Heredity (2017) 118, 486–490

www.nature.com/hdy

ORIGINAL ARTICLE

Local coexistence and genetic isolation of three pollinator species on the same fig tree species

TL Sutton, JL DeGabriel, M Riegler and JM Cook

Molecular tools increasingly reveal cryptic lineages and species that were previously unnoticed by traditional taxonomy. The discovery of cryptic species in sympatry prompts the question of how they coexist in the apparent absence of ecological divergence. However, this assumes first that the molecular taxonomy used to identify cryptic lineages delimits species boundaries accurately. This issue is important, because many diversity studies rely heavily or solely on data from mitochondrial DNA sequences for species delimitation, and several factors may lead to poor identification of species boundaries. We used a multilocus population genetics approach to show that three mtDNA-defined cryptic lineages of the fig wasp *Pleistodontes imperialis* Saunders, which pollinate Port Jackson figs (*Ficus rubiginosa*) in north-eastern Australia, represent reproductively isolated species. These species coexist locally, with about 13% of figs (where mating occurs) containing wasps from two or three species. However, there was no evidence for gene flow between them. Confirmed cases of coexisting cryptic species provide excellent opportunities for future studies of the ecological and evolutionary forces shaping both species coexistence and fig/pollinator coevolution.
Heredity (2017) **118,** 486–490; doi:10.1038/hdy.2016.125; published online 11 January 2017

INTRODUCTION

The rise of molecular taxonomy has seen an increase in the discovery of cryptic species, often revealed first by analysis of mitochondrial DNA (mtDNA) fragments (for example, Wilcox *et al.*, 1997; Hebert *et al.*, 2004; Fouquet *et al.*, 2007; Murphy *et al.*, 2011). These cryptic genetic lineages may replace each other geographically, but in cases where they coexist locally, they pose a challenge for ecologists. Conventional theory predicts that two or more species should not coexist locally in the same niche and that competitive exclusion should lead to the extinction of all but one species (Gause, 1934; Chesson, 1991; Hubbell, 2001). Explaining the local coexistence of cryptic species is therefore problematic in itself, but also influences our understanding of species interactions (for example, between plants and pollinators, or host and parasites), which were previously interpreted based on morphological data.

While molecular taxonomy may reveal cryptic lineages, the reliability of mtDNA for species delimitation has been criticised for various reasons (Rubinoff *et al.*, 2006), including the persistence of genetic structure after resumption of gene flow due to lack of mtDNA recombination (for example, Lausen *et al.*, 2008; Ruskey and Taylor, 2016), hybrid introgression (for example, Chen *et al.*, 2009; Machado-Schiaffino *et al.*, 2010) and selective sweeps caused by maternally inherited endosymbionts that can drive divergence and homogenisation across species (for example, Graham and Wilson, 2012; Xiao *et al.*, 2012; Jäckel *et al.*, 2013). These issues raise significant concerns when mtDNA is used in isolation, but they are likely to be resolved with further study of nuclear loci.

The arrays of multilocus nuclear markers provided by microsatellite loci provide an effective means of assessing reproductive isolation in coexisting cryptic mtDNA lineages. For example, Lausen *et al.* (2008) revealed high estimates of gene flow among well-differentiated mtDNA lineages of the little brown bat, *Myotis lucifugus*. Similarly, Ruskey and Taylor (2016) found that mtDNA lineages of sympatric fish were not supported by microsatellite analyses. Conversely, microsatellite analyses support mtDNA-delimited species boundaries in many taxa. Naughton *et al.* (2014) found that microsatellite data support mtDNA lineages in *Cenolia* feather stars, although introgression was observed in a small number of individuals. In addition, Donnelly *et al.* (2013) demonstrated support for mtDNA species in *Lumbricus rubellus* earthworms in South Wales. Interestingly though, divergent *L. rubellus* mtDNA lineages in Poland are not reproductively isolated (Giska *et al.*, 2015). Population genetics can thus help to elucidate the reliability of species boundaries delimited using mtDNA or only a limited number of DNA markers.

Fig trees (*Ficus*, Moraceae) and their pollinator wasps (Hymenoptera: Agaonidae) share an obligate reproductive mutualism renowned for high levels of partner specificity (Wiebes, 1979). The traditional view was that most fig species had just one pollinator wasp species. However, mtDNA analyses have helped drive the realisation that many fig species may be pollinated by multiple fig wasp species (Cook and Rasplus, 2003). Recently, there have been several reports of cryptic pollinator 'species' using the same host fig species both in sympatry and allopatry (Molbo *et al.*, 2003; Haine *et al.*, 2006; Sun *et al.*, 2011; Chen *et al.*, 2012; Darwell *et al.*, 2014). Where morphologically cryptic pollinator species coexist locally, their persistence requires ecological and/or behavioural explanation (Zhang *et al.*, 2004). For example, non-pollinating species may evolve following the rise of cheating within an existing pollinator species (Compton *et al.*, 1991; Jandér and

Hawkesbury Institute for the Environment, Western Sydney University, Penrith, New South Wales, Australia
Correspondence: Professor JM Cook, Hawkesbury Institute for the Environment, Western Sydney University, Locked Bag 1797, Penrith, New South Wales 2751, Australia.
E-mail: James.Cook@westernsydney.edu.au
Received 18 May 2016; revised 27 October 2016; accepted 30 November 2016; published online 11 January 2017

Herre, 2010). If figs often have two or more co-pollinators, then this is of major consequence for coevolutionary studies, both with regard to local ecological dynamics and speciation. Many cases of supposed cryptic fig wasp species, or at least ones that differ only subtly in morphology, have been reported recently based on mtDNA data, and we need to clarify their status to better interpret this key model system.

An extreme deviation from reciprocal partner specificity occurs with the Port Jackson fig (*Ficus rubiginosa* Desf. ex Vent.), which is pollinated by five 'species' of the *Pleistodontes imperialis* Saunders species complex along the Australian east coast (Haine *et al.*, 2006; Darwell *et al.*, 2014). Following taxonomic revision in 2002, these were considered a single species (Lopez-Vaamonde *et al.*, 2002), but were later found to resolve into distinct lineages (Haine *et al.*, 2006; Darwell *et al.*, 2014). Haine *et al.* (2006) found support for four species based on mitochondrial *cytochrome b* (*cytb*); however, the nuclear 28S rDNA and *wingless* genes only resolved two lineages. With wider sampling, Darwell *et al.* (2014), subsequently showed support for five species using nuclear ITS2 and mitochondrial COI and *cytb*.

For three of the *P. imperialis* lineages, no morphological differences are known, while the other two differ only slightly, in colour or head length (J.-Y. Rasplus, personal communication). As the host plant geographic range covers a number of biogeographic barriers (notably the Burdekin Gap, St Lawrence Gap, Dawson-McKenzie Gap and the McPherson Range) that are reflected in the mtDNA phylogeography of many animal taxa (for example, Hugall *et al.*, 2002), the interpretation of these lineages remains unclear.

We explored a potential hybrid zone around Townsville in north-eastern Queensland, where three *P. imperialis* lineages ('species 2', 'species 3' and 'species 4' *sensu* Haine *et al.*, 2006) coexist in sympatry. We used genetic markers (nuclear DNA sequences, mtDNA sequences and microsatellites) to explore patterns of gene flow between these three lineages and assess their evolutionary status. Specifically, we assessed (1) the opportunity for hybridisation (that is, the frequency of fig fruits containing offspring from different lineages), (2) nuclear microsatellite differentiation between these distinct mtDNA lineages and (3) the prevalence of hybridisation.

MATERIALS AND METHODS

Fig wasp biology

Receptive fig fruits (syconia) attract pollen-bearing female fig wasps through the emission of species-specific volatiles. The female wasp (foundress) enters the receptive syconium through a narrow opening (ostiole), which closes at the end of the receptive phase. Inside, the foundress pollinates the fig ovules, depositing a single egg into some of these, and dies within 24–48 h. Egg deposition initiates gall formation, within which the developing larva feeds on the fig seed. Offspring develop over a period of about 6–8 weeks (Harrison, 2005; Xiao *et al.*, 2008). Mature males exit their galls to locate galls containing females, and chew holes into galls in order to mate with the females within. After mating, the wingless males chew holes through the outer wall of the syconium for the females to exit through, but do not disperse themselves. Females emerge from their galls, collect pollen and exit the syconium to disperse in search of receptive syconia in which to lay eggs (Galil and Eisikowitch, 1968).

Sample collection

Ficus rubiginosa syconia were collected from eight sites around Townsville in north Queensland (19°15′24″ S, 146°49′3″ E) between November 2013 and November 2014. Near-ripe figs ($N=611$) were collected from naturally occurring trees and placed into individual plastic specimen pots. Emerged wasps were preserved in absolute ethanol prior to molecular analyses. Typically only 1–4 females lay eggs in a single syconium. Because of the high probability that any two wasps from the same syconium are siblings, only a single wasp from each syconium was used for molecular analyses. Samples from different sites and different time points were pooled for analyses.

Assessment of the potential for hybridisation

The potential for hybridisation was quantified as the proportion of syconia that produced both yellow ('species' 2) and black ('species' 3 or 4) wasps. This method provides an estimate of the minimum frequency of co-occurrence of multiple species within a syconium, and thus represents the minimum frequency of opportunities for heterospecific matings.

DNA extraction and mtDNA analyses

A subset of samples was used for DNA analyses, with sample sizes approximately proportional to the frequency of each lineage (Table 1). DNA was extracted individually from whole female insects ($N=139$) using a Chelex method (West *et al.*, 1998) to a volume of 100 µl. The *cytb* gene was amplified and sequenced in all samples using CB1/CB2 primers and the protocol described in (Jermiin and Crozier, 1994). Although COI is the standard barcoding gene in animals, most barcoding efforts for this species complex have utilised the *cytb* gene (Haine *et al.*, 2006; Darwell *et al.*, 2014) since COI amplifies inconsistently and is compromised as a marker by nuclear pseudogenes in fig wasps. DNA sequences were edited by eye in Sequencher v4.10.1 (Gene Codes, Ann Arbor, MI, USA) and aligned in Clustal X v2.0 (Larkin *et al.*, 2007).

A *cytb* haplotype phylogeny was constructed in MrBayes v3.2 (Ronquist *et al.*, 2012) to assign individuals to lineages according to Darwell *et al.* (2014). Reference sequences for each of the three lineages (Haine *et al.*, 2006; Darwell *et al.*, 2014) were included in the phylogeny, along with outgroup sequences for *Pleistodontes nigriventris* (Girault; Lopez-Vaamonde *et al.*, 2001) and another fig wasp, *Ceratosolen galili* Wiebes (Kerdelhue *et al.*, 1999). jModelTest v2 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012) was used to determine that the HKY+G (Hasegawa *et al.*, 1985) model was the most appropriate for our data. Two independent runs of four Metropolis coupled Monte Carlo Markov chains for 10 million generations were performed with a relative burn-in of 25%, and sampling every 1000 generations. The two runs were assumed to have converged if the mean s.d. of the split frequencies fell below 0.01.

Microsatellite analyses

Each individual was genotyped at nine microsatellite loci (Sutton *et al.*, 2015). These were amplified in multiplex reactions, as detailed in Sutton *et al.* (2016). All genotyping was performed on a 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA), and electropherograms were visualised and scored using GeneMapper v4.1 (Applied Biosystems). One locus (Pli10) did not amplify in *P. imperialis* sp. 3 samples, and was therefore excluded from subsequent analyses.

Table 1 mtDNA and microsatellite diversity of three sympatric *P. imperialis* species

P. imperialis species	N	*mtDNA*		*Microsatellites*		
		No. haplotypes	*Nucleotide diversity*	N_A	N_P	H_O/H_E
2	79	19	0.007±0.004	10.71±2.38	8.89±2.36	0.47/0.76*
3	39	21	0.010±0.006	4.02±1.17	2.58±1.11	0.18/0.40*
4	22	12	0.015±0.008	6.25±1.26	4.94±1.20	0.11/0.57*

Abbreviations: *N*, sample size; N_A, number of alleles (with rarefaction); N_P, number of private alleles (with rarefaction); H_O, observed heterozygosity; H_E, expected heterozygosity.
Asterisks denote significant deviation from Hardy-Weinberg equilibrium ($P<0.05$).

The number of alleles and number of private alleles with rarefaction were calculated in ADZE (Szpiech *et al.*, 2008) and deviation from Hardy-Weinberg equilibrium was tested in GENEPOP v4.2 (Raymond and Rousset, 1995; Rousset, 2008). Pairwise tests of genotypic differentiation (Goudet *et al.*, 1996) were performed in GENEPOP and pairwise population F_{ST} was calculated in ARLEQUIN.

The Bayesian clustering algorithm implemented in TESS (Chen *et al.*, 2007) was utilised to assess potential gene flow between the *cytb*-defined lineages. The CAR admixture model (Durand *et al.*, 2009) was implemented and 100 iterations were conducted, consisting of 60 000 sweeps of MCMC with a burn-in period of 10 000 sweeps. The maximum number of clusters (K_{max}) was restricted to three as our aim was to assess gene flow between the three *cytb*-defined lineages. The estimated admixture coefficients were averaged using the Greedy algorithm in CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007) and the data were subsequently visualised in DISTRUCT v1.1 (Rosenberg, 2004). For individuals whose highest cluster assignment probability from the TESS analysis was $<96\%$, lineage assignment was confirmed by also sequencing the nuclear ITS2 region (Supplementary Material).

RESULTS

At least 13.38% of *F. rubiginosa* syconia ($N=611$) contained wasps from multiple lineages, as evidenced by the presence of yellow (species 2) and black (species 3 or 4) *P. imperialis* wasps. One hundred and thirty-nine female *P. imperialis* wasps were sequenced for a 372 bp *cytb* fragment, and each individual was unambiguously assigned to one of the three known *P. imperialis* lineages found in Townsville (Supplementary Figure S1). Fragments of similar length (396 bp) were previously used by Darwell *et al.* (2014) to delimit these lineages. We found no evidence for nuclear pseudogenes in comparisons of amino-acid sequences of our samples with those of Darwell *et al.* (2014). We identified 52 unique haplotypes across three *P. imperialis* lineages and nucleotide diversity within lineages ranged from 0.007 to 0.015 (Table 1). Intraspecific *cytb* genetic divergences ranged from 0.0 to 6.5% (mean = 0.07%, 1.6 and 1.0% for species 2, 3 and 4, respectively) and interspecific divergence ranged from 8.9 to 16.1% (mean = 12.6%).

Microsatellite diversity (N_A and H_E) was greatest in *P. imperialis* sp. 2 and lowest in *P. imperialis* sp. 3, and over 50% of alleles were not shared between lineages (Table 1; Supplementary Figure S2). All lineages were significantly differentiated with pairwise F_{ST} values ranging from 0.28 to 0.54 (Table 2). Bayesian clustering analyses revealed three distinct microsatellite genetic clusters corresponding to *cytb* lineage assignments (Figure 1). Seven out of 139 individuals had maximum cluster assignment probabilities lower than 96%. Subsequent sequencing of the ITS2 region for these individuals (Supplementary Figure S3) supported their initial *cytb* lineage assignment.

Table 2 Genetic differentiation among *P. imperialis* (*cytb*) species

	sp. 2	*sp. 3*	*sp. 4*
sp. 2	—	*	*
sp. 3	0.54	—	*
sp. 4	0.28	0.34	—

Pairwise F_{ST} is given in the lower diagonal and statistical significance for tests of genotypic differentiation are given in the upper diagonal (Asterisk denotes $P<0.001$).

DISCUSSION

We used a population genetic approach to assess gene flow among three sympatric cryptic fig wasp species in north-eastern Australia. Our results revealed that $>13\%$ of syconia contained wasps from at least two mtDNA lineages. The co-occurrence of multiple species in the same syconium indicates that there is potential for hybridisation; however, we did not detect any hybrids or evidence of introgression. This study supports the reproductive isolation in local sympatry and species status of lineages delimited by Darwell *et al.* (2014), who used a few specimens from each of many sites over a wide geographic range.

Bayesian clustering analyses of female wasps indicated a clear concordance between mtDNA and multilocus microsatellite genotypes, despite seven individuals exhibiting admixed cluster assignment probabilities. It is possible that these individuals are F_1 hybrids, which should suggest the potential for gene flow among mtDNA lineages. However, we found no evidence of mtDNA introgression, which could have one of three explanations. Firstly, the frequency of multiple heterospecific foundresses entering a single syconium may be too low to facilitate repeated backcrossing with the paternal nuclear background. Alternatively, F_1 hybrids may be sterile or have reduced fitness. Molbo *et al.* (2004) found diploid F_1 hybrid males in another genus of fig wasps from Panama, suggesting a breakdown in the haplo-diploid sex determination system. Finally, these fig wasps may simply avoid mating with non-conspecifics, even though these are closely related species.

There is a growing number of reports of cryptic 'species' in the fig—fig wasp mutualism, existing both in sympatry and allopatry (Molbo *et al.*, 2003; Haine *et al.*, 2006; Moe and Weiblen, 2010; Lin *et al.*, 2011; Sun *et al.*, 2011). Moe and Weiblen (2010) identified cryptic *Ceratosolen* fig wasp 'species' in association with several widespread *Ficus* species, suggesting an extensive role for vicariance in the generation of cryptic biodiversity. This notion is supported by population genetic analyses of fig wasps in Asia and Australia (Kobmoo *et al.*, 2010; Sutton *et al.*, 2016). Despite the local coexistence of cryptic fig wasp 'species' in at least four genera (Molbo *et al.*, 2003, 2004; Haine *et al.*, 2006; Lin *et al.*, 2011; Sun *et al.*, 2011; Darwell *et al.*, 2014), true tests of hybridisation between such mtDNA lineages have only been undertaken in one of these. Molbo *et al.* (2003) identified two lineages of the fig wasp *Pegoscapus hoffmeyeri* that exhibited ~4% mtDNA divergence (they used COI, while here we use *cytb*). Subsequently, Molbo *et al.* (2004) used diagnostic microsatellite

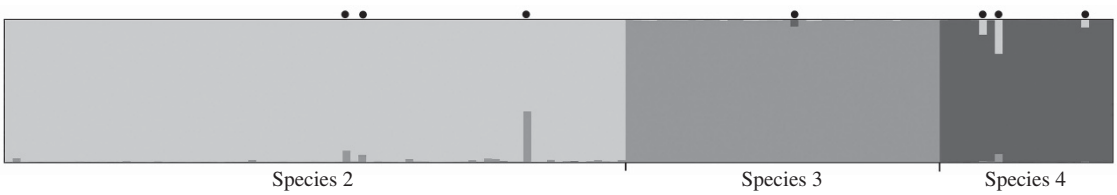

Figure 1 Genetic cluster assignments of 139 *P. imperialis* individuals based on nuclear microsatellite genotypes. Each vertical column represents an individual, and the colours indicate its probability of assignment to each of the three genetic clusters. Samples are grouped by mtDNA assignment. Circles denote individuals whose highest cluster assignment probability was <0.96.

loci to identify hybrids between these lineages in only 1% of syconia sampled, suggesting that gene flow between them is rare. Our results involve wasps from a distantly related genus to those studied by Molbo *et al.* (2004), and the consistency between studies supports the idea that mtDNA-delimited fig wasp lineages are generally reproductively isolated species.

Our data highlight the need to address the question of how cryptic species that appear to be ecological equivalents can coexist on the same resources. Zhang *et al.* (2004) proposed a model to address this fundamental ecological issue by building on the idea that stable coexistence is possible if species discriminatively direct appropriate behaviours towards conspecifics and heterospecifics (Chesson, 1991). We have shown that sympatric *P. imperialis* lineages do not exchange genes and thus represent 'true' reproductively isolated species. Future work should aim to investigate potential divergence in the intricate ecology and morphology of these species and to test the model of cryptic fig wasp coexistence proposed by Zhang *et al.* (2004).

DATA ARCHIVING

DNA sequences were deposited in GenBank with accession numbers KU316794–KU316933 (*cytb*) and KU353510–KU353516 (ITS2). Microsatellite genotype data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.54527.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Will Balmont for assistance with field sampling, and the Fryer family for access to private property in Hervey Range. TLS was supported by an Australian Postgraduate Award and the FG Swain Award (Hawkesbury Foundation).

Chen C, Durand E, Forbes F, François O (2007). Bayesian clustering algorithms ascertaining spatial population structure: a new computer program and a comparison study. *Mol Ecol Notes* **7**: 747–756.

Chen W, Bi K, Fu J (2009). Frequent mitochondrial gene introgression among high elevation Tibetan megophryid frogs revealed by conflicting gene genealogies. *Mol Ecol* **18**: 2856–2876.

Chen Y, Compton SG, Liu M, Chen X-Y (2012). Fig trees at the northern limit of their range: the distributions of cryptic pollinators indicate multiple glacial refugia. *Mol Ecol* **21**: 1687–1701.

Chesson P (1991). A need for niches? *Trends Ecol Evol* **6**: 26–28.

Compton SG, Holton KC, Rashbrook VK, van Noort S, Vincent SL (1991). Studies of *Ceratosolen galili*, a non-pollinating agaonid fig wasp. *Biotropica* **23**: 188–194.

Cook JM, Rasplus J-Y (2003). Mutualists with attitude: coevolving fig wasps and figs. *Trends Ecol Evol* **18**: 241–248.

Darriba D, Taboada GL, Doallo R, Posada D (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* **9**: 772.

Darwell C, Al-Beidh S, Cook J (2014). Molecular species delimitation of a symbiotic fig-pollinating wasp species complex reveals extreme deviation from reciprocal partner specificity. *BMC Evol Biol* **14**: 189.

Donnelly RK, Harper GL, Morgan AJ, Orozco-Terwengel P, Pinto-Juma GA, Bruford MW (2013). Nuclear DNA recapitulates the cryptic mitochondrial lineages of *Lumbricus rubellus* and suggests the existence of cryptic species in an ecotoxological soil sentinel. *Biol J the Linn Soc* **110**: 780–795.

Durand E, Jay F, Gaggiotti OE, François O (2009). Spatial inference of admixture proportions and secondary contact zones. *Mol Biol Evol* **26**: 1963–1973.

Fouquet A, Gilles A, Vences M, Marty C, Blanc M, Gemmell NJ (2007). Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. *PLoS One* **2**: e1109.

Galil J, Eisikowitch D (1968). On the pollination ecology of *Ficus sycomorus* in East Africa. *Ecology* **49**: 259–269.

Gause GF (1934). *The Struggle for Existence*. Williams & Wilkins: Baltimore.

Giska I, Sechi P, Babik W (2015). Deeply divergent sympatric mitochondrial lineages of the earthworm *Lumbricus rubellus* are not reproductively isolated. *J Mol EvolBMC Evol Biol* **15**: 217.

Goudet J, Raymond M, De Meeüs T, Rousset F (1996). Testing differentiation in diploid populations. *Genetics* **144**: 1933–1940.

Graham RI, Wilson K (2012). Male-killing *Wolbachia* and mitochondrial selective sweep in a migratory African insect. *BMC Evol Biol* **12**: 204.

Guindon S, Gascuel O (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696–704.

Haine E, Martin J, Cook J (2006). Deep mtDNA divergences indicate cryptic species in a fig-pollinating wasp. *BMC Evol Biol* **6**: 83.

Harrison RD (2005). Figs and the diversity of tropical rainforests. *BioScience* **55**: 1053–1064.

Hasegawa M, Kishino H, Yano Ta (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**: 160–174.

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc Natl Acad Sci USA* **101**: 14812–14817.

Hubbell SP (2001). *The Unified Neutral Theory of Biodiversity And Biogeography*. Princeton University Press: Princeton, NJ, USA.

Hugall A, Moritz C, Moussalli A, Stanisic J (2002). Reconciling paleodistribution models and comparative phylogeography in the Wet Tropics rainforest land snail *Gnarosophia bellendenkerensis* (Brazier 1875). *Proc Natl Acad Sci USA* **99**: 6112–6117.

Jäckel R, Mora D, Dobler S (2013). Evidence for selective sweeps by *Wolbachia* infections: phylogeny of *Altica* leaf beetles and their reproductive parasites. *Mol Ecol* **22**: 4241–4255.

Jakobsson M, Rosenberg NA (2007). CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**: 1801–1806.

Jandér K, Herre E (2010). Host sanctions and pollinator cheating in the fig tree-fig wasp mutualism. *Proceedings of the Royal Society B: Biological Sciences* **277**: 1481–1488.

Jermiin L, Crozier R (1994). The *cytochrome b* region in the mitochondrial DNA of the ant *Tetraponera rufoniger*: sequence divergence in Hymenoptera may be associated with nucleotide content. *J Mol Evol* **38**: 282–294.

Kerdelhue C, Le Clainche I, Rasplus J-Y (1999). Molecular phylogeny of the *Ceratosolen* species pollinating *Ficus* of the subgenus *Sycomorus sensu stricto*: biogeographical history and origins of the species-specificity breakdown cases. *Mol Phylogenet Evol* **11**: 401–414.

Kobmoo N, Hossaert-Mckey M, Rasplus J, Kjellberg F (2010). *Ficus racemosa* is pollinated by a single population of a single agaonid wasp species in continental South-East Asia. *Mol Ecol* **19**: 2700–2712.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H *et al.* (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

Lausen CL, Delisle I, Barclay RMR, Strobeck C (2008). Beyond mtDNA: nuclear gene flow suggests taxonomic oversplitting in the little brown bat (*Myotis lucifugus*). *Can J Zool* **86**: 700–713.

Lin R, Yeung C, Fong J, Tzeng H, Li S (2011). The lack of pollinator specificity in a dioecious fig tree: sympatric fig-pollinating wasps of *Ficus septica* in southern Taiwan. *Biotropica* **43**: 200–207.

Lopez-Vaamonde C, Dixon D, Cook J, Rasplus J (2002). Revision of the Australian species of *Pleistodontes* (Hymenoptera: Agaonidae) fig-pollinating wasps and their host-plant associations. *Zool J Linn Soc* **136**: 637–683.

Lopez-Vaamonde C, Rasplus J, Weiblen G, Cook J (2001). Molecular phylogenies of fig wasps: partial cocladogenesis of pollinators and parasites. *Mol Phylogenet Evol* **21**: 55–71.

Machado-Schiaffino G, Juanes F, Garcia-Vazquez E (2010). Introgressive hybridization in North American hakes after secondary contact. *Mol Phylogenet Evol* **55**: 552–558.

Moe A, Weiblen G (2010). Molecular divergence in allopatric *Ceratosolen* (Agaonidae) pollinators of geographically widespread *Ficus* (Moraceae) species. *Ann Entomol Soc Am* **103**: 1025–1037.

Molbo D, Machado C, Herre E, Keller L (2004). Inbreeding and population structure in two pairs of cryptic fig wasp species. *Mol Ecol* **13**: 1613–1623.

Molbo D, Machado C, Sevenster J, Keller L, Herre E (2003). Cryptic species of fig-pollinating wasps: implications for the evolution of the fig-wasp mutualism, sex allocation, and precision of adaptation. *Proc Natl Acad Sci USA* **100**: 5867–5872.

Murphy SA, Joseph L, Burbidge AH, Austin J (2011). A cryptic and critically endangered species revealed by mitochondrial DNA analyses: the Western Ground Parrot. *Conserv Genet* **12**: 595–600.

Naughton KM, O'Hara TD, Appleton B, Gardner MG (2014). Sympatric cryptic species in the crinoid genus *Cenolia* (Echinodermata: Crinoidea: Comasteridae) delineated by sequence and microsatellite markers. *Mol Phylogenet Evol* **78**: 160–171.

Raymond M, Rousset F (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J Hered* **86**: 248–249.

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S *et al.* (2012). MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* **61**: 539–542.

Rosenberg NA (2004). DISTRUCT: a program for the graphical display of population structure. *Mol Ecol Notes* **4**: 137–138.

Rousset F (2008). GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol Ecol Resources* **8**: 103–106.

Rubinoff D, Cameron S, Will K (2006). A genomic perspective on the shortcomings of mitochondrial DNA for 'barcoding' identification. *J Hered* **97**: 581–594.

Ruskey JA, Taylor EB (2016). Morphological and genetic analysis of sympatric dace within the *Rhinicthys cataractae* species complex: a case of isolation lost. *Biol J Linn Soc* **117**: 547–563.

Sun X, Xiao J, Cook J, Feng G, Huang D (2011). Comparisons of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fig wasp species and the effects of *Wolbachia* on host mtDNA evolution and diversity. *BMC Evol Biol* **11**: 86.

Sutton T, Reuter C, Riegler M, Cook J (2015). Characterisation of microsatellite markers for fig-pollinating wasps in the *Pleistodontes imperialis* species complex. *Austr J Zool* **63**: 122–126.

Sutton T, Riegler M, Cook J (2016). One step ahead: a parasitoid disperses farther and forms a wider geographic population than its fig wasp host. *Mol Ecol* **25**: 882–894.

Szpiech ZA, Jakobsson M, Rosenberg NA (2008). ADZE: A rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* **24**: 2498–2504.

West S, Cook J, Werren J, Godfray H (1998). *Wolbachia* in two insect host-parasitoid communities. *Mol Ecol* **7**: 1457–1465.

Wiebes J (1979). Co-evolution of figs and their insect pollinators. *Annu Rev Ecol Syst* **10**: 5143–4164.

Wilcox TP, Hugg L, Zeh JA, Zeh DW (1997). Mitochondrial DNA sequencing reveals extreme genetic differentiation in a cryptic species complex of neotropical pseudoscorpions. *Mol Phylogenet Evol* **7**: 208–216.

Xiao C, Jin Y, Yi Z, Cook J, Crozier R (2008). The phenology and potential for self-pollination of two Australian monoecious fig species. *Symbiosis* **45**: 91–96.

Xiao J-H, Wang N-X, Murphy RW, Cook JM, Jia L-Y, Huang D-W (2012). *Wolbachia* infection and dramatic intraspecific mitochondrial DNA divergence in a fig wasp. *Evolution* **66**: 1907–1916.

Zhang D, Lin K, Hanski I (2004). Coexistence of cryptic species. *Ecol Lett* **7**: 165–169.

Supplementary Information accompanies this paper on *Heredity* website (http://www.nature.com/hdy)