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Nuclear-Mitochondrial Barcoding Exposes the Global Pest Western Flower Thrips (Thysanoptera: Thripidae) as Two Sympatric Cryptic Species in Its Native California

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ABSTRACT Over the past three decades, Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), has become a major worldwide pest of many agricultural and horticultural crops. In response, much time, money, and effort have been put into pure and applied research focusing on the biology and control of this pest. Western flower thrips is native to Western North America and widespread in California. High levels of variation in basic biology, pest status, and resistance to insecticides bring into question the specific status of Western flower thrips. We used nuclear-mitochondrial barcoding to compare DNA sequences of nuclear and mitochondrial genes between Western flower thrips populations across California, looking for association between these unlinked loci. Sequences of D2 domain of 28S and cytochrome c oxidase I gene revealed the existence of two distinct but sympatric genetic entities, and we describe a simple polymerase chain reaction-based method for diagnosing these entities. The complete association of these nuclear and mitochondrial loci in areas of sympatry is indicative of reproductive isolation and the existence of two cryptic species, both of which key out to Western flower thrips by using morphological characters. The finding that Western flower thrips is a complex of two species has important implications for past, current, and most importantly future research on these pests.

KEY WORDS molecular identification, barcoding, tospovirus vector, 28SD2, cytochrome c oxidase I gene

Accurate identification of organisms is a fundamental requirement for valid biological research. Correct identification is paramount to accessing and assessing any existing information about a study organism. This can be particularly problematic in studies focusing on species that are part of a cryptic species complex. Such species are "cryptic" because they are difficult to distinguish morphologically (Bickford et al. 2006). One field in which accurate species identifications have proven to be critical is the control of pest arthropods (Rosen 1986, Davies et al. 2004, Armstrong and Ball 2005). Misidentification of a pest (or a natural enemy) may lead to the implementation of inappropriate control measures, resulting in considerable wasted time, money, and effort (Rosen 1986).

Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), is a major worldwide insect pest of many agricultural and horticultural crops (Kirk 2002, Kirk and Terry 2003). Western flower thrips is highly polyphagous (Lewis 1997); causes direct feeding damage to fruits, leaves, and flowers (Parella and Jones 1987, Childers 1997); and acts as a major vector of tospoviruses, most notably tomato spotted wilt virus (family *Bunyaviridae*, Genus *Tospovirus*, TSWV) (Wijkamp et al. 1995). Economic data are scarce, but in The Netherlands alone, estimates indicate annual losses from direct damage by Western flower thrips of $30 million, and from TSWV, a further $19 million (Roosjen et al. 1998, also see Kirk 2002). On a global scale, annual losses in the early 1990s attributable to TSWV were estimated at >$1 billion (Goldbach and Peters 1994), but given the continued spread of Western flower thrips (Kirk and Terry 2003) and the emergence of new varieties and species of tospoviruses that this pest may readily acquire and vector (Pappu et al. 2009), financial losses resulting from plant diseases vectored by Western flower thrips are now likely to be much higher.

Western flower thrips has become a major invasive pest of vegetable and ornamental crops, grown in open fields, shadehouses, and glasshouses, only in the past three decades. Western flower thrips is assumed to be native to Western North America and is extremely widespread throughout the state of California, where it has long been regarded as a pest (Bailey 1938). In California, it occurs across many vegetation types from the Pacific coast to the interior mountain ranges at elevations up to 3,000 m (Bryan and Smith 1956). As early as 1934, Western flower thrips was detected

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outside its native range, on naturalized California lupins in wilderness areas of New Zealand (Mound and Walker 1982). However, this New Zealand population caused no apparent problems on crops and has traditionally been recognized as a distinct "lupin strain" (Martin and Workman 1994). These distributions were apparently stable until the early 1980s when Western flower thrips began to appear throughout the eastern states of the United States and southern Canadian provinces (Kirk and Terry 2003). In 1983, Western flower thrips colonized Dutch glasshouses and from there quickly spread across Europe and into northern Africa (Kirk and Terry 2003). Western flower thrips also invaded South Africa and much of Asia, and in 1992, after 58 yr in the wilderness, Western flower thrips became a problem in glasshouses in New Zealand (Martin and Workman 1994). Globally, Western flower thrips is now probably the most important insect pest of commercial glasshouses and also has established in many outdoor crops (Kirk and Terry 2003).

Dramatically increased international movement of plant material in the 1960s and the acquisition of insecticide resistance during the 1970s have been cited as reasons why Western flower thrips became pestiferous (Kirk and Terry 2003). So called "glasshouse strains" are more resistant to insecticides than wilderness populations in California and the "lupin strain" in New Zealand (Brødsgaard 1994, Martin and Workman 1994, also see Jensen 2000). Like many thrips species (Mound 2002), Western flower thrips is also highly polymorphic. This polymorphism is demonstrated by the existence of three co-occurring color-morphs, which it has been suggested are governed by simple Mendelian genetics (Bryan and Smith 1956). However, temperature is also likely to influence color, with darker forms occurring under cooler conditions (Kirk and Terry 2003, Mound 2005). Color does not seem to affect the efficiency with which individual Western flower thrips vector TSWV (Sakimura 1962), but differences in vector efficiency have been observed between the sexes and between populations (van de Wetering et al. 1999). A study of the susceptibility of cucumber varieties to different Western flower thrips populations also revealed significant interpopulation differences in life history parameters such as rate of reproduction, developmental time, and survival (de Kogel et al. 1997). Genetic variation has been found within (Forcioli et al. 2002, Brunner and Frey 2004) and between (Gillings et al. 1996, Fang et al. 2005) Western flower thrips populations, but links between this variation and other aspects of Western flower thrips biology remain elusive.

The high levels of morphological and molecular variation, coupled with high levels of polyphagy, suggest that what we currently recognize as Western flower thrips may in fact be a complex of cryptic species. DNA-based techniques provide a powerful means of detecting cryptic species (Bickford et al. 2006). One such method that has received widespread acceptance is "barcoding" based on differences in the DNA sequence of the mitochondrial cytochrome c oxidase I gene (COI) (Hebert et al. 2003). However, delimitation of species using this single gene is somewhat contentious (Rubinoff and Holland 2005, Rubinoff et al. 2006). The original proponents of mitochondrial barcoding used mean intra- and interspecific genetic differences of a priori taxonomically well defined species, to designate 2–3% divergence between two sequences as indicative of different insect species (Hebert et al. 2003). However, this threshold level assumes a constant mutation rate across different taxa. Mutation rate in the COI gene is influenced by its physical position in the mitochondrial genome and as such, is affected by variation in mitochondrial genome arrangement. The mitochondrial genome of Thysanoptera is subject to particularly high levels of rearrangements (Shao and Barker 2003, Dowton 2004). Such lineages are expected to have multiple mutation regimes operating in different portions of the group such that 2% divergence between species might be appropriate for one group of taxa but 6% is more accurate for another; so, drawing species boundaries based solely on COI becomes ambiguous (Rubinoff et al. 2006). A more rigorous approach to delimiting species boundaries is to corroborate patterns of differentiation based on COI sequences with evidence from other independent sources of data such as morphology, ecology, or nuclear DNA sequences (Rubinoff and Holland 2005). Differences in morphology and ecology are likely to be difficult to assess in cryptic species (otherwise they would not be cryptic). However, if the partitioning of specimens by using nuclear DNA matches that of mitochondrial DNA, we may infer species boundaries with greater certainty. Such correlation between loci has been termed genotypic clustering and has been proposed as an approach to defining species (Mallet 1995, 2007). Indeed, the different modes of inheritance of nuclear and mitochondrial DNA (chromosomal and cytoplasmic, respectively) mean that congruence between nuclear and mitochondrial loci is also indicative of reproductively isolated groups (Bensch et al. 2004, Lukhtanov and Shapoval 2008), which would also lead to such groups being considered as separate species under the more traditional biological species concept (Mayr 1942). Here, we compare DNA sequences of nuclear and mitochondrial genes between Western flower thrips individuals collected from populations across its native range in California.

Materials and Methods

Specimens. Western flower thrips were collected from a range of host plants in wilderness and agricultural locations by beat sampling foliage. Thrips were preserved in 95% ethanol in labeled vials and host plants were identified. These collections were part of an ongoing statewide survey of California thrips species (Table 1). For comparison, Western flower thrips also were obtained from "wilderness" and "glasshouse" populations in New Zealand (Table 1).

Table 1. Western flower thrips (WFT) populations sampled in this study

Location	Host plant[a]	WFT specimen ID[b]	Altitude (m)	Collection date
UCR Botanical Gardens, Riverside Co.	*Quercus cedrosensis*	CAT365	370	3-V-2006
Truckee, Nevada Co.	*Eschscholzia californica*	CAT725	1,775	14-VII-2006
Granite Chief Wilderness, Placer Co.	*Wyethis* sp.	CAT718	2,027	14-VII-2006
	Unidentified shrub	CAT670	2,027	14-VII-2006
	Unidentified grasses	CAT710	2,027	14-VII-2006
S. Lake Tahoe, Alpine Co.	*Pinus* sp.	CAT665	2,394	15-VII-2006
	Unidentified grasses	CAT735	2,394	15-VII-2006
	Unidentified shrub	CAT748	2,394	15-VII-2006
	Unidentified shrub	CAT755	2,394	15-VII-2006
Lake Winnemucca, Alpine Co.	*Salix* sp.	CAT689	2,713	15-VII-2006
Lake Tahoe, Douglas Co., NV	*Lupinus* sp.	CAT662	2,117	16-VII-2006
Lancaster, Los Angeles Co.	*Solanum tuberosum*	CAT544, CAT548, CAT549	746	15-VIII-2006
	Sorghum vulgare var. *sudanensis*	CAT528, CAT2388, CAT2389, CAT2391, CAT2392	746	15-VIII-2006
	Prunus persica	CAT535, CAT537, CAT538, CAT2393, CAT2394, CAT2395, CAT2396, CAT2397, CAT2399	746	15-VIII-2006
	Prunus domestica	CAT550, CAT551, CAT553, CAT2400, CAT2401, CAT2402, CAT2403, CAT2404, CAT2405	746	15-VIII-2006
Hwy 40, 177 km E. of Barstow, San Bernadino Co.	*Senna armata*	CAT435, CAT436	685	11-IV-2007
Providence Mts SRA, San Bernadino Co.	*Brickellia incana*	CAT420	874	11-IV-2007
	Chrysothamnus paniculatus	CAT411	874	11-IV-2007
	Chilopsis linearis	CAT448	874	11-IV-2007
	Larrea tridentate	CAT390	925	11-IV-2007
	Larrea tridentate	CAT084	925	11-IV-2007
	Ephedra nevadensis	CAT405	1,342	11-IV-2007
	Lycium cooperi	CAT439, CAT440	1,284	11-IV-2007
Cal Poly San Luis Obispo, San Luis Obispo Co.	*Ribes speciosa*	CAT991, CAT992, CAT2386	123	11-V-2007
	Juncus effus	CAT1012	123	11-V-2007
	Foeniculum sp.	CAT1007	123	11-V-2007
	Vitis californica	CAT1025, CAT1026	123	11-V-2007
	Lotus scoparius var. *scoparius*	CAT999	123	11-V-2007
	Unidentified grasses	CAT1035, CAT1038, CAT1041	123	11-V-2007
Montana de Oro SP, San Luis Obispo Co.	*Heteromeles arbutifolia*	CAT827	115	1-IX-2007
Sequoia NP, Tulare Co.	Unidentified grasses	CAT504	1,981	18-V-2007
	Unidentified grasses	CAT507, CAT508, CAT2375	1,981	18-V-2007
	***Lupinus* sp.**	CAT518, CAT2376, CAT2377, CAT2378, CAT2379, CAT2380	1,981	18-V-2007
	Viola sororia	CAT462, CAT2368, CAT2369, CAT2370	1,981	18-V-2007
	Sequoiadendron giganteum	CAT521, CAT2382, CAT2383	1,981	18-V-2007
	Ceanothus integerrimus	CAT487, CAT2374	1,981	18-V-2007
	Sequoiadendron giganteum	CAT458	1,981	18-V-2007
	Arctostaphylos sp.	CAT465, CAT466, CAT467, CAT2371, CAT2372, CAT2373	1,981	18-V-2007
Salinas, Monterey Co.	*Lactuca sativa*	CAT2454, CAT2457		23-VII-2009
Spreckles, Monterey Co.	*Lactuca sativa*	CAT2460, CAT2464		23-VII-2009
Rakaia Riverbed, New Zealand	*Lupinus arboreus*	PR09-238, PR09-239		II-2007
North Canterbury, New Zealand	*Capsicum* sp.	PR09-241, PR09-242, PR09-243, PR09-244		II-2007

[a] Host plants in bold indicate that specimens of both WFT species were collected from this single plant.
[b] Underlined specimen ID numbers indicate specimens that were subsequently identified as WFTL (see text).

DNA Extraction, Amplification, and Sequencing. Whole genomic DNA was extracted from intact specimens, after which specimen carcasses were retrieved, slide-mounted, and verified morphologically (for detailed methods, see Rugman-Jones et al. 2006). Slide-mounted specimens are held at the University of California Riverside Entomology Research Museum, Riverside, CA. The polymerase chain reaction (PCR) was used to amplify 456 bp of the D2 domain of 28S (28SD2) nuclear ribosomal DNA (rDNA) ($n = 61$), 137 bp of the nuclear protein coding gene Elongation Factor 1α (EF1α) ($n = 13$) and 571 bp of the mitochondrial gene (mtDNA) cytochrome oxidase c subunit one (COI) ($n = 95$), with the following respective primer pairs: 28sF3633 (5′-TACCGTGAGGGAAAGTTGAAA-3′) with 28sR4076 (5′-AGACTCCTTGGTCCGTGTTT-3′) (Choudhury and Werren 2006), EF1 (5′-GACAACGTTGGCTTCAACGTG-3′) with EF2 (5′-ATGTGAGCAGTGTGGCAATCCAA-3′) (Palumbi 1996), and C1-J-1751 (5′-GGTCAACAAATCATAAA-

GATATTGG-3′) with C1-N-2329 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Simon et al. 1994). All PCRs were performed in 25-μl reactions in a Mastercycler ep gradient S (Eppendorf North America Inc., New York, NY). The 28SD2 PCR contained 2 μl of DNA template (concentration not determined), 1× ThermoPol PCR buffer (New England BioLabs, Ipswich, MA), 200 μM each dNTP, 6% (vol:vol) bovine serum albumin (BSA) (New England BioLabs), 1 U of *Taq* polymerase (New England BioLabs), and 0.2 μM each primer. Thermocycling was: 2 min at 95°C; followed by 38 cycles of 30 s at 94°C, 50 s at 58°C, and 1 min 15 s at 72°C; and a final 10 min at 72°C. Components of the COI PCR were as for 28SD2 but with 8% BSA and an additional 3 mM $MgCl_2$. The COI thermocycling profile was 5 min at 95°C; then 36 cycles of 30 s at 95°C, 40 s at 47°C, and 1 min at 72°C; and a final 5 min at 72°C. The EF1α PCR contained 2 μl of DNA template, 1× buffer II (Applied Biosystems, Foster City, CA), 20 μM each dNTP, 1 U of Amplitaq gold (Applied Biosystems), and 0.2 μM each primers. Thermocycling was 10 min at 95°C and then 38 cycles of 30 s at 95°C, 40 s at 48°C, and 45 s at 72°C; and a final 6 min at 72°C. Amplified DNA was cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and direct sequenced in both directions at the University of California Riverside Genomics Institute, Core Instrumentation Facility.

Sequence Analysis. Sequences were trimmed to remove PCR primers, aligned manually in BioEdit 7.0.5.3 (Hall 1999), and deposited in GenBank (Benson et al. 2008). COI sequences were translated (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) to confirm the absence of nuclear pseudogenes (Song et al. 2008), and a haplotype network was constructed using TCS version 1.21 (Clement et al. 2000), with an arbitrarily chosen connection limit of 50 steps. To investigate the wider international distribution of mitochondrial DNA (mtDNA) haplotypes, 146 existing *F. occidentalis* COI sequences were retrieved from GenBank (accessions AB276374-376, AF378685-688, AM932005, EF213765-766, EF469245-250, EF555794-889, EF591474-478, EU004552-556, EU363483-492, FN545981-994) and added to our alignment. We also included COI sequences from Western flower thrips specimens collected in Florida, Australia, and Thailand (GenBank numbers to be added). Trimming of the sequences to match those in GenBank resulted in an aligned data-matrix of 261 sequences each of 407 bp. A further 11 GenBank accessions identified as *F. occidentalis* (AM931992, AM932016-018, AM932021-23, AM932025-027, AM932029) were excluded after translation revealed that they coded for a different chain of amino acids. The larger matrix was used to produce a second haplotype network (data not shown), from which areas in which mtDNA haplotypes from our California and New Zealand specimens also were present were identified.

Species-Diagnostic PCR. Two rDNA haplotypes were found (see Results) and a one-step diagnostic PCR based on 28SD2 rDNA was designed to identify an individual's rDNA haplotype without the need for sequencing. Primer3 version 0.4.0 (Rozen and Skaletsky 2000) was used to design the forward primer *Western flower thrips28S-uni-F* (5′-CCGAATGGTGAGATTCAAGC-3′), which binds in both rDNA haplotypes and compliments the reverse primer 28sR4076, and a second internal reverse primer that only bound to one of the rDNA haplotypes *Western flower thrips28S-type2-R* (5′-GGCGCGGGCTCCACCGA-3′). Reactions were performed in 25-μl volumes containing 1 μl of DNA template, 1× ThermoPol buffer, 200 μM each dNTP, 1 U of *Taq* polymerase (New England Biolabs), and 0.2 μM each of the three primers. Thermocycling was 2 min at 95°C and then 38 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final 3 min at 72°C. PCR products were visualized on 1.2% agarose gels stained with ethidium bromide. The method was tested on all specimens for which 28SD2 sequences were already available ($n = 61$) and used to confirm the rDNA haplotype of the remaining specimens for which we only sequenced COI ($n = 34$; see Results).

Results

Sequences of 28SD2 rDNA revealed two distinct haplotypes, separating California Western flower thrips specimens into two distinct sympatric groups, discriminated by a 2-bp substitution at nucleotides 252–253 (GenBank accessions GU147942-GU148002). This same division was also evident in the EF1α sequences of a small sub-sample of specimens (GenBank accessions GU148003-GU148015). Greater variation was evident in our COI sequences (GenBank accessions GU148016-GU148130), with 20 haplotypes (Fig. 1). There were 48 variable positions of which substitutions at three of these were nonsynonymous changes (nucleotides 77, 402, and 550). The 20 haplotypes formed two distinct clusters, separated by 17 nucleotide changes (between haplotypes A and J; Fig. 1), in complete congruence with the 28SD2 rDNA division. One rDNA haplotype was characteristic of Western flower thrips individuals with a COI haplotype from mtDNA cluster 1, whereas the other rDNA haplotype was only found in individuals with a COI haplotype from mtDNA cluster 2 (Fig. 1). These two genetic profiles were reflected in the Western flower thrips specimens from New Zealand. Those from "glasshouse" populations fell in with rDNA/mtDNA type 1, and those from "wilderness" populations matched the other type.

The diagnostic PCR successfully distinguished the two rDNA haplotypes. Specimens with rDNA/mtDNA type 1 (those that aligned with the New Zealand "glasshouse strain") produced a single PCR product of ≈400 bp. In contrast, specimens with rDNA/mtDNA type 2 (those that aligned with the New Zealand "lupin strain") produced a single PCR product of ≈200 bp and often also a second faint product of ≈400 bp (Fig. 2). All 61 specimens for which 28SD2 was sequenced, and the 34 for which an rDNA haplotype could be "predicted" based on their mtDNA

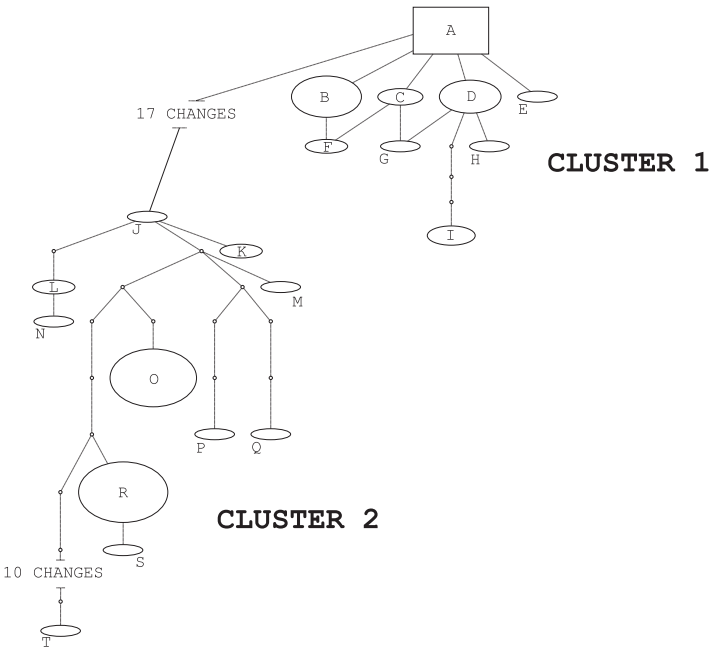

Fig. 1. Network of mitochondrial haplotypes present in our California and New Zealand samples. Haplotype size is proportional to the number of specimens sharing a haplotype. Small circles represent unobserved inferred haplotypes and lines connecting haplotypes represent a single nucleotide mutational change. The COI sequences of 95 western flower thrips individuals contained 20 haplotypes, which in turn fell into two clusters: those from cluster 1 (A–I) were characteristic of rDNA haplotype 1 and those from cluster 2 (J–T) were characteristic of rDNA haplotype 2 (see GenBank accessions).

haplotype, were correctly identified using the diagnostic PCR method.

The wider geographic distribution of Western flower thrips haplotypes identified in our California and New Zealand samples, inferred from existing GenBank COI accessions, is shown in Fig. 3. Truncating the COI data matrix, to accommodate the GenBank accessions, resulted in the loss of several variable nucleotide positions from our original matrix, and hence, the amalgamation of some of our California/New Zealand haplotypes. The overwhelming majority of COI sequences retrieved from GenBank grouped with California haplotypes from mtDNA cluster 1 (haplotypes A–I in Fig. 1), and the majority of these matched the amalgamated California haplotypes A and C (Fig. 3). Sequences falling into this group came from specimens collected in other areas of the United States (Florida, Kansas, and New York), and further afield in Australia, China, Croatia, Germany, Italy, Japan, Kenya, The Netherlands, New Zealand, Thailand, and the United Kingdom. Only three GenBank sequences matched one of our haplotypes from mtDNA cluster two (haplotypes J–T in Fig. 1). Two specimens collected in China and one from an unidentified location (accession EF469246) matched the combined haplotypes L and N (Fig. 3). Fifteen new haplotypes were detected in the GenBank sequences, of which, 14 grouped with mtDNA cluster one and were no >3bp (0.7%) different from the closest California haplotype in that cluster (Fig. 3). Thirteen of these were unique to a single specimen, the fourteenth being shared by two specimens from Italy and two from South Africa. The remaining new haplotype grouped with mtDNA cluster 2 and was shared by specimens from New Zealand and China (Fig. 3).

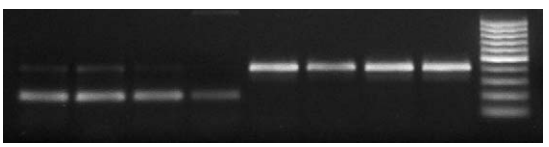

Fig. 2. Diagnostic PCR for the two Western flower thrips species. Lanes 1–4, western flower thripsL; lanes 5–8, western flower thripsG; lane 9, 100-bp DNA size standard (MBI Fermentas, Hanover, MD).

Discussion

The recent rise of Western flower thrips to the status of major worldwide pest has propagated much research into its biology and control (a Web of Science search with *Frankliniella* and *occidentalis* in the "Topic" returned 880 studies, 594 of which have been conducted during the last decade). However, any biological study is only as good as the taxonomy on which it is based. In the current study of Western flower thrips populations across California, DNA sequences of 28SD2 and COI genes revealed the existence of two distinct genetic entities. On several occasions, both types were collected from the same individual plant (Table 1). The differing modes of

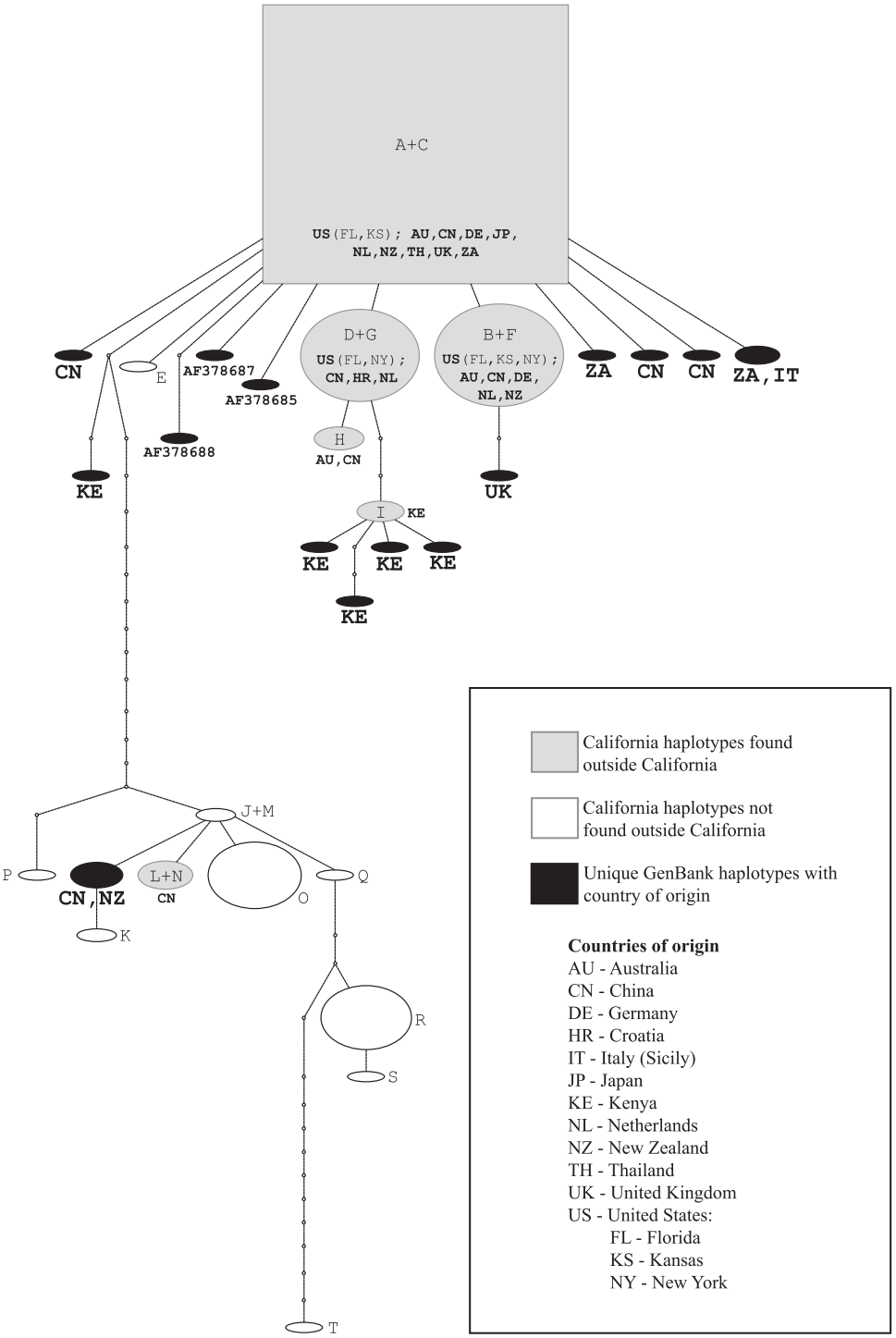

Fig. 3. Global distribution of Western flower thrips mtDNA haplotypes present in our California and New Zealand samples. The haplotype network was constructed using the 95 COI sequences used to construct Fig. 1, which were truncated to align with 146 COI sequences retrieved from GenBank and an additional 20 sequences of our own drawn from material collected outside California. Where known, the geographic distribution of each haplotype is given as a two-digit ISO country code.

inheritance of rDNA and mtDNA (chromosomal and cytoplasmic, respectively) mean that 28SD2 and COI genes cannot be physically linked (i.e., on the same DNA molecule). If our Western flower thrips specimens represented a single species, we would expect interbreeding to produce random mixing of rDNA and mtDNA haplotypes. Indeed, even with two species we may still expect to see a degree of mixing. Approximately 10% of accepted animal species are known to hybridize (Mallet 2007), and mitochondrial introgression is commonly cited as evidence for such hybridization (Shaw 2002, Bachtrog et al. 2006, Linnen and Farrell 2007, Kawakami et al. 2007, Gompert et al. 2008, Nevado et al. 2009, Spinks and Shaffer 2009, Kemppainen et al. 2009). Instead, the complete linkage disequilibrium displayed by rDNA and mtDNA loci in our specimens is indicative of two fully reproductively isolated species (Mallet 1995, Bensch et al. 2004, Monaghan et al. 2005, Lukhtanov and Shapoval 2008, Gaines et al. 2009, Sequeira et al. 2009).

One potential problem with the NuMB approach is the potential for inclusion of nonfunctional nuclear copies of COI (psuedogenes). If such psuedogenes exist and lie close to the 28SD2 region of rDNA they may be physically linked, such that recombination does not occur in this region and so the markers always occur together. We controlled for this possibility by translating each COI sequence to check for indels and stop codons, common features of pseudogenes (Song et al. 2008). None were found. A further problem may arise if there are no differences in 28SD2 sequences. This region can be highly conserved among closely related taxa, even if mitochondrial sequences are very divergent (e.g., Rugman-Jones et al. 2007). If this is thought to be a problem, a possible alternative is the nuclear protein coding gene EF1α (Caterino et al. 2000).

Cryptic species pose an obvious problem to conventional means of identification that rely solely on morphological characters. We demonstrated the utility of a simple one-step PCR for diagnosing Western flower thrips specimens as one species or the other. This method is accurate, quick, relatively inexpensive, and can be employed by technicians with basic skills in molecular biology. However, it should be recognized that we have only tested the diagnostic PCR on individuals that have (through their 28SD2 or COI sequences) been identified as one of the two Western flower thrips species. Care should be taken in applying the technique if other species are also suspected to be present in a sample (i.e., it may produce false positives). This study also highlights the importance of vouchering DNA specimens. In light of our findings, many existing studies documenting variation between Western flower thrips populations and/or individuals will necessarily be rendered unreliable or invalid, if DNA is not available to confirm which species has actually been studied.

Given the economic importance of Western flower thrips, it is surprising that the existence of two species has not been established earlier. It seems, simple laboratory crossing experiments between long-recognized "strains" have never been performed. Of course, interpretation of any crossing experiments (had they been performed) may have been confounded if some important "field" component of the isolating mechanism (e.g., conspecific sperm precedence; Price 1997, Howard 1999) was not replicated in laboratory, and/or the species were able to hybridize (Mallet 2007). As such, our findings are perhaps better evidence for the existence of a reproductive barrier in the field. Although it is not unusual to find two or more species of *Frankliniella* in the same set of flowers (Mound 2002), the coexistence of the two species at least suggests some distinct difference in their biology, for example an affiliation with different host parts (e.g., Condon et al. 2008). Future research should focus on identifying morphological, behavioral, and/or ecological differences between the two species previously recognized as *F. occidentalis.* Division of Western flower thrips specimens into two genetic species also may allow taxonomists to reexamine polymorphic "Western flower thrips" morphological traits in search of discrete diagnostic character-states.

This study highlights the importance of accurate species identification and the effectiveness of simple molecular techniques to discriminate cryptic species. Consideration of a single gene region, as advocated by proponents of mitochondrial barcoding (Hebert et al. 2003), can lead to problems in defining species boundaries. Differences in mitochondrial mutation rates across taxa (Dowton 2004) mean that an acceptable level of DNA sequence divergence in one group of taxa may be inappropriate for another (Rubinoff and Holland 2005, Rubinoff et al. 2006). In contrast, the NuMB approach advocated here removes any ambiguity from the process. If a population is totally panmictic, across a sample of individuals, regardless of levels of sequence divergence, we expect to see complete mixing of nuclear and mitochondrial barcodes. However, if individuals in that population do not interbreed freely (i.e., cryptic species), then given a sample that avoids any potential effect of genetic relatedness (e.g., one that is too small and/or from a single location) (Lukhtanov and Shapoval 2008), we expect to see congruent partitioning in the "signals" read from nuclear and mitochondrial barcodes (Bensch et al. 2004, Monaghan et al. 2005, Lukhtanov and Shapoval 2008, Gaines et al. 2009, Sequeira et al. 2009). In this study, we used a nuclear and a mitochondrial gene, but separate nuclear genes that are known to occur on different chromosomes may be equally effective.

Implications for Existing Studies. Our findings have important implications for past, current, and most importantly future Western flower thrips research. In recognition that the two species revealed in this study correspond to the "lupin" and "glasshouse" strains in New Zealand, in this section we refer to them as Western flower thripsL and Western flower thripsG, respectively. This section of the discussion is given over to several examples of studies we have found in the literature that may be interpreted differently in light of our finding. These are only examples and we are quite sure this is not an exhaustive list. Indeed, the findings of many existing studies of Western flower thrips (re-

viewed by Reitz 2009) may be applicable only to Western flower thripsG or Western flower thripsL, although unfortunately, we may never know which.

It is not clear if the global spread of Western flower thrips is a consequence of just one, or both of the species we have identified. Our sequences indicate that both Western flower thripsL and Western flower thripsG have colonized New Zealand (previously recognized as the "lupin" and "glasshouse" strains; Martin and Workman 1994), and it may be inferred from COI sequences in GenBank that both also have colonized China (Fig. 3). Given that both species can be found on the same individual host plant, and the thigmotactic nature of thrips species in general (Morse and Hoddle 2006), it seems likely that both also have been transported, as incidental passengers on commercial plant material, to many other areas. Whether both species have established in those areas is unknown. COI sequences in GenBank that match Western flower thripsG genotypes in our sample indicate that Western flower thripsG has established in Europe, Africa, Asia, and Australia (Fig. 3). However, any apparent absence of Western flower thripsL in these areas may simply reflect sampling effort, relative abundance and/or a paucity of publically available sequences. The presence of the different color-morphs may cast some light on the situation. In our specimens from California both species displayed the full range of color variation from dark to light (Bryan and Smith 1956). However, we noticed that in our sample specimens, dark forms were much more likely to be subsequently identified as Western flower thripsL (unpublished data). Perhaps dark individuals, although rare in European glasshouses (Brødsgaard 1989, Kirk 2002), indicate the presence of Western flower thripsL there. Our diagnostic PCR provides a simple means to confirm or reject this possibility, and we suggest that this is made a research priority.

Even in areas where both species have (or may have) become established, our study raises questions about the relative pest status of Western flower thripsL and Western flower thripsG. We do not know to what extent Western flower thripsL and Western flower thripsG differ in respect to important factors such as their ability to feed and reproduce on different natural and "cultivated" hosts, their efficiency as vectors of plant diseases, or their susceptibility to insecticides. In California, the only species we detected on lettuce crops (*Lactuca* sp.) in Monterey Co. was Western flower thripsG. However, both Western flower thripsG and Western flower thripsL were detected in approximately equal abundance on commercially grown plums (*Prunus domestica*) and peaches (*Prunus persica*) in southern California. A study of the performance of different Western flower thrips populations on cucumber found that a population from New Zealand produced fewer offspring than populations from The Netherlands and Italy (de Kogel et al. 1997). This may indeed be an accurate reflection of interspecific variation but the possibility is now apparent that the European populations were Western flower thripsG, and the New Zealand population was Western flower thripsL (which in itself would be interesting because it was collected as a pest of eggplant). Furthermore, it is obvious now that in their study of insecticide resistance in a "greenhouse" and "lupin strain," Martin and Workman (1994) were most likely comparing the different species. A similar study showing that "greenhouse" populations in Europe and Africa are far more resistant to insecticides than a California "field" strain (Brødsgaard 1994) may have unwittingly compared Western flower thripsG with Western flower thripsL.

Similar uncertainty also now shrouds studies documenting differences in the efficiency with which different Western flower thrips populations vector plant diseases. Sakimura (1962) compared pure-bred pale and dark forms of Western flower thrips and found the two color-morphs transmitted TSWV with equal efficiency. However, Sakimura's color-morphs originated from different populations and so yet again these differences may be representative of Western flower thripsL and Western flower thripsG. As a consequence, any true differences in the vector efficiency of the different color-morphs of just one of these species may have remained hidden. In studying differences in the competency of males and females to vector TSWV, van de Wetering et al. (1999) also found that overall, the populations with the lowest levels of TSWV transmission efficiency were two populations from the United States. Precise collection details are missing for these populations, but the possibility remains that this study was actually documenting inter- rather than intraspecific variation. Finally, the accuracy of a study simply documenting genetic variation between populations of Western flower thrips in Israel may also be suspect. Fang et al. (2005) found that *F. occidentalis* displayed much greater levels of interpopulation genetic variation than *Thrips tabaci* Lindeman. With the knowledge that Western flower thrips is two species, we should question the identity of those *F. occidentalis* populations. Some populations may have been Western flower thripsL, some Western flower thripsG, and still others a mixture of both.

Finally, the discovery that Western flower thrips is actually a complex of two cryptic species creates a problem regarding the naming of those species. Interestingly, the different color-morphs in California were once recognized as different species. Lighter forms were considered to be *F. occidentalis* and darker forms were considered to be *F. moultoni* Hood (Bryan and Smith 1956). However, as a consequence (at least in part) of the morphological variation in Western flower thrips, the ease with which it can be collected, and its wide geographic range in the western United States, 18 synonyms in total are recognized for what is currently known as *F. occidentalis* (Hoddle et al. 2009). The naming issue urgently needs resolving for the species currently considered as *F. occidentalis.*

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