

DNA Barcoding Applied to Invasive Leafminers (Diptera: Agromyzidae) in the Philippines

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Ann. Entomol. Soc. Am. 99(2): 204–210 (2006)

ABSTRACT DNA barcoding involves the sequencing of a single gene region from all species to provide a means for identifying all of life. Although appealing to many scientists, this idea has caused considerable controversy among systematists. We applied a DNA barcoding approach to outbreak populations of invasive *Liriomyza* spp. leafminer pests in the Philippines to explore the use of barcoding in a relatively well studied, economically important group. We sequenced a 527-bp portion of mitochondrial cytochrome oxidase I (COI) from 258 leafminers from 26 plant host species in the Philippines. Neighbor-joining and parsimony analysis were used to compare COI sequences from the Philippines to an extensive database of COI sequences previously obtained from samples of the invasive leafminers *Liriomyza huidobrensis* (Blanchard), *Liriomyza trifolii* (Burgess), and *Liriomyza sativae* Blanchard from locations around the world. We conclude that although a DNA barcoding approach can provide rapid species identifications, in certain instances it is likely to either overestimate or underestimate the number of species present. Only when placed within the context of considerable other data can DNA barcoding be fully interpreted and used. For economically and medically important species, which can be well studied, DNA barcoding offers a powerful means for rapid identifications.

KEY WORDS DNA taxonomy, DNA barcodes, *Liriomyza*, Agromyzidae

RECENTLY, DNA BARCODING HAS been proposed as a universal method for both identifying species and uncovering biological diversity (Hebert et al. 2003, 2004a,b; Barrett and Hebert 2005). DNA barcoding involves the collection and databasing of DNA sequence data from a single gene region (generally a 688-bp piece of mitochondrial cytochrome oxidase I [COI]) from every species. To date, published research explicitly addressing DNA barcoding has primarily focused on showing that this type of universal identification system is feasible in a variety of organisms (Hebert et al. 2003, 2004b; Barrett and Hebert 2005). Applications of DNA barcoding have been limited to a few studies (Hebert et al. 2004a), although the more general field of mitochondrial phylogeography has provided similar information on a wide range of organisms for the past decade or two (Avise 2000). Critics of DNA barcoding object to the potential abandonment of traditional morphological characters and argue that relying on a single mitochondrial gene region for identification can be misleading, particularly in the face of widespread mitochondrial polyphyly/paraphyly (Sperling 2003; Will and Rubinoff 2004). These criticisms apply most strongly to applications of barcoding used to address questions of biological di-

versity in groups that have received little traditional taxonomic study. Yet, study of just such groups, the proponents of barcoding argue, will benefit most from a barcoding approach.

With a few exceptions (e.g., forensic entomology), the application of DNA barcoding to the management of economically and medically important species has only just begun. In many insect groups, important pest species belong to species complexes comprised of morphologically cryptic species, making the traditional identification of specimens based on morphology difficult even for taxonomic specialists (Busvine 1980, Della Torre et al. 2002, Clark et al. 2005). In addition, many economically and medically important insect species are globally invasive and require immediate identification and management when discovered beyond their natural range. DNA barcoding of pest species could allow rapid identification of such populations. Because pest species are usually obvious and abundant enough for thorough sampling within natural as well as introduced ranges, an extensive phylogeographic database could be readily compiled to further enhance the information content of barcode data. Finally, because DNA barcoding can be performed on any life stage, reliable identifications of juvenile or even partial specimens are possible (Palumbi and Cipriano 1998, Symondson 2002).

One complication of using a strict DNA barcoding approach based on a single mitochondrial gene is that it encourages the adoption of fairly simplistic criteria

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for determining species limits (Sperling 2003, Will and Rubinoff 2004, Prendini 2005). In the absence of additional sources of information on species limits, mitochondrial barcoding necessarily relies on some combination of mitochondrial monophyly and genetic distances to indicate probable species. However, it is well documented that many species, as defined by other criteria, do not exhibit mitochondrial monophyly (reviewed in Funk and Omland 2003) and that mitochondrial genetic distances can vary dramatically both within and between species (e.g., Funk 1999, Scheffer and Wiegmann 2000, Scheffer and Grissell 2003). Therefore, we should not expect mitochondrial barcoding by itself to always correctly delimit species or provide correct species identifications (Sperling 2003, Will and Rubinoff 2004). Determining under which circumstances DNA barcoding can be used reliably is important, particularly when economically or medically important organisms are concerned.

To investigate the utility of barcoding for management of economically important species, we apply barcoding to invasive populations of leafmining flies for which considerable information on species limits already exists. *Liriomyza* Mik leafminers (Diptera: Agromyzidae) are notorious pests of vegetable and flower crops with outbreak populations capable of causing the loss of entire crops (Shepard et al. 1998). Most damage is caused by three polyphagous species: *Liriomyza huidobrensis* (Blanchard), *Liriomyza trifolii* (Burgess), and *Liriomyza sativae* Blanchard (Spencer 1973, Parrella 1987, Rauf et al. 2000, Andersen et al. 2002). All three species are endemic to the Americas, but each has been spread to various new locations around the world (Spencer 1973, Andersen et al. 2002, Hofsvang et al. 2005, Scheffer 2005). These three species commonly occur together, although their individual invasion histories differ. They also can be found co-occurring with many of the other 23 *Liriomyza* species reported as pests as well as with other pest leafminers (Spencer 1973). Although identification of adults of these species is possible, primarily by male genitalia dissection, this is a labor-intensive and time-consuming activity that is very difficult for nonspecialists, particularly when dealing with field collections comprising several different leafmining species. Additionally, the juvenile forms, larvae and pupae, are the life stages most commonly intercepted at international ports of entry, but they cannot be definitely identified using traditional morphological characters.

Recent phylogeographic research using sequence data from the mitochondrial COI gene has indicated that a DNA barcoding approach could be a valuable tool for the identification of pest leafminers. This research has shown that the three highly invasive species, *L. huidobrensis*, *L. trifolii*, and *L. sativae*, belong to what seem to be species complexes containing morphologically cryptic lineages. For example, *L. huidobrensis* is morphologically indistinguishable from its sister species *Liriomyza langei* Frick, although both mitochondrial and nuclear loci have diverged in the two species (Scheffer 2000, Scheffer and Lewis 2001). For *L. trifolii* and *L. sativae*, only mitochondrial data

are currently available, and although the divergences between major clades within each species are large, the question of whether these divergences represent biologically distinct cryptic species remains open. To date, worldwide surveys of invasive populations of these species have shown that only one of the major mitochondrial clades within each morphospecies is present in invasive populations (Scheffer 2005, Scheffer and Lewis 2005).

We applied DNA barcoding to recent outbreaks of invasive leafminers from vegetable growing regions in 10 provinces of the Philippines. The main objectives were to 1) determine the identity of the pest species present and 2) to evaluate the utility of DNA barcoding in an economically important group in which considerable complexity exists in mitochondrial structure. Because the primary purpose of this report is to focus on the application of DNA barcoding to an agricultural problem, detailed analysis of the geographic distributions and host use of the leafminers within the Philippines will be published elsewhere. In the current study, "species" generally refers to biological species, "morphospecies" refers to a group that seems to be one species on morphological grounds but may contain cryptic species, and "cryptic species" refers to morphologically indistinguishable but apparently biologically distinct species.

Materials and Methods

Leafminer samples were collected from 26 different host plants, primarily crops, from a total of 10 provinces in two island regions of the Philippines (Table 1). Specimens were preserved in 95% ethanol and stored at -80°C . DNA was extracted either from larval, pupal, or adult specimens by using the DNeasy insect protocol B (QIAGEN, Valencia, CA). Because DNA sequence data were to be used to identify specimens, rigorous identification of specimens (i.e., dissection of genitalia from male specimens) before DNA extraction was not undertaken, although S.J.S. visually inspected each specimen and made a note of probable species identity for most specimens.

Amplification of nearly the entire gene of mitochondrial COI was performed using the polymerase chain reaction (PCR) and the primers C1-J-1535 (AT-TGGAACITTTATATTTTATATTTGG) (Scheffer and Wiegmann 2000) and TL2-N-3014 (TCCATTGCAC-TAATCTGCCATATTA) (Simon et al. 1994). The PCR was carried out using a Mastercycler gradient thermocycler (Eppendorf Scientific, Inc., Westbury, NY) with a touchdown amplification program as follows: initial denaturation at 92°C for 2 min, followed by two touchdown cycles from 58 to 46°C (10 s at 92°C , 10 s at $58-46^{\circ}\text{C}$, 2 min at 72°C), 29 cycles of 10 s at 92°C , 10 s at 45°C , 2 min at 72°C , and a final extension step for 10 min at 72°C . A single fragment of 1533 bp resulted. PCR product was purified using the QIAquick PCR purification kits (QIAGEN).

We sequenced a 550-bp portion of the amplified fragment at the 3' end of COI by using the internal primer C1-J-2441 (5'-CCTACAGGAATTTAAATTTT-

Table 1. Samples of leafminers collected from host plants in the Philippines

Host plant (common name)	<i>L. sativae</i>	<i>L. trifolii</i>	<i>L. huidobrensis</i>	<i>L. chinensis</i>	<i>L. brassicae</i>	<i>C. horticola</i>	<i>O. phaseoli</i>
<i>Abelmoschus esculentus</i>	2						
<i>Allium cepa</i> var. <i>cepa</i>			4	7			
<i>Apium graveoleus</i> var. <i>dulce</i>			6				
<i>Arctium lappa</i>			4				
<i>Brassica juncea</i>	1	1	5				
<i>B. oleracea</i> var. <i>capitata</i>	1	1	7		4		
<i>B. oleracea</i> var. <i>italica</i>			3				
<i>B. rapa</i>	1	3	2			1	
<i>Citrullus lanatus</i> var. <i>lanatus</i>	5						
<i>Cucumis sativus</i> var. <i>sativus</i>	8						
<i>Cucurbita</i> sp.	11						
<i>Cucurbita pepo</i> var. <i>pepo</i>	3		2				
<i>Daucus carota</i> subsp. <i>sativus</i>	2	2	4				
<i>Chrysanthemum</i> sp.			4				
<i>Emilia sonchifolia</i>			4				
<i>Lactuca sativa</i> var. <i>capitata</i>			8			1	
<i>Luffa acutangula</i>	4						
<i>Lycopersicon esculentum</i> var. <i>esculentum</i>	25	8	5				
<i>Phaseolus</i> sp.	32	21	16				4
<i>Pisum</i> sp.		4	4			1	
<i>Raphanus sativus</i> var. <i>sativus</i>			1			3	
<i>Rorippa indica</i>			1				
<i>Sida</i> sp.			4			6	
<i>Solanum melongena</i>	4						
<i>S. tuberosum</i>	4						
<i>Vigna unguiculata</i>	4						
Total	107	40	84	7	4	12	4

On the island of Luzon, samples were collected from the provinces of Abra, Banaue, Benguet, Ifugao, Ilocos Sur, La Union, Mountain, Nueva Ecija, Nueva Vizcaya, and Pangasinan. On the Island of Mindanao, samples were collected from the province of Bukidnon.

TAG TTGATTAGC-3') and the external primer TLN-3017 (see above). Sequencing reactions were carried out using Big Dye Terminator sequencing kits (Applied Biosystems, Foster City, CA) and electrophoresed on an ABI 377 automated DNA sequencer (Applied Biosystems). Most sequences were confirmed by sequencing in both directions. In a few cases, in which one of the primers failed to sequence well, we used unconfirmed sequence data, but only when the electropherograms were very clean; unconfirmed sequence data were usually corroborated by identical sequences from other individuals in the study. Sequencher (Gene Codes Corp., Ann Arbor, MI) was used to assemble contigs and to align the final consensus sequences. Sequences from flies collected in the Philippines have been deposited in GenBank under accession numbers DQ150731–DQ150988. Voucher specimens associated with sequences have been deposited in the National Museum of Natural History (Washington, DC).

Sequences from 258 individuals collected in the Philippines were added to a data set of 307 COI sequences previously collected in studies of mitochondrial variation in worldwide populations of the morphospecies *L. huidobrensis/L. langei* ($n = 61$ individuals; Scheffer 2005; S.J.S. and M.L.L., unpublished data), *L. sativae* ($n = 110$ individuals; Scheffer and Lewis 2005), and *L. trifolii* ($n = 136$ individuals; S.J.S. and M.L.L., unpublished data). Identical sequences in the combined data set of 565 sequences were removed so that each sequence (hereafter "haplotype") was represented only once in subsequent analyses.

Neighbor-joining and parsimony analyses were conducted using the program PAUP*4.0b8 (Swofford 2001). During the neighbor-joining analysis, ties were broken randomly, and bootstrapping was performed with 500 replicates. Parsimony analyses were conducted using the heuristic search feature of PAUP* with 50 random addition replicates. The search was stopped at 25,000 equally parsimonious trees and a strict consensus tree was generated. All analyses were rooted using sequence data from the agromyzid *Cerodontha dorsalis* (Loew), a suitable outgroup for investigating relationships among *Liriomyza* species because it is in the same subfamily (Phytomyzinae) (Spencer and Steyskal 1986).

Results

Mitochondrial COI was sequenced from 258 specimens from the Philippines. When these sequences were combined with previously obtained sequences for *L. huidobrensis/L. langei*, *L. sativae*, and *L. trifolii*, the combined data set of 565 sequences contained 70 different haplotypes. Both neighbor-joining and parsimony analyses of a data set having each haplotype represented only once gave similar results with respect to species clusters, although only the neighbor-joining tree is presented (Fig. 1). All nodes in Fig. 1 shown with bootstrap support are also nodes that were recovered in the strict consensus of 25,000 trees resulting from parsimony analysis. Bootstrap values for all species clusters, and even major clades within species, were 100% or very close (Fig. 1).

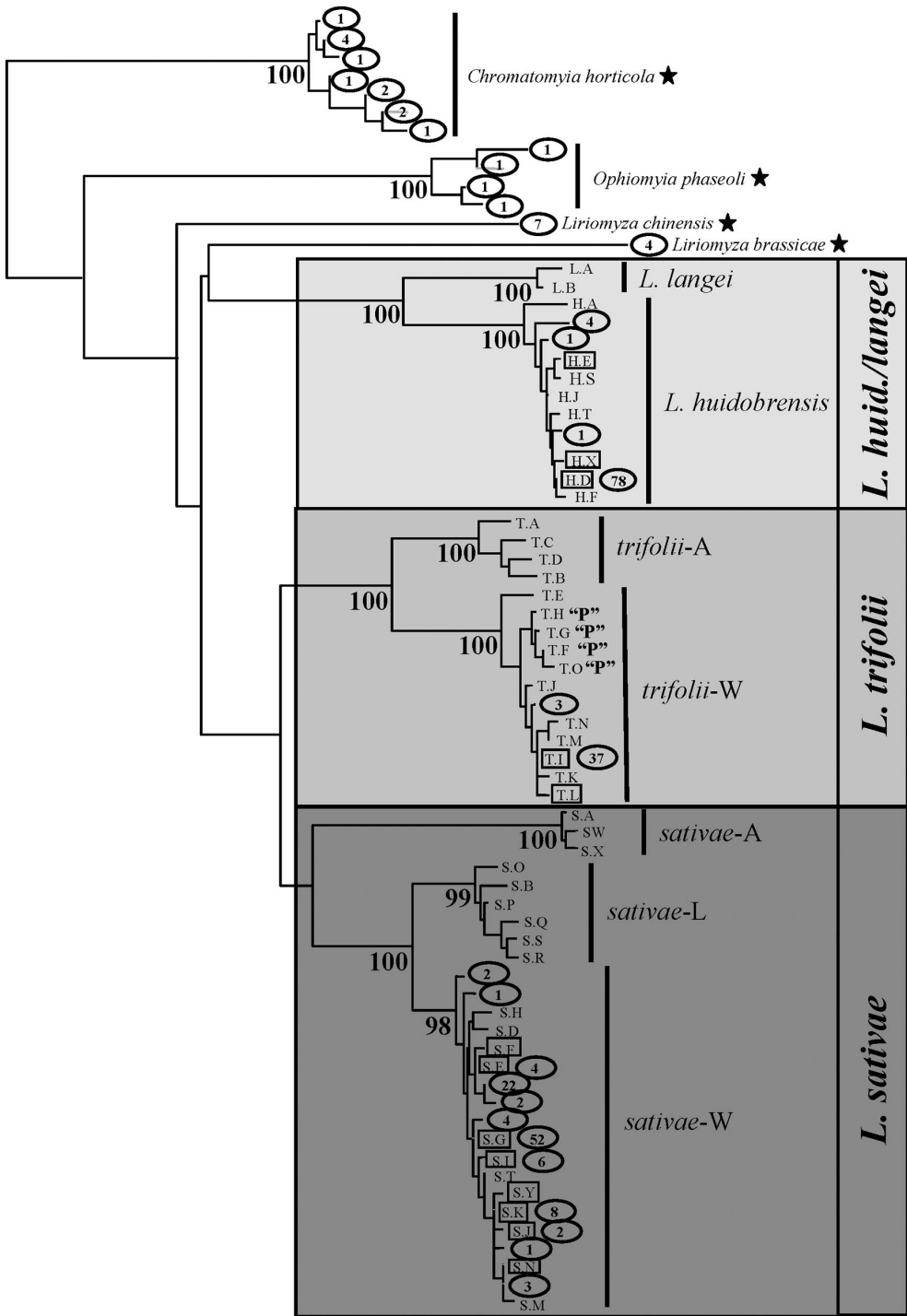


Fig. 1. Neighbor-joining tree of mitochondrial COI sequences from leafmining flies in the Philippines. Neighbor-joining bootstrap values are presented for nodes recovered in the parsimony strict consensus of 25,000 trees. Haplotypes present in the Philippines are indicated by ovals encircling the frequency of that haplotype in the Philippine samples. Named haplotypes (e.g., "T.A," "S.B") are those found previously in studies of mitochondrial variation in the three morphospecies *L. huidobrensis/langei*, *L. trifolii*, and *L. sativae* (Scheffer 2000; Scheffer and Lewis 2005; S.J.S. and M.L.L., unpublished data), and haplotypes in boxes are those that have been found previously in invasive populations. Haplotypes followed by the letter "P" within *L. trifolii* indicate a pepper-feeding clade. Stars indicate four presumptive species having haplotypes highly diverged from the three focal morphospecies.

Table 2. Uncorrected pairwise distances (percentages) among COI sequences from leafminers collected in the Philippines

	<i>L. huid</i>	<i>L. trifolii</i>	<i>L. sativae</i>	<i>L. chinensis</i>	<i>L. brassicae</i>	<i>C. horticola</i>	<i>O. phaseoli</i>
<i>L. huid</i>	0–1.3	12.0–12.7	10.4–13.6	13.5–14.5	13.6–14.7	14.3–16.9	15.2–17.2
<i>L. trifolii</i>		0–0.2	8.0–8.8	13.1–13.4	13.8–13.9	14.8–16.5	13.2–14.7
<i>L. sativae</i>			0–1.2	9.8–12.1	12.2–13.0	12.7–16.6	13.1–16.5
<i>L. chinensis</i>				0	14.9	15.7–17.8	13.7–15.1
<i>L. brassicae</i>					0	17.0–17.5	16.1–17.9
<i>C. horticola</i>						0–1.3	12.2–15.0
<i>O. phaseoli</i>							0.4–1.9

Intraspecific distances of flies collected in the Philippines shown in bold.

In both the neighbor-joining and the parsimony analyses, haplotypes representing 231 (89%) of the Philippine COI sequences clustered with the three invasive *Liriomyza* species (Fig. 1), with 84 specimens representing *L. huidobrensis*, 40 specimens representing *L. trifolii* (clade-W), and 107 specimens representing *L. sativae* (clade-W). The intraspecific uncorrected pairwise differences for the Philippine samples of these three species ranged from 0 to 1.3%. All of the mitochondrial haplotypes of these three morphospecies found in the Philippine samples belong to the mitochondrial clade within each species previously found to be globally invasive (Fig. 1).

The remaining 27 of the 258 specimens carried haplotypes that were >9.8% different from haplotypes of the three invasive polyphagous *Liriomyza* species. Neighbor-joining as well as parsimony analyses clustered these outlier haplotypes into what seem to represent four additional species. Among these four presumptive species, intraspecific variation ranged from 0 to 1.9% and interspecific variation ranged from 12.2 to 17.8% (Table 2). These sequences were compared with reference sequences currently being compiled into an agromyzid sequence database in our laboratory. Sequences from three of the additional species were found to be within 2.0% of sequences obtained previously from identified specimens of *Liriomyza brassicae* (Riley), *Liriomyza chinensis* (Kato), and *Chromatomyia horticola* (Goureau), all of which are pest species endemic to or previously reported from Asia (Spencer 1973). The fourth species, collected from beans (*Phaseolus* sp.), was at least 9.2% divergent from all other agromyzids in the data set (65 species in 18 genera), precluding a positive identification from the DNA barcoding approach. We reared adults of this species, which allowed the identification of *Ophiomyia phaseoli* (Tryon), an Old World pest of beans, based on male genitalic morphology.

It should be noted that the tree structure as presented in Fig. 1 does not necessarily represent the phylogeny of the included species because *C. dorsalis* is not an appropriate outgroup for using this data set to determine phylogenetic relationships. *O. phasioli* is in a different subfamily (Agromyzinae) from the other species and would itself be an appropriate choice for outgroup rooting of this data set. But this would not be known until the data set is used for species identifications. Because whether or not the outgroup is appropriate for phylogenetic reconstruction does not affect the interpretation of the results in terms of DNA

barcoding and species identification, for illustrative purposes, we chose to leave the analysis and presentation as originally conceived.

Discussion

Mitochondrial Variation. In most cases, introduced or invasive populations are expected to exhibit reduced genetic variation because of bottlenecks that they tend to pass through during an introduction (Nei et al. 1975). This is especially true of a marker such as the mitochondrion, which is both haploid and maternally inherited. As expected, Philippine populations of the three invasive focal species, *L. huidobrensis*, *L. sativae*, and *L. trifolii*, exhibit fewer mitochondrial haplotypes than are present within their endemic ranges (Fig. 1). This is especially true of *L. huidobrensis* and *L. trifolii* in which only a few haplotypes are present with a single haplotype for each species predominating (Fig. 1). These results are similar to findings of a previous study of invasive *L. huidobrensis* in China, which found only a single mitochondrial (cytochrome oxidase II) haplotype present in 53 specimens from nine hosts and eight locations within Yunnan province (He et al. 2002).

For *L. sativae*, although fewer haplotypes are present in Philippine populations than in the entire endemic range, genetic variation is not as reduced as in the other two focal species, and a single haplotype is not predominant. This is consistent with findings of a previous study that nearly all haplotypes within the lineage *sativae*-W can be found in geographic regions outside of the Americas (i.e., in introduced populations) (Scheffer and Lewis 2005). Why *L. sativae* seems to retain more genetic variation during introductions is not currently known, but it is an intriguing question.

DNA Barcoding. The application of DNA barcoding to invasive populations of leafminers in the Philippines illustrates the complex issues facing molecular identification. In this example, DNA barcoding assigned *Liriomyza* specimens to morphospecies, as currently defined, and even to mitochondrial lineages within species. However, these identifications rely to a large degree on the phylogenetic framework provided by prior analyses of the focal species by using both morphological as well as molecular characters (Scheffer 2000, 2005; Scheffer and Lewis 2005). If it were not already known that these morphospecies contain highly diverged mitochondrial lineages, it is likely that

DNA barcoding would result in an overestimate of the number of species present, depending on which sequences were used as reference sequences. For example, if the only reference sequences available for *L. trifolii* were T.A, T.B, T.C, and/or T.D (Fig. 1), we might conclude that a dramatically new species had been detected in the Philippines (Fig. 1, *trifolii*-W). Although we do not currently know whether the highly diverged mitochondrial clades within *L. sativae* and *L. trifolii* represent distinct biological species, it is premature to consider them as such based only on mitochondrial data. Indeed, preliminary sequence data from several nuclear genes does not show evidence of divergence between clades-A and -W in either *L. trifolii* or *L. sativae* (S.J.S. and M.L.L., unpublished data).

In addition to potentially overestimating the number of species, DNA barcoding also can underestimate the number of species by failing to recognize shallowly diverged species (Mortiz and Cicero 2004). Within *L. trifolii*, recent studies have found evidence for a genetically and behaviorally distinct species that feeds only on peppers within the clade *trifolii*-W (Fig. 1, "P") (Morgan et al. 2000; Reitz and Trumble 2002; S.J.S. and M.L.L., unpublished data). Because this pepper-feeding clade is <1% different in mitochondrial COI from other *trifolii*-W, it would probably not be recognized as a distinct species by a DNA barcoding approach despite its differing biology and apparent reproductive isolation from other *L. trifolii*.

The three focal *Liriomyza* species are all widespread and polyphagous, possibly making them more prone to potentially complicating population divergences than other species. However, reports of deep mitochondrial divergences within single species or shallow or no mitochondrial divergence between two closely related but morphologically or behaviorally distinct species are not uncommon (Avisé 2000). Additionally, a recent survey of published mitochondrial trees in animals found that 23% of animal species do not show reciprocal monophyly (Funk and Omland 2003). For these reasons, DNA barcoding based on a single mitochondrial region alone cannot be used for unambiguous species discovery and must be used with care for species identification.

In this study, DNA barcoding resulted in demarcation of four nonfocal species as distinctly different from the three focal species and from each other. The ability to judge sequences as representing something distinctly different is critical to any universal identification method and is especially important to the identification of economically or medically important species, where false positives may affect international trade and cause economic losses (Levy et al. 2001). However, confirmation of species status of the four nonfocal species as well as determination of species identity did not come about until morphological characters were checked directly (in *O. phaseoli*) or indirectly (by comparison of the sequences with sequences from previously identified specimens, in *L. brassicae*, *L. chinensis*, and *C. horticola*). Thus, DNA barcoding is most valuable when combined with tax-

onomic data from other sources, such as morphology or even behavior.

In general, DNA barcoding holds tremendous promise as a means for generating large amounts of systematic data (Hebert et al. 2003, 2004a,b). However, mitochondrial DNA does not offer a perfect metric of biological divergence (Sperling 2003, Moritz and Cicero 2004, Prendini 2005). Inferences resulting exclusively from mitochondrial barcoding may prove to be misleading, either by overestimating or by underestimating the number of species and even in some cases by misidentifying species altogether. The errors associated with DNA barcoding will be reduced as additional data are collected from particular sets of organisms, providing a context for the interpretation of mitochondrial results. Armed with such data, DNA barcoding of economically and medically important species offers a powerful means for rapid identification.

Acknowledgments

We thank A. N. Bahatan, N. S. Baucas, L. Cuanguy, T. Mangali, G. L. Sacla, and E. A. Verzola for assistance collecting leafminers. We received valuable comments on the manuscript from L. Castlebury, D. Miller, K. Omland, C. Thompson, S. Gaimari, and two anonymous reviewers.

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Received 18 March 2005; accepted 17 November 2005.