grammes involving specific crosses. Further care must be taken not to include clones of older ortets while establishing future clonal seed orchards.

1. Vasudeva, R., Rajesh Gunaga and Hanumantha, M., *Adv. For. Res. India*, 2002, **24**, 120–135.
2. Indira, E. P. and Basha, C. S., *Ann. For.*, 1999, **7**, 39–44.
3. Nagarajan, B., Mohan Varghese, Nicodemus, A., Sashidharan, K. R., Benet, S. S. R. and Kannan, C. S., Proceedings of the QFRI-IUFRO Conference Caloundra, Queensland, Australia, 1996.
4. Bhumibhamon, S., Proceedings of the Symposium IUFRO Working Group on Seed Problems, 1980, vol. 2, pp. 2–3.
5. Zobel, B. and Talbert, J., *Applied Forest Tree Improvement*, John Wiley and Sons, New York, 1984, pp. 75–116.
6. Kertadikara, A. W. S. and Prat, D., *Silv. Genet.*, 1995, **44**, 104–110.
7. Chacko, K. C., John, S. K. and Asokan, A. M., *Ann. For.*, 1997, **5**, 55–61.
8. Czabator, F. J., *For. Sci.*, 1962, **8**, 386–396.
9. Prasad, R. and Jalil, P., *J. Trop. For.*, 1986, **2**, 207–212.
10. Gupta, B. N. and Pattanath, P. G., *Indian For.*, 1975, **101**, 584–588.
11. Kosinski, G., *Arbor. Kornickie.*, 1989, **31**, 107–182.
12. Ledig, F. T., in *Conservation Biology* (ed. Soule, M. E.), Sinauer Sunderland Press, 1986, pp. 77–104.
13. Klekowski, E. J., *Trees*, 1988, **2**, 195–203.
14. Klekowski, E. J. and Godfrey, P. J., *Nature*, 1989, **340**, 389–391.
15. Aizen, M. A. and Rovere, A. E., *Int. J. Plant Sci.*, 1995, **156**, 227–231.
16. Hanumantha, M. and Vasudeva, R., *Malaysian For.*, 2001, **64**, 22–28.
17. Indira, E. P., Basha, C. S. and Chacko, K. C., *J. Trop. For. Sci.*, 2000, **12**, 21–17.
18. Gurudev Singh, B., Arjunan, M. C., Manimuthu, L., Mahadevan, M. P. and Masilamani, P., in *Genetics and Silviculture of Teak* (eds Mandal, A. K. and Ansari, S. A.), International Book Distributors, Dehra Dun, 2001, pp. 27–40.
19. Sarowat, C. H., Ph D thesis, Kassetsart University, Bangkok, Thailand, 1964.
20. Jayasankar, S., Babu, L. C., Sudhakara, K. and Unnithan, V. K. G., *Seed Sci. Technol.*, 1999, **27**, 131–139.
21. Vasudeva, R., Final project report submitted to FORTI, Karnataka Forest Department, 2000, pp. 1–102.

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Detection and phylogenetic affiliation of *Wolbachia* sp. from Indian mosquitoes *Culex quinquefasciatus* and *Aedes albopictus*

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***Wolbachia* sp. specific, 900 bp, 16S rRNA and, 650 bp, *wsp* genes were PCR amplified from two mosquito species *Culex quinquefasciatus* (strain NCCS and NIV) and *Aedes albopictus*. On the 16S rRNA based sequence analysis, *Wolbachia* sp. from *Cx. quinquefasiatus* resembled the earlier reported *Wolbachia* sp. from *Cx. pipiens* and related species from B super group whereas that of *Wolbachia* sp. from *Ae. albopictus* showed homology with members of A super group. The *wsp* gene sequence phylogeny correlated with the 16S rRNA data with *Wolbachia* sp. from *Cx. quinquefasciatus* resembling the B group with highest homology to *Cx. pipiens* and related species whereas that of *Wolbachia* sp. from *Ae. albopictus* was highly homologous to the *w*Alb A strain. The nucleotide differences between the earlier reported *w*AlbA and *Wolbachia* sp. (*w*Alb A*) studied from *Ae. albopictus* in this work were so significant that it formed a separate lineage in the A group of phylogenetic tree. These results indicated that *Wolbachia* sp. from *Cx. quinquefasciatus* were similar to previously reported species while *Wolbachia* sp. from *Ae. albopictus* would represent a novel strain of *Wolbachia* sp.**

MANY species of invertebrates are host to bacterial endosymbionts which are important in the nutritional ecology of the hosts[1] while those which are parasites can be important as agents driving evolutionary changes such as evolution of sex determination system of host[2,3]. Bacteria belonging to genus *Wolbachia* have recently been recognized to infect a high proportion of insects, mites, isopods and filarial nematodes and are maternally transmitted from parent to offspring[4]. These intracellular α-proteobacteria were reported for the first time in the ovaries of the mosquito *Culex pipiens*[5] and named as *Wolbachia pipientis*. It causes crossing incompatibility between infected males and uninfected females[6].

These bacteria have attracted scientific interest due to their ability to manipulate host reproduction, leading to distinct phenotypic effects in the host such as parthenogenesis, feminization, male killings and cytoplasmic incompatibility[7–11]. They are also known to enhance the

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fecundity or fertility of their hosts and also cause pathogenicity[5]. The ability of *Wolbachia* to modify the reproductive success of its host enables it to increase its frequency in host population without requiring horizontal transmission[12].

The manipulation of the host biology and the expected response of the host genes to such manipulations cause these infections to have important implications for the evolution of sex determination, speciation and euscociality. From the applied perspective, *Wolbachia* might be used as a weapon against pests and the diseases they carry. Mosquitoes are medically important insects that transmit a variety of diseases like malaria, filarial, dengue, yellow fever and Japanese Encephalitis. As an alternative to genetically engineered mosquitoes, these endosymbionts could be genetically transformed to modify their disease-transmitting abilities[13]. To make this approach successful it is important to understand the strain variation, if any, in *Wolbachia* sp. in mosquito populations.

Since isolation and cultivation of these bacteria outside the host cell is difficult, various workers have carried out phylogenetic analysis of *Wolbachia* using different molecular markers, viz. 16S rRNA gene[14], protein coding *ftsZ* gene essential for cell division[15], the *dnaA* gene essential for DNA replication initiation[16], the *groE* operon which encodes two bacterial heat shock proteins[17], and the *wsp* gene, which encodes a major cell surface coat protein[12]. Phylogenetic analysis of the 16S rRNA gene sequences indicated a 2% sequence divergence and separated *Wolbachia* into two distinct groups, those that infect *Culex* group of mosquitoes belong to the B group, while those which reside in *Aedes* are members of the A group[14]. Recently the phylogeny of *Wolbachia* sp., based on fast evolving gene, *wsp* gene, has been accepted[12]. *Wolbachia* sp. that infects *Cx. pipiens* complex belongs to Pip group of B super group[18]. The *wsp* gene sequences displayed an overall DNA sequence divergence of up to 23% (refs 18, 19). These bacteria are reported to infect most strains of *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. molestus*, and *Cx. pallans*. The presence of *Wolbachia* sp. in Indian isolates of *Culex* mosquitoes has been reported using cytological studies[20]. However, attempts have not been made using molecular techniques. In this study, we report the presence of *Wolbachia* sp. from *Cx. quinquefasciatus* and *Ae. albopictus* using 16S rRNA and *wsp* gene sequence-based phylogeny.

Wild female mosquitoes, *Cx. quinquefasciatus* and *Ae. albopictus*, were collected from a field near NCCS (National Centre for Cell Science, Pune) and NIV (National Institute of Virology, Pune). The laboratory-bred *Cx. quinquefasciatus* females were taken from the insectory at NIV. Well-developed ovaries were dissected out after surface sterilization of mosquitoes with 70% alcohol, suspended in physiological saline (0.85% NaCl w/v) and lysed using three freeze–thaw cycles. Rapid freezing was done at –70°C for 10 min, followed by thawing at 65°C for 10 min. Chromosomal DNA was then extracted from the lysate by the standard phenol:chloroform extraction method[21].

The *Wolbachia*-specific 16S rRNA genes from the three mosquito species were PCR amplified using primers WOLB1 [5′-TTG TAG CCT GCT ATG GTA TAA CT-3′] and WOLB2 [5′-GAA TAG GTA TGA TTT TCA TGT 3′] specific for *Wolbachia*. The amplification of *wsp* gene was done using primers wsp81F [5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′] and wsp691R [5′-AAA AAT TAA ACG CTA CTC CA-3′]. To confirm the mosquito species, the mt16S rRNA gene from them was also PCR amplified using primers MosqY1 [5′-CGC CTG TTT ATC AAA AAC AT-3′] and MosqY2 [5′-CTC CGG TTT GAA CTC AGA TC-3′] as described earlier[22]. The 16S rRNA gene PCR product of *Wolbachia* (900 bp) was sequenced using WOLB1 and WOLB2 primers, while the 650 bp *wsp* gene was sequenced using wsp81F primer. The mosquito mt16S rRNA gene fragment was sequenced with Y2 primer. The nucleotide sequences were determined using Big Dye terminator kit and the sequencing reactions were run on ABI-PRISM 310 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA).

Phylogenetic analysis of the 16S rRNA gene sequences was done at Similarity Rank program of RDP I site and BLAST-n program at NCBI site. *wsp* gene sequence analysis was done at BLAST-x program at NCBI. Multiple sequence alignment was done with closely related sequences using CLUSTAL W program at EBI site. The phylogenetic trees were constructed using Kimura-2-distances and the neighbour-joining algorithm in the MEGA 2.1 software[23]. The similarity values and the distances were calculated using the Jukes Cantor Program.

The 16S rRNA gene sequences from both *Wolbachia* strains of *Cx. quinquefasciatus*, i.e. NCCS (AF397409) and NIV (AF397410), showed 95.04% to 100% similarity with *Wolbachia* sp. from various hosts like *Cx. pipiens*, *Metaseiulus occidentalis*, *Callosobruchus chinensis*, *D. simulans*, *Nasonia vitripennis*. The sequence alignment of the two *Wolbachia* sp. 16S rRNA gene sequences from *Cx. quinquefasciatus* NCCS and NIV with closely related *Wolbachia* sp., showed 97.26% similarity among the two, whereas that from *Ae. albopictus* (AF397408), showed 98.8% to 100% similarity with six nucleotide differences with *Wolbachia* sp. from various hosts like *Diabrotica virgifera virgifera*, *Diabrotica cristata*, *Diabrotica lemniscata*, *Acalymma blandulum* and *Folsomia candida*.

A phylogenetic tree of the 16S rRNA gene sequences constructed using the Kimura-2-distances and the neighbour-joining algorithm with 598 nucleotide sequence indicated that *Wolbachia* sp. from both isolates of *C. quinquefasciatus* were grouped with its close relatives in B group, while that from *Ae. albopictus* was grouped in

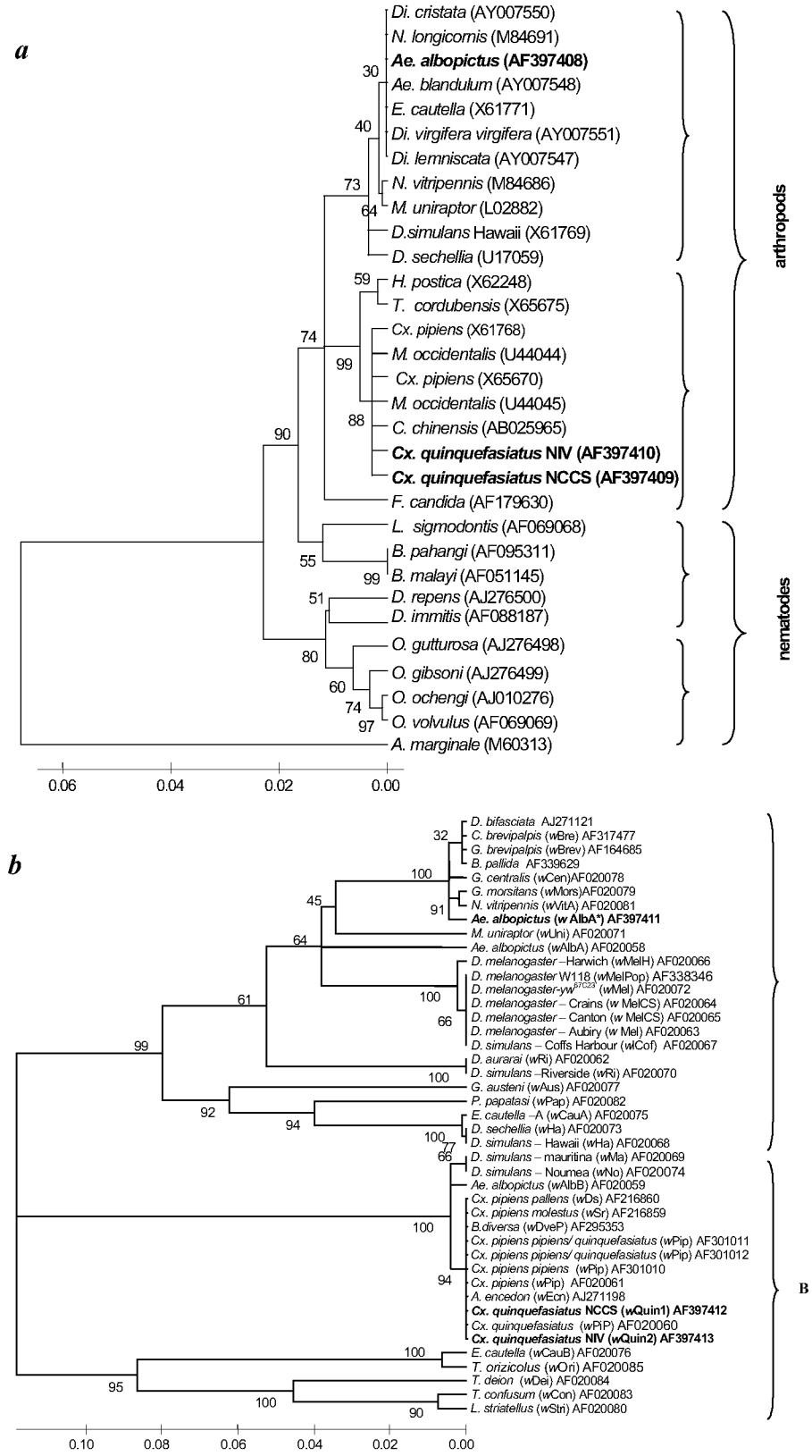

Figure 1. A midpoint rooted phylogenetic tree based on: (***a***), 16S rRNA gene sequences of *Wolbachia* (598 bp), and (***b***), *wsp* gene sequences of *Wolbachia* sp. (539 bp), constructed from Kimura 2 distances and the neighbour-joining algorithm. The numbers near the nodes indicate percentage of 500 bootstrap replicates. The scale bar indicates genetic distance. Names correspond to host species. The GenBank accession numbers are also mentioned. Newly reported data are indicated in bold.

A group (Figure 1 *a*). *Wolbachia* sp. from *Anaplasma marginale* was selected as outgroup.

The *wsp* gene sequences of *Wolbachia* sp. from both *Cx. quinquefasciatus* mosquito strains showed 100% homology with various strains of *Wolbachia* from different hosts such as: strain *w*Sr (*Cx. pipiens molestus*), strain *w*Ds (*Cx. pipiens pallens*), strain *w*Pip-cpp (*Cx. pipiens pipiens*), strain *w*Pip-cpp/q (*Cx. pipiens pipiens/quinquefasciatus*), strain *w*Pip-cpq (*Cx. pipiens quinquefasciatus*), strain *w*DevP (*Bactocera diversa*) and strain *w*Ecn (*Acraea encedon*). Multiple sequence alignment of *wsp* gene sequences of *Wolbachia* sp. from *Cx. quinquefasciatus* NCCS (AF397412 = *w*Quin1) and NIV (AF397413 = *w*Quin2) strains, with closely related *Wolbachia* sp., showed that they were identical. The *wsp* gene sequence of the *Wolbachia* strain, designated as *w*AlbA*, from *Ae. albopictus* (AF397411), showed 98.63% to 99.61% homology with various strains of *Wolbachia* from different hosts such as: *Wolbachia* sp. (*D. bifasciata*), strain *w*Brev (*Glossina brevipalpis*), *Wolbachia* sp. (*Biorhiza pallida*), strain *w*Bre (*Cx. brevipalpis*), strain *w*VitA (*N. vitripennis*), strain *w*Mors (*G. morsitans*) and strain *w*Cen (*G. centralis*). It also showed 91.88% similarity (64 positions with different nucleotides) with the previously reported *w*AlbA sequence. The sequence had three deletions, of which one was at 5′ end whereas two were at the 3′ end of sequence (data not shown). As compared to the *w*AlbB sequence, *w*AlbA* showed 125 positions with different nucleotides, thereby showing just 79.01% similarity, with six alignment gaps at 3′ end of the sequence. These differences indicated that *Wolbachia* sp. from Indian isolates of *Ae. albopictus* are different to the previously reported *Wolbachia* sp. of *Ae. albopictus*.

A phylogenetic analysis using Kimura-2-distances and the neighbour-joining algorithm using 539 nucleotide sequence of the *wsp* gene sequences indicated that *Wolbachia* sp. from both *Cx. quinquefasciatus* (NCCS and NIV) were grouped with their close relatives in B super group, whereas that from *Ae. albopictus, w*AlbA*, was grouped in A super group with its close members (Figure 1 *b*) and formed a separate lineage in the *Wolbachia* phylogeny tree. It was more close to other members of super group A than the previously reported *w*AlbA, indicating the presence of one more *Wolbachia* sp. in *Ae. albopictus*.

Phylogeny of *Wolbachia* sp. based on 16S rRNA gene indicated that *Wolbachia* sp. that infects *Culex* group of mosquitoes belongs to B group, while those which reside in *Aedes* are members of A group[14]. 16S rRNA gene sequence-based phylogenetic studies of *Wolbachia* from both *Cx. quinquefasciatus* strains (NCCS and NIV) and *Ae. albopictus* showed similar observations (Figure 1 *a*). The identification of two *Wolbachia* sp. from both *Cx. quinquefasciatus* strains was done using *wsp* gene sequence based phylogeny. Both species of *Wolbachia* from two different strains of Indian *Cx. quinquefasciatus* have shown sequence similarity with members of Pip group of B super group and correlated well with the study carried out earlier[18]. The sequence similarity between all the members of Pip group with both *Wolbachia* strains *Quin* 1 and *Quin* 2, proves that they belong to the same group.

Based on *wsp* gene phylogeny, there are two types of *Wolbachia* sp. present in *Ae. albopictus* where one belongs to Pip group of B super group while the other to the AlbA group of A super group[18]. Both these reported strains inhabited the same host *Ae. albopictus* (Houston). *Wolbachia* sp. detected in this present study belongs to A super group and formed a separate group. It showed more relatedness to Mors group members than AlbA (Figure 1 *b*). Formation of separate lineage in a phylogenetic tree indicates that *w*AlbA* might represent another strain of *Wolbachia* sp. present in *Ae. albopictus*.

1. Douglas, A. E., *Ann. Rev. Entomol.*, 1998, **43**, 409–434.
2. Hurst, G. D. D. *et al.*, *Heredity*, 1996, **77**, 177–185.
3. Juchault, P. and Mocquard, J. P., *J. Evol. Biol.*, 1993, **6**, 511–528.
4. Jeyaprakash, A. and Hoy, M. A., *Insect Mol. Biol.*, 2000, **9**, 393–405.
5. Southamer, R., Breeuwer, J. A. J. and Hurst, G. D. D., *Annu. Rev. Microbiol.*, 1999, **53**, 71–102.
6. Yen, J. H. and Barr, A. R., *Nature*, 1971, **232**, 657–658.
7. v. d. Schulenburg, J. H. G. *et al.*, *Mol. Biol. Evol.*, 2000, **17**, 584–600.
8. Southamer, R., Luck, R. F. and Hamilton, W. D., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 2424–2427.
9. Jiggins, F. M., Hurst, G. D. and Majerus, M. E. N., *Proc. R. Soc. London*, 2000, **267**, 69–73.
10. Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. and Solignac, M., *Proc. R. Soc. London*, 1992, **250**, 91–98.
11. Hurst, L. D., *Proc. R. Soc. London*, 1991, **244**, 91–99.
12. Braig, H. R., Zhou, W., Dobson, S. L. and O'Neill, S. L., *J. Bacteriol.*, 1998, **180**, 2372–2378.
13. Enserink, M., *Science*, 2000, **290**, 440–441.
14. O'Neill, S. L., Giordano, R., Colbert, A. M. E. and Karr, T. L., *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 2699–2702.
15. Holden, P. R., Brookfield, J. F. and Jones, P., *Mol. Gen. Genet.*, 1993, **240**, 213–220.
16. Bourtzis, K., Nigianaki, A., Onyango, P. and Savakis, C., *Insect Mol. Biol.*, 1994, **3**, 131–142.
17. Masui, S., Sasaki, T. and Ishikawa, H., *Zool. Sci.*, 1997, **14**, 701–706.
18. Zhou, W., Rousset, F. and O'Neill, S. L., *Proc. R. Soc. London*, 1998, **265**, 509–515.
19. Pintureau, B., Chaudier, S., Lassabliere, F., Charles, H. and Grenier, S., *J. Mol. Evol.*, 2000, **51**, 374–377.
20. Subbarao, S. K., in *Recent Developments in the Genetics of Insect Disease Vectors*, Stapes Publishing Company, Champaign, IL, 1982, pp. 313–342.
21. Sambrook, J. E., Mitsch, F. and Maniatis, T., in *Molecular Cloning – A Laboratory Manual*, Cold Spring Harbor, NY, 1989.
22. Shouche, Y. S. and Patole, M. S., *J. Biosci.*, 2000, **25**, 361–366.
23. Kumar, S., Tamura, K., Jacobsen, I. B. and Nei, M., *Bioinformatics*, 2001, **17**, 1244–1245.

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