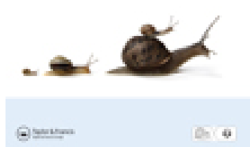

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Single strain infection of adult and larval cardamom thrips (*Sciothrips cardamomi*) by *Wolbachia* subgroup *Con* belonging to supergroup B in India

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The presence of *Wolbachia* in cardamom thrips (*Sciothrips cardamomi*), a major insect pest of cardamom (*Eletteria cardamomum*) was detected by amplification of the fast evolving outer membrane protein coding gene *wsp* of the bacterium. Studies on the identity of *Wolbachia* showed infection with only subgroup *Con* belonging to supergroup B, and both male and female thrips were infected with the same *Wolbachia* subgroup. The incidence of *Wolbachia* infection varied from 15.0 to 87.8% in thrips collected from seven cardamom growing districts of different States in India. The overall infection rate was 53.5% with 57.1% male and 50.6% female populations infected with the bacterium. DNA sequencing and phylogenetic analysis revealed that all the *Wolbachia* isolates from thrips clustered together indicating that all thrips were infected by the same *Wolbachia* strain. This is the first ever report of *Wolbachia* infection in cardamom thrips and the possibility of using the bacterium as a tool in biological control of this important insect pest is discussed.

Keywords: biological control; *Eletteria cardamomum;* endosymbiont; pest; Thysanoptera; *wsp* gene; sex infection rate

Introduction

Wolbachia, which belong to the α-protobacteria, is the most widely distributed bacterial endosymbiont in arthropods and nematodes and is believed to infect up to 70% of the world's insect species (Stouthamer et al. 1999; Rowley et al. 2004; Baldo et al. 2007; Hilgenboecker et al. 2008). The role of this bacterium in cytoplasmic incompatibility (CI), parthenogenesis, sex-determination, male-feminization, and speciation is reported in several hosts (O'Neill et al. 1992; Werren 1997; Moran et al. 2008). More than 20 groups within *Wolbachia* (Jeyaprakash & Hoy 2000) have been identified by characterizing the *Wolbachia* specific surface protein coding gene *wsp,* among which, supergroup A and B infections are reported only from arthropods (Lo et al. 2002; Li et al. 2007). Since *Wolbachia* play vital roles in insects including CI, identification, and characterization of *Wolbachia* groups in an insect species of agricultural importance will provide insight into development of novel control measures based on symbiont–host interactions.

Cardamom thrips (*Sciothrips cardamomi* Ramk.) (Thysanoptera: Thripidae) are one of the most destructive and persistent insect pests of cardamom (*Elettaria cardamomum* Maton.) (Gopakumar & Chandrasekhar 2002), a commercial spice crop of high value mainly grown in India, Guatemala, and Sri Lanka. In India, the cultivation of cardamom is restricted to South India, especially Kerala, Karnataka, and Tamil Nadu. The pest is also reported to attack other Zingiberaceae plants in Hawaii and Maui islands (Mau & Kessing 2007). Infestation by this pest results in shedding of flowers and immature capsules and formation of corky, scab-like encrustation on cardamom capsules, affecting the marketability of the produce. The pest is reported from all cardamom growing tracts of India (Devasahayam et al. 2012) and Sri Lanka (Dharmadasa et al. 2008), and found to cause up to 47% crop loss (Spices Board 2009). We report *Wolbachia* subgroup *Con* belonging to supergroup B infection in both male and female cardamom thrips collected from various cardamom growing districts in India which is the first ever report of *Wolbachia* infection in cardamom thrips.

Materials and methods

Collection of cardamom thrips

The cardamom thrips were collected from various locations of cardamom cultivation in India during 2011–2012 (Figure 1). The specimens were transferred directly to glass vials containing 70% ethyl alcohol using a fine hair brush, and stored at room temperature for further studies. We collected 13 populations of cardamom thrips from seven districts (Table 1) and a total of 159 adult insects (70 males and 89 females) and larvae from one location

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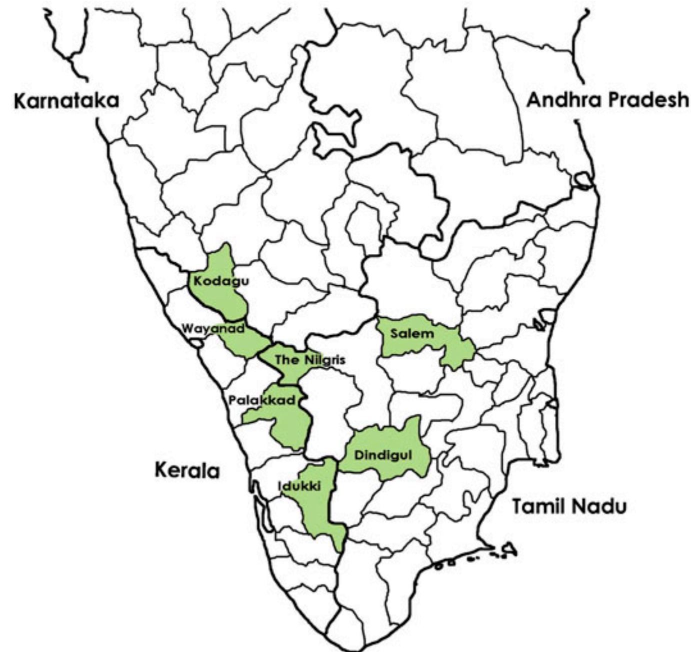

Figure 1. Map showing the collection sites of *Sciothrips cardamomi* (map not to scale).

were screened for detection of the bacterium. Before DNA isolation, the adults were sexed based on morphological characters (Asokan et al. 2011). The adult thrips were used individually, whereas the larvae were pooled (5–10 larvae) for DNA isolation and detection of *Wolbachia.*

DNA extraction

Isolation of DNA was done following the protocol of O'Neill et al. (1992) with slight modification. Briefly, individual adult thrips or 5–10 larvae were homogenized in a 1.5 ml micro tube containing 20 µl STE buffer (10 mM Tris–HCl, 100 mM NaCl, and 1 mM EDTA) with a clean, DNA-free polypropylene micro-pestle. The homogenate was incubated with 2 µl of proteinase-K (10 mg ml^{-1}) at 37 °C for 30 min, followed by incubation at 95 °C for 5 min. The homogenate was briefly centrifuged at 3000 × *g* for 5 min at room temperature and 4 µl of the supernatant (containing ~10 ng DNA μl^{-1}) was directly used as the DNA template in PCR amplifications.

PCR amplification of Wolbachia specific gene

The presence of *Wolbachia* in adult thrips and larvae was detected by amplifying the *wsp* gene with primers, 81 F and 691 R (Braig et al. 1998). The PCR assays were carried out in a reaction volume of 25 µl, containing 2.5 µl of 10× buffer, 1.0 µl of 2.5 mM dNTP's, 0.3 µl of Taq DNA Polymerase (3U μl^{-1}), 1.0 µl each of 10 mM forward and reverse primers, 13.2 µl of PCR water, and 4.0 µl of DNA template. The PCR conditions were: initial denaturation at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplification of the desired size of the DNA fragment in the PCR product was checked by electrophoresing on 0.8% agarose gel stained with ethidium bromide (5 µg ml^{-1}).

Detection of Wolbachia groups

The DNA of those thrips specimens with confirmed *Wolbachia* infection was further amplified for the *wsp* gene with primers specific for supergroup A (primers: 136F and 691R) and B (primers: 81F and 522R). The *wsp* gene amplification was observed only for supergroup B in thrips, and to determine the subgroup (SG) of *Wolbachia,* the *wsp* gene was further amplified with SG specific primers: 202 F-691 R for SG *Con*; 217 F-691R for SG *Dei*; 212 F-691 R for SG *con* + *Dei*; 183 F-691 R for SG *Pip;* and 211 F-691 R for SG *CauB* to get the desired size of amplicons (for details of primers see Zhou et al. 1998). Other PCR conditions remained the same as mentioned earlier.

DNA sequencing and phylogenetic analysis

The amplified DNA fragments from each PCR reaction were eluted using an extraction kit (Thermo Scientific Gene JET Gel Extraction Kit), following the manufacturer's instructions. The amplicons were sent to M/s Merck Millipore India Ltd, for sequencing.

Table 1. Extent of *Wolbachia* infection in cardamom thrips in India.

State	Location of collection (District)	No. of collection sites	Geographical indicators	No. of individuals screened		No. of individuals positive for *Wolbachia*		Supergroup – Subgroup	Per cent thrips population infected with *Wolbachia*
				Male	Female	Male	Female		
Kerala	Wayanad	2	11°36′18″N, 76°4′58.8″E	12	15	7 (58.3)	7 (46.7)	B–*Con*	51.9
	Idukki	5	9°51′0″N, 76°56′24″E	24	25	21 (87.5)	22 (88.0)	B–*Con*	87.8
	Palakkad	1	10°46′12″N, 76°39′0″E	5	10	0 (0.0)	3 (30.0)	B–*Con*	20.0
Tamil Nadu	Nilgiris	1	11°24′42.63″N, 76°41′ 45.24″E	5	5	3 (60.0)	3 (60.0)	B–*Con*	60.0
	Salem	1	11°39′0″N, 78°9′36″E	8	10	2 (25.0)	1 (10.0)	B–*Con*	16.7
	Dindigul	1	10°21′0″N, 77°57′0″E	6	14	1 (16.7)	2 (14.3)	B–*Con*	15.0
Karnataka	Kodagu	2	12°25′14.88″N, 75°44′ 22.92″E	10	10	6 (60.0)	7 (70.0)	B–*Con*	65.0

Figures in parentheses show per cent infection.

DNA sequences were subjected to a basic local alignment search tool (BLAST) search to identify sequences deposited in GenBank that had significant homology. The *wsp*, supergroup B and *Con* sequences of cardamom thrips collected from various locations were deposited in GenBank (accession numbers: KF378593–KF378603) and the sequences were aligned with corresponding gene sequences of reference taxa retrieved from the GenBank using MUSCLE incorporated in MEGA5 (Tamura et al. 2011). Sequence data for all reference supergroups and subgroups of *Wolbachia* were included for phylogenetic analyses. Gaps in alignment were treated as missing data and the phylogenetic trees were constructed by the neighbor-joining method and *p*-distance. Bootstrap analysis was done with 2000 replicates. *Wolbachia* strains with GenBank accession Nos. AF020063 and AF020072 belonging to supergroup A were used as out groups and a rooted tree was constructed.

Statistical analysis

We used a binary mixed model approach with presence or absence of infection as the response, sex as a fixed effect and geographic location as a random effect to study the significant effect of sex as well as to estimate the amount of variation explained by geographic location. The analysis was done using the generalized linear model procedure (PROC GLIMMIX) of SAS® 9.3 software for statistical analysis (SAS 2011).

Results

Wolbachia detection

PCR amplification of the *wsp* gene from adult and larval cardamom thrips with specific primers (81F/691 R) produced the desired size of DNA fragment (~600 bp) confirming the presence of *Wolbachia* in thrips (Figure 2). The bacterium was detected in both male and female thrips in all locations except one (Palakkad) where it was absent from males. The infection status varied from 0.0 to 87.5% in males and 20.7 to 88.0% in females, and the mean infection status in adult male and female thrips was 57.1 and 50.6%, respectively. We observed a sex ratio of 29 males to 71 females ($n = 100$) in field population of Kodagu district. The level of infection in various populations of thrips differed greatly and was lowest (15.0%) in Dindigul district and highest (87.8%) in Idukki district (Table 1). However, there was no gender effect on the occurrence of infection in different populations from various locations as the *p*-value is very high ($F = 0.01$; $p = 0.93$; $df = 1, 6$). The overall infection level of *Wolbachia* in thrips populations was 53.5%. When DNA from thrips that tested positive for *Wolbachia* was amplified using supergroup specific *wsp* primers, amplicons corresponding to supergroup B (~442 bp) were obtained. Supergroup A was not amplified from any of the thrips tested. To characterize the *Wolbachia* present in cardamom thrips, we followed the classification of Zhou et al. (1998). Using their primers, we only got amplification for DNA fragments (~488 bp) corresponding to the *Con* subgroup (Figure 2). DNA from larvae also showed similar amplification for *Con* (not shown in Figure 2). This confirmed infection of male, female and larval thrips populations with the same *Wolbachia* strain.

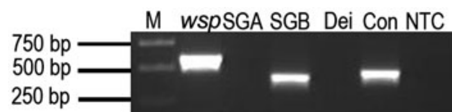

Figure 2. PCR amplification of the *wsp* gene for detection of *Wolbachia* groups in *Sciothrips cardamomi*. Lanes 2–6 indicate amplification of the *wsp* gene, supergroups and subgroups. Lane 1 – molecular weight marker; Lane 2 – *wsp* gene; Lane 3 – supergroup A; Lane 4 – supergroup B; Lane 5 – subgroup *Dei*; Lane 6 – subgroup *Con*; Lane 7 – non template control.

Phylogenetic analysis

The sequence data generated for the *wsp* surface protein using *wsp* specific primers and the primers specific to supergroup B and the *Con* subgroup were deposited in GenBank with accession numbers KF378593–KF378603 (Table 2). A BLAST search for the sequences indicated that the *Wolbachia* of *S. cardamomi* isolated from different locations were similar to *Wolbachia* strains belonging to the supergroup B detected in *Conotrachelus nenuphar* (Herbst), *Pandemis dumetana* (Treitschke), *Orius nagaii* (Yasunaga), and *Oryzaephilus surinamensis* (Linnaeus). The *Wolbachia* sequences of 12 isolates from *S. cardamomi* along with 24 strains from other arthropod hosts retrieved from GenBank were subjected to phylogenetic analysis. The phylogram (Figure 3) showed that all the *Wolbachia* isolates detected in the study from thrips collected from different agro-ecosystems clustered together showing 99% similarity among them indicating that irrespective of geographical isolation, all thrips were infected by the same *Wolbachia* strain, *wScar*. The *wScar* strains shared a close relationship with other *Wolbachia* strains like *wCne2*, *wCne1*, *wDumB*, *wNag1*, *NFRI55,* and Clone RE-04-C245. These closely related *Wolbachia* strains were reported from insect hosts belonging to different orders such as Coleoptera, Hemiptera, and Lepidoptera from China, Japan, and USA (Table 2). The *Wolbachia* strain from a Thysanopteran used in the present phylogentic analysis was divergent from our strains reported here (Figure 3).

Table 2. *Wolbachia* strains used for phylogenetic analysis.

Host insect	Order	*Wolbachia* strain	Location	GenBank accession number
Sciothrips cardamomi	Thysanoptera	*w*Scar-BHAG-wsp	Bhagamandala, India	KF378597
S. cardamomi	Thysanoptera	*w*Scar-CRC-WSP	Appangala, India	KF378593
S. cardamomi	Thysanoptera	*w*Scar-IRUL-wsp	Irulam, India	KF378598
S. cardamomi	Thysanoptera	*w*Scar-NALL-wsp	Nallanur, India	KF378603
S. cardamomi	Thysanoptera	*w*Scar-BHAG-GrB	Bhagamandala, India	KF378601
S. cardamomi	Thysanoptera	*w*Scar-CRC-GrB	Appangala, India	KF378596
S. cardamomi	Thysanoptera	*w*Scar-Irul-GrB	Irulam, India	KF378595
S. cardamomi	Thysanoptera	*w*Scar-NALL-GrB	Nallanur, India	KF378604
S. cardamomi	Thysanoptera	*w*Scar-BHAG-con	Bhagamandala, India	KF378602
S. cardamomi	Thysanoptera	*w*Scar-CRC-con	Appangala, India	KF378600
S. cardamomi	Thysanoptera	*w*Scar-IRUL-con	Irulam, India	KF378599
S. cardamomi	Thysanoptera	*w*Scar-NALL-con	Nallanur, India	KF378594
Drosophila melanogaster	Diptera	wMel	USA	AF020063
D. melanogaster	Diptera	wMel	USA	AF020072
Ephestia cautella	Lepidoptera	wCauB	USA	AF020076
Laodelphax striatellus	Homoptera	wStri	China	AF020080
Tribolium confusum	Coleoptera	wCon	USA	AF020083
Trichogramma deion	Hymenoptera	wDei	USA	AF020084
Culex pipiens	Diptera	wPip	USA	AF020061
Callosobruchus chinensis	Coleoptera	wBruOri	Japan	AB038339
Larval dryinid wasp	Hymenoptera	wDry	Japan	AB046721
Tagosedes orizicolus	Hemiptera	wOri	Costa Rica	AF020085
Trichogramma kaykai	Hymenoptera	wKayA	The Netherlands	AF071912
Torymus bedeguaris	Hymenoptera	wBed	The Netherlands	AF071915
Phlebotomus perniciosus	Diptera	wPrn	Italy	AF237884
Bactocera pyrifoliae	Diptera	wPyr	Thailand	AF295349
Coquillettidia crassipes	Diptera	wCra	Thailand	AF317478
Armigeres kesseli	Diptera	wKes	Thailand	AF317489
Culex sitiens	Diptera	wSit	Thailand	AF317491
Bemisia tabaci	Hemiptera	wBtab1	Greece	AJ291372
Orius nagaii	Heteroptera	wNag1	Japan	AB094368
Conotrachelus nenuphar	Coleoptera	wCne1	USA	GU013550
C. nenuphar	Coleoptera	wCne2	USA	GU013551
Franklinothrips vespiformis	Thysanoptera	wVes	Japan	AB045314
Pandemis dumetana	Lepidoptera	wDumB	China	EU399650
Oryzaephilus surinamensis	Coleoptera	NFRI55	Japan	AB469919
Melanitis leda	Lepidoptera	Clone RE-04-C245	USA	KC137219

Discussion

We employed molecular tools involving the *wsp* gene for the identification of *Wolbachia* and *Wolbachia* subgroup-specific primers for characterizing the bacteria, associated with cardamom thrips. In this study, only supergroup B infection was detected. Similar observations on infection with only *Wolbachia* supergroup B were reported in the predatory thrips, *Franklinothrips vespiformis* (Crawford) (Arakaki et al. 2001), *Leptothrips mali* (Fitch), and in many other insects (Nirgianaki et al. 2003). The present findings also support the earlier reports that arthropods were only infected with supergroup A or B (Lo et al. 2002; Li et al. 2007). Subgroup analysis of *Wolbachia* using specific primers showed the presence of the *Con* subgroup in thrips. The clustering of all the *Wolbachia* strains of *S. cardamomi*, as evidenced in the present phylogenetic analysis, showed that the cardamom thrips were infected only by subgroup *Con* of *Wolbachia.* The presence of *Wolbachia* subgroup *Con/Rug* was detected in many insects, including the predatory thrips, *L. mali* (Nirgianaki et al. 2003). In this study, thrips populations collected from different agro-ecosystems showed varying levels of infection, ranging from 15.0 to 87.8%. Kumm and Moritz (2008) also observed both infected and non infected individuals in the thrips, *Suocerathrips linguis* (Mound and Marullo), and *Gynaikothrips ficorum* (Marchal). Even though *Wolbachia* association is very common in arthropods (Werren 1997), its prevalence within a species may vary from very low to high (Hilgenboecker et al. 2008). In our study, more than half of the male and female thrips were infected by *Wolbachia* with a slightly higher infection rate (57.1%) in males than in females (50.6%); the overall infection rate was 53.5%. The infection rate of *Wolbachia* in populations of other species of thrips reported ranged from 0% [*Frankliniella occidentalis* (Pergande) *and Thrips tabaci*

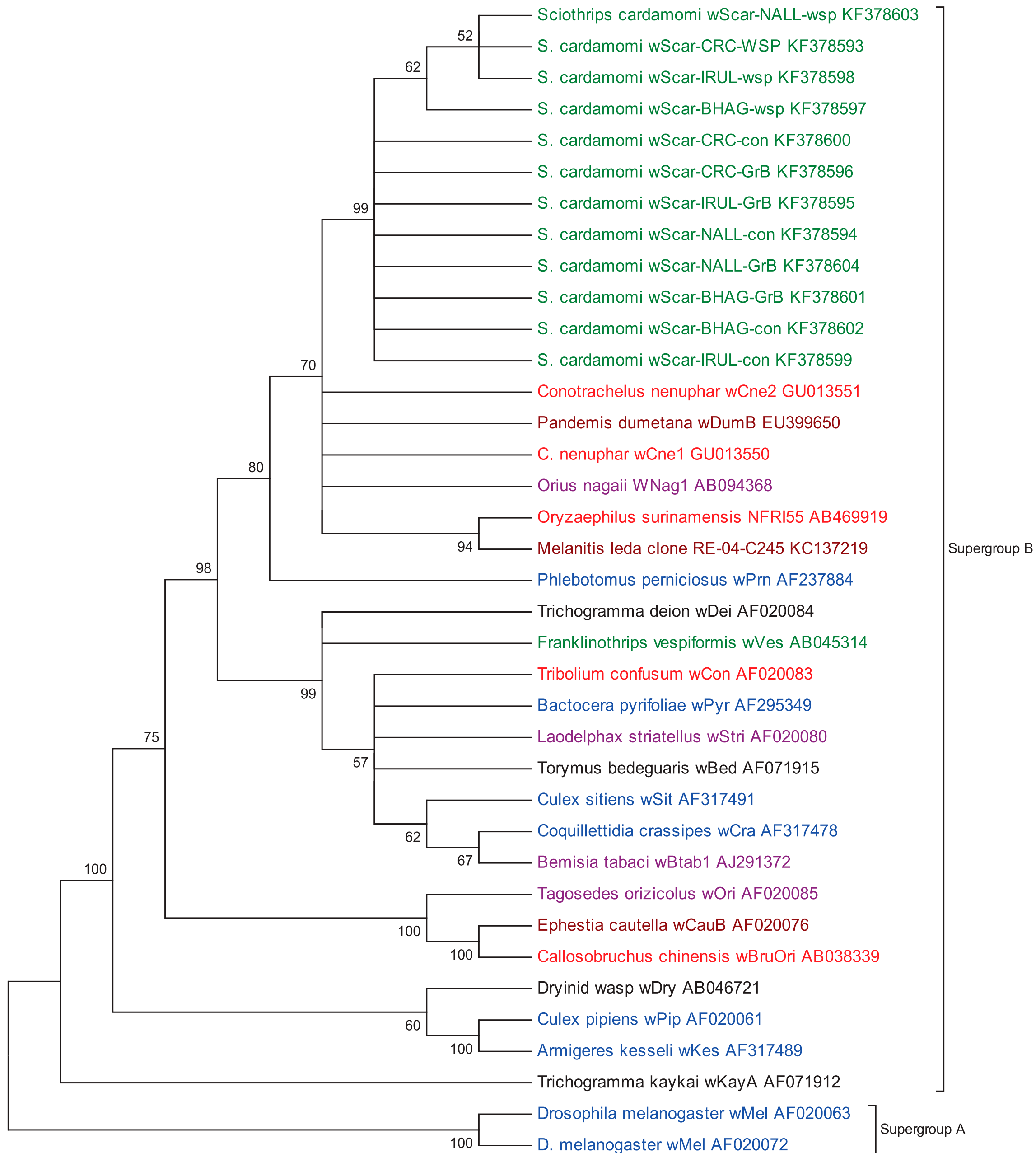

Figure 3. Neighbor-joining tree based on analysis of wsp sequences from different insects. Numbers above or below the nodes indicate bootstrap values (>50%) generated after 2000 replications. wsp sequences obtained from GenBank are shown with their accession numbers and strain name in the figure. Wolbachia supergroup A was used as the outgroup. In online version, the branches are colored according to insect orders: Thysanoptera (Green); Lepidoptera (Maroon); Coleoptera (Red); Diptera (Blue); Hemiptera (Purple); Hymenoptera (Black).

(Lindeman)] to 100% [*Echinothrips americanus* Morgan, *Hercinothrips femoralis* (Reuter), *and Parthenothrips dracaenae* (Heeger)] (Kumm & Moritz 2008). Fluctuations ranging from 43.0 to 80.5% in infection levels by *Wolbachia* have been reported in many insects (Jeyaprakash & Hoy 2000; Nirgianaki et al. 2003; Li et al. 2007). Infection of male and female cardamom thrips with the same *Wolbachia* subgroup is unlikely to rule out the possibility of cytoplasmic incompatibility in the thrips. However, *Wolbachia* utilizes mechanisms other than reproductive parasitism to maintain itself within populations and these mechanisms may be at least as common and important to *Wolbachia* as reproductive parasitism (Hughes et al. 2011).

Single infection by *Wolbachia* makes the expansion capacity of a species more powerful (Li et al. 2007).

Cardamom thrips occur in all cardamom growing tracts in India and their successful establishment was also reported in cardamom growing tracts in Sri Lanka (Dharmadasa et al. 2008). Even though, cardamom thrips were described as early as 1935 in India (Ayyar 1935), the species has not further evolved through speciation as evidenced by the recent work of Asokan et al. (2013). The single strain *Wolbachia* infection in cardamom thrips may play a role in further speciation in future as reported in *Bemisia tabaci* (Gennadius), infected by single and multiple *Wolbachia* strains (Li et al. 2007). Interestingly, the *wsp* sequences of the cardamom thrips in this study did not share any close relationship with any of the reported *wsp* strains from thrips species. However, in our BLAST searches, they shared a close relationship with the strains reported from insects belonging to Coleoptera, Lepidoptera, and Hemiptera. Similar observations were also made by Zhang et al. (2010) in their *Wolbachia* studies on *C. nepuphar*. There was no geographical clustering of our present isolates in phylogenetic analyses demonstrating the uniqueness of these *w*Scar isolates.

Knowledge of the effect of *Wolbachia* on fitness and reproduction of hosts has increased the attention on symbionts as potential biocontrol agents (Bourtzis 2008; Ahantarig & Kittayapong 2011). A symbiont could be eliminated from a host to remove their possible beneficial role in the host or transferred to a host for taking advantage of endosymbiont infection. Disinfection of the bacteria and cross mating with the naturally infected populations, or super infecting the populations with other *Wolbachia* strains may provide further insight into the exact role of *Wolbachia* in reproduction of cardamom thrips. The single strain infection of *Wolbachia* in cardamom thrips as reported in this study could be utilized to develop a *Wolbachia*-based biocontrol strategy against this serious insect pest.

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