

MOLECULAR IDENTIFICATION OF PARASITOID, *Encarsia formosa* GAHAN IN *Bemisia tabaci* (GENNADIUS) AND DETERMINATION OF ITS SECONDARY ENDOSYMBIONTS

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Abstract

In this study two pairs of primers based on mitochondrial cytochrome oxidase subunit 1 (mtCOI) region and 28S ribosomal RNA (rRNA) gene region were used for identifying very tiny and morphologically indistinguishable parasitoid *Encarsia formosa* (Gahan) which are specific to this insect. The fragment amplified by these primer pairs were 860 and 650 bp in length. Species specificity test showed that all *E. formosa* specimens were detected with no cross reactions with other aphelinid species, including *E. sophia* (Girault & Dodd), *E. luteola*, *E. Inaron* and *E. Nigricephala*. Using phylogenetic cladogram by the sequences analysis of both mtCOI and 28S rRNA genes could be detected in *E. formosa* accurately in all replicates. *Cardinium* and *Wolbachia* secondary endosymbiont were also detected in *E. Formosa* used by PCR amplification as well as sequence analysis of 16S-23S rDNA gene region. The molecular technique developed here would be useful for rapid and precise species identification, determination of the host spectrum and more effective utilization of *E. formosa*. This research work has been performed from January 2011 to June 2012 at the insect molecular physiology lab in the Republic of Korea.

Keywords: Molecular identification, mtCOI, *Encarsia formosa*, Secondary endosymbiont, *Bemisia tabaci*.

Introduction

Whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) has become one of the predominant agricultural and horticultural pest species throughout many subtropical and tropical regions of the world (Anthony *et al.*, 1995; De Barro, 1995; Qiu *et al.*, 2007). The control of whiteflies by traditional chemical means has become difficult because of ground water contamination and pest resistance and thus forcing efforts toward alternative means of control (Landa *et al.*, 1994). Aphelinids are common parasitoids of whiteflies, and members of the genus

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Encarsia have been used with success in several biocontrol programs. *Encarsia* is the cosmopolitan parasitoid, among all 107 *Encarsia* species documented as parasitoids of whiteflies and at least eight are currently under study for biological control (Lenteren *et al.*, 1997). *Encarsia formosa* Gahan became one of the most successful natural enemies in biological control after its use to control the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (van Lenteren and Woets, 1988). *E. formosa* is applied on a large scale in the greenhouse industry with an estimate of approximately 5,000 ha in more than 20 of the 35 countries that have a greenhouse industry (van Lenteren *et al.* 1996).

Although no phylogeny of *Encarsia* species has been proposed, *E. formosa* and *E. luteola* Howard are considered closely related, if not merely population variation of the same species (Schauff *et al.*, 1996). *Encarsia luteola* shares many of the same hosts as *E. formosa*, including *B. argentifolii* and *T. vaporariorum* (Schauff *et al.*, 1996). *E. luteola* and *E. formosa* are both widespread in the New World (Polaszek *et al.*, 1992; Schauff *et al.*, 1996) and have been introduced to one or more locations in the Old World for biological control of *Bemisia* spp. and *T. vaporariorum* (Gerling and Rivnay 1984; Rivnay and Gerling 1987; Polaszek *et al.*, 1992; Huang and Polaszek, 1998).

Maternally inherited bacterial endosymbionts are common among arthropods (Douglas, 1989; Werren *et al.*, 1995; Hurst and Jiggins, 2000; Russell *et al.*, 2003; Duron *et al.*, 2008), with the most widespread bacterial endosymbiont, *Wolbachia*, recently estimated to infect 66% of arthropod species (Hilgenboecker *et al.*, 2008). Infection by multiple symbionts is reasonably common (Duron *et al.*, 2008). Most studies have focused on multiple coinfecting strains of a single symbiont, the most common symbiont, *Wolbachia* (Mouton *et al.*, 2004), and/or comparing populations of hosts that are naturally infected with different symbiont combinations (Narita *et al.*, 2007; Ros and Breeuwer, 2009). *Encarsia inaron* (Hymenoptera: Aphelinidae) is an introduced parasitic wasp that has both *Wolbachia* and *Cardinium* endosymbionts (Perlman *et al.*, 2006). *Wolbachia* is an α -Proteobacteria that has been shown to maintain its prevalence within many arthropod taxa through a variety of reproductive manipulations (Werren *et al.*, 2008). *Cardinium* is a more recently described symbiont that is not closely related to *Wolbachia*, yet is capable of a similar array of reproductive manipulations (Hunter *et al.*, 2003). In *E. inaron*, *Wolbachia* was shown to cause CI, whereby matings between infected males and uninfected females do not produce viable female offspring (White *et al.*, 2009). *Cardinium*, however, did not cause CI, nor did it interact with the *Wolbachia*-induced CI phenotype.

Therefore, symbiont influence was investigated on host fitness as a potential explanation for the persistence of *Cardinium* in the wasp population.

Although *E. formosa* and *E. luteola* between the two species are quite similar in morphology, there are distinct behavioral differences. *E. formosa* is a thelytokous species, which rarely produces males because of the presence of *Wolbachia* (Zchori-Fein *et al.*, 1992), whereas males are common in *E. luteola* (Gerling and Rivnay, 1984). Parasitism of *T. vaporariorum* by *E. formosa* causes melanization of the puparia, whereas parasitism by *E. luteola* does not induce melanization of immatures (Gerling and Rivnay, 1984). The two species differ in host feeding, percent parasitism, and total number of *B. argentifolii* nymphs killed, although the significance of these differences depends on the strain of *E. formosa* and poinsettia cultivar used (Heinz and Parrella, 1994). It is unlikely that the taxonomic problem of distinguishing *E. formosa* and *E. luteola* will be resolved using classic morphological techniques. Rather, it has been suggested that morphometrics or other methods will be necessary to overcome these difficulties (Polaszek *et al.*, 1992). Here the morphological characters have been reviewed that can be used to distinguish *E. Formosa* and then to characterize the amount of genetic variation within and between the species, and to present a molecular assay that rapidly distinguishes *E. formosa*.

Materials and method

Collection of *Encarsia formosa* samples: Adult individuals of *E. formosa* and parasitized nymphs of *B. tabaci* were collected from tomato and cucumber host plants in greenhouses of Kyungpook National University, Daegu, Andong, Weiseong and Sangju in Korea (Table 5). The adult *E. formosa* and its immature stages in the nymphs of *B. tabaci* were collected with tomato and cucumber leaves in 2012, immediately preserved a part of samples in 99% ethanol after collection, and stored them at -20°C for further molecular analysis. A rest of immature stages as well as adults with cucumber and tomato leaf were kept on wet cotton for observing its lifecycle under the microscope.

DNA extraction and PCR amplification

Total genomic DNA was extracted from individual *E. formosa* according to Dellaporta *et al.* (1983) and Palmer *et al.* (1998) using Invitrogen Purelink Genomic DNA mini kit for further use. After removing the sample from ethanol had been washed with double-distilled water to remove alcohol, the extracted DNA template were directly used for PCR amplification or were kept at -20°C for later use (Jahan *et al.*, 2011).

Primer design and PCR amplification

Table 1. List of primers used for *Encarsia formosa* identification.

Gene	Primer Name	Primer Direction	Primer Sequence (5' to 3')	Size (bp)	Reference	Tm. (°C) cycles
mtCOI	C1-J-2195	Forward	TTGATTTTTTGGTCA	860	Simon et al. 1994	52°C
	L2-N-3014	Reverse	TCCAGAAGT TCCAATGCACTAATC TGCCATATTA			35
28S rRNA	LR-J-12887	Forward	CCGGTTTGA ACTCA GATCATGT	720	Simon et al. 1994	55°C
	LR-N-13398	Reverse	CGCCTGTTTAACAA AAACAT			35

E. formosa and its' harbored secondary endosymbionts were determined using the genomic DNA which was extracted from collected parasitoid of whitefly from different places in Korea with the primers listed in Tables 1 and 2, using Polymerase Chain Reaction (PCR). All PCR reaction mixture performed in 20 µl volume that included 1 µl of each primer (Forward and Reverse), 1 µl of DNA template and 17 µl smart buffer which were supplied by the manufacturer (Smart taq pre-mix). All PCR reactions were carried out on the PTC-200 DNA engine thermal cycler (MJ Research PTC-200 DNA Engine Thermal Cycler PCR).

Table 2. List of primers used for secondary endosymbiont detection in *E. formosa*.

Secondary Endosymbiont	Targeted Gene	Primer Direction	Primer Sequence (5' to 3')	Size (bp)	Reference	Annealing Temp.(cycle)
<i>Arsenophonus</i>	23S rDNA	Forward Reverse	CGTTTGATGAATT CATAGTCAAA GGTCCTCCAGTTA GTGTTACCCAAC	~600	Thao and Baumann, 2004	60°C (30)
<i>Cardinium</i>	16s rDNA	Forward Reverse	GCGGTGTA AAAATG AGCGTG ACCTMTTCTTAAC TCAAGCC	~400	Weeks et al., 2003	58°C (35)
<i>Hamiltonella</i>	16s rDNA	Forward Reverse	TGAGTAAAGTCTG GAATCTGG AGTTCAAGACCGC AACCTC	~700	Zchori-Fein & Brown, 2002	58°C (35)
<i>Rickettsia</i>	16s rDNA	Forward Reverse	GCTCAGAACGAA CGCTATC GAAGGAAAGCAT CTCTGC	~900	Gottlieb et al., 2006	60°C (30)
<i>Wolbachia</i>	16s rDNA	Forward Reverse	CGGGGGAAAAAT TTATTGCT AGCTGTAATACAG AAAGTAAA	~625	Zhou et al., 1998; Heddi et al., 1999	55°C (35)

PCR condition

The mixtures with mtCOI and 28S rRNA used for PCR reactions were performed in a 20 µl mixture containing 5 x SuperTaq PCR buffer (10 mM Tris-HCL, 40 mM KCl, 1.5 mM MgCl₂, pH 9.0), 2.5 mM dNTPs, 0.5 µM of each primer, 1 unit of SuperTaq DNA polymerase (SuperBio Co, Korea) and 1 µg of DNA as a template. The mixtures were amplified in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) with a 1 min initial denaturation at 95°C, 35 cycles (1 min at 94°C, 30 sec at 52-55°C, 2 min at 72°C), and finally by a 5 min extension at 72°C. For symbiont detection, species specific primers were also amplified with 5 min initial denaturation at 95°C, 30-35 cycles (30-60 sec at 92-95°C, 0.5-1 min at 55-60°C, 1 min at 72°C), and finally by a 10 min extension at 72°C which are listed in Table 3.

Table 3. PCR reaction used to detect secondary endosymbiont in *E. formosa*.

Endosymbiont	Targeted Gene	Pre-denaturation	Denaturation	Cycling conditions		
				Annealing	Extension	Cycles
<i>Arsenophonus</i>	23SrDNA	95°C (5 min)	95°C (30 sec)	60°C (30 sec)	72°C (45 sec)	30
<i>Cardinium</i>	16SrDNA	95°C (5 min)	94°C (1 min)	58°C (1 min)	72°C (1 min)	35
<i>Fritschea</i>	23SrDNA	95°C (5 min)	95°C (30 sec)	60°C (30 sec)	72°C (45 sec)	30
<i>Hamiltonella</i>	16SrDNA	95°C (5 min)	94°C (1 min)	58°C (1 min)	72°C (1 min)	35
<i>Rickettsia</i>	16SrDNA	95°C (2 min)	92°C (30 sec)	60°C (30 sec)	72°C (30 sec)	30
<i>Wolbachia</i>	16SrDNA	95°C (5 min)	95°C (30 sec)	55°C (30 sec)	72°C (1 min)	35

Sequencing of mtCOI and 28S rRNA gene of selected *E. formosa* samples

Five *E. formosa* samples (Table 5) that produced the most diverse polymorphic patterns were selected for mitochondrial cytochrome oxidase subunit I (mtCOI) and 28S rRNA gene-sequence analysis. Extracted DNAs from collected parasitoid of whiteflies were used in PCR amplification of the mtCOI gene (~860 bp) (Frohlich *et al.*, 1999; Maruthi *et al.*, 2004) and 28S rRNA gene (~720 bp) (Simon *et al.* 1994). The sequences of 763 bases for mtCOI and 634 bases for 28S rRNA were analyzed by parsimony and distance methods (Swofford, 2002), along with selected mtCOI and 28S rRNA sequences from the NCBI database.

Identification of parasitoid of whiteflies and endosymbionts

Parasitoid of *B. tabaci* was determined by amplification of mtCOI and 28S rRNA gene fragments from the extracted genomic DNA samples (Khasdan *et al.*, 2005). The presence of 6 secondary endosymbionts such as *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia* and *Wolbachia* in *E. formosa* was determined using specific primer sets of each endosymbiont by amplification of either 16S or

23S rDNA gene fragments (Chiel *et al.*, 2007). Specific primer sets of *E. formosa* and endosymbionts were listed on the Tables 1 and 2. The amplified PCR products were visualized on a 1.0% agarose gel containing ethidium bromide. Expected PCR products were excised from the gel and purified using the Wizard PCR preps DNA purification system and sequenced either directly or by cloning into the pGEM-T easy plasmid vector (Promega, Madison, WI, USA).

DNA sequence analysis

Sequences of the PCR products were determined using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and analyzed using a 3730XL DNA Sequencer (Applied Biosystems, Foster City, USA). Databases were searched using the BLAST algorithm (Altschul *et al.*, 1997; Schäffer *et al.*, 2001) in NCBI, and sequences were aligned using the MUSCLE program (Edgar, 2004). Mitochondrial COI and 28S ribosomal RNA sequences of *E. formosa* were analyzed using Bayesian MrBayes 3.0 software. Four Metropolis-coupled Markov Chain Monte Carlo (MCMC) chains were run until standard divergence of the split frequencies become lower than 0.01 (Ronquist and Huelsenbeck, 2003). All sequences were analyzed over 10 million generations, and four sequences were sampled every 100 generations. The first 25% of burn-in (SUMP and SUMT) cycles were discarded prior to the construction of consensus tree, which were visualized by MEGA 4.0 (Tamura *et al.*, 2007).

Results

Identification of *Encarsia formosa*: Collected five populations of *E. formosa* from 5 different regions of Korea in 2012 (Table 5) were analyzed. PCR amplification of mtCOI and 28S rRNA gene regions with target DNA fragments showed that important parasitoid of whiteflies were identified in all examined populations. All the collected populations from different regions in Korea were clearly *E. formosa* (Figure 1).

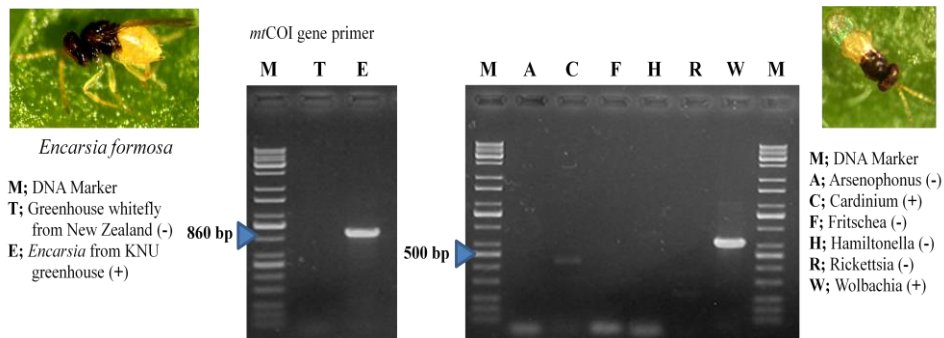


Fig. 1. PCR amplification for identification of *Encarsia* parasitoid and its' endosymbionts.

Table 4. Sequence of mtCO1 region of *E. formosa* in Korea.

Name of Specimen	Sequence of Mitochondrial Cytochrome oxidase subunit 1 (mtCOI) and 28S rRNA region of parasitoid <i>E. formosa</i>	T _m (°C)	Length (bp)
<i>E. formosa</i> (mtCOI gene)	TTTCTCATATAATTAATAATGAAAGATTAAAAAAG AAGTTTTGGAATTATAGGAATAATTTATGCAATAAT TTCAATTGGTTTATTAGGTTTTATTGTTTGAGCTCAT CATATATTTACTATTGGTATAGATATTGATACTCGAGC TTATTTTACATCAGCTACAATAATTATTGCAATTCCA ACAGGAATTAATAATTTTATAGATGATTAGCATCAATG AATGGAATAAAAAATTTATTTAAATGTATCAAATTTAT GATTAATAGGATTTATTTTTTTTATTACTGTAGGTGG ATTAACAGGGATTATATTATCAAATCTTCTATTGAT ATTGTTTTACATGATACTTATTATGTAGTTGCTCATT TCATTATGTATTATCTATAGGAGCTGTATTGCTATTT TTGGTAGATTTATTTATTGATTTCCCTATAATATTTGGT TTATCATTAATCAAAAATGATTAATAATCAATTTT TTATAATGTTTATTGGTGTAATTTAACTTTTTTTCCT CAACATTTTTTAGGATTAAGTGAATACCACGTCGT TATTCAGATTATCCAGATTCTTATTTATGTTGAAATTT AATTCATCTATTGGTTCATTAATTCATTATTTAGAA CTTTATTTTTTTTTTTTATTATTTGAGATAGATTAATT TCAATTCGTTAATAATTTTTATAAAAAATTTAAATA ATTCTATTGAATGATTAATAAATTATCCTTCTAGATCT CATTCTTTTTATGAAAT	52	763
<i>E. formosa</i> (28S rRNA gene)	CGTGTGCTTGATAGTGCAGCTCTAAGTTGGTGGA AACTCCATCTAAGGCTAAATATCGCCACGAGACCG ATAGCGAACAAGTACCGTGAGGGAAAGTTGAAAA GAACTTTGAAGAGAGAGTTCAATAGTACGTGAAAC CGTTCAGGGGTAAACCTGAGAAACCCAAAAGATC GAATGGGGAGATTCATCGTCAACCTCTTTGGCTTAC GTGTGGGTGCGATGATCGGGACCTCGGTCTCGCGT CACGCGCTCGCTGCGGCATGTCCGGAGGGGTCGGC GTGCACTTCTCCCTGGTAGGACGTGCGGACCCGT TGGGTGCTTGTCTACGGTCCGGTTGGTTCGACTGGC GAGTCACGTGCAAACGCGATCTCGCCAGACCACTG GTTTGCCCGATTGGCTGCCCGACGGTATTGAACGAT AATTAACATGGTATTGGGCCGCACAAAGTGC GTTC AGGCCCGTCGTCAGGCTCGTCTCGCTCGGATTTTC GAACCTGGTTTTCGTTGTCGAGTGCTTGACGTGGCT GTTGGGCGACGGTGTCTCGGACTGGCTCTAAATA TTCGAACGGATATGCCTGTCCGGCAGCCTACTGCTT TGGGTACTTTCAGGACCCGTCTTGAACACGGACC AA	55	634

Identification of secondary endosymbionts in *E. formosa* populations

To find out the relationship between *E. formosa* and pattern of endosymbiont infection in different population of *E. formosa*, the presence of 2 endosymbiotic bacteria in all examined populations from Korea were observed by PCR analysis of 16S or 23S rDNA sequences (Fig. 1). *Cardinium* and *Wolbachia* were detected in all the tested populations. *Arsenophonus*, *Hamiltonella* and *Rickettsia* were not detected in any populations of collected *E. formosa* from Korea. In this study, all examined populations were infected with two secondary endosymbionts (Table 5). In addition, *Cardinium* were always co-infected with *Wolbachia* but never co-infected with others (Table 5).

Table 5. Profile of secondary endosymbiotic bacteria in *Encarsia formosa* of Korea.

Species Parasitoid	Host	Locations	Endosymbiotic Bacteria					
			A	C	F	H	R	W
<i>E. formosa</i>	<i>B. tabaci</i>	Daegu, Korea	-	+	-	-	-	+
<i>E. formosa</i>	<i>B. tabaci</i>	KNU greenhouse, Korea	-	+	-	-	-	+
<i>E. formosa</i>	<i>B. tabaci</i>	Andong, Korea	-	+	-	-	-	+
<i>E. formosa</i>	<i>B. tabaci</i>	Weiseong, Korea	-	+	-	-	-	+
<i>E. formosa</i>	<i>B. tabaci</i>	Sangju, Korea	-	+	-	-	-	+

Arsenophonus (A), *Cardinium* (C), *Fritschea* (F), *Hamiltonella* (H), *Rickettsia* (R), *Wolbachia* (W)

Life cycle observation of *E. formosa*

Some cucumber and tomato leaves were collected with adult *E. formosa*, adult *B. tabaci* and parasitized nymphs of whitefly for keen observation which we reared in control environment of our laboratory (Figure 2).

Phylogenetic analysis of whiteflies and different endosymbionts

The Neighbour-joining phylogenetic tree reconstruction based on 21 mitochondrial cytochrome oxidase subunit-I (mtCOI) and twenty three 28S ribosomal RNA sequences of different closest *Encarsia* which shown in Figure 3 and 4. It revealed that all collected parasitoid from Korea are *E. formosa*. Mitochondrial COI sequences of all *E. formosa* of different countries were clustered in a single clade individually with high distance from each other; different species of *Encarsia* clustered in different clades on the phylogenetic tree (Fig. 3). It is clear that collected all parasitoid from Q biotype of *B. tabaci* in Korea are *E. formosa*.

High genetic variance among all collected sequence of *Encarsia* in same way by constructed Neighbour-joining phylogenetic tree based on twenty three 28S

rRNA sequences of different morphologically indistinguishable *Encarsia* were compared which shown in Fig. 4. It exposed that ribosomal RNA (16S rRNA) sequences of collected *E. formosa* were clustered in same clades of *E. formosa* (Fig. 4).

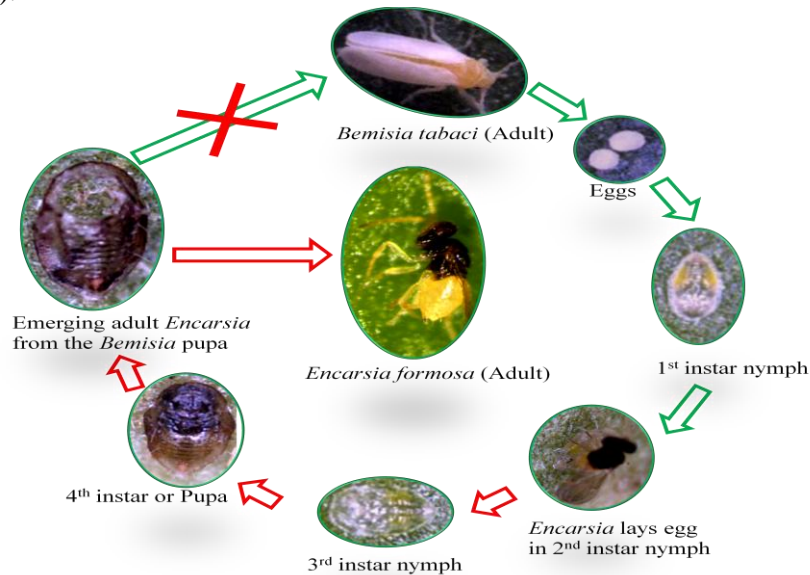


Fig. 2. Life cycle of *Encarsia formosa* using the host Q biotype of *Bemisia tabaci*.

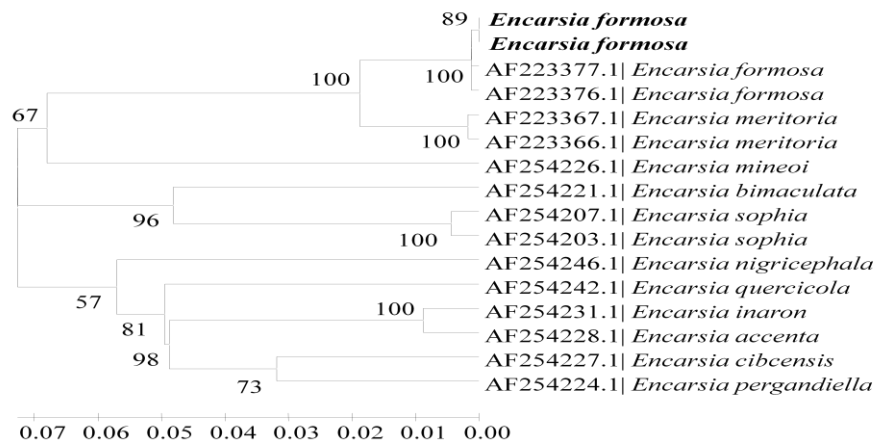


Fig. 3. Phylogenetic tree based on DNA fragment (~650 bp) of 28S ribosomal RNA gene sequences of different *Encarsia*. The numbers placed at each node indicate the bootstrap support for values >50. The horizontal branch length is drawn to scale, which indicates the horizontal distance of 0.00-0.07. Bold letters indicate the samples of the present study.

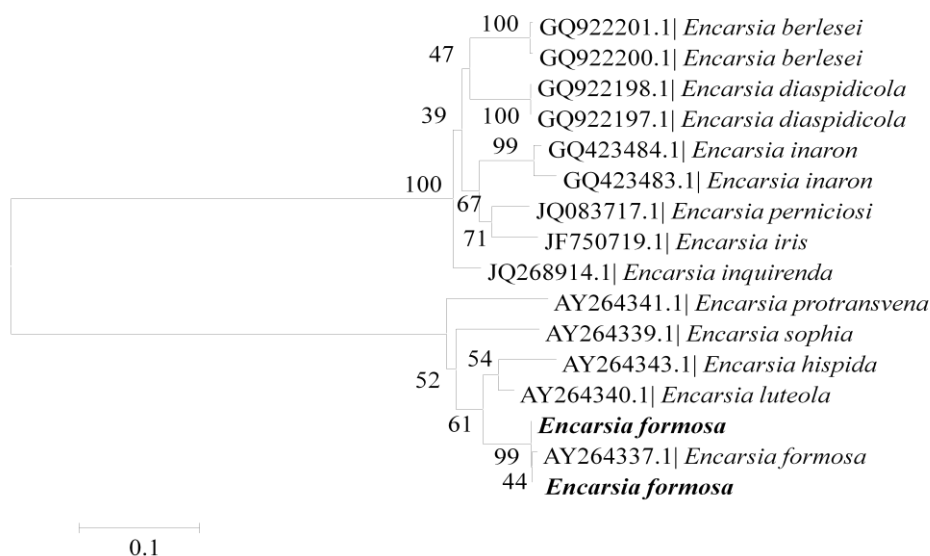


Fig. 4. Phylogenetic tree based on DNA fragment (~860 bp) of mtCOI gene sequences of different *Encarsia*. The numbers placed at each node indicate the bootstrap support for values >50. The scale bar indicates the horizontal distance of 0.1. Bold letters indicate the samples of the present study.

Discussion

Encarsia formosa, *E. Sophia*, *E. nigricephala* and *E. luteola* are difficult to distinguish based on morphology alone. The required slide mounting techniques for the tiny insects are very laborious and difficult too. For example, the amount of cellular reticulation on the mesosoma, may be difficult to detect in slide mounted preparations. Based on a sampling of two and six individuals per species, the mtCOI and D2 expansion region of 28S rDNA provided sufficient genetic variation to characterize and unambiguously distinguish these species (Babcock and Heraty, 2000). In an attempt to unambiguously classify specimens of *E. formosa* and *E. luteola*, identified two reciprocal molecular markers mtCOI and 28S rRNA. The D2 region of 28S rDNA was sequenced for five populations of *E. formosa*. All of these sequences were identical and they showed 100% similarity with other *Encarsia formosa* sequences of National Center for Biotechnology and Information (NCBI) database. The observed amount of sequence variations between *E. formosa* and other *Encarsia* were substantial and supported by Babcock and Heraty (2000).

A parsimony analysis based upon the 28S-D2 gene region was performed using 11 individuals from four species of the *luteola* species group and rooting with the sister genus *Encarsiella*. Of 635 aligned bp, 43 were parsimony-informative characters. A single most-parsimonious tree (Fig. 3) was recovered from the analysis with a length of 130 steps, consistency index of 0.923, and retention index of 0.875. All branches of this tree are well supported by bootstrap and jackknife analyses and the tree is stable to successive approximations character weighting. The six populations of *E. formosa* formed a monophyletic clade, as do the two populations each of *E. luteola* and *E. meritoria*. *Encarsia hispidula* is placed as sister taxon to those taxa indicated above, and *E. formosa* and *E. luteola* were sister taxa. These are the same relationships, including a monophyletic *luteola* group, that result from a more extensive analysis including additional *Encarsia* species (unpublished data). The patterns of little within-species sequence variation and substantial between-species variation enabled us to develop two reciprocal molecular markers from the 28S-D2 gene region that unambiguously distinguish *E. formosa* and *E. luteola*.

Encarsia inaron coinfects with *Cardinium* and *Wolbachia* (White *et al.*, 2010) that fully matched with present observation, *Cardinium* and *Wolbachia* were always present in all tested *E. formosa* population. Although, recently Ros *et al.* (2012) showed that recombination of *Wolbachia* with *Cardinium* in *Bryobia* spider mite that also similar with present findings about endosymbiont harbored in *E. formosa*.

The reciprocal molecular markers developed here in order to provide a rapid assay for characterizing large numbers of individuals. Because reliable morphological characters were available to separate more distantly related species from *E. formosa* and *E. luteola*, a combined morphological and molecular approach is suggested.

Conclusion

The molecular techniques used in this study could easily be identified from the test groups of individuals for potential cross contamination in insectary and other colonies. This application may prove useful in quality control programs of large rearing facilities; particularly those in which *E. formosa* are being produced for biological control worldwide. In this study, we used two pairs of primers based on mtCOI region and 28S rRNA gene region for identifying *E. formosa* which are specific to *E. formosa*.

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