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INVASION NOTE

Restructuring of a mutualism following introduction of Australian fig trees and pollinating wasps to Europe and the USA

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Abstract Figs and fig-pollinating wasps are obligate mutualists that require each other to complete sexual reproduction. However, landscapers can establish populations of fig trees outside their native ranges by propagation through exported seeds, seedlings or cuttings. Once mature, these trees could be colonized by pollinating wasps and/or various non-pollinating wasps that also develop in figs. In recent decades, the Australian endemic *Ficus rubiginosa* has been planted widely in the Mediterranean region and in parts of the USA. Observation of ripe fruit production suggested that a pollination mutualism has been re-established by pollinating wasps colonizing trees in the plant's introduced range. We therefore used sampling of pollinators from mainland Spain, Tenerife and California (USA) and molecular studies to characterize the restructured mutualism and compare it with the native range. In the native range, the plant is pollinated by five wasp species that form the *Pleistodontes imperialis* complex. However, all wasps in the introduced ranges belonged to just one of these species (*P. imperialis* sp. 1). Moreover, their mtDNA diversity was close to zero and the sequences clearly link them with the native southern population of this species. None of the > 20 non-pollinating wasp species from the native range were found in the introduced ranges. In summary, the restructured mutualism has been dramatically simplified, lacking all non-pollinating wasps and all but one pollinator species from the native range. Moreover, the one pollinator species to establish successfully shows a drastic reduction in genetic diversity relative to its source population.

Keywords *Cytb* · DNA barcoding · *Ficus rubiginosa* · ITS2 · *Pleistodontes imperialis* · Symbiotic interactions

Introduction

Symbiosis is a key factor in the evolution of global biodiversity, and many important ecosystem

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functions, such as pollination and nitrogen fixation depend strongly on symbiotic interactions between species. Although some symbiotic interactions can be diffuse, involving loose sets of species, many others are intimate and highly partner-specific (Leigh Jr 2010). In such cases, the ability of a given species to establish and successfully reproduce in a new range depends heavily on the presence and availability of viable partner species. A classic example is the obligate mutualism between fig trees (*Ficus* species) and their tiny pollinating wasps in the family Agaonidae (Hymenoptera) (Cook and Rasplus 2003).

There are over 750 *Ficus* (Moraceae) species and each is pollinated by only a small number (often one, but up to five) of highly host-specific agaonid wasp species (Wiebes 1979; Cook and Rasplus 2003; Darwell et al. 2014). The wasps are the only pollen vectors for the figs, and the wasp offspring can develop only inside fig fruits, where each wasp larva feeds on one fig flower. *Ficus* is a pantropical genus and figs often act as keystone species by providing resources for a wide variety of frugivorous animals (Shanahan et al. 2001). Figs are also highly valued culturally and used extensively in plantings of parks, roadsides and gardens throughout the warmer parts of the world. This has led to several species being planted extensively outside their natural ranges, including on different continents. Most introduced populations of fig trees also harbour fig wasp species. However, the high host specificity of the wasps, and the fact that most *Ficus* sections are restricted to particular continents or biogeographic regions, act as major barriers to establishment of new pollination mutualism partnerships. Nevertheless, in some cases, the known pollinators of a given fig species have been deliberately introduced following the establishment of mature fig trees in a new country (e.g. Pemberton 1934; reviewed in McKey 1989).

Ficus rubiginosa Desf. ex. Vent. is endemic to eastern Australia. It is a popular species in the horticultural trade, both within and outside of Australia. In fact, it has been planted widely around the globe, in coastal regions of Spain and in the Canary Islands, as well as in the USA, New Zealand, Italy and Israel (Gardner and Early 1996; Starr et al. 2003). For a long time, it was thought to be pollinated by a single species, *Pleistodontes imperialis* Saunders (see Lopez-Vaamonde et al. 2002), but Haine et al. (2006) used *cytochrome b* sequences to reveal four well-differentiated mitochondrial lineages that were suggested to be separate species. Darwell et al. (2014) subsequently identified a complex of five *P. imperialis* species, supported by data from two mitochondrial markers and the nuclear internal transcribed spacer 2 (ITS2) locus. Interestingly, Darwell et al. (2014) also showed that *P. imperialis* sp. 1 has disjunct northern and southern populations, separated by > 1000 km. This study was supported by a more recent population genetic study (Sutton et al. 2016), which suggests that the two disjunct *P. imperialis* sp. 1 populations could represent incipient species.

In this study, we take advantage of the remarkable microcosm of the fig to study reassembly of a symbiotic interaction outside of its natural range. Specifically, we explore the species and genetic diversity of wasps pollinating the Australian endemic *F. rubiginosa* in its introduced range in mainland Spain, the Canary Islands and San Diego, USA to determine (1) the wasp species present and (2) their likely source region.

Materials and methods

Sample sites and specimen collection

Mature fig fruits were collected from *Ficus rubiginosa* trees in the introduced range in mainland Spain (Carboneras, Cartagena, Málaga, Mojácar, Moraira and Murcia), Tenerife (Canary Islands), and San Diego (USA) between March 2005 and June 2011. *Pleistodontes imperialis* wasps either emerged naturally from the fruits or were dissected out from figs that were first preserved in ethanol. All wasps were stored in 95% ethanol at − 20 °C.

DNA extraction

DNA was extracted from wasps using a Chelex extraction procedure detailed in West et al. (1998). A single whole female wasp representing each sample was ground up using a pipette tip and added to a 100 µl Chelex (Bio Rad) solution. Tubes were centrifuged for 2 min at 13,000 rpm and incubated for 3 h at 56 °C, then 15 min at 96 °C. Tubes were then vortexed for 10 s, centrifuged again for 2 min at 13,000 rpm, and the supernatant stored in a new tube for PCR.

Cytochrome b sequencing

A 444 bp segment of mitochondrial *cytb* was amplified using the CB1/CB2 primers (Jermiin and Crozier 1994). PCRs were performed in a Touchgene Gradient thermocycler (Techne) in 22 µl reactions consisting of 2 µl DNA template, 1X Biomix (Bioline), 1.5 µM $MgCl_2$ and 0.5 µM of each forward and reverse primer. The PCR cycling conditions were: 3 min at 95 °C followed by 30 cycles of 15 s at 94 °C, 20 s at 45 °C and 30 s at 72 °C, and a final extension of 72 °C for 5 min. All PCR products were purified and sequenced by Macrogen (Korea) using the same primers employed in the original PCR.

ITS2 sequencing

The nuclear ITS2 region was sequenced for five Spanish wasps (including wasps from both mainland Spain and Tenerife) and four wasps from San Diego using the ITS2F/ITS2R primers (White et al. 1990). In addition, we sequenced ITS2 for eight other wasps collected previously from the native range of *F. rubiginosa* (Sutton et al. 2016, 2017). DNA was amplified in 22 µl reactions, comprising 2 µl DNA template, 1X Biomix (Bioline), 0.5 µM $MgCl_2$, and 0.35 µM of each forward and reverse primer. Thermal cycling conditions were as follows: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 40 s at 72 °C, and a final extension of 72 °C for 10 min. PCR products were sequenced by Macrogen.

Data analyses

DNA sequences were aligned and edited using BioEdit v. 7.0.9.0 (Hall 1999). Cytochrome b and ITS2 haplotype phylogenies were constructed using MrBayes v. 3.2 (Ronquist et al. 2012) to assign the introduced wasps to one of the five species identified by Darwell et al. (2014). Reference sequences representing each of the five species were included in each phylogeny (Darwell et al. 2014). The most appropriate substitution model for each data set was determined using jModelTest v. 2 (Guindon and Gascuel 2003; Darriba et al. 2012). The *cytb* haplotype tree was constructed using the symmetrical model (Zharkikh 1994) with gamma distributed rate variation. The ITS2 haplotype tree was constructed using the Kimura 2-parameter model (Kimura 1980). We performed two independent runs of four Metropolis coupled Monte Carlo Markov chains for 10 million generations, sampling every 1000 generations and with a relative burn-in period of 25%. It was assumed the two runs had converged if the standard deviation of the split frequencies of the two runs was below 0.01.

We used DnaSP v. 5 (Librado and Rozas 2009) to estimate nucleotide diversity (π) and gene diversity for introduced and native *P. imperialis* sp. 1 (south and north populations; Sutton et al. 2016). We also calculated Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) to test for deviations from equilibrium (e.g. demographic expansion, selection).

Results

Cytochrome b phylogeny and diversity

Sequences of 333 bp were obtained for 97 individual wasps from various locations in mainland Spain, Tenerife and San Diego (GenBank accession numbers KJ796700–KJ796788, MG025546–MG025553). Six unique sequences were found for wasps from the introduced ranges. Three haplotypes could be determined fully at all positions, and differed from each other by either one or two base pairs (i.e. 0.3 to 0.6% sequence divergence). The other three haplotypes had double peaks at a small number of sites (7/333; 2.1%). As these haplotypes were almost identical to the others at unambiguous positions, we do not present further analysis of them here. All three fully resolved introduced haplotypes were placed unambiguously within *P. imperialis* species 1; i.e. only one of the five native pollinator species was found in the introduced range. Moreover, the introduced wasps all clustered with *P. imperialis* sp. 1 sequences from the native southern Australian population (Fig. 1). One haplotype (Introduced01) was dominant in mainland Spain (57/61 wasps; 93%) and Tenerife (23/31 wasps; 74%). This was also the most common haplotype in *P. imperialis* sp. 1 (south) in the native range (22/100 wasps; 22%), as well as being the only haplotype in the five San Diego samples. Two haplotypes (Introduced01 and Introduced02) were found in Málaga, Spain, and all three introduced haplotypes were found in Tenerife.

Nucleotide and gene diversity measures were estimated after assignment of individuals to one of

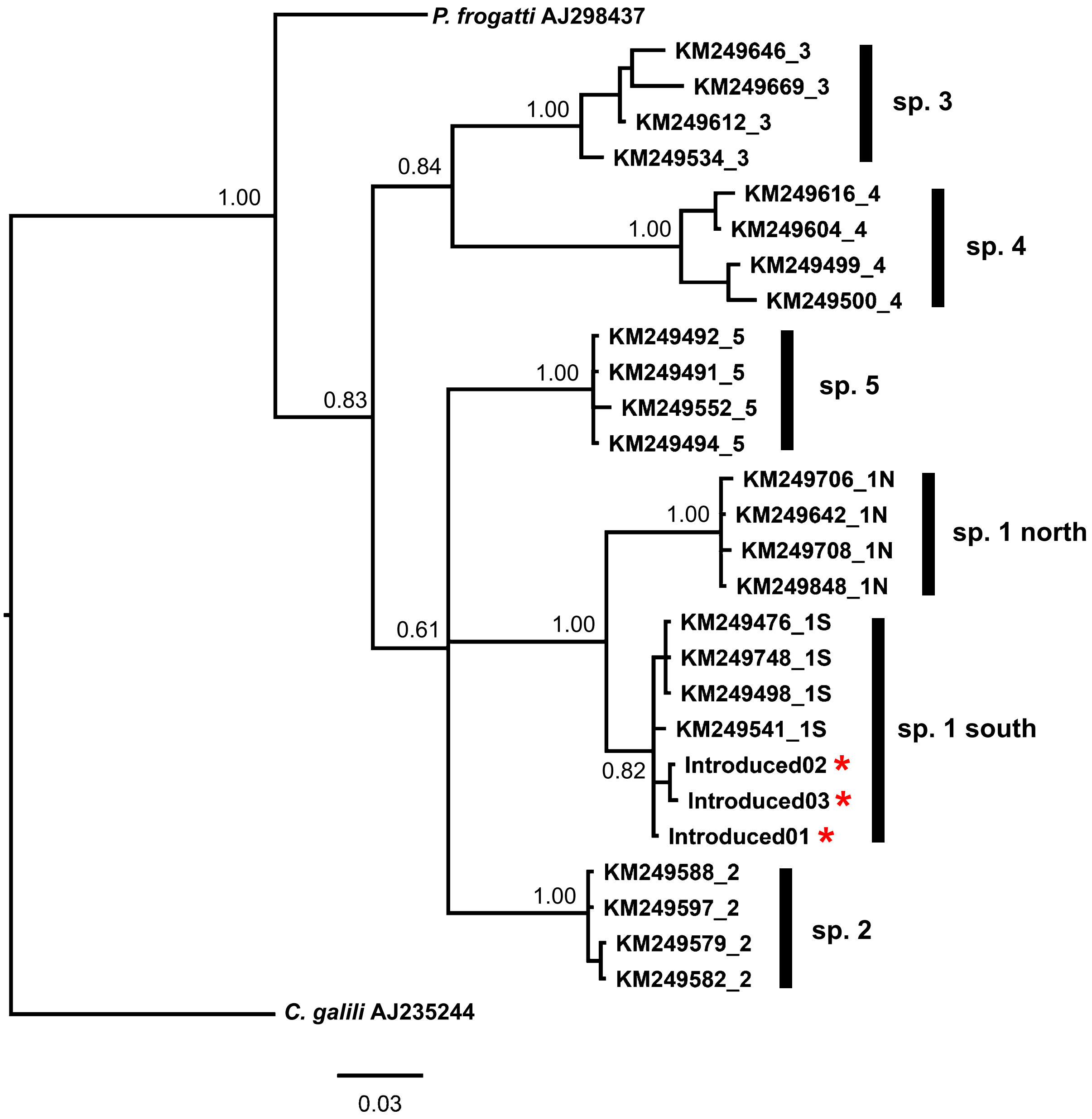

Fig. 1 Consensus Bayesian topology based on 333 bp of the *cytb* gene for *Pleistodontes imperialis.* Introduced wasp haplotypes (red asterisks) from 97 individuals, group with the southern species 1 population in the native range. Posterior node probabilities are indicated for the five species, and branch lengths are proportional to the number of substitutions per site. Native wasp sequences from Darwell et al. (2014). Tree routed using the outgroups *Ceratosolen galili* (Kerdelhué et al. 1999) and *P. froggatti* (Lopez-Vaamonde et al. 2001)

the three true haplotypes. Introduced Spanish wasps were far less diverse than sp. 1 in either the south or north populations in the native range (Table 1). Species 1 (south) had the greatest nucleotide (0.008 ± 0.005) and gene (0.925 ± 0.017) diversity, whereas individuals from San Diego had zero diversity. Significant negative values of *D* and *Fs* indicate a recent population expansion in the sp. 1 populations in the native range, although no such changes in population size were detected in the introduced wasps.

Table 1 Cytochrome b variation, and demographic analyses of *Pleistodontes imperialis*

	Sp. 1 south ($N = 100$)	Sp. 1 north ($N = 34$)	Spain ($N = 61$)	Tenerife ($N = 31$)	San Diego ($N = 5$)
π	0.008 ± 0.005	0.005 ± 0.004	0.0004 ± 0.0007	0.0015 ± 0.0015	0.000 ± 0.000
Gene diversity	0.925 ± 0.017	0.547 ± 0.104	0.126 ± 0.057	0.422 ± 0.093	0.000 ± 0.000
Tajima's D	− 2.25	− 2.35	− 1.08	− 0.02	0
	($P < 0.001$)	**($P < 0.001$)**	($P = 0.08$)	($P = 0.45$)	($P = 1$)
Fu's F_s	− 26.69	− 4.42	− 1.73	0.06	N/A
	($P < 0.0001$)	**($P < 0.02$)**	($P = 0.10$)	($P = 0.43$)	

Native wasps from the south and north populations of sp. 1; introduced waps from mainland Spain, Tenerife and San Diego. Nucleotide (π) and gene diversity values ± SD. Significant values shown in bold. Data for sp. 1 wasps in the native range were obtained using sequences from Sutton et al. (2016; GenBank accessions KT189199–KT189330)

ITS2 phylogeny

There was considerable base ambiguity in the middle portion of the fragment sequenced, perhaps due to the existence of multiple paralogous ITS sequences, as has been reported for several animal taxa. Consequently, sequences were trimmed from 344 bp to 138 bp, for which alignment and comparison was unambiguous (see Online Resources 1–3 for alignments). All pollinator species were resolved clearly; only one nuclear ITS2 haplotype was identified for the nine wasps from Spain and San Diego. Although the native Australian southern and northern sp. 1 populations group together in the tree that includes other species in the complex, individuals could be assigned to population simply based on a two-bp indel at sequence position 113 (Fig. 2; Online Resource 4). From this, it was apparent that all samples from the introduced range shared the same haplotype as the southern *P. imperialis* sp. 1 population, providing nuclear DNA support for the above results using mitochondrial *cyt b*.

Discussion

The reassembly of symbiotic interactions between figs and their pollinating wasps in introduced ranges is critical for the production of ripe figs with seeds and hence, for fig reproduction. This is also important for wider ecological interactions, as figs can provide important year-round resources for diverse frugivores (McKey 1989; Shanahan et al. 2001). Frugivores can aid the spread of plants in introduced ranges and, if environmental conditions support seedling recruitment, introduced figs may become invasive. For example, the seeds of *Ficus microcarpa*, native to Asia but cultivated widely, can be spread long distances by birds in its introduced range (Williams 1939). Consequently, it has become an aggressive invader in Hawaii, where its pollinator has also been introduced, and is considered to pose a high threat to native flora (Starr et al. 2003). *Ficus rubiginosa* has also been introduced in several countries and its fruits attract local frugivores in at least parts of its introduced range (Starr et al. 2003). Both pollinator wasps (Gardner and Early 1996) and some non-pollinating wasps (Early 2000) have been reported in *F. rubiginosa* figs in New Zealand, along with observations of seedlings. Interestingly, Gardner and Early (1996) argued that pollinator wasps probably reached New Zealand by natural long-distance dispersal from Australia, making this case clearly different to wasp arrivals in northern hemisphere locations such as California or Spain.

Our study revealed a severe reduction in pollinator diversity in the *F. rubiginosa* pollination mutualism following its introduction to Spain, Tenerife and San Diego. We detected only one of the five cryptic *Pleistodontes imperialis* obligate pollinator species, and all individuals ($N = 97$) were assigned to the sp. 1 southern population. Given the small sample size obtained from San Diego, however, we cannot rule out the coexistence of other *P. imperialis* species. Although we do not know the history of wasp introductions in Spain and San Diego, *P. imperialis* previously became locally established in Hawaii just

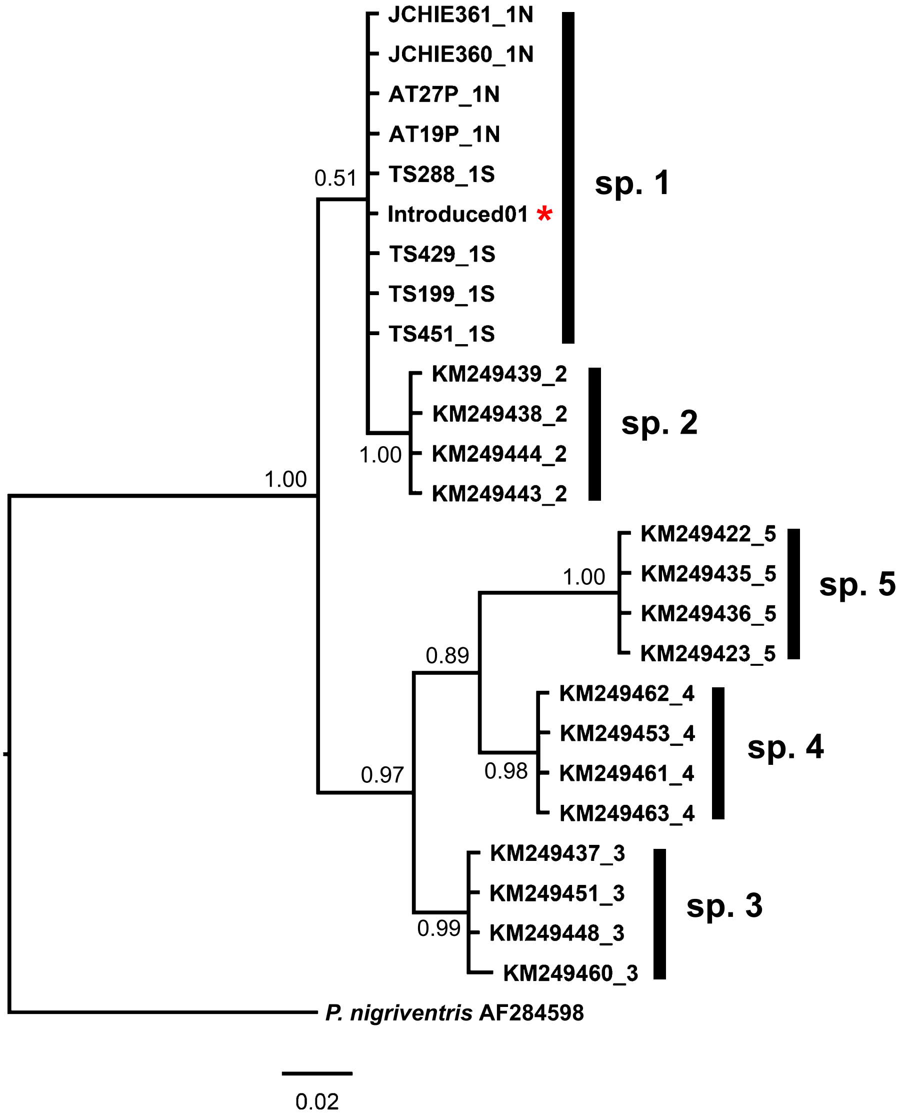

Fig. 2 Consensus Bayesian topology based on 138 bp ITS2 sequences for *Pleistodontes imperialis* individuals. The introduced wasp haplotype (red asterisk), shared by all nine individuals sampled, group with sp. 1 from the native range. Posterior node probabilities are indicated for the five species, and branch lengths are proportional to the number of substitutions per site. Tree routed using the outgroup *P. nigriventris* (Lopez-Vaamonde et al. 2001). *Pleistodontes imperialis* sp. 2–4 sequences obtained from Darwell et al. (2014)

1 year following its release (Anonymous 1924). Pollination mutualisms have also been established in some other countries where *F. rubiginosa* has been planted, e.g. in New Zealand (Gardner and Early 1996), as well as Israel (D. Eisikowitch, pers. comm.), but the identity, diversity and genetic status of the pollinating wasps are currently uncertain. Nuclear ITS2 sequences have been shown to provide a reliable complement to the mitochondrial *cytb* in fig wasps (e.g. Darwell et al. 2014; Sutton et al. 2016), and in our study both ITS2 and *cytb* phylogenies support the placement of the introduced wasps with the southern population (New South Wales) of *P. imperialis* sp. 1 (Sutton et al. 2016), which is the most likely source population for the introduced wasps. Interestingly, if pollinators have reached New Zealand by natural long-distance dispersal (Gardner and Early 1996), we would predict them to be *P. imperialis* sp. 1 since this is the only species found throughout most of the host plant's range in New South Wales (e.g. Darwell et al. 2014; Sutton et al. 2016), with the other four species being found farther north, mainly in Queensland.

We detected far lower genetic diversity in wasps in the introduced range than in either of the two sp. 1 populations in the native range. In fact, there was hardly any variation in the introduced populations, although the low genetic diversity revealed in the San Diego wasps may be partly attributable to small sample size. Species 1 south and north populations in the native range showed evidence for a recent population expansion, but the introduced wasps did not. As the introduced wasp populations are probably less than 100 years old, possibly much less, there may have been insufficient time for mutation to replace mtDNA variation that was lost through the colonization process. Ornamental *F. rubiginosa* trees have been planted in many locations along the Spanish coastline, and represent a fragmented habitat, although some areas already have large numbers of both trees and pollinators. However, the initial populations of pollinators would probably have been bottlenecked at colonization and then subjected to genetic drift and subsequent loss of genetic diversity following introduction (Frankham et al. 2010).

The Spanish and San Diego wasps had almost identical *cytb* haplotypes, differing by no more than 0.6%. By comparison, we found sp. 1 south and north populations in the native range had up to 3.3 and 5.1% intraspecific *cytb* divergences, respectively, and a maximum divergence between wasps from these two different populations of 7.5%. It is therefore likely that the introduced pollinators in all regions studied came from the same area in the south of the native range of the host plant. It is also possible that the wasps went through serial colonization events outside of Australia after one original export from the native range. Further studies targeting populations in other countries (e.g. Israel, Italy, New Zealand) and using a wider range of genetic markers (e.g. microsatellites) could be used to test this hypothesis. However, we also cannot exclude the possibility that more than one species (or population) of pollinator was translocated, but that the others have become extinct or have not been sampled. The latter seems unlikely, however, given the broad geographic sampling area of this study and the fact that different members of the *P. imperialis* complex co-exist in the same sites and even figs in parts of the native range (Sutton et al. 2017).

The fig/pollinator mutualism is also exploited by often diverse communities of non-pollinating fig wasps (NPFW) that develop in fig fruits as gall-inducing seed predators or parasitoids. These parasitic species may (West and Herre 1994) or may not (Bronstein 1991) be in direct competition with fig wasps for fig flowers, but either way, are important components of the system and influence its coevolution (Kerdelhué and Rasplus 1996). Dunn et al. (2008), for example, concluded that parasitism pressure has contributed to pollinator preference for oviposition in central fig ovules. This preference allows outer ovules to develop into fig seeds and, thus, allows for both male and female reproductive functions of the fig to persist.

Introduced species often leave their natural enemies behind (Torchin et al. 2003), and NPFW may also be present in lower densities in introduced ranges (Wang et al. 2015). In our study, we found no NPFW, although in its native range *F. rubiginosa* hosts over 20 NPFW species (Segar et al. 2013; Darwell and Cook 2017), several of which parasitize the pollinator galls. A few of these species have colonized New Zealand, probably by natural long-distance aerial dispersal (Early 2000), but (as for the pollinators) wasp arrivals in northern hemisphere populations would seemingly require human mediation. This makes an interesting comparison with *F. microcarpa*, which is probably the fig species planted most widely around the world as an urban tree. Many *F. microcarpa* populations beyond its native range of Asia and Australasia now have a few of the 43 wasp species associated with this host plant (Wang et al. 2015). In particular, the pollinator and two phytophagous species are often the first wasps recorded in introduced populations. This may reflect the fact that they are common and widespread in the native range and, therefore, the species most likely to be present in transported figs (Wang et al. 2015). In addition, none of these three species (in contrast to e.g. a parasitoid) requires the presence of any other wasp species in order to establish a new population.

The other way for introduced plants to gain insect associates is when local species adopt the new host plant. There are major barriers to this process in fig wasps due to the importance of coevolved insect and plant traits (Cook and Segar 2010), such that it is most likely when the introduced range includes closely related fig species with similar wasps. In a global survey of *F. microcarpa*, new colonists were very rare, occurring only occasionally in one country (South Africa) that had local figs from the same subgenus (Wang et al. 2015). On this basis, rapid recruitment of new wasp species by *F. rubiginosa* via host shifts seems very unlikely in the northern hemisphere.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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