

# Molecular barcoding for central-eastern European *Crioceris* leaf-beetles (Coleoptera: Chrysomelidae)

## Research Article

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**Abstract:** Among *Crioceris* leaf-beetles, the two most widespread species (*Crioceris asparagi* and *C. duodecimpunctata*) are serious invasive plant pests, while another two (*C. quatuordecimpunctata* and *C. quinquepunctata*) are rare species restricted to steppe-like habitats in Eurasia. The aim of the research was to check the genetic distinctiveness of these four species and develop barcodes for their molecular identification using the mitochondrial Cytochrome Oxidase I (COI) gene and two nuclear markers: Elongation Factor 1- $\alpha$  (EF1- $\alpha$ ) and Internal Transcribed Spacer 1 (ITS1). The identification of each species was possible and reliable with the use of COI and ITS1 markers. EF1- $\alpha$  was omitted in analyses due to its high level of heterozygosity (presence of multiple PCR products). *C. duodecimpunctata* and *C. quatuordecimpunctata* were shown to be sister taxa, but the similar genetic distances between all of the species indicate that these species originated almost simultaneously from a common ancestor. Identification of two separate clades in populations of *C. quatuordecimpunctata* suggested that the clades are isolated and can be considered as separate conservation units.

**Keywords:** *Asparagus* beetles • COI • EF1- $\alpha$  • ITS1 • Species identification • Pest • Steppe • Phylogeny • Taxonomy • Conservation units

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## 1. Introduction

The subfamily Criocerinae (about 1400 species) is comprised of three groups of genera: *Lema*-group (4 genera), *Lilioceris*-group (4 genera), and *Crioceris*-group (11 genera) [1-3]. In the Palearctic region 6 genera and 212 species occur from all three of these groups. The genus *Crioceris* Müller, 1764 belongs to the later group, *Crioceris*. The *Crioceris*-group probably originated at the Cretaceous–Paleocene boundary, which is supported by the time of origin of their host plants (Asparagaceae) [3]. The genus *Crioceris* contains up to 22 species naturally inhabiting only the Palearctic region, while only one species, *C. nigroornata* Clark, also extends to the Oriental region [3-5]. In Europe, 8 species have been

identified but only four of them are known to inhabit the central and eastern part of this continent: *C. asparagi* (Linnaeus 1758), *C. duodecimpunctata* (Linnaeus 1758), *C. quatuordecimpunctata* (Scopoli 1763) and *C. quinquepunctata* (Scopoli 1763). All *Crioceris* species are oligophagous or monophagous and feed mostly or exclusively on *Asparagus* L. (mainly on *A. officinalis* L.) [6]. *Asparagus* is a perennial salt-tolerant herbaceous plant that has both wild and domesticated varieties. It is native to the region east of the Mediterranean Sea and to the Middle East [7]. The theory about its origins in central and eastern European steppes has been questioned [8]. However, evidence is provided for the natural occurrence of *asparagus* in this area by the fact that *C. quatuordecimpunctata* and *C. quinquepunctata*

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live only on wild-growing plants. Among the European *Crioceris* species, two species, *C. asparagi* and *C. duodecimpunctata*, were introduced into North America [9] and Hawaii [10], where they have become serious pests of cultivated asparagus [11]. Therefore, in these countries studies to identify or develop control agents for these beetles have been undertaken (e.g., [12]). On the other hand, in Australia, where *Asparagus asparagoides* (L.) is a non-native weed, *Crioceris* beetles were used as a control agent for that plant [13]. The other two species, *C. quatuordecimpunctata* and *C. quinquepunctata*, inhabit the steppes and xerothermic habitats of central and eastern Europe and central and eastern Asia. Because of the loss of such areas, the two species have become rare and endangered in Europe, but so far they have not been protected. There are distinct color variations in *Crioceris* species, resulting in from two (*C. quatuordecimpunctata*), to seven (*C. quinquepunctata*) to about thirty varieties (*C. asparagi* and *C. duodecimpunctata*), aberrations, or forms for each species named by various authors [14,15]. These color differences, however, have no taxonomic significance [14,15]. All *Crioceris* beetles have similar eggs and larvae (immature life stages not yet described for *C. quinquepunctata*). Three of the European species, *C. duodecimpunctata*, *C. quatuordecimpunctata* and *C. quinquepunctata*, are similar in terms of morphology and ecology that may suggest that they are closely related. They are often found in the same localities and habitats, which suggests that their speciation was sympatric. No hybrids of these taxa are known, but hybridization cannot be excluded. *Crioceris duodecimpunctata* and *C. quatuordecimpunctata* are probably sister species, because they differ in the number of black spots (12 and 14, respectively), on the elytra, and in the presence of black spots on the pronotum. Traditionally, differences in coloration were often used for species separation, but such characteristics are often unreliable. Another problem with speciation of *Crioceris* is the genetic isolation of the geographically separate *C. quatuordecimpunctata* and *C. quinquepunctata* populations. These two species consist of highly isolated populations, which can probably be considered as separate conservation and/or taxonomic units [16,17]. Genetics of *Crioceris* leaf-beetles is understudied. Except for studies using single species in phylogenetic analyses on Phytophaga [18] based on nuclear rDNA and the phylogeny of Chrysomelidae based on Elongation Factor 1- $\alpha$  (EF1- $\alpha$ ) [19] or rDNA [20] little research has been completed in this area. Furthermore, four *Crioceris* species (*C. asparagi*, *C. duodecimpunctata*, *C. macilenta*, *C. paracentesis*) were studied cytologically and it

was discovered that all four species possessed  $2n=16$  chromosomes, which is probably the ancestral state for this genus [22]. The entire mitochondrial genome of *C. duodecimpunctata* was published in 2003 [21].

The economic importance of the widespread *C. asparagi* and *C. duodecimpunctata*, the rarity of the steppe-inhabiting *C. quatuordecimpunctata* and *C. quinquepunctata*, and the difficulties in distinguishing their eggs and larvae makes the development of DNA barcodes for these species vital. Genetic barcoding is a taxonomic technique that uses the DNA sequences of a chosen marker, either the mitochondrial Cytochrome Oxidase I (COI) gene for animals, the chloroplast tRNA<sup>Leu</sup> UAA (trnL) intron for plants, or the rDNA Internal Transcribed Spacers for fungi, to identify an individual as belonging to a particular species [23-28]. Applications include identifying insect eggs and larvae (which typically have fewer diagnostic characters than adults), insect debris (beetle fragments) or signs of beetle activity (e.g. faeces). DNA barcoding is also recommended before inferring the phylogeny and biogeographic history of studied species and populations.

To discover relations among central and eastern European *Crioceris* species and develop barcodes for them, we performed a molecular study.

## 2. Experimental Procedures

### 2.1 Sampling

Specimens of the three studied *Crioceris* species (*C. duodecimpunctata*, *C. quatuordecimpunctata* and *C. quinquepunctata*) and of *C. asparagi* as the closely related outgroup (from the same genera) were collected during several field trips in 2009 and 2010 in Poland and/or Ukraine (Table 1). Additionally, single specimens of *Clytra laeviuscula* (Ratzeburg, 1837), *Mimosestes ulkei* (Horn, 1873), *Donacia bicolor* Zschach, 1788, *Lysathia ludoviciana* (Fall) and *Altica litigata* (Fall) were used as more distantly related outgroups. Specimens were first preserved in 99% ethanol and then stored at -22°C. Five specimens per species, mostly from different localities, were used in analyses.

### 2.2 Laboratory procedure

Whole insect bodies were used for DNA extraction using a Nucleospin Tissue kit (Macherey-Nagel). Amplification of fragments of a widely used animal barcode, mitochondrial Cytochrome Oxidase I (COI) gene, was performed using primer pairs C1-J-2183 and TL2-N-3014 [29]. Additionally, EF1- $\alpha$  and Internal Transcribed Spacer 1 (ITS1) nuclear markers, also frequently used in phylogenetic studies of insects, were amplified using

| Species                        | Symbol | Country | Locality                    | Number of specimens / populations | Collector    |
|--------------------------------|--------|---------|-----------------------------|-----------------------------------|--------------|
| <i>C. asparagi</i>             | CA     | Poland  | Upper Oder valley (Silesia) | 3                                 | MAM          |
|                                |        | Poland  | Noteć valley                | 2                                 | DK,ŁK,MAM    |
| <i>C. quinquepunctata</i>      | C5     | Ukraine | Dniestr valley              | 5                                 | DK,ŁK,MAM,VR |
|                                |        | Poland  | Oder valley                 | 2                                 | DK,ŁK,MAM    |
| <i>C. duodecimpunctata</i>     | C12    | Poland  | Vistula valley              | 1                                 | DK,ŁK,MAM    |
|                                |        | Ukraine | Dniestr valley              | 2                                 | DK,ŁK,MAM,VR |
|                                |        | Poland  | Oder valley                 | 2                                 | DK,ŁK,MAM    |
| <i>C. quatuordecimpunctata</i> | C14    | Poland  | Vistula valley              | 1                                 | DK,ŁK,MAM    |
|                                |        | Ukraine | Dniestr valley              | 2                                 | DK,ŁK,MAM,VR |

**Table 1.** Collecting localities of studied leaf-beetles. Collectors: MAM –Mazur; DK –Kubisz; ŁK –Kajtoch, VR –Rizun.

primer pairs EFs149 and EFα1R [30,31] and ITS1 and ITS2 [32]. The concentration of the reagents used for the amplification of COI, ITS1 and EF1-α markers and the cycling profile for PCR have been described previously [33,34]. After purification (NucleoSpin Extract II, Macherey-Nagel), the PCR fragments were sequenced using a BigDye Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems) and ran on an ABI 3100 Automated Capillary DNA Sequencer. All newly obtained sequences were deposited in GenBank (Accession nos. JF775778–JF775816). Sequences of other leaf-beetles were also downloaded from GenBank, including *Mimosestes ulkei* and *Donacia bicolor* for COI alignments (AB499964, EU880600) and, *Lysathia ludoviciana* and *Altica litigata* for ITS1 alignments (EU682397, EU682395). We failed to amplify ITS1 for *Clytra laeviuscula*.

### 2.3 Data analysis

The sequences were checked and aligned using BioEdit v.7.0.5.2 [35] and ClustalX software [36]. For the analyses, the appropriate nucleotide substitution model was first determined using MrModeltest 2.3 [38] in conjunction with PAUP\* [37] and using Akaike Information Criterion to select the best-fit model. Two methods for phylogeny reconstruction were used, Bayesian inference (BI) and maximum parsimony (MP). The former was run using MrBayes 3.1 [39] with one cold and three heated Markov chains for 3 000 000 generations and trees were sampled every 100<sup>th</sup> generation (according to [40]). Each simulation was run twice. Convergence of Bayesian analyses was estimated using Tracer v. 1.5.0 (41) and appropriate number of sampled trees were discarded as 'burn-in', and the remainder used to reconstruct a 50% majority rule consensus tree. Maximum parsimony was computed using PAUP\* 4.0b10 [37]. For all MP analyses, heuristic search with tree bisection-reconnection (TBR)

branch swapping and random addition sequences with MaxTrees=500 were conducted with 500 random addition replicates. Gaps were treated as fifth character state. Node support was assessed with the bootstrap technique using 5000 pseudoreplicates and TBR branch swapping. Tree reconstruction was performed separately for each marker. All trees were visualized with TreeView 1.6.6 [42]. Pairwise distances were calculated using MEGA v.5 [43] and Kimura 2-parameter model (K2P) to provide valuable information for taxon circumscription within the ingroup (*Crioceris* genus) (see [44]). Haplotypes were determined using DnaSP v.5 [45].

## 3. Results

In 788 bp of COI and 441 bp of EF1-α, no indels were observed. The EF1-α sequences were highly heterozygous in *C. duodecimpunctata* (41 variable characters), *C. quatuordecimpunctata* (50) and *C. quinquepunctata* (50) and slightly heterozygous in *C. asparagi* (4). This high level of heterozygosity indicates that the amplification of double or multiple PCR products is occurring (two or more copies of the same gene or a gene and a pseudogene(s)), therefore EF1-α was not included in further analyses. The ITS1 sequences were variable structurally among the *Crioceris* species, the analysis revealed many indels leading to different sequence lengths in particular species (from 606 bp to 650 bp). Standard genetic indices for all the studied *Crioceris* species are presented in Table 2.

The best nucleotide substitution model for MrBayes analyses of the COI and ITS1 respectively were as follows: GTR+G+I (proportion of invariable sites I=0.458; gamma distribution shape parameter G=0.894; -lnL=3717.25; AIC=7454.50) and the GTR+I (the proportion of invariable sites I=0.366; -lnL=1710.76; AIC=3439.52).

| Species                        | [L] | [V] | [S] | [L]     | [V] | [S] |
|--------------------------------|-----|-----|-----|---------|-----|-----|
|                                | COI |     |     | ITS1    |     |     |
| all studied <i>Crioceris</i>   | 788 | 213 | 211 | 669     | 144 | 144 |
| <i>C. asparagi</i>             | 788 | 6   | 6   | 606     | 0   | 0   |
| <i>C. duodecimpunctata</i>     | 788 | 24  | 8   | 650     | 0   | 0   |
| <i>C. quatuordecimpunctata</i> | 788 | 11  | 8   | 620     | 0   | 0   |
| <i>C. quinquepunctata</i>      | 788 | 1   | 1   | 616-620 | 2   | 2   |

**Table 2.** Standard genetic indices of genetic markers (COI and ITS1) calculated for studied *Crioceris* species. L – sequence length; V – number of variable sites; S – number of segregating sites.

| <i>Crioceris</i> species    | <i>asparagi</i> | <i>quinquepunctata</i> | <i>duodecimpunctata</i> | <i>quatuordecimpunctata</i> |
|-----------------------------|-----------------|------------------------|-------------------------|-----------------------------|
| <i>asparagi</i>             | 0.0-0.1/0.0     |                        |                         |                             |
| <i>quinquepunctata</i>      | 18.6/20.4       | 0.0-0.2/0.0            |                         |                             |
| <i>duodecimpunctata</i>     | 18.7/23.2       | 20.3/10.3              | 0.1-2.4/0.0             |                             |
| <i>quatuordecimpunctata</i> | 18.4/21.1       | 17.0/7.8               | 16.9/10.2               | 0.0-1.3/0.0-0.4             |

**Table 3.** Average pairwise distances of genetic markers (COI and ITS1; %): interspecific (across the *Crioceris* species) and intraspecific (for the studied specimens of a particular species).

Maximum parsimony heuristic searches resulted in six COI trees [length=682 steps; consistency index (CI)=0.7023; retention index (RI)=0.8613] based on 234 parsimony-informative characters; and two ITS1 trees [length=199 steps; (CI)=0.9598; (RI)=0.9903] based on 484 parsimony-informative characters. Maximum parsimony and Bayesian methods resulted in similar topologies.

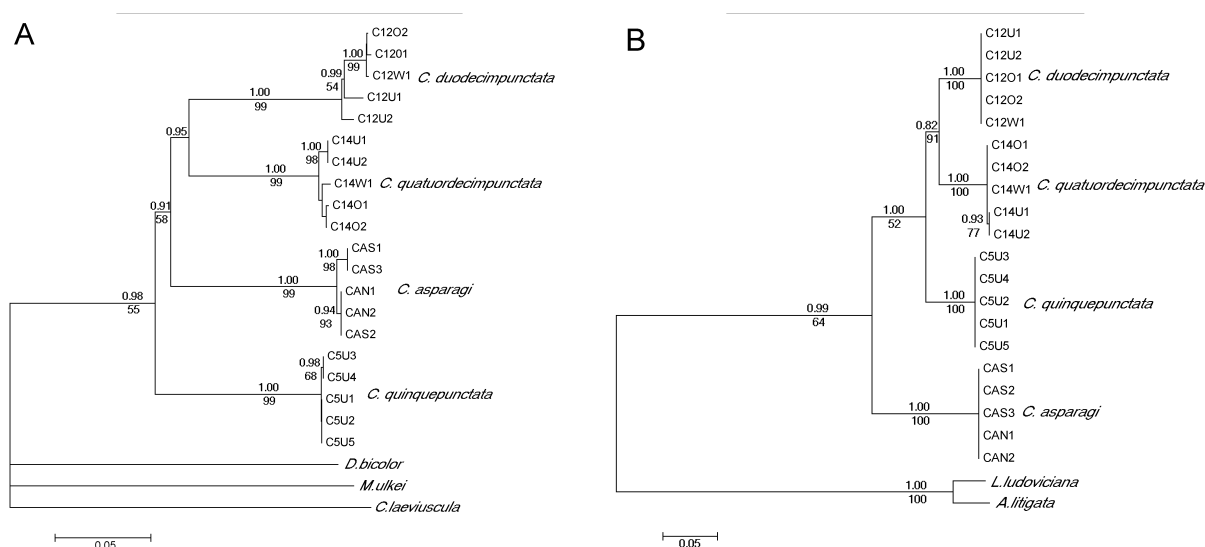
The COI and ITS1 phylogenetic trees (Figure 1) showed that the *Crioceris* species formed a monophyletic clade. To investigate the validity of monophyly of *Crioceris* genus as well as that of the single species, the COI histogram based on the pairwise K2P distance matrix was built (Figure 2). All studied species formed a well-defined entity with respect to outgroups, with K2P distances ranging from 16.9 to 20.3 (mtDNA; Figure 2) and from 7.8 to 23.2 (ITS1; Table 3) and the boundaries between species were clearly defined (Figure 1 and 2). The distances between members within each taxon were always much lower than those among the four taxa (Figure 2 and Table 3). The greatest intraspecific mitochondrial distances were found between the Polish and Ukrainian populations of *C. duodecimpunctata* and *C. quatuordecimpunctata*.

The mitochondrial phylogenetic tree (Figure 1) shows that the studied *Crioceris* beetles are related as follows: (*quinquepunctata* (*asparagi* (*duodecimpunctata*, *quatuordecimpunctata*))), but this order is supported only by Bayesian posterior probabilities. In the ITS1 phylogenetic tree, the species order was slightly different: (*asparagi* (*quinquepunctata* (*duodecimpunctata*,

*quatuordecimpunctata*))). However, on both of these trees, *C. quatuordecimpunctata* is clearly separated into two subclades: Polish and Ukrainian. Similarly, in the case of *C. duodecimpunctata*, specimens from Polish and Ukrainian localities possess different mtDNA haplotypes, but not nuclear (ITS1) genotypes. In the case of *C. asparagi*, some clades are present on the COI tree, but they are not correlated with the geographic origin of the studied specimens and they have the same nuclear (ITS) genotypes.

## 4. Discussion

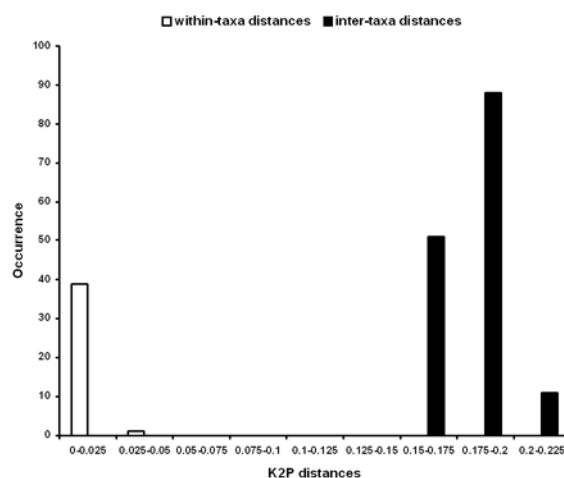
Phylogenetic inferences support the monophyly of the *Crioceris* genus. Genetic distances consistent with the intra-specific level are confirmed by the barcoding approach. Also the monophyly of the species as currently defined is supported, as the mtDNA clades (and ITS1 clades) exhibit strong identities based on barcoding evidence. Among the three species most similar in terms of morphology (*C. duodecimpunctata*, *C. quatuordecimpunctata* and *C. quinquepunctata*), only *C. duodecimpunctata* and *C. quatuordecimpunctata* appeared to be sister species, being most closely related in respect to both mitochondrial DNA and nuclear ITS1 marker. Surprisingly, *C. quinquepunctata*, and not *C. asparagi*, turned out to be the most external species according to COI, but on the ITS1 phylogenetic tree *C. asparagi* was outermost. However, mitochondrial distances



**Figure 1.** Phylogenetic bayesian trees of four *Crioceris* species (CA - *C. asparagi*, C5 - *C. quatuordecimpunctata*, C12 - *C. duodecimpunctata* and C14 - *C. quinquepunctata*) and outgroups constructed using COI (A) and ITS1 (B) markers. U – Ukrainian populations; Polish populations: W – from lower Vistula valley, O – from lower Oder valley, N – middle Noteć valley and S – Silesia. Upper numbers indicate posterior probabilities of Bayesian inference, lower numbers – bootstrap values for maximum parsimony trees (shown only if above 0.50 and 50%, respectively).

were at similar levels among all of the studied *Crioceris* species. In respect of ITS1 only, *C. asparagi* appeared to be the most distant (by a factor of 2) from the other *Crioceris* species. Similar levels of mitochondrial genetic differences among all the *Crioceris* species can be explained by the simultaneous speciation of all of the studied asparagus beetles. The large distance of *C. asparagi* in respect of ITS1 may have resulted from the presence of many indels which differentiated *C. asparagi* from the other species, but this does not necessary mean that this species is more distinct from the other *Crioceris*. On the other hand, the almost complete absence of intraspecific diversity in this marker can be understood in terms of concerted evolution of tandemly arrayed ribosomal DNA [46,47]. The ancestor of these species (and probably of all other *Crioceris* species) must have divided within a short time frame into progenies or species.

The low numbers of specimens used per species in this study do not permit any conclusions about differences in the genetic diversities of the studied asparagus beetles to be made. However, *C. asparagi* seems to be the least diversified in respect of all the markers. This probably results from its high natural or man-induced mobility and expansion across most of the world. In this species, the level of gene flow is probably high, which prevents its genetic differentiation. The lack of geographic differentiation of *C. asparagi* populations shows that neither COI nor ITS1 can be used for the identification of the source populations



**Figure 2.** Barcoding K2P distances histograms at the within - (in white) and among (in black) - taxa levels.

of the common invasive asparagus beetles in North America or Australia. For *C. duodecimpunctata*, some differentiation in the mitochondrial marker was found, which suggests that COI could be helpful in the detection of the ancestral populations of these introduced populations. More extensive studies, with more specimens from many localities across the species range should be undertaken to test the usefulness of COI for identifying the source populations of these two species, that are serious pests outside their native ranges.

The identification of separate clades in both of the used markers for the Polish and Ukrainian populations of *C. quatuordecimpunctata* provides some evidence that these populations are isolated and probably can be treated as separate evolutionary units. To check if geographically isolated populations of this steppe species, rare and threatened in central and eastern Europe, truly consist of separate conservation units, further research on many populations is needed. Conservation units have been so far identified for the central and eastern European populations of the xerothermic weevil *Centricnemus leucogrammus* (Germ.) [48], and that pattern is likely to be common to many steppe-xerothermic beetles and other invertebrates.

The mitochondrial barcode test showed that the COI gene varies greatly among the *Crioceris* species and can be useful for species identification, especially in those species where eggs or other juvenile stages are not described. It could also be helpful in the identification of pest species (*C. asparagi* and *C. duodecimpunctata*) in North America and the threatened populations of *C. quatuordecimpunctata* and *C. quinquepunctata* in central and eastern Europe. Additionally, ITS1 markers provide evidence that all of the four studied species are clearly different with respect to their nuclear DNA. No signs of a possibility hybridization event were found, and the considerable genetic distances among the species also demonstrated that they have been separated from each other for a long period of time.

The utility of the COI mitochondrial gene as a DNA barcoding system for the identification or verification of the taxonomic status of species has been tested in many animal groups and has shown promising results in some cases (e.g., for leaf-beetles and weevils: [49-52]) and bugs [53,54]), but also some constraints in others (e.g., [25,55,56]). Similar research on the application of DNA barcoding for testing taxonomic and phylogenetic species concepts of closely related species of leaf-beetles were performed e.g. on *Oreina* genus [57,58] and *Bruchidius* genus [59]. As opposed to *Oreina* leaf-beetles, which some morphological species were not valid species and others were species complexes [57,58] and similarly to *Bruchidius* beetles [59], *Crioceris* species turned out to be monophyletic and clearly distinct from each other in respect to their mtDNA. This validation was also supported by the unequivocal differentiation of the species with respect to the ITS1 nuclear marker. The mtDNA clades exhibit strong identities based on barcoding evidence and provide useful guidelines to define species boundaries among *Crioceris* leaf-beetles.

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