

Parthenogenesis-Inducing *Wolbachia* in *Trichogramma kaykai* (Hymenoptera: Trichogrammatidae) Originates from a Single Infection

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ABSTRACT To determine the degree of within-population diversity of a parthenogenesis-inducing *Wolbachia* symbiont in the parasitic wasp *Trichogramma kaykai* Pinto, Platner & Stouthamer, we established >200 isofemale lines of this (partly) parthenogenetic species from the Californian Mojave Desert. These were screened with *Wolbachia*-specific polymerase chain reaction primers. An infection rate of 12.4% was found. We then generated 45 DNA sequences for the *ftsZ* gene and compared these with 19 sequences from other *Trichogramma* species. We found a single nucleotide position diagnostic for the *T. kaykai* symbiont. Phylogenetic analyses indicated that this diagnostic position is a derived character and that all the *Wolbachia* symbionts in *T. kaykai* are very closely related; this suggests that all of them originate from a single, ancestral infection.

KEY WORDS *Trichogramma*, *Wolbachia*, DNA sequencing, bacterial symbiont, genetic variation, parthenogenesis

THE PROKARYOTE *Wolbachia* is a common endosymbiont in arthropods (Werren et al. 1995a). It is typically transmitted vertically (i.e., from mother to offspring via the egg cytoplasm). Like certain other vertically transmitted symbionts (Hurst 1993), it has evolved the ability to increase its own transmission by altering the host's reproductive characteristics. Three distinct types of such host manipulation have so far been reported for *Wolbachia*. First, cytoplasmic incompatibility, in which the symbiont sabotages the fertilization of uninfected eggs by infected males (O'Neill et al. 1992, Brecuwer and Werren 1990). Second, in isopods it causes feminization and egg production in genetic males (Juchault et al. 1994). Third, in several Hymenoptera it induces parthenogenesis. In these haplodiploids, the symbiont restores diploidy (hence, female gender) in eggs that normally would have developed as (haploid) males. The phenomenon has been reported in various parasitoids (Stouthamer et al. 1993, Zchori-Fein et al. 1995, Van Meer et al. 1995), but it has been best studied in the egg parasitoid genus *Trichogramma* (Stouthamer et al. 1990; Stouthamer et al. 1993, Stouthamer and Kazmer 1994).

Recent studies have elucidated the phylogeny of the various *Wolbachia* strains using sequences of PCR-amplified ribosomal (O'Neill et al. 1992, Rousset et al. 1992, Stouthamer et al. 1993) or protein-coding (Werren et al. 1995b) genes. These studies have firmly established that the 3 distinct effects of *Wolbachia* do not follow any phylogenetic pattern. They also dem-

onstrated the existence of 2 monophyletic groups of strains, types I and II. However, the genetic variability of *Wolbachia* in a single host population has hardly been investigated. The only observations of this kind concern hosts that exhibit cytoplasmic incompatibility (Brecuwer et al. 1992, Werren et al. 1995b). A particularly well-studied example of this is *Drosophila simulans*, in which 4 different *Wolbachia* variants are found, in some cases producing double infections (i.e., infections with >1 *Wolbachia* strain) (Mercot et al. 1995). In cytoplasmic incompatibility, such multiple infection is to be expected; after an initial cytoplasmic incompatibility *Wolbachia* has spread to fixation, the population is available again for invasion by a 2nd cytoplasmic incompatibility *Wolbachia* (Rousset and Solignac 1995). Although multiple infections of parthenogenesis-inducing *Wolbachia* are not known, the same is likely not to be true for them; once parthenogenesis is established, a new strain probably will no longer have a mechanism by which to invade.

On the other hand, a host species in which the parthenogenesis *Wolbachia* is not established in the entire population, like most species of *Trichogramma*, might still be invaded by additional parthenogenesis-causing strains. Also, in analogy with organelle evolution, the *Wolbachia* lineages in different populations of the same host species might diverge and mix upon secondary contact, or genetic diversity might arise within a single population. All these processes would result in a nonuniform *Wolbachia* population. This possibility has never been investigated; type I DNA sequences from several parthenogenetic *Trichogramma* species have been published (Rousset et al. 1992, Stouthamer et al. 1993, Werren et al. 1995b), but

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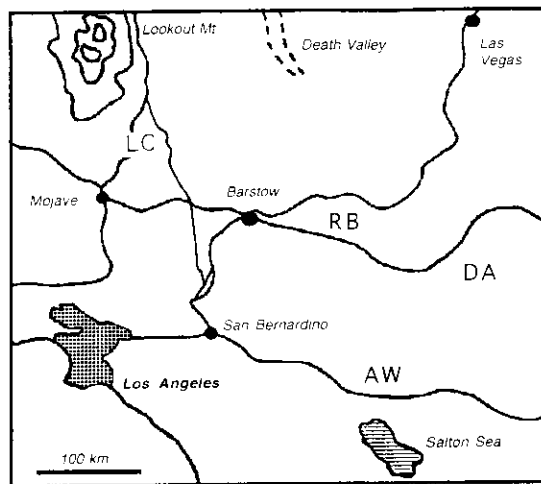


Fig. 1. Study area, with collection sites for *T. kaykai* indicated. AW, Dillon Road; DA, Danby; LC, Last Chance Canyon; RB, Road to Barstow.

usually no attempt has been made to assess genetic variability within a single host species. Sequences from >1 locality are known only for *Trichogramma deion* Pinto and Oatman (Stouthamer et al. 1993), among which a certain degree of diversity was detected. However, from each of the 4 localities only single clones were sequenced, so it is unclear if this reflects among- or within-population diversity.

The object of this study is to determine the degree of genetic diversity in the *Wolbachia* present in parthenogenetic individuals of a related species, *Trichogramma kaykai* Pinto, Platner & Stouthamer. This species parasitizes eggs of the lycacid butterfly *Apodemia mormo* (Felder & Felder) on *Eriogonum inflatum* in the Californian Mojave Desert (Pinto et al. 1997). As in most parthenogenetic *Trichogramma* species, the *Wolbachia* infection has not gone to fixation, but sexual and parthenogenetic individuals live side by side in the same population. In fact, gene flow occurs between the infected and the uninfected parts of the population because parthenogenetic females will mate with males and fertilize some of their eggs (Stouthamer and Kazmer 1994).

Materials and Methods

Collecting Wasps and Maintaining Cultures. *Apodemia* eggs were collected from *E. inflatum* at 4 localities in the Mojave Desert (southern California): Last Chance Canyon (Kern County), Dillon Road (San Bernardino County), Road to Barstow (San Bernardino County), and Danby (San Bernardino County). These localities are hereafter referred to as LC, AW, RB, and DA, respectively (see Fig. 1). Eggs were placed in gelatin capsules and left to emerge. Eggs from which female parasitoids emerged were assigned their locality code and a number. Because usually >1 female emerged from a host, each female was identified with an additional number. For example, the 2nd

female emerging from the 29th host from Danby would be referred to as DA-29-2. These single females were then allowed to parasitize eggs of *Mamestra brassicae* (L.). The F₁ was evaluated to decide whether lines were parthenogenetic or not, using a female/total offspring ratio of 0.9 or higher as the criterion. After this, all isofemale lines were cultured continuously on *Ephesttia kuehniella* Zeller. Most lines were identified morphologically by J. D. Pinto (University of California, Riverside); for the remaining lines, a PCR-based technique was used (Van Kan et al. 1996; unpublished data). Voucher specimens have been placed in the collection of the Department of Entomology of the University of California, Riverside. Cultures were maintained at 25°C, 60–70% RH, and a photoperiod of 16:8 (L:D) h.

Molecular Techniques. DNA was extracted from 1–5 individuals from each line, which were ground in 100 µl of a 5% suspension of Chelex-100 (Bio-Rad, Hercules, CA) with 3 µl proteinase K (20 mg/ml), and incubated for at least 2 h at 56°C, followed by 5 min at 95°C. In some cases, a phenol–chloroform extraction and ethanol–NaAc precipitation was done following Stouthamer et al. (1993).

All lines, both sexual and parthenogenetic, were checked for the presence of *Wolbachia* using diagnostic PCR of the *ftsZ* gene, as described in Holden et al. (1993). A minority of the lines were checked using the primers and protocol from Werren et al. (1995b). Each PCR reaction was carried out in a 50-µl reaction volume, using, apart from standard conditions, 0.5 U SuperTth DNA-polymerase, 5 pmol of each primer, and a final [Mg²⁺] of 2.8 mM. As a control, PCR reactions also were performed on DNA from the field host *Apodemia*, as well as the 2 laboratory hosts, *M. brassicae* and *E. kuehniella*.

Polymerase chain reaction products were not sequenced directly because initial attempts produced gels with multiple signals. Instead, we used the following cloning procedure. PCR products were first excised from agarose gels, extracted by the freeze-squeeze technique (Sanbrook et al. 1989), and cloned in a pMOSBlue T-tailed vector (Amersham Life Science, Little Chalford, England). Recombinants were checked for the presence of the correct insert by rapid direct colony screening, as recommended by the manufacturer. Positive colonies were then grown overnight in liquid medium, and plasmid DNA was isolated using QIAprep spin columns (QIAGEN). The insert was sequenced in both directions on an ABI automated sequencer. To minimize sequencing errors, the electropherograms were checked by eye. From most lines, a single clone was sequenced, with the exception of LC-128 (9 clones), AW-11-1 (2 clones), AW-7-3 (2 clones), and LC-10-3 (2 clones).

Sequence Analysis. After deletion of primers, all 45 type I *ftsZ* sequences were aligned against those from 12 other *Trichogramma* species and (to be used as an outgroup) a *Protocalliphora* species. These sequences have been reported by Werren et al. (1995b) and Schilthuisen and Stouthamer (1997); GenBank accession numbers U28198–28202, U74471–U74485. Be-

cause deletions were virtually absent, alignment could be carried out manually.

When type II sequences were encountered (divergent *Wolbachia* strains, unrelated to the type I normally found in *Trichogramma* [Stouthamer et al. 1993]), these were aligned separately against some other type II sequences, namely from *Drosophila melanogaster*, *Nasonia giraulti*, *N. longicornis*, and *N. vitripennis* (from Werren et al. (1995b); GenBank accession numbers U28189, U28182, U 28187, and U28188).

The type I alignment was submitted to the EMBL Nucleotide Sequence Database and can be obtained from <ftp://ftp.ebi.ac.uk/pub/databases/cmb/align>, under reference number DS25907. A single type I sequence from *T. kaykai* and 2 type II sequences from *Ephesia* have been deposited at GenBank (accession numbers U59696, U62125, and U62126, respectively).

The use of 2 sets of primers gave type I sequences of different lengths: 716 bp (primers from Holden et al. 1993) and 955 bp (primers from Werren et al. 1995b), with an overlap of 624 bp. The resulting stretches of missing data on both ends of the alignment were left untrimmed. The data were then entered in PAUP 3.1.1 (Swofford 1993) and analyzed with a heuristic search for the optimal tree, treating gaps as 5th base. We used 10,000 random addition sequence replicates, without branch swapping, to prevent bias toward a particular topology in the resulting most parsimonious trees. Trees were rooted with the *Protocalliphora* sequence, on the basis of the tree in Werren et al. (1995b). Consistency indices (Kluge and Farris 1969) also were calculated. Topological stability of the type I trees was investigated with T-PTP testing (Faith 1991). This was done with test version d53 of PAUP* 4.0, using the same search settings as described above, although the number of random addition replicates was limited to 100, to avoid prohibitively lengthy computing. The results are here included with permission from the author of PAUP*4.0, D.L. Swofford. The small size of the type II data matrix permitted an exhaustive search. The resulting trees were rooted with the *Trichogramma cordubensis* sequence, and a strict consensus was calculated.

Results

PCR Assay. The PCR assay showed the presence of *Wolbachia* in only 1 of the 3 lepidopteran hosts, the laboratory host *E. kuehniella*. Specific PCR and sequence analysis of 2 clones demonstrated that this was a type II strain. It is probably identical to the type II strain that was reported from a culture of this species in Antibes (Rousset et al. 1992).

Among 208 *T. kaykai* lines cultured and PCR-tested, 35 (17%) were parthenogenetic and PCR-positive. This percentage is an overestimate of the infection rate, because multiple lines from 1 host (e.g., DA-29-1, DA-29-2) were not maintained in culture when sexual. Correction for this yields 19 infected mothers of a total of 154 (i.e., an estimated degree of infection of 12.4%). We did not find any PCR-negative, parthenogenetic lines. In 2 cases, however, PCR products were derived

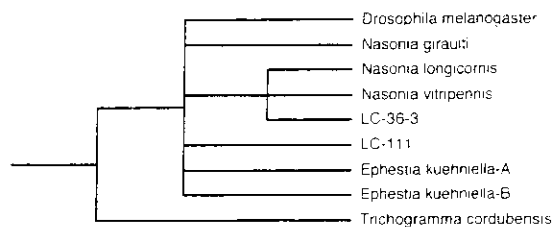


Fig. 2. Strict consensus tree over all most parsimonious trees for the type II sequences (obtained from sexual lines and possibly result from contamination).

from sexual lines. Sequence analysis showed these to belong to type II-strain *Wolbachia*.

Phylogenetic Analysis. Two hundred and thirteen most parsimonious trees (length 124) were generated in an exhaustive search on the type II data. In the consensus tree (Fig. 2), all 4 type II sequences encountered during this study (2 from *E. kuehniella*, 2 from sexual *T. kaykai* lines) are compared with each other and with other type II sequences.

The analysis of the type I data indicated the presence of a single diagnostic nucleotide for the *T. kaykai* symbionts: at position 850 all clones from this species carried a G, and all other sequences (*Trichogramma* and the outgroup) had a T. In fact, the majority rule consensus over 2,500 most parsimonious trees (length = 169; consistency index = 0.93 [Fig. 3]), showed that all 45 *T. kaykai* clones tend to form a monophyletic group because of this synapomorphy, although occasionally a sequence from 1 of the other *Trichogramma*, which lack this character state, also fell in this clade. The T-PTP value of 0.01 also gave significant support for a monophyletic origin for all *T. kaykai* sequences.

Within *T. kaykai* itself, little variability was observed. All type I sequences showed a high degree of similarity ($\approx 99\%$). Differences were found at 82 of 1,047 positions, but because these were all single substitutions or shared by only 2 or 3 sequences, they were interpreted as PCR artifacts, suggesting a polymerase infidelity of $\approx 0.26\%$ (90 changes in 34,941 bases sequenced). This corresponds to infidelities found in other studies (e.g., Stewart et al. 1995).

Discussion

The type-II tree (Fig. 2) suggests the presence in LC-111 of a sequence similar to the one in *E. kuehniella*. This is a common phenomenon in *Trichogramma* cultures reared on this host, and ongoing research is to determine whether this is the result of contamination or horizontal transmission (M.M.M. Van Meer and J. Witteveldt, personal communication). The sequence from LC-36-3 is very similar to the *N. vitripennis* sequence, which also might suggest contamination, because this species is used in our laboratory. This seems a likely explanation, because isofemale lines founded by sisters of LC-36-3 all proved PCR-negative.

been picked up. Some pairs of clones tend to be grouped together, but this is caused by the PCR artifacts mentioned previously and should not be regarded as evidence for intrapopulation structuring.

In summary, our data suggest that all the parthenogenesis-inducing *Wolbachia* strains in *T. kaykai* are descendants of a single ancestral infection. Any subsequent infections with other *Wolbachia* strains have either not occurred or have not become established.

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