

MOLECULAR MARKERS IN CLASSICAL BIOLOGICAL CONTROL OF THE CITRUS LEAFMINER: TAXONOMIC AND ECOLOGICAL EVALUATIONS

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INTRODUCTION

The success or failure of a biological control project could depend on the correct identification of the pest or the natural enemy. The most successful natural enemies are highly host-specific. Therefore, the correct identification is especially important when either the pest or the natural enemy has closely related species that are morphologically indistinguishable (cryptic species). Rosen (1986), in his chapter on cryptic species and biological control, presents some classic examples where misidentification of pests and natural enemies were the cause of costly failures of different biological control projects.

The Asian citrus leafminer, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), was first detected in Florida citrus in 1993 (Heppner, 1993). Two *Ageniaspis* populations (one from Australia and one from Taiwan) were introduced into Florida between 1994 and 1996 as part of a classical biological control project against the citrus leafminer (Hoy and Nguyen, 1997). The two populations cannot be distinguished morphologically, and taxonomic specialists identified both as *Ageniaspis citricola* Logvinoskaya. *Ageniaspis* adults of the two populations from the citrus leafminer are small (0.8 to 1.0 mm long) and morphologically similar. Morphological characters of the antennae, mandibles, and aedeagus were compared with the use of light and scanning electron microscopy, but the Taiwan and Australian populations of *Ageniaspis* could not be distinguished with these features.

Valid parasitoid species may differ only in their behavior and physiology (Fernando and Walter, 1997). Some biological differences were detected between the Taiwanese and the Australian populations. The Taiwanese population was more difficult to rear under laboratory and greenhouse conditions and fewer numbers of adults emerged from this colony than from the Australian colony. Adults from the two populations had different abilities to retain water (net water loss rates), with the Taiwanese adults losing moisture more rapidly (Yoder and Hoy, 1998). This implies a higher humidity requirement by adults of the Taiwan population.

Preliminary reciprocal crosses suggested the *Ageniaspis* populations from Taiwan and Australia do not interbreed (Alvarez, 2000; Hoy *et al.*, 2000). However, crossing studies have many inherent limitations as a diagnostic test for species differentiation, and reproductive isolation *per se* does not necessarily imply that the non-crossing populations are different species. Neither sterility nor viability of offspring resulting from cross mating are always a useful criterion of species status (Paterson, 1988).

Molecular techniques could solve, in part, the problem of inadequate systematics for some natural enemy groups. Here we report the use of several molecular tools to resolve taxonomic and ecological questions regarding the biotype or cryptic species status of two *Ageniaspis* populations. We also used these tools to analyze establishment patterns of the two populations, detect colony contamination, and test the two *Ageniaspis* populations and their host for the presence of the endosymbiont bacteria *Wolbachia*.

STUDY 1: USEFULNESS OF THE MITOCHONDRIAL CO1 AND RIBOSOMAL ITS2 DNA SEQUENCES IN SEPARATING *AGENIASPIS*

The taxonomic level at which specific genes or nucleotide regions are useful varies across taxa. Different genes have been used in order to evaluate the genetic relatedness of closely related species or populations. In theory, rapidly evolving genes and nucleotide regions are useful for comparisons of closely related taxa (Kocher *et al.*, 1989). However, highly conserved genes, such as CO1 and *Actin*, also have been used successfully as molecular markers for closely related taxa (Danforth *et al.*, 1998; Hoy *et al.*, 2000).

Our first objective was to assess the relative phylogenetic usefulness of a segment of the mitochondrial protein coding gene CO1 and the non-coding ribosomal region ITS2 for the Taiwan and Australian populations of *Ageniaspis* and for *A. fuscicollis* Dalman, a species present only in Europe, assuming *a priori* that if the Australian and Taiwanese *Ageniaspis* populations were the same species their sequences should be placed in the same clade regardless of the kind of phylogenetic analysis conducted, while *A. fuscicollis* sequences should be placed in a different clade.

Second, since we know that there are hundreds or thousands of copies of ribosomal DNA (rDNA) genes in tandem arrays in the eukaryotic nuclear genome and that these copies have been shown to be different in several organisms (deer ticks: Rich *et al.*, 1997; mosquitoes: Onyabe and Conn, 1999; nematodes: Hugall *et al.*, 1999; and crayfish: Harris and Crandall, 2000), we also investigated whether the level of intra- and inter-genomic variation within the ITS2 region of *Ageniaspis* is sufficiently great so as to affect the phylogenetic separation between the Australian and Taiwanese *Ageniaspis* populations. Complete information about the materials and methods of this research can be found in Alvarez and Hoy (2002).

RESULTS

ITS2 Length Variation

The primer pair (5.8SF and 28SR) amplified the ribosomal ITS2 region from all *Ageniaspis* populations. The length of the 5.8S (65 bp) and 28S (24 bp) gene segments flanking the ITS2 sequences was identical for all 20 clones from the three *Ageniaspis* populations, and the only variable DNA region was the ITS2.

Variability in ITS2 length was observed within and between individuals of the three *Ageniaspis* populations examined (Table 1). These consistent differences in length could be used to separate the Australian and Taiwan populations without the necessity of sequencing. The ITS2 PCR product was useful for identifying colony contamination and for determining which population had established in the field in this classical biological control project.

ITS2 Sequence Variation

Mean sequence divergence of ITS2 variants within and between each *Ageniaspis* population was estimated with PAUP by uncorrected P , which is the proportion of nucleotide sites differing between two compared sequences (see Table 2 for mean uncorrected P values within and between individuals and GC content). There was an identical variant (uncorrected $P = 0.0$) present in two different individuals of the Taiwan population (clones *pJM* 16 and *pJM* 20). However, all clones obtained from a single individual were different, and no single variant was common to all wasps in any *Ageniaspis* population.

Table 1. Sequence and length divergence of ITS2 variants from different *Ageniaspis* populations and species, and from two pteromalid species (*Trichomalopsis dubius* and *Nasonia vitripennis*).

Species (wasp number)	Clone	GenBank accession numbers	ITS2 Length (bp)	GC Content (%)	Sequence divergence (uncorrected P)	
					within individuals	between individuals
AcA (1) a ¹	pJM1	AF291439	714	40.90	0.0014	0.0037
	pJM2	AF291440	713	40.81		
AcA (2) a	pJM3	AF291441	712	41.01	0.0047	
	pJM4	AF291442	706	41.22		
	pJM5	AF291443	712	40.73		
AcT (1) a ²	pJM12	AF291444	509	46.37	0.0118	0.0066
	pJM13	AF291445	510	46.08		
	pJM31	AF291455	509	46.56		
AcT (2) a	pJM16	AF291446	509	46.36	0.0059	
	pJM17	AF291447	508	46.65		
AcT (3) a	pJM18	AF291448	512	46.29	0.0020	
	pJM19	AF291449	510	45.88		
AcT (4) a	pJM20	AF291450	509	46.36	0.0020	
	pJM21	AF291451	510	46.08		
AcT (5) a	pJM23	AF291452	509	46.37	0.0092	
	pJM24	AF291453	508	45.47		
	pJM26	AF291454	509	46.17		
Af (1) a ³	pJM8	AF291456	347	38.90	0.0117	
	pJM9	AF291457	337	38.87		
	pJM22	AF291458	347	38.62		
<i>T. dubius</i>		U02961	411	48.42		
<i>N. vitripennis</i>		U02960	502	48.29		

¹AcA (1) a= *Ageniaspis citricola* Australian population (wasp number 1) clone a.²AcT (1) a= *Ageniaspis citricola* Taiwan population (wasp number 1) clone a.³Af (1) a= *Ageniaspis fuscicollis* (wasp number 1) clone a.

Table 2. Mean ITS2 sequence divergence (uncorrected P) among different *Ageniaspis* populations and species, and two pteromalid species (*T. dubius* and *N. vitripennis*).

	Australian <i>Ageniaspis</i>	Taiwanese <i>Ageniaspis</i>	<i>A. fuscicollis</i>	<i>T. dubius</i>
Australian <i>Ageniaspis</i>	—			
Taiwanese <i>Ageniaspis</i>	0.2844	—		
<i>A. fuscicollis</i>	0.4118	0.3706	—	
<i>T. dubius</i>	0.5483	0.4741	0.4050	—
<i>N. vitripennis</i>	0.5448	0.4684	0.3987	0.02975

Sequence divergences among Australian individuals (0.0037) and among Taiwan individuals (0.0066) (Table 2) were approximately 8 and 4.5 times lower than the mean sequence divergence between the two species *Trichomalopsis dubius* (Ashmead) and *Nasonia vitripennis* (Walker) (0.02975). Therefore, if by chance the most divergent ITS2 sequences from an Australian and from a Taiwan individual were used for phylogenetic studies, these two individuals would still be grouped in separate clades. The mean sequence divergence between the Australian and Taiwanese populations (0.2844) is greater than that found for two different species: *T. dubius* and *N. vitripennis* (0.02975).

ITS2 Phylogenetic Analysis

Complete ITS2 sequences of the ribosomal DNA for 20 *Ageniaspis* clones (12 Taiwanese, five Australian, and three *A. fuscicollis*) were determined and positively confirmed as ITS2 with a BLAST search of GenBank. ITS2 sequences were phylogenetically informative in separating the three *Ageniaspis* populations and the topologies of the four phylogenetic trees were similar. Because of this similarity, the maximum-likelihood consensus bootstrap tree is the only one shown here (Fig. 1). All trees consistently show that the Australian and Taiwan individuals unambiguously cluster into separate clades, and that these clades always were supported by bootstrap values of 100% for the minimum evolution, MP, and ML trees.

DISCUSSION

Despite the level of intra-individual variation found in this work, the sequences of the ITS2 region were phylogenetically informative and separated the three populations (*A. citricola* Australian, Taiwanese, and *A. fuscicollis*). Intra-individual sequence variation in the ITS2 regions of *Ageniaspis* populations from Australia and Taiwan was sometimes greater than the variation between individuals in each population. However, bootstrap values strongly supported (100%) the nodes separating three population groups for all phylogenetic analyses tested (minimum evolution, MP, and ML trees).

ITS2 sequence differences between the Australian and Taiwanese populations are much greater (100 less 31%, or 69% difference) than those found between the two different pteromalid species (15.6%) tested in this study, which is consistent with previous conclusions that these two populations are cryptic species (Alvarez, 2000; Hoy *et al.*, 2000). If ITS2 variants within individuals had differed as much as those between populations, the data would not support the hypothesis that

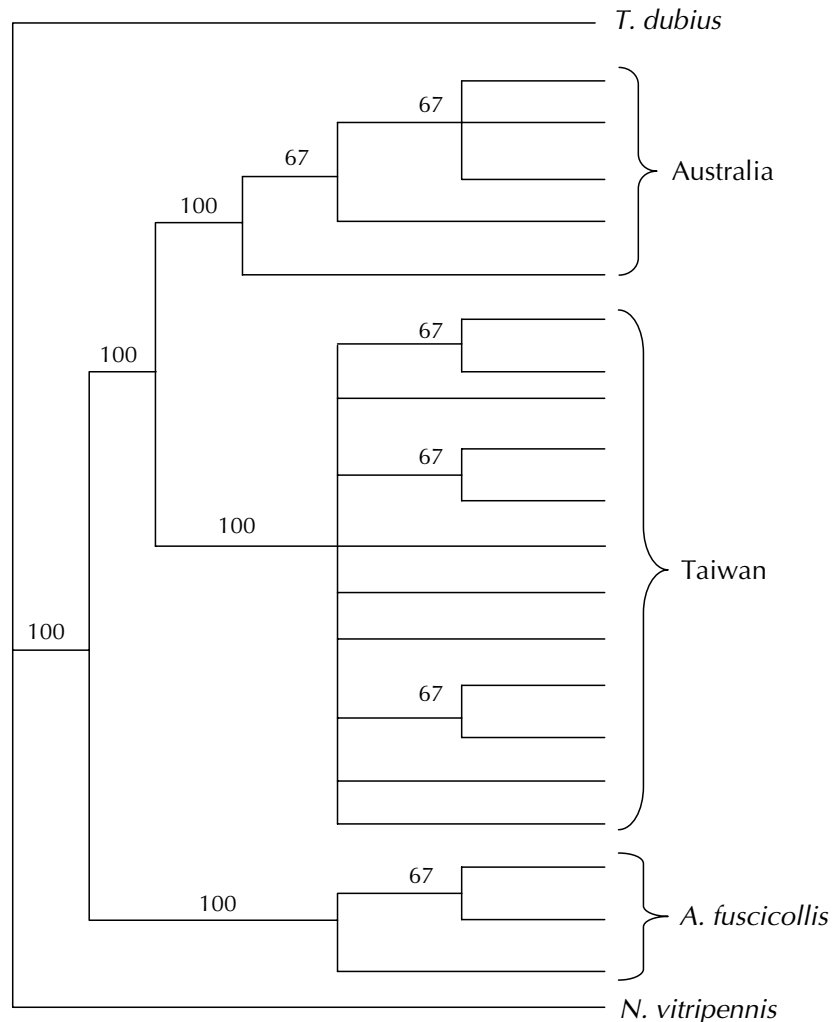


Figure 1. A maximum-likelihood consensus bootstrap tree based on rDNA ITS2 sequence data of *Ageniaspis citricola* (two populations: Taiwan and Australia) and *Ageniaspis fuscicollis*. The tree was rooted using the sequences of *Trichomalopsis dubius* and *Nasonia vitripennis*. Numbers are bootstrap percentages (100 replications) for clades supported above the 50% level.

they represent cryptic species, based on this analysis. Such variability prevented Onyabe and Conn (1999) from confirming the presence of cryptic species of the mosquito *Anopheles numeztovari* Gabaldon (found previously by other authors) because the ITS2 variants within individual mosquitoes from Brazil often differed as much as those between localities.

Finding a good molecular marker can be a complicated process, especially for phylogenetic studies of closely related species. Caterino *et al.* (2000) advocated that everyone should use a few genes and DNA regions (including the COI) for insect phylogenetics to coordinate efforts in insect molecular systematics and improve our ability to compare results across taxa. However, the hypothesis that the COI gene should be a good marker for closely related species should be evaluated carefully for each taxon. The length and sequence of the COI mitochondrial regions of all *Ageniaspis* tested were greater than 99.6% similar and therefore not informative (Alvarez, 2000). The COI section thus may not be a good marker for closely related encyrtid wasps.

A few recent phylogenetic analyses using ITS2 sequences consider the fact that intragenomic variation may exist, but some authors examine only one sequence from few individuals (Fenton *et al.*, 1994; McLain *et al.*, 1995; Xu and Qu, 1997). While we examined two to three variants from several individuals from each population, this is still a very small sample of potential ITS2 sequences considering that some insects are known to have 700 copies of ribosomal DNA (Collins *et al.*, 1989). Intragenomic sequence variation was sometimes greater than the variation between individuals in both the Australian and Taiwan *Ageniaspis* populations. This variability did not affect this phylogenetic reconstruction, but it may be possible to find cases other than that of Onyabe and Conn (1999) where such intragenomic variation could affect the conclusions of a phylogenetic analysis. For unknown reasons, it was more difficult to clone the Australian variants than the Taiwanese variants. Therefore, it is possible that we have underestimated variability in the ITS copies of the Australian population.

Examining the genetic variability of all ITS2 copies within an individual organism could be achieved only by sequencing the entire genome. However, when inferring phylogenetic affiliations between populations or cryptic species, we recommend examining several clones from each individual and obtaining these clones from more than two individuals from each population, so that this potential variability is considered. If this recommendation were followed, it would make phylogenetic analyses with the ITS2 region relatively more expensive than analyses with mitochondrial genes, which typically are present as one genotype within an individual, or with single copy nuclear genes, which may consist of only one or two alleles.

STUDY 2: RAPD-PCR USEFUL FOR DISCRIMINATING BETWEEN THE TWO AGENIASPIS POPULATIONS

By the time that the Taiwanese population of *Ageniaspis* was imported and released into Florida in 1996, the Australian population had colonized most of Florida's 850,000 acres of citrus groves and parasitism of citrus leafminer pupae was found to be as high as 99% at some sites (Hoy and Nguyen, 1997). For this reason, the fate of the Taiwan population was unknown.

The objective of this work was to determine whether the Taiwan population of *A. citricola* has established in Florida's citrus groves, and assess relative frequencies of the two populations at 10 different release sites, if establishment was confirmed. As mentioned before, the two populations could be distinguished by cloning and sequencing *Actin* genes and ITS2 ribosomal regions. However, since this study involved sampling many insects per site and several research sites, use of cloning and sequencing would be impossible because of the cost and the amount of labor involved. Random Amplified Polymorphic DNA (RAPD) is a Polymerase Chain Reaction (PCR)-based fingerprinting technique that does not require prior knowledge of DNA sequence (Williams *et al.*, 1990), and provides a rapid way of identifying genetic markers to distinguish closely related species (Wilkerson *et al.*, 1995). RAPD-PCR is rapid, inexpensive and easy when studies involve many insects, as in this case. RAPD markers have been criticized as a tool for molecular identification of species, however, because the results are difficult to reproduce (Ellsworth *et al.*, 1993). Therefore, the consistency of RAPD markers for identification of *Ageniaspis* populations was tested through time and between different reactions. RAPD-PCR also was used to monitor the purity of the two *Ageniaspis* colonies reared independently in separate facilities.

MATERIALS AND METHODS

DNA Extraction and RAPD-DNA Amplification

Genomic DNA was extracted by the Chelex 100 (Bio-Rad Laboratories, Richmond, CA) method (Edwards and Hoy, 1993), with some modifications, from 20 individual *Ageniaspis* pupae selected at random from each site for a total of 200 pupae (see the section on Sample Collection and Places of Collection below). Only one pupa per pupal chain was used for DNA extraction because this species is polyembryonic (Edwards and Hoy, 1998).

RAPD-PCR amplifications were performed as indicated in Saiki (1989) in a Perkin-Elmer DNA Thermal Cycler model 480 (The Perkin-Elmer Corporation, Norwalk, CT), for 45 cycles, each cycle consisting of melting at 94 °C for 30 s, annealing at 36 °C for 30 s, and extension at 72 °C for 1 min 30 s. The three arbitrary primers used in this study were decamers A16 (AGCCAGCGAA), 188 (GCTGGACATC), and 196 (CTCCTCCCC). RAPD-PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light. Only distinct bright bands were scored.

Sample Collection and Places of Collection

Control DNA was extracted from Australian and Taiwanese *Ageniaspis* individuals collected in Queensland (Australia), and Taichung, Taiwan, respectively. Field *Ageniaspis* individuals used in this study were collected in seven counties in Florida between June 1997 and September 1998. The sites chosen for this survey were sites where the Taiwanese wasps had been released. Collection sites, number of individuals collected and dates of collection are in Table 3.

Table 3. Collection localities and dates for *Ageniaspis* populations in Florida determined by RAPD-PCR, 1997-1998.

Date	Locality	County	Number of Individuals
1997			
Oct. 21	Labelle	Hendry	20
1998			
June 30	Homestead (2 sites)	Dade	40
July 20	Ft. Pierce (2 sites)	St. Lucie	40
July 22	Stuart (2 sites)	Martin	40
July 21	Labelle	Hendry	20
July 23	Gainesville	Alachua	20
Sept. 21	Lakeland	Polk	20

Monitoring Colony Purity

Because contamination is a common problem in colonies of minute parasitoids, RAPD-PCR was used to characterize *Ageniaspis* individuals from Australian and Taiwanese colonies reared independently in separate greenhouses at the University of Florida and at the quarantine facility of the Division of Plant Industry (DPI), Department of Agriculture and Consumer Services, Gainesville. DNA was extracted from 10 adult wasps selected at random from each colony (Taiwan and Australian) every month from September 1997 to September 1998. DNA banding patterns were then compared to patterns produced with DNA extracted from Australian and Taiwanese wasps to verify colony purity.

RESULTS AND DISCUSSION

Field Results

All primers regularly produced fixed and unique banding patterns that enabled the identification of the Taiwan and Australian *Ageniaspis* populations in a straightforward manner. The banding patterns were determined and sizes of the markers calculated for each primer. These markers were used to compare the 600 banding patterns (200 individuals by three primers) produced by individuals collected from the field. DNA from all 200 *Ageniaspis* pupae from field samples exhibited banding patterns identical to those of *Ageniaspis* from Australia. These results thus provide no evidence that the Taiwan population had established in Florida by the end of 1999. However, future monitoring of *Ageniaspis* populations in Florida might provide evidence of establishment of the Taiwanese population.

Monitoring Colony Purity

Monthly DNA banding patterns from 10 adult wasps selected at random from the Taiwan and Australian colonies matched the expected patterns for Taiwan and Australian wasps, respectively, for the first 11 months. Unexpectedly, the 12th RAPD-PCR results showed that two Taiwan populations reared at the DPI and in one greenhouse at the University of Florida had been lost and replaced by the Australian population sometime between August 1998 and September 1998. Without this molecular assay method we might not have discovered this problem before conducting additional biological and ecological experiments and could have made erroneous conclusions. One explanation for the contamination of the Taiwan colony could be that Australian adults emerging in plastic bags in the laboratory (see Smith and Hoy, 1995 for rearing methodology) were mislabeled or released in the wrong greenhouse. Because of the difficulty in rearing the Taiwan population of *Ageniaspis*, every time the colony density was low, we exchanged *Ageniaspis* material with the DPI and this could explain why both colonies converted to the Australian population so rapidly.

Contamination problems are common when rearing small, morphologically similar parasitoids such as *Trichogramma* and *Aphytis* (Dourojeanni, 1990; Fernando and Walter, 1997). Therefore, we recommend monitoring parasitoid colonies and preserving voucher specimens as often as possible. In this way, it would be possible to monitor parasitoid colonies and populations in order to identify the possible explanations of different biological control project outcomes.

STUDY 3: DIFFERENT *WOLBACHIA* STRAINS INFECT THE TWO CRYPTIC SPECIES OF *AGENIASPIS*

Wolbachia bacteria infect the reproductive tissues of many arthropods and are transmitted cytoplasmically (maternally) (Jeyaprakash and Hoy, 2000; Werren *et al.*, 1995a). These bacteria modify the reproduction of their hosts in different ways, including reproductive incompatibility and loss of males in some hymenopteran parasitoids (Cook and Butcher, 1999).

Werren *et al.*, (1995a), with the use of Standard PCR, reported that about 17% of 154 arthropod species tested positive for *Wolbachia* using the *ftsZ* primers. A recent survey conducted using a more sensitive technique called Long PCR and the *wsp* primers found 76% of 62 arthropod species in 13 orders were positive for *Wolbachia*, indicating that *Wolbachia* infection may be more widespread than previously estimated (Jeyaprakash and Hoy, 2000). In this survey, an individual *Ageniaspis* of the Australian population tested positive for the *wsp* gene, which encodes a *Wolbachia* surface protein (Braig *et al.*, 1998). This finding led us to question whether both *Ageniaspis* populations were infected and whether the *Wolbachia* strains were the same. In addition, we wondered whether *Wolbachia* strains in *Ageniaspis* and their citrus leafminer host were sufficiently similar that horizontal transfer between host and parasitoid could be inferred.

Long PCR products of the *wsp* gene were cloned, sequenced, and analyzed phylogenetically using individuals of the Australian and Taiwan *Ageniaspis* populations, and of their lepidopteran host, the citrus leafminer.

MATERIALS AND METHODS

DNA was extracted from individual adults of *Ageniaspis* collected in Queensland (Australia) or Taichung (Taiwan), and from citrus leafminer collected from a greenhouse colony at the University of Florida, Gainesville. DNA was extracted by the Pure Gene method (Minneapolis, Minnesota) following manufacturer's instructions, and amplified by the Long PCR (Jeyaprakash and Hoy, 2000) with the modified 81F and 691R *wsp* primers of Zhou *et al.* (1998):

WspF 5' -TGGTCCAATAAGTGATGAAGAACTAGCTA-3'

and

WspR 5'-AAAAATTAAACGCTACTCCAGCTTCTGCAC-3'.

Long PCR was performed in a 50 ml volume using three linked files as in Jeyaprakash and Hoy (2000). Long PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light.

Cloning and Sequencing

Long PCR products were ligated into a TOPO 2.1 vector, and transformed into competent One Shot cells (Invitrogen), with subsequent ampicillin selection, following manufacturer's instructions. Plasmid DNA was extracted using a Qiagen Plasmid Mini-prep Kit (Valencia, CA), following manufacturer's instructions. All products were incubated and digested with EcoR1 and visualized on a 1% agarose gel to verify that the inserts corresponded to the expected size of the PCR products. Colonies containing the plasmids with the inserts were recultured in 50 ml LB medium. Plasmids were extracted using Qiagen Plasmid Midi-prep Kit (Valencia, California). One DNA insert from the Australian *Ageniaspis* individuals, three from the Taiwanese *Ageniaspis* individuals, and two from the citrus leafminer individuals were sequenced on both strands using a Perkin-Elmer Applied Biosystems ABI PRISM Automated DNA sequencer located at the University of Florida ICBR Core Facility.

Sequence Alignment and Phylogenetic Analysis

Sequences were aligned using the default parameters within CLUSTAL W (Thompson *et al.*, 1994). Aligned sequences were then manually refined in PAUP, based on the alignment produced by Zhou *et al.* (1998). The phylogenetic analysis was performed after excluding the 41 bp third hypervariable region (positions 499-540) of the gene because of the poor alignment in this region (Braig *et al.*, 1998). The resulting alignment included 576 bases and has been deposited in the EMBL alignment database and is available by FTP from <ftp://ftp.ebi.ac.uk/pub/databases/embl/align/>. GenBank accession numbers are in Table 4.

Table 4. *Wolbachia* strains from two cryptic species of *Ageniaspis* and from their host, the citrus leafminer (*Phyllocnistis citrella* Stainton).

Host	Clone	GenBank Accession Numbers	Type	Closest Sequence ¹	Reference Strain Within Group	% Divergence From Reference Strain
<i>Ageniaspis</i> "citricola" From Taiwan	wTai-1	AF291459	B	wStri	wCon	2.23
	wTai-2	AF291460	A	wCof	wMel	0.95
	wTai-3	AF291461	A	wRi	wRi	7.30
<i>A. "citricola"</i> From Australia	WAus	AF291462	B	wStri	wCon	2.21
<i>Phyllocnistis citrella</i>	wCIm-1 and 2	AF291463 AF291464	A	wCof	wMel	0.95

¹The closest *Wolbachia* *wsp* sequences producing significant alignments in BLAST

The closest *wsp* sequences producing significant alignments in BLAST for each of the *Ageniaspis* and citrus leafminer *Wolbachia* were determined and the reference *wsp* sequence for each group was retrieved from the GenBank (Zhou *et al.*, 1998). Phylogenetic analyses were performed with the six *wsp* sequences from this study and the three closest reference *wsp* sequences of Zhou *et al.* (1998). The data set was analyzed in PAUP 4.0b (Swofford, 1999), using the Neighbor-joining algorithm (Saito and Nei, 1987), with the optimality criterion set for distance and Kimura 2-parameter. The reliability of the branching order was determined by 1000 bootstrap replications (Felsenstein, 1985). The *Wolbachia* present in the *Ageniaspis* and their citrus leafminer host were named using the system of groups and subgroups proposed by Zhou *et al.* (1998).

RESULTS AND DISCUSSION

Both *Ageniaspis* individuals tested from the *Ageniaspis* cryptic species and the citrus leafminer tested positive for *Wolbachia* by Long PCR. Cloning and sequencing of the *wsp* gene fragment revealed a triple infection (two A- and one B- *Wolbachia*) in the *Ageniaspis* individuals from Taiwan, and a single infection in the Australian *Ageniaspis* and in the citrus leafminer.

These *Wolbachia* sequences were named following the accepted abbreviation style in which *w* precedes the first three letters of the species name of the host (Rousset and de Stordeur, 1994). However, because the two tested organisms in this study share the same first three letters (cit) in the species names (*Ageniaspis citricola* and *Phyllocnistis citrella*), we have named the *Wolbachia* from *P.*

citrella as *wClm* for citrus leafminer (Table 4). The *Wolbachia* from the two cryptic *Ageniaspis* species were named according to the place of origin (*wAus*, for the *Wolbachia* from the Australian (but originally from Thailand) *Ageniaspis* and *wTai* for the *Wolbachia* from the Taiwan *Ageniaspis*).

A BLAST search of GenBank performed with the six *wsp* sequences revealed four that were unique. The two *wsp* sequences amplified from the citrus leafminer (*wClm*-1 and *wClm*-2) and one amplified from the Taiwan individual (*wTai*-2) were identical. The closest *Wolbachia wsp* sequences producing significant alignments in BLAST for each of the *Ageniaspis* and citrus leafminer *Wolbachia* are presented in Table 4. The sequences of the reference *Wolbachia* strain within each group (Zhou *et al.*, 1998) were used for the phylogenetic analysis (Table 4). For example, the closest sequence producing significant alignments to the three identical *Wolbachia* sequences was the A-*Wolbachia wCof* from *Drosophila simulans* Sturtevant (Coffs Harbour strain). This *wCof Wolbachia* sequence had been placed previously in the Mel group. Therefore, the sequence used for this phylogenetic study was the reference strain in this group, the A-*wMel* strain of *Drosophila melanogaster* (yw67c23).

The phylogenetic analysis of the *Ageniaspis* and citrus leafminer *wsp* sequences identified three as A- and two as B- *Wolbachia*. The three identical sequences (two from the citrus leafminer, identified in the tree as *wClm* and one from the Taiwan *Ageniaspis*, identified as *wTai*-2) clustered together within the A- Mel group, suggesting horizontal transfer could have occurred between the citrus leafminer host and its parasitoid (Fig. 2). These three sequences exhibited 0.95% sequence divergence from the reference A-*wMel* strain. Another A-*Wolbachia* sequence from the Taiwan *Ageniaspis* (*wTai*-3) displayed 7.3% sequence divergence from the reference A-*wRi Wolbachia* strain. According to the proposed naming system of Zhou *et al.* (1998), each named *Wolbachia* group is defined by the *wsp* sequence similarity of its members and this similarity should be greater than 97.5%. Therefore, the *wTai*-3 was placed in a new group named A-Tai.

The *Wolbachia* sequence *wTai*-1 and *wAus* clustered together within the B- Con group and the divergences of these sequences from the reference *wCon Wolbachia* were 2.3% and 2.1%, respectively.

Double *Wolbachia* infection (presence of A and B *Wolbachia*) previously was considered rare, occurring only in 5.8% of 154 insects tested, and the random expectation for frequency of double infection was estimated as 1.2% (Werren *et al.*, 1995a). Infection with two A- and two B- *Wolbachia* also was detected in one species, *Tribolium confusum* Jacquelin du Val (Werren *et al.*, 1995b). Jeyaprakash and Hoy (2000) suggested that sequences of 59% (17 of 29) of the *Wolbachia*-positive arthropods could not be directly sequenced due to multiple *Wolbachia* infections within individuals. They concluded that multiple infections could be detected more frequently with the use of Long PCR, which is more sensitive than Standard PCR. In this study, the Taiwanese *Ageniaspis* individuals tested with the use of Long PCR, had a triple *Wolbachia* infection. A second species (the Australian *Ageniaspis*) may well have had a double *Wolbachia* infection. Two bright and close bands were observed in the Long PCR product when visualized in the agarose gel (data not presented). However, despite analyzing more than 20 white bacterial colonies that apparently contained the plasmids with *wsp* inserts, only one clone could be obtained and sequenced. Therefore, it is possible that the number of *Wolbachia* types in the Australian *Ageniaspis* has been underestimated.

Phenotypic effects, if any, due to infection with *Wolbachia* are unknown in the three species. Both *Ageniaspis* populations have males and females, so these *Wolbachia* do not cause thelytoky as has been shown in some other hymenopteran species (Stouthamer, 1997). Because these two populations of *Ageniaspis* have different strains of *Wolbachia*, the populations appear to have been reproductively isolated for a long time. Different *Wolbachia* infections in closely related parasitoids have been implicated in parasitoid speciation (Vavre *et al.*, 1999). For example, reproductive isolation between two

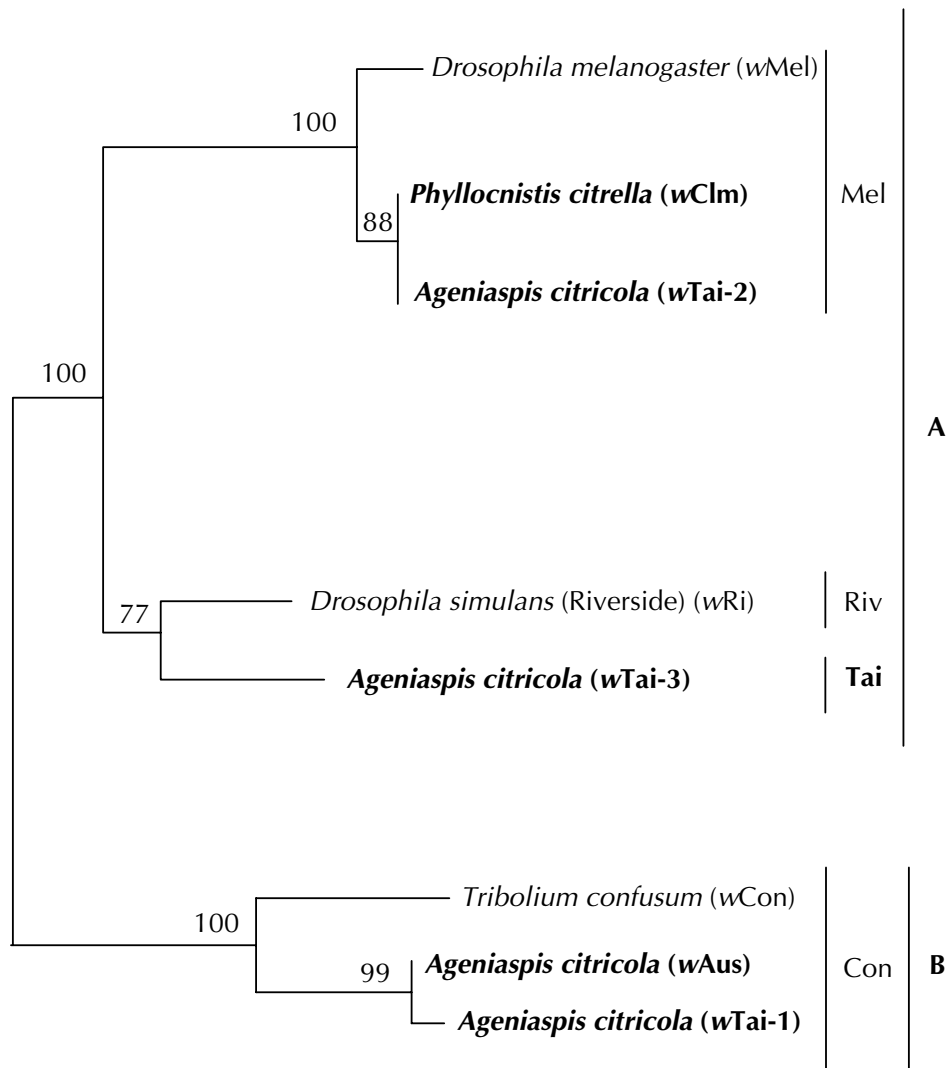


Figure 2. Phylogenetic tree based on the *wsp* sequence data of *Wolbachia* strains from two cryptic species of *Agenesipis* (one from Taiwan and one from Australia but originally from Thailand) and from their host, the citrus leafminer (*Phyllocnistis citrella* Stainton). Name of the host is followed by the strain name in parentheses and by the group designation according to Zhou *et al.* (1998). The tree was generated by the Neighbor Joining algorithm (Saito and Nei, 1987) using the Kimura distance and is midpoint rooted. Numbers are bootstrap percentages of 1,000 replications.

parasitoids, *Nasonia vitripennis* (Walker) and *Nasonia giraulti* Darling (both Hymenoptera: Pteromalidae) carrying different *Wolbachia* previously has been shown to be due to the *Wolbachia* because cured populations could interbreed (Bordenstein and Werren, 1998).

The data on *Wolbachia* strains, along with data on the ITS2, *Actin* genes, and RAPD-PCR analyses (Alvarez and Hoy, 2002; Hoy *et al.*, 2000) are congruent with the hypothesis that the Australian and Taiwan *Agenesipis* are cryptic species. Jager and Menken (1994), with the use of RAPD-PCR, reported the presence of cryptic species in *Agenesipis fuscicollis* Dalman obtained from different hosts at the same geographic location in Europe, implying sympatric speciation had occurred. This suggests that the number of species of *Agenesipis* might prove to be greater than the current nine if other “populations” were to be examined with molecular methods.

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