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Cryptic diversity, reproductive isolation and cytoplasmic incompatibility in a classic biological control success story

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Molecular genetics and symbiont diagnostics have revolutionized our understanding of insect species diversity, and the transformative effects of bacterial symbionts on host life history. *Encarsia inaron* is a parasitoid wasp that has been shown to harbour two bacterial endosymbionts, *Wolbachia* and *Cardinium*. Known then as *E. partenopea*, it was introduced to the USA in the late 1980s from populations collected in Italy and Israel for the biological control of an ornamental tree pest, the ash whitefly, *Siphoninus phillyreae*. We studied natural populations from sites in the USA, the Mediterranean and the Middle East as well as from a *Cardinium*-infected laboratory culture established from Italy, with the aims of characterizing these populations genetically, testing reproductive isolation, determining symbiont infection status in their native and introduced range, and determining symbiont role. The results showed that the two *Encarsia* populations introduced to the USA are genetically distinct, reproductively isolated, have different symbionts and different host–symbiont interactions, and can be considered different biological species. One (‘*E. inaron*’) is doubly infected by *Wolbachia* and *Cardinium*, while only *Cardinium* is present in the other (‘*E. partenopea*’). The *Cardinium* strains in the two species are distinct, although closely related, and crossing tests indicate that the *Cardinium* infecting ‘*E. partenopea*’ induces cytoplasmic incompatibility. The frequency of symbiont infection found in the native and introduced range of these wasps was similar, unlike the pattern seen in some other systems. These results also lead to a retelling of a successful biological control story, with several more characters than had been initially described. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **117**, 217–230.

ADDITIONAL KEYWORDS: *Cardinium* – cryptic species – cytoplasmic incompatibility – *Encarsia inaron* – *Encarsia partenopea* – invasive species – molecular systematics – parasitic wasps – *Siphoninus phillyreae* – *Wolbachia*.

INTRODUCTION

Parasitoid wasps rival insect herbivores as the most diverse guild of animals. Described species in superfamilies Chalcidoidea and Ichneumonoidea alone number in the tens of thousands, and these are generally considered to represent only a fraction of their true diversity. Recent estimates suggest that there could be as many as 500 000 species of parasitoid wasps just in the megadiverse Chalcidoidea

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(Heraty, 2009). Explosive radiation in this group has resulted in extraordinary morphological diversity (Heraty *et al.*, 2013), but the small size, convergence and reduction or loss of morphological traits has often led to limited differentiation at the species level (Heraty, 2004). The advent of molecular systematics and of an integrative approach to taxonomy (Dayrat, 2005; Padial & De La Riva, 2010) has given systematists much greater resolution in delimiting species in this problematic group (Chesters *et al.*, 2012; Gebiola *et al.*, 2012). In this context, past applications of the best systematic practices that were based entirely on morphology may need to be revisited with the support of new molecular genetic information. Classical biological control of arthropods, in which a specialist natural enemy is introduced to an area where its host or prey has become a pest, is one such important application of systematics. Proper taxonomic identification of both pest and prospective enemy populations is a crucial component of a successful introduction programme. For example, the misidentification of the pest in its native range may result in importation into quarantine of a natural enemy that does not recognize the pest as a host, while the conflation of two entities as one natural enemy can result in missed opportunities to evaluate a better candidate. Even within a single, host-specialized natural enemy species, the genetic, behavioural and endosymbiont-associated attributes of populations can differ significantly across the native range, making matches between hosts and effective parasitoids difficult (Hunter, 1999; Heraty, 2009). Furthermore, cryptic species can interfere with each other through ineffective mating, decreasing their ability to lower pest populations (Stouthamer *et al.*, 2000). More generally, introductions made for the purposes of biological control serve as natural experiments where the history and circumstances of the introduction are known, and the effects of the introduction on the species biology, population genetic structure and microbial associates such as intracellular symbionts can be studied (e.g. Fauvergue *et al.*, 2012).

Maternally inherited intracellular bacterial symbionts can be used in addition to host genes as auxiliary sources of genetic information that can be useful in taxonomy and systematics (Gebiola *et al.*, 2012). Even more importantly, these symbionts are agents of rapid and durable evolutionary change, sometimes causing major shifts in host phenotype, or reproductive isolation and divergence from other populations or sibling species (Breeuwer & Werren, 1990; Shoemaker, Katju & Jaenike, 1999; Bordenstein, O'Hara & Werren, 2001; Himler *et al.*, 2011). Documenting the presence, identity and role of symbionts in host taxa not only provides clues to gene flow and genetic resources for systematic relationships, but also offers insight into the evolutionary relationships among groups, as well as into host reproduction and life history.

The parasitoid wasp *Encarsia inaron* Walker (= *E. partenopea* Masi) (Hymenoptera: Aphelinidae) is the biological control agent credited with a textbook case of successful biological control of the ash whitefly, *Siphoninus phillyreae* (Haliday), a pest of numerous ornamental trees and fruit crops (Bellows *et al.*, 1990). *Encarsia inaron* was described with minimal detail by Walker (1839) as *Aphelinus inaron*. However, the only type specimen under the name *inaron* at the Natural History Museum in England (NHM) that could be assigned to the genus *Encarsia* was badly damaged, with only the antennae remaining intact (Graham, 1976). By comparing these antennae with those of two *Encarsia* females of the Haliday collection in Dublin, labelled *inaron* by Walker, Graham (1976) designated a lectotype for *E. inaron*, although this specimen had a damaged gaster. Furthermore, Graham (1976) stated that *E. inaron* is similar to the description of *Encarsia aleyrodis* (Mercet). After examining type material of *E. aleyrodis* and material of *E. partenopea* from the type host and locality (Portici, southern Italy), Polaszek, Evans & Bennett (1992) synonymized both species under *E. inaron*. The synonymy was justified by a large degree of variation in the gaster coloration (from entirely yellow to brown) that did not appear consistent enough to associate with the described species.

In 1989 and 1990, two populations of what was then known as *E. partenopea*, one from southern Italy and a second from Israel, were cultured first at the University of California, Riverside (UCR) quarantine facilities and later at the California Department of Food and Agriculture's Biological Control Insectary in Sacramento. Field releases began in southern California in autumn 1989. Initially, the two colonies were kept separate, and the wasps from Israel were released throughout California except for San Diego County, the only location where the wasps from Italy were released (Gould, Bellows & Paine, 1995; Gerling, Rottenberg & Bellows, 2004). The rationale was that the wasps from Israel were from a region that more closely matched the warmer, central valleys and deserts of California than the region in which the Italian wasps were collected (Pickett *et al.*, 1996). However, the efforts to keep the two colonies separate soon collapsed due to their proximity in adjacent UCR greenhouses (C. Pickett & J. Gould, pers. comm.), and official documents (UCR quarantine records) confirm that the two colonies became mixed in 1991 and released throughout California regardless of their geographical origin. In 1992, *E. partenopea* was synonymized with

E. inaron, and this name then applied to both populations released in the USA (Polaszek *et al.*, 1992). In this year, wasps collected from established populations in California were redistributed to Tucson, Arizona (David Byrne, pers. comm.), and in 1994 to North Carolina (McDonald *et al.*, 1995). Since its introduction, this wasp has expanded its range in the USA and has been found on ash whitefly in Florida (Stocks & Hodges, 2010) and in Nevada (this study). In all three locations where it was released (California, Arizona and North Carolina), *E. inaron* established and reduced the ash whitefly to non-pest status (Perlman *et al.*, 2006).

The important role of intracellular bacterial symbionts in the reproductive biology of parasitoid wasps was not known at the time of the importation of *E. inaron*, and yet both populations bore symbionts. One population of wasps investigated in the current study and collected in Tucson, Arizona, was found to be infected by two bacterial endosymbionts, *Cardinium* and *Wolbachia*. Crosses between cured and infected wasps showed that one or both symbionts caused cytoplasmic incompatibility (hereafter 'CI'; Perlman *et al.*, 2006). In unidirectional CI, three of the possible crosses between symbiont-infected and uninfected males and females produce normal numbers of offspring, but uninfected females mated with infected males produce few or no offspring (Werren, Baldo & Clark, 2008). White *et al.* (2009, 2011) disentangled the role of the two symbionts in the Arizona wasp population, showing that *Wolbachia* causes CI and *Cardinium* does not manipulate reproduction. The second population investigated in the current study was collected from the type locality of *E. partenopea* in Italy, the same location from which the wasps released in San Diego County had been collected. Once established in the laboratory, we observed consistent differences in the colour of the gaster between the Italian population and the population established in the field in Arizona, and also observed that *Cardinium* alone infects this second population.

The specific objectives of this study were to (1) genetically characterize the Italian population (hereafter IT) in comparison with the population established in Arizona (hereafter AZ), along with field material collected from the native range; (2) assess the symbiont infection status and characterize the symbionts in all laboratory and field populations; (3) determine the role of *Cardinium* in the IT population; (4) assess the level of reproductive compatibility between the IT and AZ populations; and (5) assess their species status. The ultimate goal of the project was to better understand the influence of symbionts, genetic structure, geography and historical introduction on the biology of the *E. inaron* species complex.

MATERIAL AND METHODS

Insect cultures and sampling

Encarsia inaron was established in the laboratory at the University of Arizona from two locations as described above, one collected from ash whitefly in Tucson, AZ, USA, in 2002, and one collected in Portici, southern Italy, in 2005. The University of Arizona was the sole site of laboratory investigations for this study. This wasp is a solitary endoparasitoid of whiteflies, and was cultured on sweetpotato whiteflies (*Bemisia tabaci*, 'MEAM1' or 'B' genetic group; De Barro *et al.*, 2011) on cowpea (*Vigna unguiculata*) in our laboratory as described by Perlman *et al.* (2006). The parasitoids lay both male and female eggs in first- to third-instar whitefly nymphs. Single adult wasps emerge ~ 2 weeks later. Upon verification of *Cardinium* infection in the culture from Italy, an antibiotic-cured culture was initiated by treating females with rifampicin for three generations (following Hunter, Perlman & Kelly, 2003); symbiont status of both infected and cured lines from Italy and Arizona was confirmed throughout using diagnostic PCR. Additional material for molecular and morphological analyses was obtained from two locations in Israel, two locations in Italy, one location in Turkmenistan and three locations in Iran (Table 1, Supporting Information, Fig. S1).

Insect genetic analyses

To assess genetic divergence between the IT and AZ *E. inaron*, fragments of the mitochondrial gene cytochrome oxidase c subunit I (COI) and the D2 expansion region of the 28S rDNA gene (28S-D2) were sequenced. Wasp DNA was extracted using either a Qiagen QiAmp DNA MicroKit following the manufacturer's instructions or a Chelex-proteinase K protocol as in White *et al.* (2009). COI was amplified using the forward primers C1-J-2183 or C1-J-2195 (Simon *et al.*, 1994) and with the reverse primer TL2-N-3014 (Simon *et al.*, 1994). 28S-D2 was amplified with primers D2F and D2R (Campbell, Steffen-Campbell & Werren, 1993). PCR conditions for COI and 28S-D2 were as described by Gebiola *et al.* (2009). PCR products were cleaned and sequenced in both directions at Macrogen Korea, at the University of Arizona Genetics Core and at Polo GGB (Perugia, Italy). Chromatograms were assembled using BioEdit 7.0 (Hall, 1999) and edited manually. COI sequences were verified for protein coding frameshifts and nonsense codons using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Sequences obtained from populations included in the molecular analysis (Table 1) were deposited in GenBank (accession numbers GQ423483-GQ423487 and KR338369-KR338462). For 28S-D2,

Table 1. Specimens used for molecular analyses of insect and symbiont characterization

Code	Species	Sex	Country	Locality	Collection date	Host	Plant
INAZ1	*E. inaron*	♂	USA	Tucson, AZ	Oct. 2002	*Siphoninus phillyreae*	*Punica granatum*
INAZ2	*E. inaron*	♂	USA	Tucson, AZ	Oct. 2002	*Siphoninus phillyreae*	*Punica granatum*
INAZ3	*E. inaron*	♂	USA	Tucson, AZ	Oct. 2002	*Siphoninus phillyreae*	*Punica granatum*
INAZ4	*E. inaron*	♂	USA	Tucson, AZ	Oct. 2002	*Siphoninus phillyreae*	*Punica granatum*
INAZ5	*E. inaron*	♂	USA	Tucson, AZ	Oct. 2002	*Siphoninus phillyreae*	*Punica granatum*
INEL1	*E. inaron*	♂	Israel	Yoqneam Moshava	Oct. 2011	*Siphoninus phillyreae*	*Punica granatum*
INEL2	*E. inaron*	♀	Israel	Yoqneam Moshava	Oct. 2011	*Siphoninus phillyreae*	*Punica granatum*
INEL3	*E. inaron*	♀	Israel	Yoqneam Moshava	Oct. 2011	*Siphoninus phillyreae*	*Punica granatum*
INEL4	*E. inaron*	♀	Israel	Yoqneam Moshava	Oct. 2011	*Siphoninus phillyreae*	*Punica granatum*
INEL5	*E. inaron*	♂	Israel	Yoqneam Moshava	Oct. 2011	*Siphoninus phillyreae*	*Punica granatum*
INIR1	*E. inaron*		Iran	Mashhad	6 Jul. 2013	*Siphoninus phillyreae*	*Punica granatum*
INIR2	*E. inaron*		Iran	Mashhad	6 Jul. 2013	*Siphoninus phillyreae*	*Punica granatum*
INIR5	*E. inaron*		Iran	Kashmar	Jul. 2013	*Siphoninus phillyreae*	*Punica granatum*
INIR6	*E. inaron*		Iran	Kashmar	Jul. 2013	*Siphoninus phillyreae*	*Punica granatum*
INIR7	*E. inaron*		Iran	Kashmar	Jul. 2013	*Siphoninus phillyreae*	*Punica granatum*
INIR3	*E. inaron*		Iran	Gorgan	12 Jun. 2013	*Siphoninus phillyreae*	*Fraxinus* sp.
INIR4	*E. inaron*		Iran	Gorgan	12 Jun. 2013	*Siphoninus phillyreae*	*Fraxinus* sp.
INIS1	*E. partenopea*	♀	Israel	Tel Aviv	13 Oct. 2011	*Bemisia tabaci*	
INIS2	*E. partenopea*	♀	Israel	Tel Aviv	13 Oct. 2011	*Bemisia tabaci*	
INIS3	*E. partenopea*	♀	Israel	Tel Aviv	13 Oct. 2011	*Bemisia tabaci*	
INIS4	*E. partenopea*	♀	Israel	Tel Aviv	13 Oct. 2011	*Bemisia tabaci*	
INIS5	*E. partenopea*	♀	Israel	Tel Aviv	13 Oct. 2011	*Bemisia tabaci*	
INIS6	*E. partenopea*	♀	Israel	Tel Aviv	17 Oct. 2006	*Bemisia tabaci*	
INIS7	*E. partenopea*	♀	Israel	Tel Aviv	17 Oct. 2006	*Bemisia tabaci*	
INIS8	*E. partenopea*	♀	Israel	Tel Aviv	17.Oct. 2006	*Bemisia tabaci*	
INIS9	*E. partenopea*	♀	Israel	Tel Aviv	17 Oct. 2006	*Bemisia tabaci*	
INIS10	*E. partenopea*	♀	Israel	Tel Aviv	17 Oct. 2006	*Bemisia tabaci*	
INIT1	*E. partenopea*	♀	Italy	Caserta	10 Oct. 2004	*Bemisia tabaci*	*Solanum nigrum*
INIT2	*E. partenopea*	♂	Italy	Caserta	10 Oct. 2004	*Bemisia tabaci*	*Solanum nigrum*
INIT3	*E. partenopea*	♂	Italy	Caserta	10 Oct. 2004	*Bemisia tabaci*	*Solanum nigrum*
INIT4	*E. partenopea*	♂	Italy	Caserta	10 Oct. 2004	*Bemisia tabaci*	*Solanum nigrum*
INIT5	*E. partenopea*	♀	Italy	Caserta	10 Oct. 2004	*Bemisia tabaci*	*Solanum nigrum*
INIT6	*E. partenopea*	♂	Italy	S. Maria a Vico	15 Oct. 2004	*Trialeurodes vaporariorum*	*Pelargonium grandiflorum*
INIT7	*E. partenopea*	♂	Italy	S. Maria a Vico	15 Oct. 2004	*Trialeurodes vaporariorum*	*Pelargonium grandiflorum*
INIT8	*E. partenopea*	♀	Italy	S. Maria a Vico	15 Oct. 2004	*Trialeurodes vaporariorum*	*Pelargonium grandiflorum*
INIT9	*E. partenopea*	♀	Italy	S. Maria a Vico	15 Oct. 2004	*Trialeurodes vaporariorum*	*Pelargonium grandiflorum*
INIT10	*E. partenopea*	♀	Italy	S. Maria a Vico	15 Oct. 2004	*Trialeurodes vaporariorum*	*Pelargonium grandiflorum*
INIT11	*E. partenopea*	♀	Italy	Portici	30 Sep. 2012	*Aleyrodes elevates*	*Ficus carica*
INTK1	*E. partenopea*	♀	Turkmenistan	Ashgabad	27 Jun. 2012	*Aleyrodes singularis*	*Lactuca serriola*
INTK2	*E. partenopea*	♀	Turkmenistan	Ashgabad	27 Jun. 2012	*Aleyrodes singularis*	*Lactuca serriola*
INTK3	*E. partenopea*	♂	Turkmenistan	Ashgabad	27 Jun. 2012	*Aleyrodes singularis*	*Lactuca serriola*

sequences of species belonging to the *E. inaron* species group, namely *E. inaron* from London (UK), type locality of *E. inaron*, *Encarsia* sp. near *inaron*, *Encarsia estrellae* Manzari & Polaszek, *Encarsia azimi* Hayat, *Encarsia* sp. near *azimi* and *Encarsia dichroa* (Mercet) (Manzari *et al.*, 2002) were also included. As outgroup taxa, *Encarsia* species were chosen based on the availability of both 28S and COI sequences, including *Encarsia formosa* Gahan, *Encarsia hispida* De Santis, *Encarsia luteola* Howard, *Encarsia protransvena* Viggiani and *Encarsia sophia* Girault & Dodd.

The COI sequences were aligned manually and the 28S-D2 sequences were aligned using the G-INS-I algorithm in MAFFT 7 (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). Phylogenies were obtained using maximum likelihood (ML) in RAxML 7.0.4 (Stamatakis, 2006) and Bayesian inference (BI) in MrBayes 3.2 (Ronquist *et al.*, 2012) on a supermatrix partitioned as 28S-D2, COI codon positions 1 and 2, COI codon position 3, and implementing the GTR+G evolutionary model, according to the best partitioning scheme found by PartitionFinder (Lanfear *et al.*, 2012). ML trees were obtained after 1000 multiple inferences on the original alignment, starting from a random parsimonious tree and default initial rearrangement settings and number of rate categories. ML branch support was based on 1000 rapid bootstrap pseudoreplicates, and clades were considered as supported when bootstrap was >70%. For BI, two parallel runs of four simultaneous Monte Carlo Markov chains (MCMCs) were run for two million generations, and trees were sampled every 1000 generations. Convergence of the separate runs was checked using the average deviation of split frequencies diagnostic (< 0.01), and the potential scale reduction factor (close to 1.00 for all parameters). The burnin value was set at 25% of sampled topologies, and post-burnin trees were summarized as a 50% majority rule consensus tree with posterior probabilities as nodal support and the threshold for clade acceptance set at 0.95. Uncorrected intra- and interspecific *p*-distances based on COI were calculated using MEGA4 (Tamura *et al.*, 2007).

Symbiont infection status

A sample of 12 individuals from each laboratory culture (cured IT, cured AZ, *Wolbachia*–*Cardinium*-infected AZ and *Cardinium*-infected IT), all specimens collected in the field and used for molecular analyses (Table 1) and ten specimens from Reno, Nevada, USA (collected on 10 September 2009 on ash whiteflies attacking a hawthorn tree, *Crataegus* sp.) were screened for both *Cardinium* and *Wolbachia* by diagnostic PCR following the protocol described by White *et al.* (2009) except using NEB (New England Biolabs) *Taq* polymerase and corresponding buffer (which contains $MgCl_2$). For *Cardinium*, we used primers Ch-F/Ch-R that yield a product of 394 bp for *Cardinium*-infected individuals (Zchori-Fein & Perlman, 2004). For *Wolbachia*, we used primers V1–V6 (O'Neill *et al.*, 1992) that amplify a 900-bp fragment of the hypervariable region of 16S rDNA. Products were visualized with SYBR green as described by White *et al.* (2009).

Symbiont characterization

Cardinium was characterized by sequencing a fragment of 16S rDNA and of the gyrase B gene (gyrB). 16S rDNA was amplified with *Cardinium*-specific primers CLOf and CLOr (Weeks, Velten & Stouthamer, 2003). Gyrase B was amplified using the nested-PCR approach described by Yamamoto & Harayama (1995) with the specific primers gyr125F and gyr1023R (Zchori-Fein *et al.*, 2004) in the second PCR. However, due to failures in some amplifications, new specific primers that amplify a 910-bp fragment were designed: 349F (5′-TTGCATGGTGTAGGGGTKTC-3′) and 1259R (5′-CGTTCGGAACARTCTGCTAA-3′), which were used in a direct PCR with an annealing temperature of 54 °C. *Cardinium* gyrB from AZ wasps was previously sequenced (GenBank accession no. DQ317669; Perlman *et al.*, 2006). Gyrase B sequences were also used for phylogenetic analyses to determine the closest relatives of the bacterial strains identified. ML and BI analyses were performed as described for insect genes, and the best partitioning scheme found by PartitionFinder was codon position 1+2 and codon position 3, implementing the GRT+G and GTR+G+I models, respectively. MCMCs were run for one million generations.

For populations infected by *Wolbachia*, this symbiont was characterized at the strain level by using multilocus sequence typing (MLST) by characterizing the sequence of five housekeeping genes (Baldo *et al.*, 2006), as well as by sequencing the wsp gene (81F and 631R; Zhou, Rousset & O'Neill, 1998) and 16S rDNA with *Wolbachia*-specific primers wolF and wolR (O'Neill *et al.*, 1992).

CI crosses

To test for CI, all four possible crosses among the *Cardinium*-infected and uninfected IT *E. inaron* were conducted, with the uninfected female × *Cardinium*-infected male being the predicted CI cross. The crosses were conducted using the procedure optimized for *E. inaron* by White *et al.* (2009) with the modifications and details described below.

Experimental arenas were prepared by infesting 7-day-old cowpea plants with a moderate density of adult whiteflies in a large fabric cage in the greenhouse (102 × 76 × 90 cm) overnight, after which the adult whiteflies were removed by compressed air and the plants stored in the greenhouse for whitefly nymph development. Eighty leaf discs from leaves chosen at random from the infested plants were cut and placed on agar in 35-mm dishes 1 day before the start of the experiment, when most whitefly nymphs were first or second instar.

Wasp pupae were isolated from *Cardinium*-infected and uninfected laboratory colonies, and upon adult emergence were sexed and released according to cross type onto whitefly-infested cowpea plants caged in inverted, screened, 3.8-L plastic jars for 48 h, where mating occurred more readily than in vials. Twenty females were randomly selected from cages of each of the four crossing types, and each individual was assigned to a leaf disc bearing whitefly nymphs for 24 h, and was then transferred to a second disc for one more day of oviposition as described by White *et al.* (2009). This method ensured reasonable numbers of progeny with which to assess CI. Following wasp removal, leaf discs were incubated in an environmental chamber at 27 °C, 16:8-h light–dark and 65% relative humidity until wasp progeny emergence. Emerged wasps were sexed and counted. Replicate females were excluded from the analysis if they died before the completion of the second day of oviposition or were lost during transfer between leaf discs.

Encarsia inaron, like most Hymenoptera, is haplodiploid and produces males from unfertilized eggs. In the crossing test results, a lack of daughters produced by any particular female could indicate unsuccessful mating or female embryo death as a result of CI. Spermathecae were therefore dissected and observed for sperm as described by White *et al.* (2009) in eight of nine mothers that produced no daughters.

Analyses of variance of the number of female progeny and the number of male progeny in the four treatments were used to test the CI hypothesis, using ANOVA in R (R Development Core Team, 2010). We predicted significantly fewer female progeny in the putative CI cross (infected males × uninfected females) than in the other treatments. In haplodiploid organisms, only female embryos develop from fertilized eggs and are killed by CI. For female progeny, the prediction was tested with the full model, and also with a contrast of the putative CI vs. the other three crosses. The CI hypothesis predicts one of two outcomes for male progeny. In haplodiploid organisms, CI may result in female mortality, in which case male offspring number is not expected to be different among crosses. It may also result in the CI-affected embryo losing the entire set of paternal chromosomes and developing as a haploid male (the 'male development' type of CI; Vavre *et al.*, 2002), in which case we would expect a significantly greater number of males in the putative CI cross than in the other crosses.

Interspecific crosses

To test for gene flow between the AZ and IT populations, we performed the four possible crosses using cured AZ and cured IT cultures in mass matings on whole plants in screened 3.8-L jars. Whitefly-infested plants and parasitoids were obtained as described above. Three replicate jars were used per cross. Twenty virgin females and ten males were released in each jar. Upon adult emergence, all wasps present in the jars were collected, sexed and counted.

RESULTS

Genetic analyses

ML and BI based on the concatenated dataset resulted in trees of identical topology and suggested that the morphospecies *E. inaron* can be divided into two main groups: one including populations from Arizona, Israel (Yoqneam Moshava) and Iran, and the other including those from Israel (Tel Aviv), Italy and Turkmenistan (Fig. 1). Populations in the first group share their 28S-D2 sequence with *E. inaron* collected in London, the type locality of *E. inaron*, so hereafter we refer to this clade as '*E. inaron*'. A single polymorphism (A/T at position 219 of the alignment) distinguishes *E. inaron* from other populations from Israel, Italy and Turkmenistan, which in turn have the same 28S sequence as a sample collected from Portici (Italy), the type locality of *E. partenopea*. Furthermore, the sample from Portici clustered with other samples from Italy, whereas samples from Israel (Tel Aviv) and Turkmenistan form a separate clade. Therefore, we refer to these two clades as '*E. partenopea*' and '*E. partenopea* B', respectively. Uncorrected *p*-distance based on COI among '*E. partenopea*' and '*E. partenopea* B' is 1.8%, whereas the distance between '*E. inaron*' and either '*E. partenopea*' or '*E. partenopea* B' is 3.6%. Interestingly, a single COI haplotype was recovered for *E. inaron* from Israel (Yoqneam Moshava), USA and Iran.

Symbiont infection status

The expected *Cardinium* and *Wolbachia* infection status was confirmed for all samples from each laboratory strain, i.e. both cured strains were

uninfected by both symbionts, whereas the IT infected strain ('*E. partenopea*') was infected by *Cardinium* only, and the AZ strain ('*E. inaron*') was infected by both *Wolbachia* and *Cardinium*. Among field samples, '*E. partenopea*' and '*E. partenopea* B' populations were infected only by *Cardinium*, but the symbiont was not fixed. Indeed, *Cardinium* was found in 5/10 specimens from Tel Aviv (Israel), 5/10 specimens from Italy and 2/3 samples from Turkmenistan. In contrast, *Cardinium* was fixed in the two laboratory cultures. In '*E. inaron*', *Wolbachia* was fixed in all field populations as well as in the laboratory culture, whereas 11/15 specimens from Yoqneam Moshava (Israel) and 4/10 from Reno, Nevada, were infected by *Cardinium*.

Symbiont characterization

We identified two different strains of *Cardinium*, one infecting *E. inaron* and one infecting '*E. partenopea*' and '*E. partenopea* B' clades. The two strains are identical at the 16S rDNA region sequenced, but differ by 3 out of 792 nt of the gyrase B gene. The closest relative of these two strains for both gyrase B (Fig. 2) and 16S (data not shown) is *Cardinium* infecting *Bemisia tabaci*, with *Cardinium* infecting other *Encarsia* species being part of the same monophyletic assemblage (Fig. 2).

The *Wolbachia* MLST on Arizona, Nevada and Iran populations of '*E. inaron*' resulted in a single and novel sequence type (ST #431) due to a novel gatB allele, and the *wsp* gene further confirmed the presence of a single *Wolbachia* strain (data not shown). The *Wolbachia* symbiont infecting the '*E. inaron*' clade belongs to the B supergroup and its closest relative is found in the mosquito *Mansonia africana*.

CI crosses

The crossing results support the hypothesis that *Cardinium* induces CI in '*E. partenopea*'. There were statistically significant differences in female production across treatments ($F_{3,34} = 7.50$, $P < 0.001$), and the putative CI cross (infected males × uninfected females) produced very few female offspring (mean of 0.60 females ± 0.60 SE) relative to the other three crosses (mean of 8.21 females ± 1.00 SE, ANOVA contrast of the putative CI cross vs. the other three treatments: $F_{1,36} = 19.49$, $P < 0.0001$; Fig. 3). The average number of male offspring did not differ among treatments, however (mean of 5.63 males ± 0.54 SE, $F_{3,34} = 1.30$, $P = 0.29$; Fig. 3). These results are consistent with the 'female mortality' rather than the 'male development' type of CI. Most of the mothers with no female offspring were in the putative CI cross (9/10 across treatments). All spermathaecae dissected from this cross ($n = 8$) contained sperm, indicating that the females had mated successfully, and supporting CI, rather than mating failure, as an explanation for the male-only broods.

Interspecific crosses

We found no evidence of gene flow between '*E. inaron*' (AZ) and '*E. partenopea*' (IT). No daughters were produced from either the IT♂ × AZ♀ or the AZ♂ × IT♀ crosses, whereas an average of 1131.3 ± 392.4 (SE), and 583.0 ± 373.7 (SE) males were produced per jar, respectively. In the intraspecific crosses slightly female-biased sex ratios were produced. In the IT × IT cross, an average of 1558.3 ± 351.8 (SE) progeny were produced per jar, of which 61.4% were female. In the AZ × AZ cross an average of 896.3 ± 496.6 (SE) were produced per jar, of which 59.1% were female.

DISCUSSION

Several lines of evidence indicate that the morphospecies *E. inaron* includes at least two species that correspond to the taxonomic concepts of *E. inaron* and *E. partenopea*. There is genetic distinctiveness between samples from London (UK), the type locality of *E. inaron*, and samples from Portici (Italy), the type locality of *E. partenopea*, and this degree of difference is consistent with species-level differentiation found in other chalcidoid taxa. In general, 28S-D2 is highly conserved, if not invariant between closely related species of various chalcidoids that are well differentiated biologically and at COI (Heraty *et al.*, 2007; Gebiola, Bernardo & Burks, 2010). In one case, Palearctic and Nearctic species of the genus *Pnigalio* (Eulophidae) had the same 28S-D2 sequence (Gebiola *et al.*, 2010). The single 28S-D2 polymorphism that distinguishes '*E. inaron*' and '*E. partenopea*' is consistent with what has already been observed in Eulophidae, where variation in COI can correspond to a single diagnostic polymorphism in 28S-D2 (Gebiola *et al.*, 2009; Nugnes *et al.*, 2015). As for COI, the relatively low divergence found between the three lineages (< 4%) is similar to or even higher than that found between cryptic species of other parasitoid wasps (Heraty *et al.*, 2007; Nugnes *et al.*, 2015). Furthermore, the crosses between populations from Arizona and Italy, which belong to the '*E. inaron*' and '*E. partenopea*' clades, respectively, were found to be entirely reproductively isolated. At present, the only way to distinguish the two species with certainty is by gene sequences, as it seems that no discrete morphological character can support the distinction. Although

Figure 1. BI majority-rule consensus tree for the concatenated 28S-D2–COI dataset. Posterior probability values > 0.95 are shown above branches, along with bootstrap values > 70% for the topologically identical ML tree. ns, Not significant. Scale bar, 0.3 changes per nucleotide position.

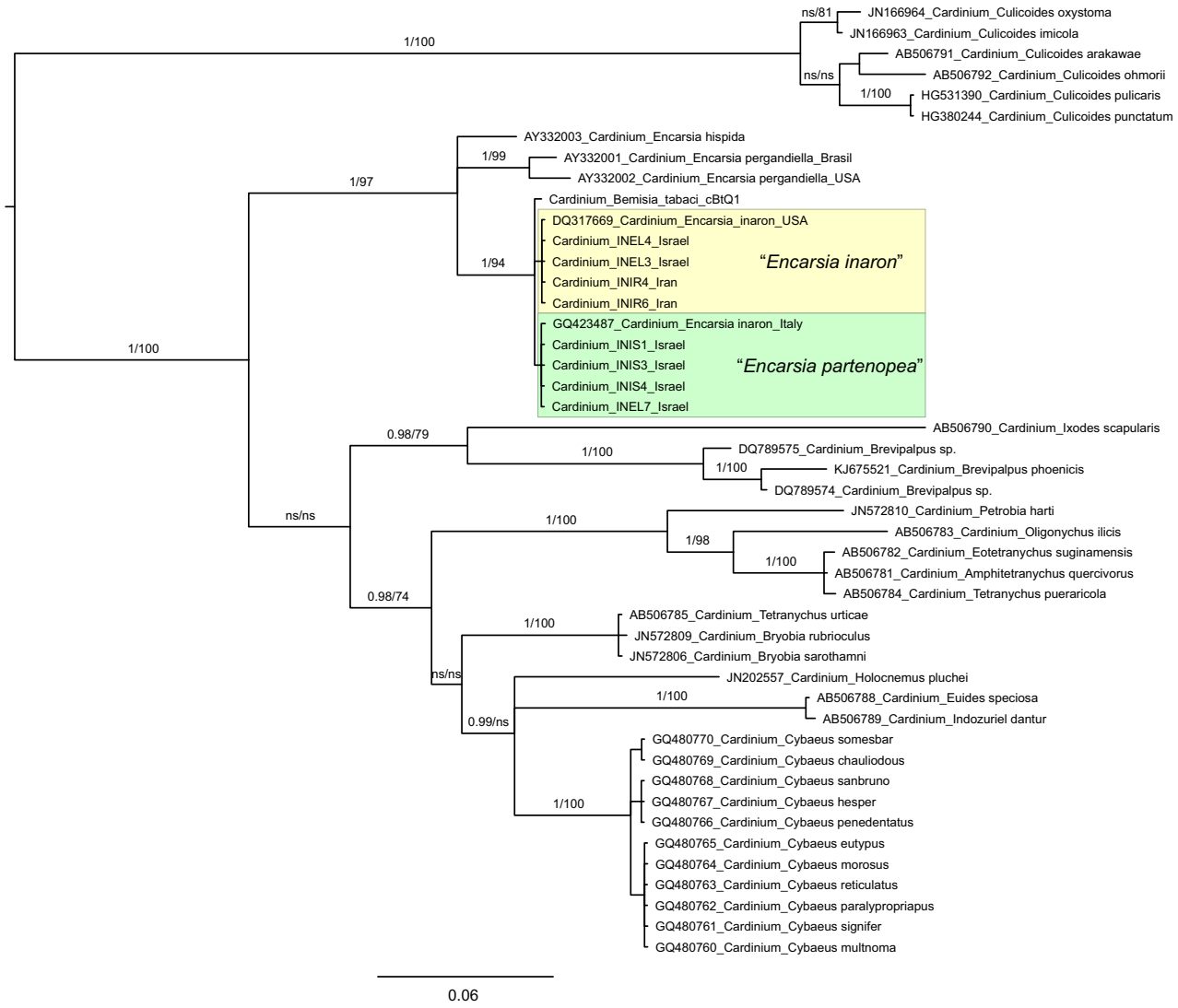

Figure 2. BI majority-rule consensus tree for *Cardinium* gyrase B gene. Posterior probability values > 0.95 are shown above branches, along with bootstrap values > 70% for the topologically identical ML tree. ns, Not significant. Scale bar, 0.06 changes per nucleotide position.

we found that the gaster colour is a useful character in the laboratory under constant temperatures, we also noticed that it may be temperature dependent and is not generally a diagnostic character for this species complex when specimens are collected in the field, consistent with Polaszek *et al.* (1992) and Laudonia & Viggiani (1993). As a taxonomic revision of this species complex is beyond the objectives of this study, a separate study will aim to document morphological differences, if any, that could further support the genetic distinctiveness, as well as to resolve the species status of '*E. partenopea* B'.

The genetic and biological evidence for reproductive isolation of '*E. inaron*' and '*E. partenopea*' is supported by the symbiont infection status, which differs between the two species. *Encarsia inaron* samples collected from American, Mediterranean and Middle Eastern sites were infected by both *Wolbachia* and *Cardinium*, showing that the pattern first documented in the AZ population brought into the laboratory (Perlman *et al.*, 2006; White *et al.*, 2009) is widespread in this species. Additionally, both '*E. inaron*' and '*E. partenopea*' are infected with species-specific strains of *Cardinium* that, although closely related, induce different phenotypes. A previous study showed *Cardinium* to be asymptomatic in '*E. inaron*' (where *Wolbachia* causes CI; White *et al.*, 2009), but we found that the *Cardinium* in

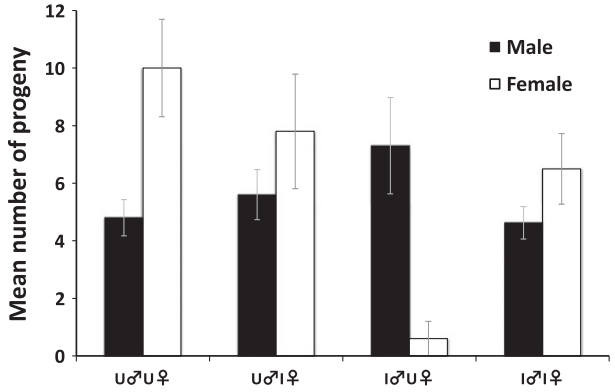

Figure 3. The mean number of male and female progeny per mated female produced in the crosses of *Cardinium*-infected (I) and uninfected (U) IT *E. inaron*. The number of female progeny is significantly lower in the putative CI cross, I♂U♀, than in any of the other crosses ($F_{1,36} = 19.49$, $P < 0.0001$), while the number of male progeny does not differ significantly across cross types ($F_{3,34} = 1.30$, $P = 0.29$). $n = 10$ in all but the I♂I♀ treatment ($n = 8$).

'*E. partenopea*' caused the female mortality type of CI. While CI-*Cardinium* has been reported now in several studies in mites (Gotoh, Noda & Ito, 2007; Ros & Breeuwer, 2009) and in a planthopper (Nakamura *et al.*, 2012; Zhang, Zhao & Hong, 2012), it is still comparatively uncommon compared with CI-*Wolbachia*, and the current study appears to be only the second where it has been shown in parasitoid Hymenoptera, with the first documented in the congener *E. pergandiella* Howard (Hunter *et al.*, 2003).

Interestingly, while fixed in our laboratory cultures, *Cardinium* appears not to be fixed in either species in the field. This might be expected for the apparently asymptomatic infection in *E. inaron* but is surprising for the CI-inducing strain in '*E. partenopea*'. In general, given high vertical transmission rates and strong CI, CI symbionts that are at a frequency greater than the threshold determined by fecundity costs should sweep to a stable equilibrium that is often close to fixation (Hoffmann, Turelli & Harshman, 1990). This result suggests that one of the three parameters that can influence the stable equilibrium (CI strength, maternal transmission rates, fecundity relative to uninfected females) is lower under field conditions than in the laboratory (Hoffmann *et al.*, 1990).

We also found symbiont infection status and frequency to be similar in the native and introduced ranges of '*E. inaron*', the one species that established in the USA. *Wolbachia* was fixed in field populations sampled in both Old World sites and in Arizona and Nevada, while *Cardinium* was at intermediate frequency wherever it was sampled. This is interesting because symbionts have been lost in other examples of introductions, for example in invasive ants such as the Argentine ant *Linepithema humile* (Mayr), the little fire ant *Wasmannia auropunctata* (Roger) and several fire ant *Solenopsis* species (Shoemaker *et al.*, 2000; Tsutsui *et al.*, 2003; Reuter, Pedersen & Keller, 2004; Rey *et al.*, 2013). Whether bottlenecks due to small founding populations, or differing selective pressures in the new habitat are more important in determining symbiont loss is an open question (Shoemaker *et al.*, 2000), but preservation of the symbiont composition in the current example, where intentional introductions would probably have led to larger founding population sizes, lends some support to the bottleneck hypothesis.

'*Encarsia inaron*' is distributed in Israel, Iran, Japan, New Zealand and the UK (this study; Manzari *et al.*, 2002; Babcock *et al.*, 2001), and has a very low diversity of mtDNA, with only one COI haplotype recovered so far. Our data suggest Israel is the origin of the '*E. inaron*' material introduced in the USA and released in California (except for San Diego County), Arizona and North Carolina, in accordance with the written record. It also suggests a relatively recent sweep in the mtDNA, possibly driven by CI-*Wolbachia*. Mitochondrial sweeps may be observed when an arthropod population is invaded by a symbiont that rapidly spreads. Because both host mtDNA and the symbiont are maternally inherited, the mtDNA haplotype may hitchhike through the population to fixation. Consequently, this selective sweep will lead to a loss of mtDNA diversity in part of the host species distribution (Hurst & Jiggins, 2005). '*Encarsia partenopea*' is distributed in India, Israel, Italy and Turkmenistan (this study), and there is evidence that the two species are sympatric in Israel. Indeed, the wasps originally introduced to the USA ('*E. inaron*') were collected from ash whiteflies at the same location in Tel Aviv where subsequently wasps from *B. tabaci* ('*E. partenopea*') were collected (D. Gerling, pers. comm.). Furthermore, within samples of '*E. inaron*' collected some 70 km further north, in the Jezreel valley, there is at least one '*E. partenopea*' individual. At present, we do not know if both wasp species established in the USA. Literature suggests that only '*E. inaron*' (from the original colony from Israel) became established, although under the laboratory conditions of this study '*E. partenopea*' females had higher fecundity. If so, the key for the prevalence of '*E. inaron*' could have been the original expansion of the mass-rearing of the colony from Israel (Bellows *et al.*, 1992; Gould, Bellows & Paine, 1992). An alternative explanation could be that climatic adaptation played a large role in establishment, as individuals collected

from the drier Mediterranean climate of Israel (i.e. '*E. inaron*') may have been more successful in the interior valleys of California as well as in Arizona and in the south-eastern USA.

This study is therefore also a cautionary tale for modern biological control practice and underlines the importance of (1) genetic characterization of introduced natural enemy populations, (2) testing for nuclear genetic and symbiont-associated barriers to reproductive compatibility among introduced populations, and (3) characterization and testing of the role of intracellular and potentially gut symbionts for agent fitness. Indeed, the current study supports the utility of both molecular tools and laboratory experiments in delineating and characterizing introduced agents and their symbionts for biological control of pests. The molecular tools employed here were not available to the ecologists and systematists involved in the introduction of the two wasp populations for biological control at that time, but we can now show that the wasps introduced to the USA for the biological control of the ash whitefly represent two genetically distinct and reproductively isolated species. That these species are infected by a CI-*Wolbachia* and a CI-*Cardinium*, respectively, reinforces the concerns expressed by Perlman *et al.* (2006), even though, in the current example, we found species barriers that would have limited CI effects between species. Mixing of two populations of the same species with different CI symbiont infection status, however, may cause reproductive failure in quarantine or after release in the new area. This may be detrimental in any situation where intentional introductions are made: for biological control, as described here, or for conservation, where threatened or endangered populations are augmented by populations from elsewhere (Frankham, 2010). Even without any expectation of the direct suppression of reproduction associated with CI, however, it is useful to consider the influence of symbionts on host fitness. Commonly, CI symbionts persist at high frequency because uninfected individuals produce few progeny due to CI, but they rarely benefit the host. The energetic costs of bearing the symbiont itself may significantly reduce host fecundity, and less than perfect vertical transmission will produce uninfected females that are then affected by CI (Perlman, Kelly & Hunter, 2008). When a symbiont in a host population causes reproductive manipulation, antibiotic treatment in quarantine before release may be considered, as it may improve the fitness of the agents, while after releases no manipulation is possible. On the other hand, antibiotic treatment may be detrimental if some intracellular symbionts or gut microbiota perform other functions important for host success. Ideally, fitness should be compared between symbiotic and aposymbiotic cultures prior to release. More generally, the study shows the utility of applying molecular tools to recent examples of historical introductions to understand how host and symbionts may be influenced by the introduction process. Here, by showing that symbionts may be conserved when insects are intentionally introduced, our results support the probable role of bottlenecks in the established pattern of symbiont loss in new habitats.

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REFERENCES

Babcock C, Heraty JM, De Barro PJ, Driver F, Schmidt S. 2001. Preliminary phylogeny of *Encarsia* Förster (Hymenoptera: Aphelinidae) based on morphology and 28S rDNA. *Molecular Phylogenetics and Evolution* **18:** 306–323.

Baldo L, Hotopp JCD, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR, Hayashi C, Maiden MCJ, Tettelin H, Werren JH 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology* **72:** 7098–7110.

Bellows TS, Paine TD, Arakawa KY, Meisenbacher C, Leddy P, Kabashima J. 1990. Biological control sought for ash whitefly. *California Agriculture* **44:** 4–6.

Bellows T, Paine T, Gould J, Bezark L, Ball J, Bentley W, Coviello RJ, Downer J, Elam P, Flaherty D, Gouveia P, Koeler C, Molinari RH, O'Connell NV, Perry E, Vogel G. 1992. Biological control of ash whitefly: a success in progress. *California Agriculture* **46:** 24–28.

Bordenstein S, O'Hara PF, Werren JH. 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409:** 707–710.

Breeuwer JA, Werren JH. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346:** 558–560.

Campbell B, Steffen-Campbell J, Werren JH. 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Molecular Biology* **2:** 225–237.

Chesters D, Wang Y, Yu F, Bai M, Zhang TX, Hu HY, Zhu CD, Li CD, Zhang YZ. 2012. The integrative taxonomic approach reveals host specific species in an encyrtid parasitoid species complex. *PLoS ONE* **7:** e37655.

Dayrat B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society* **85:** 407–416.

De Barro PJ, Liu SS, Boykin LM, Dinsdale AB. 2011. *Bemisia tabaci*: a statement of species status. *Annual Review of Entomology* **56:** 1–19.

Fauvergue X, Vercken E, Malausa T, Hufbauer RA. 2012. The biology of small, introduced populations, with special reference to biological control. *Evolutionary Applications* **5:** 424–443.

Frankham R. 2010. Challenges and opportunities of genetic approaches to biological conservation. *Biological Conservation* **143:** 1919–1927.

Gebiola M, Bernardo U, Monti MM, Navone P, Viggiani G. 2009. *Pnigalio agraules* (Walker) and *Pnigalio mediterraneus* Ferrière and Delucchi (Hymenoptera: Eulophidae): two closely related valid species. *Journal of Natural History* **43:** 2465–2480.

Gebiola M, Bernardo U, Burks R. 2010. A reevaluation of the generic limits of *Pnigalio* Schrank (Hymenoptera: Eulophidae) based on molecular and morphological evidence. *Zootaxa* **2484:** 35–44.

Gebiola M, Gómez-Zurita J, Monti MM, Navone P, Bernardo U 2012. Integration of molecular, ecological, morphological and endosymbiont data for species delimitation within the *Pnigalio soemius* complex (Hymenoptera: Eulophidae). *Molecular Ecology* **21:** 1190–1208.

Gerling D, Rottenberg O, Bellows TS. 2004. Role of natural enemies and other factors in the dynamics of field populations of the whitefly *Siphoninus phillyreae* (Haliday) in introduced and native environments. *Biological Control* **31:** 199–209.

Gotoh T, Noda H, Ito S. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity* **98:** 13–20.

Gould JR, Bellows TS, Paine TD. 1992. Evaluation of biological control of *Siphoninus phillyreae* (Haliday) by the parasitoid *Encarsia partenopea* (Walker), using life-table analysis. *Biological Control* **2:** 257–265.

Gould JR, Bellows TS, Paine TR. 1995. Preimaginal development, adult longevity and fecundity of *Encarsia inaron* (Hym: Aphelinidae) parasitizing *Siphoninus phillyreae* (Hom: Aleyrodidae) in California. *Entomophaga* **40:** 55–68.

Graham MD. 1976. The British species of *Aphelinus* with notes and descriptions of other European Aphelinidae (Hymenoptera). *Systematic Entomology* **1:** 123–146.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acid Symposium Series* **41:** 95–98.

Heraty J. 2004. Molecular systematics, Chalcidoidea and biological control. In: Ehler R, Mateille T, eds. *Genetics, evolution and biological control*. CAB International, London, UK: Wiley-Blackwell, 39–71.

Heraty J. 2009. Parasitoid biodiversity and insect pest management. In: Foottit RG, Adler PH, eds. *Insect biodiversity*. Oxford, UK: Wiley-Blackwell, 445–462.

Heraty JM, Woolley JB, Hopper KR, Hawks DL, Kim JW, Buffington M. 2007. Molecular phylogenetics and reproductive incompatibility in a complex of cryptic species of aphid parasitoids. *Molecular Phylogenetics and Evolution* **45:** 480–493.

Heraty JM, Burks RA, Cruaud A, Gibson GAP, Liljeblad J, Munro J, Rasplus JY, Delvare G, Jansta P, Gumovsky A, Huber J, Woolley JB, Krogmann L, Heydon S, Polaszek A, Schmidt S, Darling DC, Gates MW, Mottern J, Murray E, Dal Molin A, Triapitsyn S, Baur H, Pinto JD, van Noort S, George J, Yoder M. 2013. A phylogenetic analysis of the megadiverse Chalcidoidea (Hymenoptera). *Cladistics* **29:** 466–542.

Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, Chiel E, Duckworth VE, Dennehy TJ, Zchori-Fein E, Hunter MS. 2011. Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* **332:** 254–256.

Hoffmann AA, Turelli M, Harshman LG. 1990. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* **126:** 933–948.

Hunter MS. 1999. Genetic conflict in natural enemies: a review, and consequences for the biological control of arthropods. In: Hawkins BA, Cornell HV, eds. *Theoretical approaches to biological control*. Cambridge: Cambridge University Press, 231–258.

Hunter MS, Perlman SJ, Kelly SE 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proceedings of the Royal Society B: Biological Sciences* **270:** 2185–2190.

Hurst GDD, Jiggins FM. 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences* **272:** 1525–1534.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment Software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30:** 772–780.

Lanfear R, Calcott B, Ho SYW, Guindon S. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* **29:** 1695–1701.

Laudonia S, Viggiani G 1993. Effetto della temperatura sulla colorazione degli adulti di *Encarsia partenopea* Masi (Hymenoptera: Aphelinidae). *Bollettino Del Laboratorio Di Entomologia Agraria "Filippo Silvestri"* **50:** 141–146.

Manzari S, Polaszek A, Belshaw R, Quicke DLJ. 2002. Morphometric and molecular analysis of the *Encarsia*

inaron species-group (Hymenoptera: Aphelinidae), parasitoids of whiteflies (Hemiptera: Aleyrodidae). *Bulletin of Entomological Research* **92:** 165–175.

McDonald RC, Robbins NS, Baker RR, Bellows TS. 1995. Release and colonization of *Encarsia inaron* (Walker) (Hymenoptera: Aphelinidae) to control ash whitefly, *Siphoninus phillyreae* (Haliday) (Homoptera: Aleyrodidae), in North Carolina. NCDA Beneficial Insect Lab Annual Report of Activities: 37–45.

Nakamura Y, Yukuhiro F, Matsumura M, Noda H. 2012. Cytoplasmic incompatibility involving *Cardinium* and *Wolbachia* in the white-backed planthopper *Sogatella furcifera* (Hemiptera: Delphacidae). *Applied Entomology and Zoology* **47:** 273–283.

Nugnes F, Gebiola M, Monti MM, Gualtieri L, Giorgini M, Wang J, Bernardo U. 2015. Genetic diversity of the invasive gall wasp *Leptocybe invasa* (Hymenoptera: Eulophidae) and of its *Rickettsia* endosymbiont, and associated sex-ratio differences. *PLoS ONE* **10:** e0124660.

O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences of the United States of America* **89:** 2699–2702.

Padial J, De La Riva I. 2010. A response to recent proposals for integrative taxonomy. *Biological Journal of the Linnean Society* **101:** 747–756.

Perlman SJ, Kelly SE, Zchori-Fein E, Hunter MS. 2006. Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*. *Biological Control* **39:** 474–480.

Perlman SJ, Kelly SE, Hunter MS. 2008. Population biology of cytoplasmic incompatibility: maintenance and spread of *Cardinium* symbionts in a parasitic wasp. *Genetics* **178:** 1003–1011.

Pickett CH, Ball JC, Casanave KC, Klonsky KM, Jetter KM, Bezark LG, Schoenig SE. 1996. Establishment of the ash whitefly parasitoid *Encarsia inaron* (Walker) and its economic benefit to ornamental street trees in California. *Biological Control* **6:** 260–272.

Polaszek A, Evans GA, Bennett FD. 1992. *Encarsia* parasitoids of *Bemisia-Tabaci* (Hymenoptera, Aphelinidae, Homoptera, Aleyrodidae) – a preliminary guide to identification. *Bulletin of Entomological Research* **82:** 375–392.

R Development Core Team. 2010**.** R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/.

Reuter M, Pedersen JS, Keller L. 2004. Loss of *Wolbachia* infection during colonisation in the invasive Argentine ant *Linepithema humile*. *Heredity* **94:** 364–369.

Rey O, Estoup A, Facon B, Loiseau A, Aebi A, Duron O, Vavre F, Foucaud J. 2013. Distribution of endosymbiotic reproductive manipulators reflects invasion process and not reproductive system polymorphism in the little fire ant *Wasmannia auropunctata*. *PLoS ONE* **8:** e58467.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61:** 539–542.

Ros VID, Breeuwer JAJ. 2009. The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobia sarothamni*. *Heredity* **102:** 413–422.

Shoemaker DD, Katju V, Jaenike J. 1999. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. *Evolution* **53:** 1157–1164.

Shoemaker DD, Ross KG, Keller L, Vargo EL, Werren JH. 2000. *Wolbachia* infections in native and introduced populations of fire ants (*Solenopsis* spp.). *Insect Molecular Biology* **9:** 661–673.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87:** 651–701.

Stamatakis A. 2006. RAxML-VI-HPC: maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22:** 2688–2690.

Stocks I, Hodges G. 2010. Ash whitefly, Siphoninus phillyreae (Haliday), a new exotic whitefly (Hemiptera: Aleyrodidae) in central Florida, and Encarsia inaron, its parasitoid (Hymenoptera: Aphelinidae). Division of Plant Industry. http://www.freshfromflorida.com/content/download/23897/486234/siphoninus-phillyreae.pdf

Stouthamer R, Jochemsen P, Platner G, Pinto J. 2000. Crossing incompatibility between *Trichogramma minutum* and *T. platneri* (Hymenoptera: Trichogrammatidae): implications for application in biological control. *Environmental Entomology* **29:** 832–837.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution* **24:** 1596–1599.

Tsutsui ND, Kauppinen SN, Oyafuso AF, Grosberg RK. 2003. The distribution and evolutionary history of *Wolbachia* infection in native and introduced populations of the invasive argentine ant (*Linepithema humile*). *Molecular Ecology* **12:** 3057–3068.

Vavre F, Fleury F, Varaldi J, Fouillet P, Bouletreau M. 2002. Infection polymorphism and cytoplasmic incompatibility in Hymenoptera–*Wolbachia* associations. *Heredity* **88:** 361–365.

Weeks AR, Velten R, Stouthamer R. 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proceedings of the Royal Society B: Biological Sciences* **270:** 1857–1865.

Werren JH, Baldo L, Clark ME. 2008. *Wolbachia*, master manipulators of invertebrate biology. *Nature Reviews Microbiology* **6:** 741–751.

White JA, Kelly SE, Perlman SJ, Hunter MS. 2009. Cytoplasmic incompatibility in the parasitic wasp *Encarsia*

inaron: disentangling the roles of *Cardinium* and *Wolbachia* symbionts. *Heredity* **102:** 483–489.

White JA, Kelly SE, Cockburn SN, Perlman SJ, Hunter MS. 2011. Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. *Heredity* **106:** 585–591.

Yamamoto S, Harayama S. 1995. PCR amplification and direct sequencing of gyrb genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Applied and Environmental Microbiology* **61:** 3768.

Zchori-Fein E, Perlman SJ. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Ecology* **13:** 2009–2016.

Zchori-Fein E, Perlman S, Kelly SE, Katzir N, Hunter MS. 2004. Characterization of a 'Bacteroidetes' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of "Candidatus *Cardinium hertigii*". *International Journal of Systematic and Evolutionary Microbiology* **54:** 961–968.

Zhang XF, Zhao DX, Hong XY. 2012. *Cardinium* – the leading factor of cytoplasmic incompatibility in the planthopper *Sogatella furcifera* doubly infected with *Wolbachia* and *Cardinium*. *Environmental Entomology* **41:** 833–840.

Zhou W, Rousset F, O'Neill S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society B: Biological Sciences* **265:** 509–515.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Map showing collection locations of the different parasitoid populations.