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Molecular Entomology

Molecular Survey for the Invasive Leafminer Pest *Liriomyza huidobrensis* (Diptera: Agromyzidae) in California Uncovers Only the Native Pest *Liriomyza langei*

SONJA J. SCHEFFER,1,2 MATTHEW L. LEWIS,1 STEPHEN D. GAIMARI,3 AND STUART R. REITZ4


**ABSTRACT** *Liriomyza huidobrensis* (Blanchard) is a highly destructive invasive leafminer pest currently causing extensive damage to vegetable and horticultural crops around the world. *Liriomyza langei* Frick is a leafminer pest native to California that cannot currently be morphologically distinguished from *L. huidobrensis*. We used a DNA-barcoding approach, a published PCR-RFLP method, and a new multiplex PCR method to analyze 664 flies matching the morphological description of *huidobrensis–langei*. We found no evidence for the presence of *L. huidobrensis* in our extensive samples from California. In addition to the new molecular method, this work is important because it provides definitive data that the California "pea leafminer" is currently, and has probably always been, *L. langei*. These data will also be important in the event that the highly invasive *L. huidobrensis* ever becomes established.

**KEY WORDS** molecular diagnostics, multiplex PCR, invasive species, crop pest

The polyphagous leafmining fly *Liriomyza huidobrensis* (Blanchard) has become a notorious pest of a wide variety of vegetable and flower crops that number in the hundreds (Spencer 1973, Reitz and Trumble 2002). Originally described from Argentina (Blanchard 1926), since the 1980s, *L. huidobrensis* has become invasive and has spread to Europe, the Middle East, Asia, and North America (Canada; van der Linden 1990; Weintraub and Horowitz 1995; Head et al. 2000; Scheffer 2000; Scheffer et al. 2001, 2006; He et al. 2002). Newly established populations are often devastating, and it is currently considered one of the world’s most destructive agromyzid pests.

In 1951, Frick described a leafminer from peas in California as *Liriomyza langei* Frick (Frick 1951, Parrella 1982). Upon further examination, this species appeared to be morphologically indistinguishable from *L. huidobrensis* and was synonymized with *L. huidobrensis* by Spencer (1973). Scheffer (2000) found that South American and Californian *L. huidobrensis* s.l. differed substantially in mitochondrial cytochrome oxidase sequence data. These molecular differences were subsequently corroborated by differences in two independent nuclear genes, and the name *L. langei* Frick was revived for the California (plus Hawaii) populations (Scheffer and Lewis 2001). Invasive populations around the world sampled to date have invariably carried mitochondrial haplotypes belonging to the South American *L. huidobrensis* (e.g., those from Canada, China, Indonesia, Italy, Israel, Japan, Korea, the Philippines, South Africa, Sri Lanka, and Taiwan; Scheffer 2000; Scheffer et al. 2001, 2006; He et al. 2002; Takano et al. 2005 as reported in Takano et al. 2008). Most recently, Takano et al. (2008) found clear evidence of partial reproductive isolation between two colonies of flies molecularly identified as *L. langei* and *L. huidobrensis*. Currently, there is no evidence of these two species occurring in sympatry in any part of the world.

California is one of the primary vegetable-growing regions within the United States. Although *L. langei* has been known in California as “the pea leafminer” (at times as *L. huidobrensis*) and reported as a polyphagous pest since at least the 1930s (Chaney 1995), it was not until the 1990s that its pest status in California changed from minor secondary pest to an economically important crop pest (Chaney 1995, Reitz and Trumble 2002). It has been suggested that this change has come about due to the presence of a genetically distinct and more pestiferous population in the central coastal valleys that is spreading southward (Morgan et al. 2000, Reitz and Trumble 2002). Given the invasive nature of South American *L. huidobrensis* and the fact that currently it cannot be distinguished morphologically from *L. langei*, the question of whether invasive *L. huidobrensis* is present but un-
documented in California has never been adequately addressed. The purpose of this study was to conduct a molecular survey to determine whether *L. huidobrensis* is present but masquerading as *L. langei* in vegetable-growing regions of California. To accomplish this, we used molecular methods to screen extensive samples of adult flies collected from several regions and crops known to harbor *L. langei*. We paid particular attention to the populations in the central coastal valleys, where the change in pest status of presumed *L. langei* was first observed (Chaney 1995, Reitz and Trumble 2002).

**Materials and Methods**

Members of the California Department of Food and Agriculture collected leafmining flies by sweep-netting various crop hosts during the period 3–24 October 2006 in 11 counties of California: Fresno, Imperial, Monterey, Riverside, San Benito, San Diego, San Luis Obispo, San Mateo, Santa Barbara, Santa Cruz, and Ventura. Specimens were preserved in ethanol and stored at −80°C. Because multiple leafminer species were present, samples were sorted by S.J.S. to include only flies matching the external morphological description of *L. langei* and *L. huidobrensis*.

Two molecular methods were used to screen flies for evidence of *L. huidobrensis*. First, total genomic nucleic acids were extracted from the abdomens of individual male flies, and mitochondrial cytochrome oxidase I was amplified and sequenced. In the second method, female abdomens were pooled, and the resulting pooled genomic nucleic acids were screened using two PCR-based screening procedures. Males and females were treated differently to save genitalia of individual male specimens for morphological analysis while at the same time processing a large number of specimens while minimizing the time and expense of sequencing all specimens in the study.

**Males and DNA Sequencing.** Male specimens were dissected and the abdomens removed and subjected to the DNeasy tissue extraction procedure (Qiagen, Valencia, CA) without grinding. The essentially intact heads and thorax of each fly were retained as voucher material to be investigated further if anomalous results, evidence of *L. huidobrensis*, or both, were discovered during the screening. Female abdomens within each pooled sample were ground together for extraction using a micropestle and subjected to the DNeasy tissue extraction procedure.

Two independent PCR-based screening procedures were used to test pooled female extractions for the presence of *L. huidobrensis* DNA. The first method is a published PCR-RFLP procedure that uses a single pair of primers to amplify a 1,031-bp fragment of COI and COII in both *L. huidobrensis* and *L. langei* (Scheffer et al. 2001). Restriction digests of the amplification fragment using EcoRV and SpeI in separate reactions result in diagnostic bands of differing lengths for *L. huidobrensis* and *L. langei* that can be visualized on an agarose gel (for full details see Scheffer et al. 2001).

We tested positive controls (several independent DNA extractions of a single *L. huidobrensis* pooled with four *L. langei*) to ensure that any *L. huidobrensis* present would be detected even at the lowest frequency possible using this procedure. Although initial results using the positive controls were acceptable (data not shown), during the course of the actual screening, we detected unacceptable variation in the strength of the positive controls.

For this reason, we developed a second screening procedure using a CO site-specific primer PCR test to determine whether *L. huidobrensis* DNA was present in the pooled samples. This PCR test uses three primers specifically designed to be used together to amplify either a 356-bp piece of COI of *L. langei* or a 1,798-bp piece of COI and COII of *L. huidobrensis*, depending on which species’ DNA is present in a genomic template. The forward primer LirHL-F-1 (5′-TATAG TGYAAAACGGAGC-3′) was designed to perfectly match a region of COI of both *L. langei* and *L. huidobrensis*. The reverse primer Lang-R-2 (5′-ATAAT GYTGGTATAGGACCC-3′) was designed to perfectly match *L. langei* COI sequence and also to be different from *L. huidobrensis* sequence. During PCR, the use of this primer in combination with the forward primer, LirHL-F-1, selectively amplifies a 356-bp piece of only *L. langei* COI even in the presence of *L. huidobrensis* DNA. A second reverse primer, Huid-R-3 (5′-CCCTG CCGTAGCAACCA-3′), was designed similarly to selectively amplify a 1,798-bp piece of *L. huidobrensis* COI and COII. When all three primers are used in a single PCR reaction containing *L. langei*, *L. huidobrensis*, or both, species-specific DNA amplification
takes place. Because of differences in the length of the species-specific amplification products, visualization on agarose gels results in size-specific bands that are diagnostic for each species (Fig. 1, L. langei: Lanes A and F; L. huidobrensis: Lanes G and H). If both species are present in the same samples, both diagnostic bands will be present; we used multiple positive controls consisting of one L. huidobrensis to four L. langei to ensure sensitivity of the test to dilute L. huidobrensis DNA (Fig. 1, Lanes B–E).

Results

More than 1,000 agromyzid flies were received from the California Department of Food and Agriculture. Field crops and weeds from which the leafminer samples were swept included beans, broccoli, Brussels sprouts, cabbage, cauliflower, celery, cheeseweed, lettuce, mustard, peas, and spinach. Agromyzid samples from several of the sampled counties, Fresno, Imperial, Riverside, San Diego, and Ventura, contained no flies matching the external description of L. langei or L. huidobrensis, and are not considered further in this study.

We screened a total of 48 male specimens and 616 female specimens from the counties of Monterey, San Benito, San Luis Obispo, San Mateo, Santa Barbara, and Santa Cruz (Table 1). All DNA sequences from male specimens clustered with 100% bootstrap support with those previously obtained from L. langei (Fig. 2). Within the L. langei clade, there was no obvious clustering of sequences either by county of origin or by crop–weed association. Uncorrected pairwise distances between the L. huidobrensis and L. langei clades ranged from 4.8 to 5.0%. Distances within

Table 1. California county and crops from which screened flies were sweep-netted during October 2006

<table>
<thead>
<tr>
<th>County</th>
<th>Crop</th>
<th>No. of females screened</th>
<th>No. of males screened</th>
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<tr>
<td>Monterey</td>
<td>Broccoli, cauliflower, cheeseweed, lettuce</td>
<td>141</td>
<td>10</td>
</tr>
<tr>
<td>San Benito</td>
<td>Broccoli, cabbage, celery, lettuce, spinach</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td>San Luis Obispo</td>
<td>Broccoli, cabbage, cauliflower, celery, lettuce, spinach</td>
<td>129</td>
<td>0</td>
</tr>
<tr>
<td>San Mateo</td>
<td>Beans, Brussels sprouts, mustard, peas</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>Santa Barbara</td>
<td>Peas</td>
<td>76</td>
<td>8</td>
</tr>
<tr>
<td>Santa Cruz</td>
<td>Broccoli, lettuce, spinach</td>
<td>183</td>
<td>11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>616</strong></td>
<td><strong>48</strong></td>
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L. langei ranged from 0 to 1.4% and within L. huidobrensis from 0 to 0.7%, although this latter estimate does not reflect variation across the native range of L. huidobrensis (S.J.S. and M.L.L., unpublished data).

Limiting the analysis to only the DNA barcode region of COI yielded essentially the same results as with the full-length dataset: the two species formed monophyletic clusters with 100% bootstrap support for each species. All California specimens clustered with L. langei (phylogram not shown).

Using the taxon-specific priming approach for pooled female specimens, the resulting PCR bands in almost all cases indicated only the presence of L. langei. Anomalous bands were obtained in a few cases, and for these samples the voucher heads and thoraxes were extracted and sequenced. In no case was L. huidobrensis DNA recovered, although in a couple of cases the DNA indicated the presence of an additional, unidentified species (this is not surprising, as several nonpest agromyzid species in California are externally quite similar to L. langei or L. huidobrensis).

Discussion

Our molecular survey of 664 male and female flies matching the external description of L. huidobrensis

Fig. 2. Neighbor-Joining phenogram of L. langei COI sequences from specimens collected in California as well as previously published COI sequences from L. huidobrensis collected in South and Central America and elsewhere. Bootstrap values for the L. langei and L. huidobrensis clusters indicated on the branches. Within the L. langei cluster, previously published sequences are shown in bold. All of the sequences from L. huidobrensis and from L. bryoniae have been previously published; GenBank accession numbers are indicated on the figure for each published sequence.

- 0.001 substitutions/site
and *L. langei* found no evidence for the presence of *L. huidobrensis* in California. The counties from which leafminer samples were obtained were those known to or likely to contain “pea leafminers.” That only *L. langei* DNA was found in these counties (with the exception of a few highly divergent sequences from unidentified species) indicates that *L. huidobrensis* was not present in these regions in detectable numbers during October 2006. Given the multivoltine life cycle of *L. huidobrensis*, it remains possible that this species could be present and undetected during yet unsampled temporal periods. However, our results are consistent with those of Scheffer (2000; S.J.S. and M.L.L., unpublished data) that have found only *L. langei* sequence data in samples from other times and locations within California. To date, there is no evidence that *L. huidobrensis* populations have ever established and spread within California.

Our samples of *L. langei* originated in six different counties and were collected from 11 different crops. No evidence for geographic structure or clustering by crop was seen, consistent with the general view of *L. langei* as a polyphagous pest with at least moderate dispersal abilities.

The new multiplex PCR screening method using pooled samples is an ideal approach to screening large samples of morphologically similar but distinct species (or populations) that can be diagnosed using sequence data. By aligning sequences from identified specimens from both groups, divergent sites can be found from which group (taxon)-specific primers can be designed. The primer in one direction should be appropriate for both groups, while primers specific to one or the other of the two groups should be designed such that PCR amplifications for the two groups will be of different sizes, each diagnostic for one or the other group. One caveat concerning this method is that false-positive bands may be obtained if nontarget specimens are inadvertently analyzed.

Pooling specimens before DNA extraction allows greater efficiency in terms of both money and time, due to a reduction in the numbers of both genomic extractions and PCR reactions; by pooling five female specimens per extraction, we cut costs and bench time by ∼80%, thereby allowing us to screen more individuals. In theory, this pooled approach could be used to screen very large numbers of samples at one time. However, in the case of agricultural pests, especially when screening for rare or newly invasive species, there is a need to be able to corroborate any positive bands resulting from the test, particularly because of the possibility of the inclusion of nontarget species. In our case, we retained the head and thorax of each female such that if a positive *L. huidobrensis* band was obtained, we could go back to the heads and thoraxes for that pooled sample and sequence for corroboration. Obviously, the more specimens have been pooled, the more voucher material has to be individually sequenced for confirmation of positive bands, so there is a trade-off between the number of specimens pooled in a given extraction and the ability to confirm important results.

In summary, we used mitochondrial COI sequences and two PCR-based screening methods in an extensive, but temporally limited, investigation of whether the invasive *L. huidobrensis* is present in California. Samples originated in a number of different counties and were associated with a variety of crop species. From our collections made in 2006, no evidence for the presence of *L. huidobrensis* was found. This information and the genetic variation we have documented provide important baseline data in the event of the introduction and possible establishment of the highly invasive *L. huidobrensis*.

Acknowledgments

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