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Research article

EF-1α DNA Sequences Indicate Multiple Origins of Introduced Populations of *Essigella californica* (Hemiptera: Aphididae)

Thomas Théry,[1,2] Eckehard G. Brockerhoff,[3] Angus J. Carnegie,[4] Rui Chen,[5] Stephen R. Elms,[6] Maurice Hullé,[7] Richard Glatz,[8,9] Jaime Ortego,[10] Ge-Xia Qiao,[5] Évelyne Turpeau,[7] and Colin Favret[1]

[1]Department of Biological Sciences, Biodiversity Centre, University of Montreal, 4101 rue Sherbrooke Est, Montreal, QC, H1X 2B2 Canada (thomasjcthery@gmail.com; ColinFavret@AphidNet.org), [2]Corresponding author, e-mail: thomasjcthery@gmail.com, [3]Scion, New Zealand Forest Research Institute, Christchurch 8440, New Zealand (Eckehard.Brockerhoff@scionresearch.com), [4]NSW Forest Science, NSW Department of Primary Industries, Parramatta, NSW 2150, Australia (angus.carnegie@dpi.nsw.gov.au), [5]Chinese Academy of Sciences, Institute of Zoology, Beijing 100101, P. R. of China (chrui11@live.cn; qiaogx@ioz.ac.cn), [6]HVP Plantations, Churchill, VIC 3842, Australia (Stephen.Elms@hvp.com.au), [7]Université Rennes 1, Institut de Génétique, Environnement et Protection des Plantes (IGEPP), Le Rheu 35653, France (maurice.hulle@rennes.inra.fr; evelyne.turpeau@inra.fr), [8]School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, SA 5064, Australia (richard@dess.net.au), [9]Terrestrial Invertebrates, South Australian Museum, Adelaide, SA 5000, Australia, and [10]Instituto Nacional de Tecnología Agropecuaria (INTA), EEA Mendoza, Luján de Cuyo 5507, Mendoza, Argentina (ortegojaime@yahoo.com.ar)

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Abstract

Aphids in the pine-feeding Nearctic genus *Essigella* (Sternorrhyncha, Aphididae, Lachninae) have been introduced in Europe, North Africa, Oceania, and South America. Mitochondrial, nuclear, and endosymbiont DNA sequences of 12 introduced populations from three continents confirm they all belong to *Essigella californica* (Essig, 1909). Intron sequence variation of the nuclear gene *EF-1α* has revealed the existence of four distinct groups. Group I gathers one population from China, where the species is newly reported, and several from Europe (France and Italy); Group II is represented by one population from Argentina; Group III includes two populations from Southern Australia with one from New Zealand; and Group IV corresponds to five populations from Eastern and South-Eastern Australia. These results indicate that introduced populations of *E. californica* have at least four source populations. They also show that intron variation of *EF-1α* can be a method to discriminate populations of asexually reproducing aphids.

Key words: asexual lineage, silvicultural pest, invasive species, Lachninae, population discrimination

Essigella Del Guercio, 1909 (Aphididae, Lachninae, Eulachnini) (Chen et al. 2016) is a Nearctic genus of aphids living on the needles of Pinaceae (Sorensen 1994). *Essigella californica* (Essig, 1909) is the only species introduced outside North America (Sorensen 1994). It is recorded from France (Turpeau and Remaudière 1990), Spain (Seco Fernández and Mier Durante 1992), Australia (Carver and Kent 2000), New Zealand (Carver and Kent 2000, Flynn et al. 2003), Brazil (Zonta de Carvalho and Noemberg Lazzari 2000), Madeira (Aguiar and Ilharco 2001), Italy (Barbagallo et al. 2005), Tunisia (Boukhris-Bouhachem et al. 2007, Blackman and Eastop 2016), Argentina (Ortego and Mier Durante 2012), and Great Britain (Reid et al. 2015). After examination of the source material, the record from Malta (Mifsud et al. 2009) was discarded due to misidentification, the specimen belonging to the genus *Eulachnus*, not *Essigella*. *Essigella californica* has been recorded on over 34 different species of *Pinus* and on some other Pinaceae (Blackman and Eastop 1994, Watson and Appleton 2007). Though the genus is not usually economically important in its native distribution (Sorensen 1994), nor is it known to vector plant viruses (Carver and Kent 2000), *E. californica* was recorded as causing yellowing and defoliation in France (Turpeau and Remaudière 1990) and in New Zealand (Carver and Kent 2000). Although it is not considered a significant pest in New Zealand (Watson et al. 2008), in Australia, *E. californica* has been associated with severe chlorosis and defoliation across much of the commercial *P. radiata* D. Don plantation estate and is considered a significant silvicultural pest in that territory (May and Carlyle 2003; May 2004; Eyles et al. 2011; Stone et al. 2013a,b). Damage by *E. californica* was estimated to cause losses of up to AU$21 million per annum to the Australian forest industry (May 2004), which led to a biological control program using

Diaeretus essigellae Starý and Zuparko, 2002 (Hymenoptera, Braconidae) (Kimber et al. 2010) and the development of resistance breeds of *P. radiata* for commercial deployment (Sasse et al. 2009).

COI is a mitochondrial gene well known for its use as a DNA barcode in animals (Hebert et al. 2003a,b; Hajibabaei et al. 2006). *COI* is employed in aphid species identification, notably in pest control and in phylogenetic analyses (Lee et al. 2011, Cœur d'acier et al. 2007). However, *COI* shows limits in some aphid groups, sometimes not being precise enough in species delimitation (Cœur d'acier et al. 2014; Lee et al. 2011, 2014). Thus, other genes have been investigated to improve species resolution. The gene *Gnd* of the obligate bacterial endosymbiont *Buchnera aphidicola* and the mitochondrial gene *ATP6* (Chen et al. 2013, Lee et al. 2014) were successfully tested. Contrary to mitochondrial genes, many nuclear genes are more stable (Simon et al. 2010). They are often useful in phylogenetic analyses of higher-level arthropod taxa (Caterino et al. 2000, Simon et al. 2010). For example, the nuclear gene *EF-1α* has been employed in the phylogeny of Hexapoda (Djernæs and Damgaard 2006). Moreover, the variation of the *EF-1α* exon–intron structures has proven to be efficient in low-level phylogenetic studies as well (Simon et al. 2010). It was successfully used in phylogenetic reconstructions in several groups of insects (Cho et al. 1995, Condamine et al. 2013, Lin et al. 2013, Cooper et al. 2014) and notably in aphids (Moran et al. 1999, Normark 1999, Von Dohlen et al. 2006, Kim and Lee 2008).

DNA analyses have revealed the existence of cryptic species of aphids (Depa et al. 2012, Lee et al. 2015). Often, the only biological distinction between morphologically identical species is their preferred host (Heie 1986, Lee et al. 2015, Mróz et al. 2015). *Essigella* is a genus with a difficult taxonomy. Species are morphologically similar and several show high intraspecific variation (Sorensen 1994). For these reasons, *Essigella californica* can be morphologically confused with *E. hoerneri* Gillette & Palmer, 1924 and *E. pini* Wilson, 1919 (Sorensen 1994, Barbagallo et al. 2005). However, several elements permit distinction between those species in North America. *Essigella californica* occurs in the same geographic vicinity as *E. hoerneri* but usually does not colonize pines of subsection Cembroides as does *E. hoerneri* (Sorensen 1994, Blackman and Eastop 2016). As a result, if the host is well-identified, there are few risks of misidentification between the two aphid species. In contrast, the respective native geographic ranges of *E. californica* and *E. pini* do not overlap (Sorensen 1994), making their species identification straightforward. However, these two species are known to share several pine species as hosts (Sorensen 1994, Barbagallo et al. 2005); thus, host identity is less useful for identification outside North America. The difficulty in confirming the identity of invasive populations of *Essigella* species has important repercussions for pest management, especially insofar as an authoritative identification is needed when searching for potential biological control agents.

Intraspecific morphological variation based on host plants was documented in *E. californica* by Sorensen (1994). This variation was considered either as being purely intraspecific (Sorensen 1994), or suggestive that *E. californica* was actually one of a complex of species (Zonta de Carvalho and Noemberg Lazzari 2000). No molecular systematic study has been carried out on the genus *Essigella*, and the existence of cryptic species within the putative *E. californica* complex has not been fully evaluated. Additionally, because of the possible confusion between *E. californica* and other *Essigella* species, and because the identity of the introduced populations was deduced only by morphology, there is no confirmation that those populations belong to a single species and that this species is indeed *E. californica*.

In this study, we used four genes, *ATP6*, *COI*, *EF-1α*, and *Gnd*, from 12 introduced populations of *Essigella* in order to confirm if they belong to the same species and if so, that this species is *E. californica*. For this purpose, our results were compared with sequences from four North American populations of *E. californica* and from three other species-level taxa, *E. fusca* ssp. *voegtlini* Sorensen, 1994, *E. hoerneri*, and *E. pini*.

Materials and Methods

Taxon Sampling

North American specimens of *Essigella* were collected during the summers of 2012 and 2013 (North Carolina and California). Overseas specimens were collected between 2012 and 2015 from Argentina, Australia, China, France, Italy, and New Zealand (Table 1). All

Table 1. Collecting data of specimens and Genbank accession numbers of DNA sequences

Species	Country	Locality	Host plant	GenBank accession numbers			
				ATP6	*COI*	*EF-1α*	*Gnd*
E. californica	Argentina	Malargüe, Mendoza	*Pinus* sp.	KY288967	KY288911	KY288929	KY288948
E. californica	Australia	Adelaide (SA)	*Pinus* sp.	KY288966	KY288910	KY288928	KY288947
E. californica	Australia	Benalla (V)	*Pinus* sp.	KY288965	KY288909	KY288927	KY288946
E. californica	Australia	Bombala (NSW)	*Pinus patula*	KY288964	KY288908	KY288926	KY288945
E. californica	Australia	Churchill (V)	*Pinus* sp.	KY288963	KY288907	KY288925	KY288944
E. californica	Australia	Hamilton (V)	*Pinus* sp.	KY288962	KY288906	KY288924	KY288943
E. californica	Australia	Mt Mitchell (NSW)	*Pinus radiata*	KY288976	KY288920	KY288938	KY288957
E. californica	Australia	Whiporie (NSW)	*P. elliottii x P. caribaea*	KY288977	KY288921	KY288939	KY288958
E. californica	China	Mt. Weibao (Yunnan)	*Pinus yunnanensis*	KY288980	KM501336	KY288942	KY288961
E. californica	France	Le Rheu (Ille et Vilaine)	*Pinus radiata*	KY288978	KY288922	KY288940	KY288959
E. californica	Italy	Turin (Piedmont)	*Pinus strobus*	KY288979	KY288923	KY288941	KY288960
E. californica	New Zealand	Christchurch (Canterbury)	*Pinus resinosa / wallichiana*	KY288970	KY288914	KY288932	KY288951
E. californica	USA	San Bernardino Co. (CA)	*Pinus coulteri*	KY288973	KY288917	KY288935	KY288954
E. californica	USA	Ventura Co. (CA)	*Pinus* sp.	KY288975	KY288919	KY288937	KY288956
E. californica	USA	Ventura Co. (CA)	*Pinus attenuata*	KY288971	KY288915	KY288933	KY288952
E. californica	USA	Placer Co. (CA)	*Pinus ponderosa*	KY288972	KY288916	KY288934	KY288953
E. hoerneri	USA	San Bernardino Co. (CA)	*Pinus monophylla*	KY288974	KY288918	KY288936	KY288955
E. fusca voegtlini	USA	Los Angeles Co. (CA)	*Pinus coulteri*	KY288968	KY288912	KY288930	KY288949
E. pini	USA	Swain Co. (NC)	*Pinus rigida*	KY288969	KY288913	KY288931	KY288950

specimens were preserved in 95% ethanol after collecting and subsequently kept at −20 °C until DNA extraction. Species identifications were made with the published keys to the species of the genus *Essigella* by Sorensen (1994) and Blackman and Eastop (1994). Specimens were also compared with authoritatively identified reference material, including type specimens and material in the Sorensen Collection (Essig Museum of Entomology, Berkeley, CA). All voucher specimens are slide-mounted in Canada Balsam and are deposited in the Ouellet-Robert Collection of the University of Montreal (QC, Canada); Chinese specimens are deposited in the National Zoological Museum of China, Institute of Zoology, Chinese Academy of Sciences, Beijing (People's Republic of China).

DNA Extraction, Amplification, and Sequencing

We made at least two separate extractions for each collection sample. Nondestructive DNA extractions were performed using the DNeasy Blood & Tissue kit of QIAGEN and the protocol of Favret (2005). PCR amplifications were performed at the Biodiversity Centre (University of Montreal, Montreal, QC, Canada) using Thermocycler Eppendorf Mastercycler ProS, with Phire Green Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Our protocols follow, with some modifications described in Table 2, those of Lee et al. (2014) for *ATP6*, Inbar et al. (2004) for *COI*, Favret and Voegtlin (2004) for *EF-1α*, and Chen et al. (2013) for *Gnd*. Primers for each gene are the same as those published in these references. Amplicons were sequenced in both directions with the same primers at the Génome Québec Innovation Centre and the McGill University (Montreal, QC, Canada).

Data Analysis

Chromatograms of each gene were edited using Geneious 9 software (Kearse et al. 2012). Obtained sequences were compared with those in GenBank in order to confirm their general identity. Sequences were aligned and compared with Bioedit Version 7.2.5 (Hall 1999) using the ClustalW multiple alignment program (Thompson et al. 1994). Interspecific sequence divergence between species corresponds to Kimura 2 parameter (K2P) distance using MEGA 6.0 (Tamura et al. 2013).

We located the *EF-1α* introns by cross-referencing our *Essigella* sequences with transcript sequences from *Acyrthosiphon pisum* (Harris, 1776) (http://www.aphidbase.com: ACYPI006711-RA).

Results

Mitochondrial and *Buchnera* Genes

COI, *ATP6*, and *Gnd* were sequenced and analyzed for each population. The amplicon lengths were 658 base pairs (bp), 663 bp, and 749 bp for *COI*, *ATP6*, and *Gnd*, respectively (see Table 1 for GenBank accession numbers). All of the introduced populations of *Essigella* were genetically homogeneous for both mitochondrial genes (*COI* and *ATP6*) and the *Buchnera* gene (*Gnd*). These sequences were compared with those of North American populations of *E. californica*, *E. hoerneri*, *E. fusca voegtlini*, and *E. pini*. The *Gnd* sequences for the introduced populations matched perfectly those of *E. californica* in North America. The sequences of *COI* and *ATP6* of the introduced populations also closely matched those of North American *E. californica*. The North American *E. californica* population collected in Ventura Co. on an unidentified *Pinus* shows one nucleotide substitution in *COI*. This population and the North American one collected in Placer Co. on *Pinus ponderosa* Douglas ex Lawson 1836 each show one nucleotide substitution in *ATP6* at two different loci. Sequence divergence between introduced populations and *E. hoerneri* were 2.8%, 5.2%, and 5.7% for *COI*, *ATP6*, and *Gnd*, respectively. Rates between introduced populations and *E. fusca voegtlini* were 3.3%, 4.8%, and 8.8% for *COI*, *ATP6*, and *Gnd*, respectively. Rates between introduced populations and *E. pini* were 3.7%, 5.8%, and 9.5% for *COI*, *ATP6*, and *Gnd*, respectively. Rates between *E. hoerneri* and *E. fusca voegtlini* were 3.3%, 6.0%, and 10.3% for *COI*, *ATP6*, and *Gnd*, respectively, and those between *E. hoerneri* and *E. pini* were 3.7%, 7.2%, and 11.3% for *COI*, *ATP6*, and *Gnd*, respectively. The rates between *E. fusca voegtlini* and *E. pini* were 2.6%, 6.0%, and 8.8% for *COI*, *ATP6*, and *Gnd*, respectively. Moreover, a sequence of *COI* from British *Essigella* published on GenBank (accession number KM888108) is identical to our sequences from introduced *E. californica*.

Comparison of *EF-1α* Sequences

First, we analyzed *EF-1α* sequences of all *E. californica* specimens together. In that case, sequence length was 785 bp including introns and 610 bp without introns (see Table 1 for GenBank accession numbers). As with *COI*, *ATP6* and *Gnd*, *EF-1α* sequences were essentially identical between all the *E. californica* populations with or without introns except for the presence of a single nucleotide substitution in three populations, two from Australia and one from New Zealand. That substitution is located at position 195 of exon 5. Second, we analyzed *EF-1α* sequences of all *Essigella* species together. Sequences lengths, including the introns, were 767 bp for *E. californica* and *E. hoerneri*, 746 bp for *E. fusca voegtlini*, and 775 bp for *E. pini*. The lengths of sequences without introns were 610 bp for all species. *EF-1α* exon sequence divergence between introduced populations of *Essigella californica* and *E. hoerneri*, *E. fusca voegtlini*, and *E. pini* was 0.3%, 1.5%, and 1.3%, respectively; respective sequence divergence including the introns was 0.3%, 2.1%, and 1.8%.

EF-1α DNA sequence traces indicated the presence of heterozygosity in seven populations of *E. californica* and in the one of *E. pini*.

Table 2. Primers and PCR protocols

Genes	Primers sequences	Initial denaturation	No. of cycles	Denaturation	Annealing	Elongation	Final elongation
Lep-F1	ATTCAACCAATCATAAAGATATTGG	94 °C for 1 min	35	94 °C for 4 min	45 °C for 1 min	72 °C for 90 s	72 °C for 2 min
Lep-R1	TAAACTTCTGGATGTCCAAAAAATCA						
BamHI	SWATWATGCCWGGWGCGCGGGCCGC	98 °C for 1 min	35	98 °C for 20 s	51 °C for 40 s	72 °C for 40 s	72 °C for 3 min
ApaI	CWCCAAAATAATCWCKTTGWGCTTG						
tRNALysAf2	GACTGAAAAGCAAAGTAATGATCTCT	94 °C for 3 min	35	94 °C for 30 s	55 °C for 30 s	65 °C for 1 min	None
CO3WWRD	TCWCGAATWACATCWCGTCATCA						
EF1-F	GAACGTGAACGTGGTATCAC	98 °C for 30 s	35	98 °C for 10 s	51 °C for 20 s	72 °C for 20 s	72 °C for 2 min
EF1-R	TGACCAGGGTGGETTTCAATAC						

Table 3. Heterozygosity patterns and locations

Location in *EF-1α* sequence	Argentina	Australia						China	France	Italy	New Zealand	USA (California)			
		Adelaide (SA)	Benalla (V)	Bombala (NSW)	Churchill (V)	Hamilton (V)	Mt Mitchell (NSW)	Whiporie (NSW)				*Pinus attenuata*	*Pinus coulteri*	*Pinus ponderosa*	*Pinus* sp.
Intron 3	T	T	T	T	T	T	T	T	W	W	W	T	T	T	T
Intron 4	T	T	T	T	T	T	T	T	K	K	K	T	T	T	T
Exon 51. 135	C	Y	C	C	C	Y	C	C	C	C	C	Y	C	C	C
Exon 51. 195	R	G	A	A	A	G	A	A	A	A	A	G	R	A	R
Intron 5	A	A	A	A	A	A	A	A	R	R	R	A	A	A	A
Intron 5	C	C	C	C	C	C	C	C	Y	Y	Y	C	C	C	C

The evidence is apparent in the form of clear and consistent double peaks in the sequencing chromatograms. In particular, there are six apparent heterozygous sites across multiple samples in the *E. californica* populations. The first two sites are located in introns 3 and 4, the next two are located in exon 5 at positions 135 and 195 and represent silent substitutions. The last two heterozygous loci are located in intron 5. The presence or absence and the nature of the heterozygous sites in populations of *E. californica* reflect four distinct groups (see Table 3). Group I: populations from France, Italy, and China each displayed a [1:A/T, 2:G/T, 3:C, 4:A, 5:A/G, 6:C/T] pattern; Group II: the Argentinean population displayed a [T, T, C, A/G, A, C] pattern; Group III: two populations from southern Australia (Adelaide and Hamilton) and one from New Zealand displayed a [T, T, C/T, G, A, C] pattern; Group IV: five eastern and southeastern Australian populations (Benalla, Bombala, Churchill, Mt Mitchell, and Whiporie) displayed a [T, T, C, A, A, C] pattern. Among the North American populations of *E. californica*, one displayed the same pattern as that of Group IV and three displayed that of Group II. The Group I and Group III patterns were not recovered in our North American samples.

Discussion

COI, ATP6, and *Gnd*

The comparison of the K2P distances of *COI* indicates that the introduced populations of *Essigella* all belong to *E. californica*. Indeed *COI* sequence divergence observed between the different studied species were from 2.8% to 3.7% and were superior to the rate of 2% accepted in barcoding studies to separate two species (Hebert et al. 2003b). Meanwhile, the divergence between the various populations of *E. californica* never exceeded 0.2%, corresponding to a single nucleotide substitution. The separation between our four species was also confirmed with results obtained with *ATP6* and *Gnd*. However, the divergences observed for those three genes are lower than those observed between species in other genera of Eulachnini (*Eulachnus* and *Cinara*). In fact, interspecific distance values of *COI* for *Cinara* found by Chen et al. (2012) are 8.7% (± 2.2%) to 10.4% (± 2.4%) and those by Chen et al. (2013) are 8.9% (± 2.1%). For the genus *Eulachnus*, sister genus to *Essigella*, Chen et al. (2012, 2013) calculated values of 7.7% (± 0.9%) to 9.5% (± 1.4%) and 7.4% (± 0.9%).

K2P distances in *Gnd* seem also lower in *Essigella*. For *Gnd*, our results were between 5.7% and 11.3% whereas Chen et al. (2013) found values of 30.8% (± 5%) for *Cinara* and 16.9% (± 4.6%) for *Eulachnus*. We found no published values of sequence divergence of *ATP6* in the two other cinarine genera, but Lee et al. (2014) published an average value of 8.3% based on 20 other aphid species, whereas we found that in *Essigella* they were between 4.8% and 7.2%.

Our lower values could be explained by the small number of species we compared with our samples of *E. californica*. Moreover, *E. hoerneri* is known to be phylogenetically close to *E. californica*. It is likely that comparing additional species of *Essigella* would yield values closer to those obtained by others authors with *Cinara* and *Eulachnus*. Having confirmed that all introduced populations belong to *E. californica*, comparison of our *COI* sequences with those available in GenBank (KM888108) confirms the presence of the species in Great Britain (Reid et al. 2015).

Heterozygosity of *EF-1α* Sequences

Although heterozygous loci are often seen as hindrances in molecular systematics, in our case polymorphic sites provided important

information. Normark (1999) also observed polymorphic sites in *EF-1α* sequences in *Trama*, another genus of Lachninae. Our results showed the same phenomenon, appearing as obvious double peaks, almost or identical in height, on our sequencing chromatograms. Like Normark (1999), we checked if each double peak was present in at least one other specimen of the same colony. Because *E. californica* is asexual in the territories where it was introduced, there is no sexual recombination in those populations. In such conditions, each heterozygous pattern will be specific to one population (Birky 1996, Schwander et al. 2011). Our study indicates that introduced populations of *E. californica* have at least four origins, and that at least four introductions occurred around the world: one in South America, one in Eurasia (Europe and China), and two in Oceania. However, it is hard to believe that a single introduction occurred in China, Italy, and France. It seems more likely that these countries received *E. californica* separately, although our data are not able to confirm it.

Retracing the Routes of Introduction

The heterozygous sites found in some introduced populations of *E. californica* were also found in North America. On the one hand, the *EF-1α* pattern found in five Australian populations (Benalla, Bombala, Churchill, Mt Mitchell and Whiporie) is also found in one populations of North America (populations collected on *Pinus ponderosa*). Sequences of *COI* and *Gnd* of this North American population matched perfectly with those of Group IV. However, this population showed a difference of one nucleotide in the *ATP6* sequence in comparison with that found in Group IV. We made a similar observation with the Group II, displaying the same *EF-1α* pattern as that found in the three other North American populations (on *P. attenuata* Lemmon 1892, on *P. coulteri* D. Don 1836 and on *Pinus* sp.). Two of them (on *P. attenuata* and on *P. coulteri*) had *COI*, *ATP6*, and *Gnd* sequences identical to those found in Group II. The other (on *Pinus* sp.) had a difference of one nucleotide in the *COI* sequence and a difference of one nucleotide in the *ATP6* sequence in comparison with those of Group II. Even though our results suggest at least four origins for introduced populations of *E. californica*, the small differences we found in *COI* and in *ATP6* sequences indicate that our results may not be precise enough to prove a direct link between introduced and particular North American populations. Higher resolution population genetic methods such as microsatellites (Llewellyn et al. 2003, Li et al. 2015), AFLP (amplified fragment length polymorphism; Dieni et al. 2016), or RFLP (restriction fragment length polymorphism; Piffaretti et al. 2013) may be more useful in locating the North American points of origin of the various introduced populations.

Heterozygous base pairs in the introns of the nuclear gene *EF-1α* can discriminate populations in asexual aphid species. In our case, they showed that introduced populations of *E. californica* have at least four origins and that *E. californica* was introduced at least four times outside Western North America, its native territory. It also confirms that the species is now present in Europe, Oceania, South America, and Asia.

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