. Assuming that complete association with Cucurbitaceae is the ancestral condition, we can estimate a divergence time scale for the Diabroticites examined in this study. For example, the average COI distance between *Acalymma* and *Diabrotica* species of 16.02% (calculated from Table 2) divided by an assumed sequence divergence rate of 1.71% per million years for Coleopteran COI sequences (Brower, 1994), yields an estimated divergence time of 9.37 million years ago (Ma) between these genera and 8.09 Ma (average distance = 13.84%) between the *Diabrotica* species group. Both time frames are within Metcalf's (1979) hypothesized origin of a Diabroticite progenitor 20 Ma. The inclusion of Old World *Aulocophora* in future studies may also provide additional insight into the co-evolutionary relationship between these beetles and Cucurbitaceae as well as a time

scale for the divergence of the different tribes, genera, and species within this entire group.

Conclusions

In conclusion, the molecular phylogeny obtained in this study supports morphological and allozyme based taxonomic assignments of *Diabrotica* at the species group level (*virgifera* and *fucata*) but differs from the allozyme study at lower taxonomic levels. Results are dependent upon both the taxa sampled and the resolving ability of the DNA sequences selected. Thus, only an initial view of *Diabrotica* phylogeny is presented in this work. It would be advantageous in future studies to include additional taxa in both the *virgifera* and *fucata* species groups as well as taxa from the third *Diabrotica* group, *signifera*, for which little information exists. Additional biological information is also necessary to assist in the interpretation of future results because little is known about most *Diabrotica*, particularly non-pest species. Given the inferred oligophagous (monophagous) characteristic of the *virgifera* species group (Krysan & Smith, 1987) larval associations with grasses, an evolutionary study based on the phylogenetic relationships between the preferred larval hosts and/or female host-plant oviposition choices as well as the insects themselves would be of significant value. Correlating the results of such a study may provide insights as to why some species have become or have the potential to become pests. Beyond *Diabrotica* it would also be of value to expand phylogenetic studies into related genera to confirm whether the genus is indeed monophyletic. Krysan et al. (1989) raised this question because their allozyme based phenogram showed the *fucata* clade to be a sister group of the Diabroticite genera *Acalymma* and *Paranapicaba*. Likewise, it may be beneficial to expand phylogenetic examination across other genes such as other mitochondrial protein coding genes (Simon et al., 1994), nuclear ribosomal genes like 18S, or nuclear protein coding genes like elongation factor-1a (Caterino et al., 2000).

Experimental procedures

Insects and DNA extraction

Two individuals from beetles representing thirteen species of *Diabrotica* and two of *Acalymma* (outgroup) were collected from several localities for

molecular analysis, thirty in total (Table 3). Specimens were identified using available dichotomous keys (Munroe & Smith, 1980; Krysan, 1986; Krysan & Smith, 1987) with representative vouchers verified by J. L. Krysan (USDA-ARS retired) and preserved in 95% ethanol or frozen (–80 °C). DNA was extracted using a modification of Black & DuTeau's (1997) CTAB (hexadecyltrimethylammonium bromide) extraction protocol. The thorax and legs of individual beetles were ground in 500 ml CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 0.02 M EDTA (pH 8.0), 2.0% CTAB, and 0.2% b-mercaptoethanol), and 5 ml of 20 mg/ml of proteinase K was then added to each sample. After vortexing the mixture, samples were held at 65 °C for 1 h vortexing at 20 min intervals. Samples were then cooled to room temperature before adding 15 ml of 50 mg/ml RNase A, vortexing, and incubation at 37 °C for 2.5 h (samples were vortexed at 30 min intervals). After incubation with RNase A, samples were centrifuged at 10 000 x g for 5 min at room temperature. The supernatant was transferred to a fresh tube, where 500 ml of a chloroform:isoamyl alcohol (24:1) was added. The mixture was vortexed and then centrifuged for 15 min at 10 000 x g. The upper aqueous layer was then transferred to a fresh tube where DNA was precipitated with 500 ml of 100% isopropanol (–20°C). The mixture was gently inverted five times and placed at 4 °C for at least 2 h followed by centrifugation at 10 000 x g at 4 °C for 30 min. The supernatant was removed and the DNA pellet was washed twice with 700 ml of 70% and 100% ethanol (–20 °C), respectively. After the final wash and ethanol removal, the DNA pellet was air dried and resuspended overnight in 100 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 7.6)).

PCR amplification, cloning, and sequencing

Polymerase chain reaction (PCR) was used to amplify two target regions in the mitochondrial and nuclear genomes: COI and ITS- 2, respectively. The COI gene was amplified in two overlapping fragments. The first fragment was amplified using the forward primer 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′, named C1-J-1718 (Simon et al., 1994) and the reverse 5′-CCCGGTAAAATTAAAATATAAACTTC- 3′, named C1-N-2191 in (Simon et al., 1994), which amplifies a central portion of the COI gene. The second overlapping COI fragment was amplified using the forward primer 5′-CAACATTTATTTTGATTTTTTGG-3′ (named C1-J-2183 in Simon et al., 1994) and reverse 5′-TCCAATGCACTAATCTGCCATATT- 3′ (named TL2-N-3014 in Simon et al., 1994), which amplifies the central and 3′ fragments of the COI gene along with a 5′ portion of the t-RNA-Leu gene. PCR of both COI gene fragments was done in 50 ml reaction volumes

containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM dNTPs, 0.8 μM of each primer, 1.25 U of AmpliTaq polymerase (Perkin Elmer, Branchburg, NJ), 3.5 mM $MgCl_2$, and 6 ml of DNA template (diluted 1 : 10 from the original CTAB extraction). PCR of the reaction mixtures was done using either a GeneAmp PCR system 2400 or 9600 (Perkin Elmer, Branchburg, NJ) with the following temperature profile for both gene fragments: 94 °C for 2 min; thirty-five cycles of 94 °C for 1 min, 52 °C for 30 s, 72 °C for 1 min, and a final extension step of 72 °C for 8 min. All amplifications had a negative control containing no DNA template. Amplification of ITS-2 was done using primers designed by Navajas et al. (1998). The forward primer 5′-GGGTCGATGAAGAACGCAGC- 3′ and the reverse 5′-ATATGCTTAAATTCAGCGGG- 3′ are defined in the highly conserved 5.8S and 28S flanking rDNA regions, respectively. ITS-2 PCR amplifications were done in 50 μl reaction volumes using the same reaction mixture proportions and thermocyclers described for COI gene amplification. The PCR temperature profile was also nearly identical, the exception was an annealing temperature of 55 °C.

Target PCR amplicons were purified via electrophoresis on low-melt 1.0% TAE (0.04 M Tris-acetate, 0.001 M EDTA (pH 8.0)) agarose gels stained with ethidium bromide. Target DNA was briefly visualized over a UV transilluminator and excised from the gel. Purified PCR amplicons were then cloned directly into pCR 2.1 TOPO plasmid vector (TA cloning kit, Invitrogen Corp., Carlsbad, CA) using the manufacturer's protocol. Positive clones were sequenced in both directions using a dye primer sequencing protocol at the University of Nebraska, DNA Sequencing Core Research Facility (Lincoln, NE) using a Li-Cor model 4000 l DNA sequencer (Li-Cor, Inc. Lincoln, NE). Sequences were deposited in GenBank with corresponding accession numbers listed in Table 3.

Phylogenetic construction

COI and ITS-2 sequence data were aligned using the Clustal W algorithm (Thompson *et al*., 1994) as implemented in GCG 10.1 for UNIX (Genetics Computer Group Inc., Madison, WI) using default parameters. For the protein coding COI gene, sequences were also translated to amino acids (using GCG 10.1) based on invertebrate codon usage of mitochondrial DNA to assist in manual adjustments, which were made by eye. MacClade (version 3.08, Maddison & Maddison, 1999) was used to compute sequence statistics such as variation among nucleotide positions within the codon as well nucleotide transformation frequencies. Genetic distances for individual data sets were estimated using Tamura–Nei distance (Tamura & Nei, 1993).

The data (individual and combined sequences) was also tested for phylogenetic structure that is significantly different from random using the PTP test (Faith, 1991) with 10 000 random matrices and randomizing in-group taxa using PAUP (v. 4.0b4a).

Both data sets (COI and ITS-2) were tested for heterogeneity to assess whether combining of COI and ITS-2 is appropriate for further phylogenetic analysis using the partition homogeneity test developed by Farris et al. (1994, 1995) and implemented within PAUP. Tests were performed with 1000 iterations.

Minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) phylogenetic analyses were conducted using the version of PAUP previously described. Each was performed using the heuristic search option employing step-wise addition with 100 random taxon addition sequence replicates. MP analysis was conducted using equal weight and with a weighting scheme of down-weighting third codon position transitions by a factor of 2 with respect to transversions (estimated by maximum likelihood on a neighbor-joining tree (Saitou & Nei, 1987)). The model of DNA substitution for ME and ML was determined using the program MODELTEST (v. 3.04) (Posada & Crandall, 1998). MODELTEST uses hierarchical likelihood ratio tests to determine the fit of General Time Reversible (GTR) family of substitution models (sixty-four total) that best fits the data. Parameters (base composition, substitution rates, proportion of invariable sites, and the gamma shape parameter) for the chosen model were estimated by maximum likelihood on the neighbour-joining algorithm (Saitou & Nei, 1987). Node support for MP and ME analyses was assessed using 1000 bootstrap pseudoreplicates, due to computational constraints only 100 bootstrap replicates were performed for the ML analysis. Gaps were treated as missing data.

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Figures

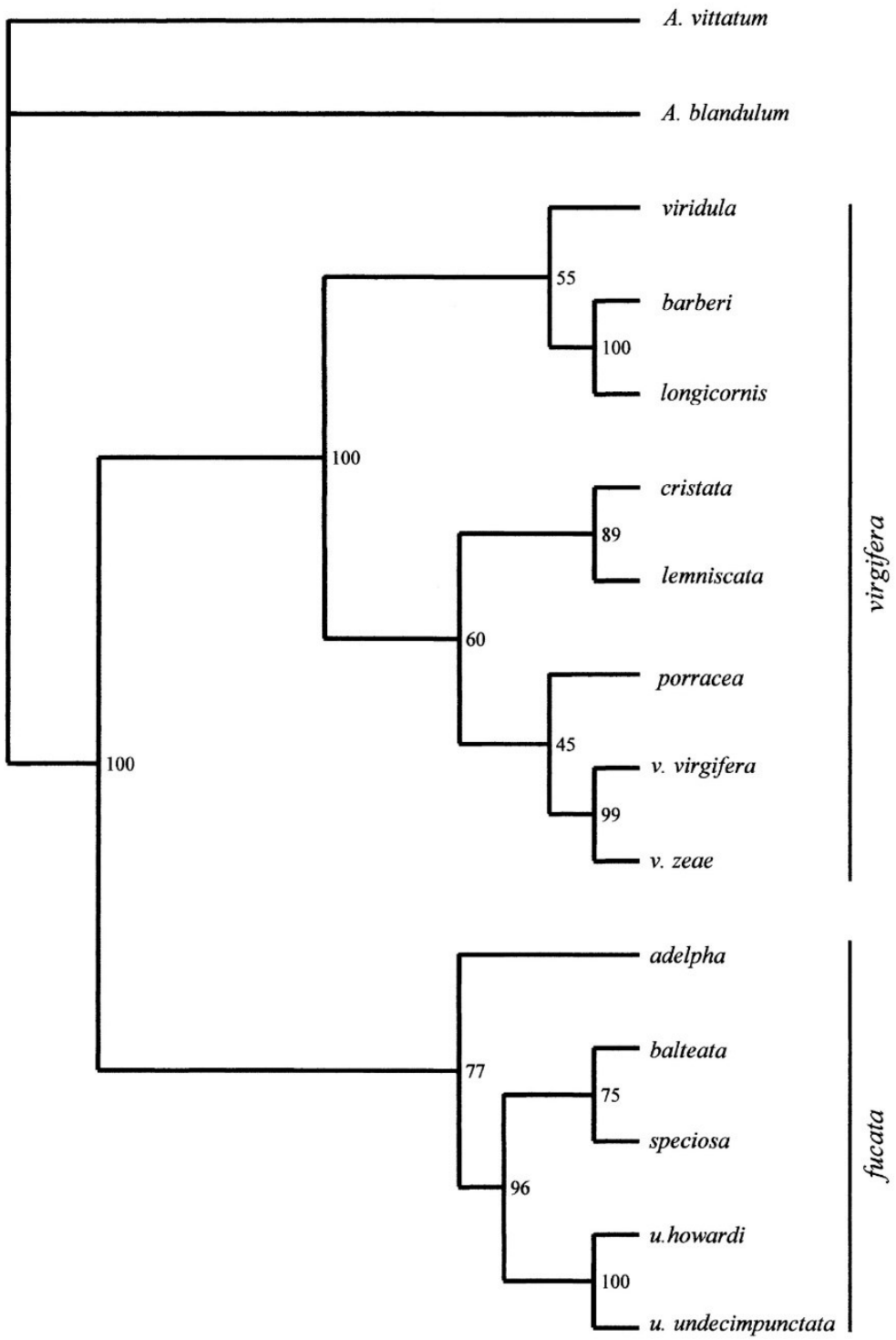

Figure 1. Unweighted parsimony tree (1154 steps; Consistency Index = 0.609, Retention Index 0.584) resulting from analysis of combined COI and ITS-2 data sets (1682 characters). Bootstrap percentages of 1000 replicates are shown above the branches. Morphological species group labels are shown to illustrate taxonomic congruence.

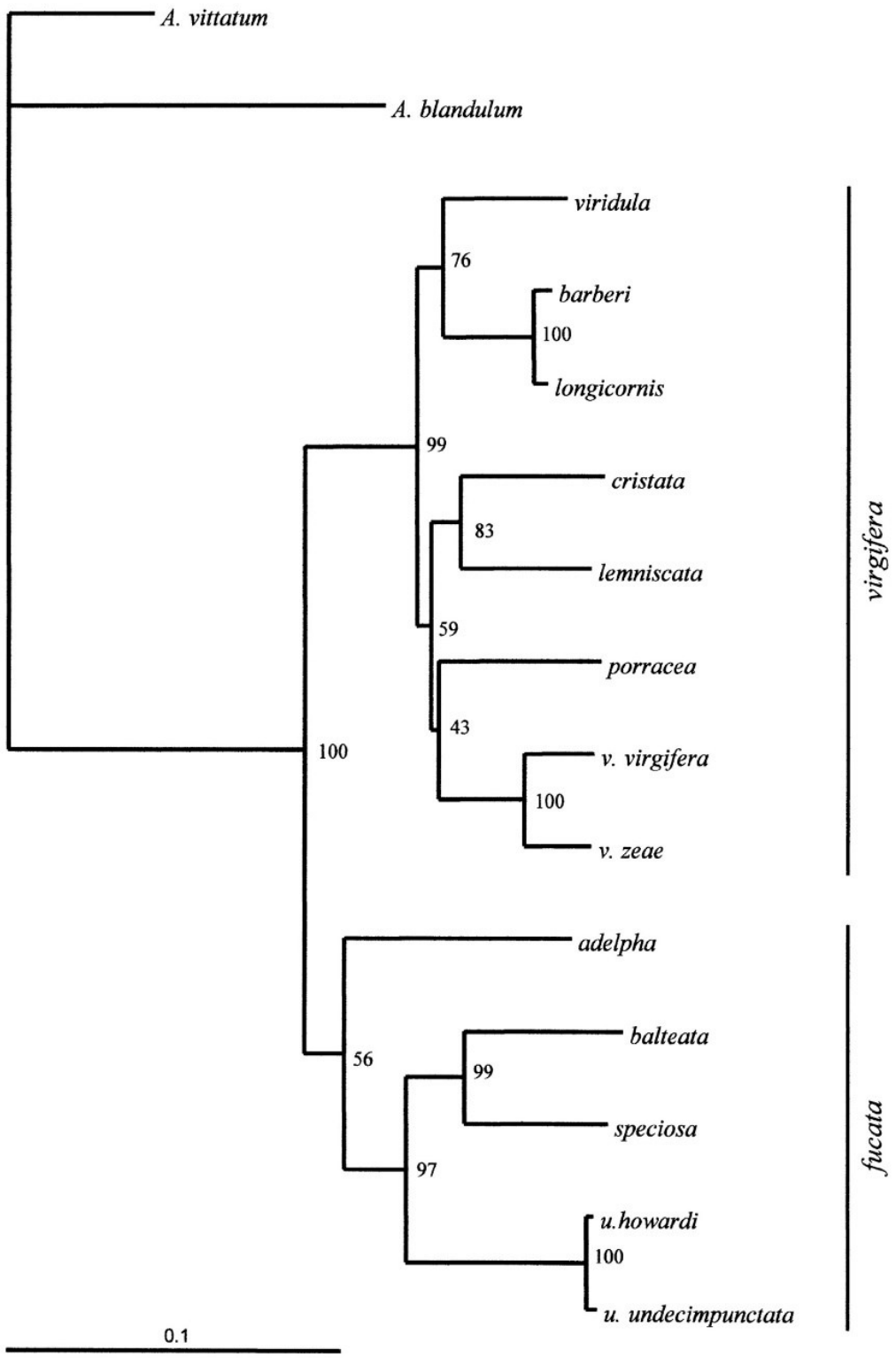

Figure 2. Minimum evolution tree for the combined COI and ITS-2 data sets (1682 characters) under the GTR + I + G model (see results section for parameter values). Bootstrap percentages of 1000 replicates are shown above the branches. Branch lengths are proportional to inferred distances. Morphological species group labels are shown to illustrate taxonomic congruence.

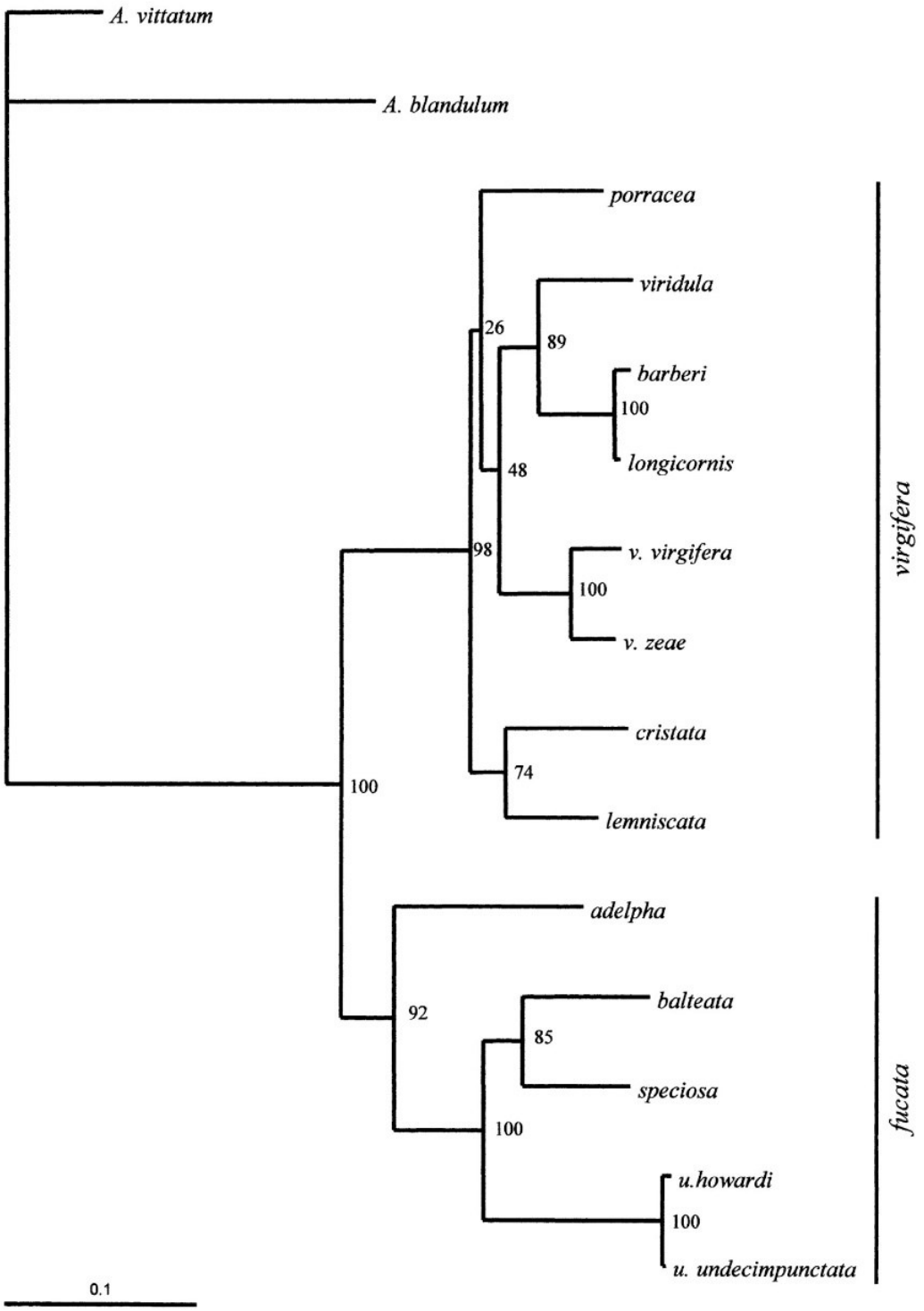

Figure 3. Maximum-likelihood tree (Ln likelihood = –7539.79) for the combined COI and ITS-2 data sets (1682 characters) under the GTR + I + G model (see results section for parameter values). Bootstrap percentages of 100 replicates are shown above the branches. Branch lengths are proportional to inferred distances. Morphological species group labels are shown to illustrate taxonomic congruence.

Table 1. Base composition for COI and ITS sequences.

Codon nucleotide position	Base composition				variable sites	% Indels
	A	C	G	T		
COI pos. 1	27.95–28.93 (28.45)	13.41–15.03 (14.55)	25.06–26.20 (25.83)	29.84–32.27 (31.18)	18.18	1
COI pos. 2	16.40–17.05 (16.82)	23.18–23.69 (23.42)	16.14–16.63 (16.34)	43.18–43.74 (43.42)	3.86	1
COI pos. 3	44.09–48.29 (46.03)	5.47–8.86 (7.00)	1.37–5.45 (3.52) 41.	36–44.87 (43.45)	74.09	1
All **COI** sites	29.70–31.29 (30.43)	14.39–15.79 (14.99)	14.58–15.83 (15.23)	38.48–39.70 (39.35)	32.05	1
All **ITS-2** sites	28.86–33.14 (32.32)	12.99–15.14 (13.93)	17.16–21.43 (19.15)	33.81–35.59 (34.60)	21.55	47*

* Nucleotide sites that had an indel with respect to A. vittatum. Base composition ranges across taxa are given with means in parentheses.

Table 2. Genetic distances based on Tamura and Nei's method; ITS-2 (above diagonal) and COI (below diagonal).

	Acalymma		virgifera species group								fucata species group				
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.
1. A. blandulum		0.091	0.223	0.223	0.226	0.222	0.227	0.232	0.229	0.237	0.202	0.202	0.211	0.215	0.192
2. A. vittatum	0.126		0.164	0.164	0.162	0.166	0.164	0.168	0.165	0.168	0.147	0.144	0.154	0.162	0.131
3. D. cristata	0.177	0.145		0.011	0.023	0.026	0.026	0.029	0.023	0.032	0.074	0.064	0.079	0.085	0.055
4. D. lemniscata	0.184	0.146	0.088		0.020	0.023	0.023	0.026	0.020	0.029	0.071	0.061	0.075	0.082	0.055
5. D. barberi	0.162	0.136	0.107	0.098		0.003	0.009	0.012	0.006	0.027	0.068	0.058	0.062	0.069	0.055
6. D. longicornis	0.162	0.136	0.107	0.094	0.011		0.012	0.015	0.009	0.030	0.065	0.055	0.059	0.066	0.052
7. D. v. zeae	0.179	0.141	0.090	0.082	0.093	0.090		0.003	0.009	0.029	0.071	0.064	0.072	0.079	0.055
8. D. v. virgifera	0.176	0.136	0.105	0.103	0.092	0.089	0.047		0.012	0.032	0.074	0.067	0.075	0.082	0.058
9. D. viridula	0.170	0.135	0.105	0.104	0.078	0.075	0.102	0.098		0.026	0.077	0.068	0.072	0.079	0.058
10. D. porracea	0.179	0.147	0.113	0.091	0.089	0.090	0.096	0.089	0.095		0.081	0.071	0.085	0.092	0.055
11. D. speciosa	0.181	0.151	0.146	0.138	0.143	0.139	0.146	0.139	0.142	0.157		0.009	0.030	0.037	0.022
12. D. balteata	0.184	0.156	0.142	0.148	0.141	0.141	0.150	0.144	0.146	0.152	0.098		0.021	0.027	0.013
13. D. u. howardi	0.183	0.149	0.138	0.134	0.124	0.125	0.138	0.139	0.125	0.133	0.112	0.118		0.006	0.022
14. D. u. undecimpunctata	0.182	0.146	0.140	0.133	0.124	0.125	0.134	0.138	0.125	0.134	0.106	0.114	0.005		0.029
15. D. adelpha	0.170	0.151	0.140	0.137	0.132	0.133	0.141	0.141	0.145	0.147	0.122	0.135	0.136	0.133	

Table 3. Collection sites for Diabrotica and Acalymma (outgroup) specimens examined with corresponding sequence accession numbers.

Species	Collection locality and host	GenBank accession numbers.	
virgifera clade		**COI**	**ITS-2**
D. barberi Smith and Lawrence	USA: Iowa: Clinton County on Zea mays L.	AF278544	AF278559
D. cristata (Harris)	USA: Nebraska: 9-Mile Prairie, Lancaster County on Andropogon gerardii Vitman	AF278545	AF278560
D. lemniscata LeConte	USA: New Mexico- near Kit Carson Museum, Colfax County on Curcurbita foetidissima HBK	AF278546	AF278561
D. longicornis (Say)	USA: Nebraska: Nuckolls County on Curcurbita foetidissima HBK	AF278547	AF278562
D. porracea Harold	Panama: Cordillera on Zea mays L.	AF278548	AF278563
D. virgifera virgifera LeConte	USA: Kansas: Wallace County on C. foetidissima HBK	AF278549	AF278564
D. virgifera zeae Krysan and Smith	USA: Texas: Bell County on Zea mays L.	AF278550	AF278565
D. viridula (Fabricius)	Panama: Cordillera on Zea mays L.	AF278551	AF278566
fucata clade			
D. adelpha Harold	Panama: Chiriqui on Glycine sp.	AF278552	AF278567
D. balteata LeConte	USA: Florida: Indian River County laboratory colony host unknown	AF278553	AF278568
D. speciosa Germar	Brazil: Sete Lagoas on Glycine max L. AF278554	AF278569	
D. undecimpunctata howardi Barber	USA: New Mexico: near Kit Carson Museum, Colfax County on Curcurbita foetidissima HBK	AF278555	AF278570
D. undecimpunctata undecimpunctata Mannerheim	USA: California: Alameda County laboratory colony, host unknown	AF278556	AF278571
Outgroup			
A. blandulum (LeConte)	USA: Nebraska: Nuckolls County on Curcurbita foetidissima HBK	AF278543	AF278558
A. vittatum (Fabricius)	USA: Nebraska: Lancaster County on Curcurbita pepo L.	AF278542	AF278557