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# Isolation and identification of culturable bacteria from honeydew of whitefly, *Bemisia tabaci* (G.) (Hemiptera: Aleyrodidae)☆

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ABSTRACT

*Bemisia tabaci* (G.) is an important pest and a vector of Gemini viruses infecting plants. During the process of feeding *B. tabaci* excretes honeydew which is rich in nutrients, and an excellent medium for microbial growth. Recent report proved that volatile emitted by the honeydew associated bacteria of aphid, *Acyrthosiphon pisum* Harris was involved in natural enemy calling. Thus understanding the honeydew associated bacteria is of paramount importance from the non-chemical method of insect pest management. In this perspective, very less information is available on bacteria associated with the honeydew excreted by *B. tabaci*. Therefore, in the present study we have isolated and characterized three culturable bacteria from the honeydew of *B. tabaci* viz. *Bacillus endophyticus*, *Bacillus niacini* and *Roseomonas* species by employing 16Sr DNA BLASTx analyses which revealed that both *B. endophyticus* and *B. niacini* had high similarity (>99%) to the respective species, while *Roseomonas* sp. showed only 95% similarity to the existing *Roseomonas* sp. specificity of honeydew

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association of *Roseomonas* sp. was confirmed by developing specific primers as this genus is reported from immunocompromised persons and recently from ticks and mites. The present study also indicated the possible host-plant origin of these honeydew associated bacteria.
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## Introduction

Among the agriculturally important insect pests, sap sucking ones are assuming greater importance in post *Bacillus thuringiensis* (*Bt*) phase and impending climate change. In this regard, sap sucking insects like thrips, aphids, mealybugs, leafhoppers, whiteflies inflict damage by direct feeding and as a vector of many plant viruses. Among them, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is devastating globally as a vector for a number of Gemini viruses (Brown, 1990; Jones, 2003). It is highly polyphagous infesting crops such as cotton, tomato, soyabean, tobacco, radish etc., which primarily feed on the phloem sap that is rich in sugars. Like other homopterans *B. tabaci* also excretes copious amount of honeydew (complex mixture of sugars, organic acids, amino acids and some lipids), which is also an excellent medium for microbial growth (Mittler, 1958; Bargen et al., 1998; Leroy et al., 2011b; Thibout et al., 1993). During its development, immature stages such as nymphs actively participate in feeding phloem sap and excrete large amount of sugary rich honeydew, which helps in developing sooty mold thus the reduction of host plant photosynthesis (Byrne and Bellows, 1991). The nutritional quality of honeydew to natural enemies depends on plant species and its physiological status, (Blackmer and Byrne, 1999; Crafts-Brandner, 2002), which is also influencing the whitefly parasitoid species feeding on plant and insect nectar (Burger et al., 2005; Stapel et al., 1997). That is how honeydew plays an important role in tri-trophic interaction (plant–pest–natural enemy), being excellent source of nutrients for certain kind of ants which in turn protect them from the natural enemies.

Various non-chemical insect pest management strategies available are Biological control (Fariaa and Wraight, 2001), *Bacillus thuringiensis* (Bt) (Agrawal and Bhatnagar, 2003), Nuclear Polyhedrosis Virus (NPV) (Erayya et al., 2013) etc. However, natural enemy calling is an ingenious way in developing a non-chemical insect pest management strategy, whereas a non-prey food such as honeydew used by predators and parasitoids explores implications for biological pest control programs (Wackers et al., 2008). Mandour et al. (Mandour et al., 2005) demonstrated that whitefly parasitoid *Eretmocerus* sp. nr. *furuhashii* (Aphelinidae: Hymenoptera) is attracted more towards trehalulose and trehalose, which are the main components of host honeydew. Thus honeydew will serve as food and host searching cue (kairomone) for the parasitoids. Predators and parasitoids of aphids exploit honeydew as a host-location kairomone and an oviposition stimulus (Bargen et al., 1998; Hagvar and Hofsvang, 1991). The volatiles produced by the honeydew associated bacteria in aphid, *A. pisum* enhanced the efficacy of the hoverfly, *Episyrphus balteatus*, by driving prey location and ovipositional preferences (Leroy et al., 2011a). It is important to find alternative pesticide free management strategies for *B. tabaci* considering high levels of resistance developed for various classes of insecticides (Kranthi et al., 2002; El Kady and Devine, 2003). In this regard, it is also essential to identify the microflora particularly bacteria associated with honeydew for its utility in managing *B. tabaci*. Therefore, in the present study, we are reporting for the first time the culturable bacteria associated with the honeydew of *B. tabaci* of which no information is available. Further identifying the volatiles from these bacteria of *B. tabaci* honeydew will open up a new avenue in eco-friendly management of the same and also on other insect pests.

## Materials and methods

### *Stock culture*

*B. tabaci* was reared on cotton (*Gossypium hirsutum*) according to Salvucci et al. (Salvucci et al., 1997) in wooden cages (2′ × 2′) at room temperature (27–30 °C) with RH 70–90%.

*Collection of honeydew*

Cotton leaf infested with nymphs was placed on to the petriplate lid with the abaxial surface exposed. Honeydew was collected from the nymphs on the sterile petriplate inside the laminar hood and stored in −20 °C until further use.

*Isolation and identification of honey associated culturable bacteria*

Honeydew was serially diluted (1:10) using sterile double distilled water and was spotted on Nutrient Agar, Yeast Extract Agar and Potato Dextrose Agar, individually (Thomas et al., 2012) and incubated at 28 ± 2 °C and 37 ± 2 °C. Observations were made on colony growth till seven days and were purified by employing single colony isolation. Morphological analyses were carried based on Bergey's Manual of Systematic Bacteriology (Logan et al., 2009; Schreckenberger et al., 2007; Nagel and Andreesen, 1991).

*DNA isolation and Polymerase Chain Reaction (PCR)*

Genomic DNA was extracted according to Sambrook et al. (Sambrook et al., 1989) and was treated with 5 μl of RNase. The bacterial isolates were characterized by employing 16Sr RNA gene specific primers (Table 1) resulting in an amplicon size of 1500 bp. Polymerase Chain Reaction (PCR) was carried out in 25 μl total reaction volume, that contained the following components: 5–10 μl of template (50 ng/μl), 20 pmol of each primer, 10 mM Tris–HCl (pH-8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 U of Taq DNA polymerase (Fermentas Life Sciences, EU) and the rest was made up with DNase and RNase free water (Sigma, USA). PCR was carried out in a thermal cycler (Veriti, Applied Biosystems, USA) with the following cycling parameters; 94 °C for 4 min as initial denaturation followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s 72 °C for 45 s and 72 °C for 20 min as final extension. The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10 μg/ml) and visualized in a gel documentation system (UVP, UK).

*Cloning and sequencing*

PCR amplified products were eluted and ligated into T/A cloning vector pTZ57R/T (Fermentas Life Sciences, EU) and transformed into *Escherichia coli* cells (DHA5α). The cells were spread on LB agar containing X-gal (300 μg/ml), IPTG (120 μg/ml) and ampicillin (100 μg/ml) and incubated at 37 °C overnight. Blue/white selection was carried out on the following day and plasmids were isolated from the positive clones using GeneJET™ Plasmid Miniprep Kit (Fermentas Life Sciences, EU) according to manufacturer's protocol. Sequencing was carried out in an automated sequencer (ABI Prism 310; Applied Biosystems, USA) using M13 Universal primers in both directions. Homology search was carried out using BLAST (http://www.ncbi.nlm.nih.gov) and the differences in nucleotide sequences of various bacteria were determined employing the sequence alignment editor 'BioEdit' and further analyzed employing MEGA 5.0 (Tamura et al., 2011). The Neighbor-Joining (NJ) trees were constructed using the Kimura-2-parameter (K2P) distance model (Kimura, 1980; Saitou and Nei, 1987). All the sequences generated in the present study were deposited in NCBI-GenBank. The other GenBank accession numbers used to construct the trees are HM770880, HM770881, JX290085, JX307684, KC153529, JF281737, AM231587, AJ786000, JN377653, AY220740, AY360348, AF538712, EF368368, EU867313, AY150050, AY150046, X73820, JX966451, KC153529 and JX290085, JX307684.

**Table 1**
Primers employed in the current study.

| Primer name | Sequence | PCR amplicon | Reference |
|---|---|---|---|
| 16SP0<br>16SP6 | 5′-GAAGAGTTTGATCCTGGCTCAG-3′<br>5′-CTACGGCTACCTTGTTACGA-3′ | 1500 bp | Brown (1990) |

**Table 2**
Details of 16S rDNA sequences for honeydew associated bacteria of *B. tabaci* with its nucleotide identity percentage.

| Sl. No. | Nucleotide identity | NCBI-GenBank accession number | Bacterium | Isolate # |
|---|---|---|---|---|
| 1 | 99% | KC818119 | *Bacillus niacini* | BTH#1 |
| 2 | 99% | KC818118 | *Bacillus endophyticus* | BTH#2 |
| 3 | 95% | KC818120 | *Roseomonas* sp. | BTH#3 |

## Results and discussion

In the present study we have isolated three culturable bacteria viz. *Bacillus endophyticus*, *Bacillus niacini* and *Roseomonas* sp. by 16S rDNA sequencing. BLAST search for these isolates showed the highest hit for the respective species and the sequences were deposited with NCBI-GenBank (Table 2). The phenotypic characteristics of these three isolates are as follows: *B. niacini* (isolate BTH#1) is a Gram positive, motile, long chain. On Nutrient Agar, the colonies were smooth with light beige center surrounded by translucent areas of variable extension and showed positive to both oxidase and urease tests. *B. endophyticus* (isolate BTH#2) was Gram positive, non-motile, rods. On Nutrient Agar colony appeared as slimy white of 1–3 mm in diameter and was positive for oxidase and negative for urease test. On the other hand, *Roseomonas* sp. (isolate BTH#3) was Gram negative, motile, cocci, pale-pink mucoid with 1–3 mm diameter. It was positive for both oxidase and urease tests (Table 3) (Logan et al., 2009; Schreckenberger et al., 2007; Nagel and Andreesen, 1991).

Sequence comparison of *B. endophyticus* sequence with those deposited with NCBI-GenBank showed 99.5% similarity with seven mismatches out of 1455 bp. This sequence similarity was further supported by the 100 bootstrap values in the Maximum Parsimony (MP) tree (Fig. 1b). Recent studies reported the presence of *B. endophyticus* in the foregut of white leg shrimp, *Litopenaeus vannamei* larvae (NCBI-GenBank accession numbers HM770880 and HM770881). Further sequence comparison revealed that, the *B. endophyticus* 16Sr DNA sequences showed 80% and above percent similarity with those of *B. endophyticus* isolated from soil, polluted water etc. (Supplementary Fig. 1). Similar comparisons were made for *B. niacini* 16Sr DNA sequences from our study with the sequence of *B. niacini* isolated from the mealybug gut (NCBI-GenBank accession number JX966451), which revealed 98.33% similarity with 16 mismatches out of 902 bp, which was supported by 100 bootstrap value of the MP tree (Fig. 1a). Considering the potential of bacteria from aphid honeydew in attracting and enhancing the efficacy of natural enemies (Leroy et al., 2011a), these bacterial strains could become candidates for commercial bio-control agent. Extensive studies that have been carried out on the isolation and characterizations of various endosymbiotic bacteria associated with *B. tabaci* are listed in Supplementary Table 1. However, the present study is the first report on isolation and characterization of bacteria associated with the honeydew from *B. tabaci*. In the present study, we tried to track the possible origin of these honeydew associated bacteria. In this connection, the above mentioned three honeydew associated bacteria were not reported previously as an endosymbionts from *B. tabaci* (Supplementary Table 1). However, the previous studies on plant tissues proved that many of the endosymbionts associated with the insects were originally from the vascular tissues of the plant and picked up by the insects while sucking the phloem sap (Reva et al., 2002). In this regard, both *B. endophyticus* and *B. niacini* have been reported previously as endophytes of cotton

**Table 3**
Distinguishing phenotypic characteristics of the honeydew associated bacteria of *B. tabaci*.

| Sl. No. | Bacterial isolate | Description | | | | | |
|---|---|---|---|---|---|---|---|
| | | Gram staining | | Oxidase test | | Urease test | |
| 1 | *Bacillus niacini* (BTH#1) | P[a] | – | SP[b] | – | SP[b] | – |
| 2 | *Bacillus endophyticus* (BTH#2) | P[a] | – | P[a] | – | – | N[c] |
| 3 | *Roseomonas* sp. (BTH#3) | – | N[c] | SP[b] | – | P[a] | – |

[a] P = Positive.
[b] SP = Slightly positive.
[c] N = Negative.

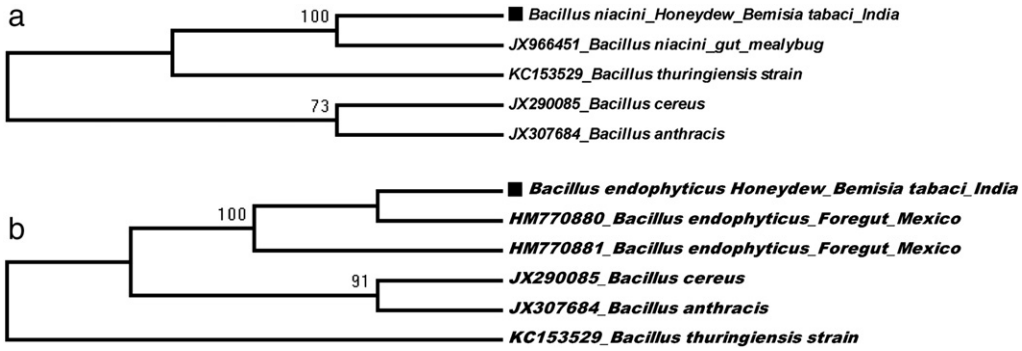


**Fig. 1.** (a) Phylogenetic tree showing the evolutionary position of *B. endophyticus* with other two NCBI-GenBank accessions reported from foregut of shrimp in Mexico. (b) Maximum Parsimony (MP) tree showing the evolutionary relationship of *B. niacini* from the current study with JX966451, isolated from mealybug gut. (a&b) The tree was constructed with MP algorithm in MEGA 5. 0 (Tamura et al., 2011) with 1000 bootstrap replicates. *B. cereus*, *B. anthracis* and *B. thuringiensis* are served as the out groups. Bootstrap values ≥65% are shown. The isolate obtained in the present study indicated in shaded rectangle.

and maize (Reva et al., 2002). Thus our studies revealed the possibility of host plant origin of these honeydew associated bacteria viz. *B. endophyticus* and *B. niacini*. However, in the case of *Roseomonas* sp. BLAST search showed a score of 95%, which only indicated the Genus, *Roseomonas*. Hence, we retrieved all the available 16Sr DNA sequences reported for *Roseomonas* species and were compared. The phylogenetic analysis showed that, *Roseomonas* sp. isolated in the present study is closely related to *Roseomonas cervicalis* which was earlier isolated from ticks and marine sponges in China and India respectively (Liu et al., 2010); NCBI-GenBank Accession Number—JF281737). None of the members of the Genus *Roseomonas* were reported as endophytes of plants. The sequence analysis clearly indicated that the *Roseomonas* sp. which was reported in the current study could be a novel species of the Genus *Roseomonas* (Fig. 3). We designed the *Roseomonas* sp. specific primers (Table 4) in order to determine the origin. Thus our primers produced an amplicon of approximately 700 bp directly from diluted honeydew as a template. Further cloning and sequencing were carried out and the BLASTx results of the sequences clearly demonstrated the presence of *Roseomonas* sp. in the honeydew. (See Fig. 2.)

Approaches such as *Bt*-transgenics and the futuristic RNA interference etc. hold enormous potential in insect pest management programs (Asokan et al., 2012). Recently, Pascal et al. (Leroy et al., 2011a)

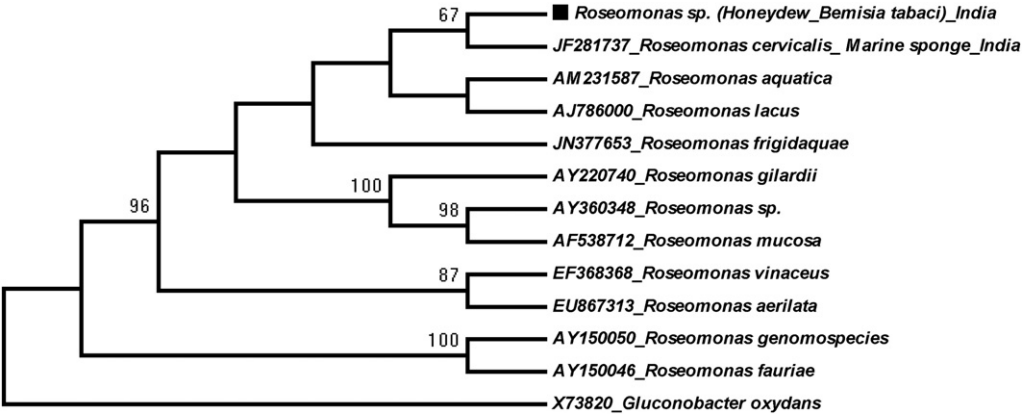


**Fig. 2.** MP tree based on 16SrDNA gene sequences of *Roseomonas spp*. Tree constructed employing MEGA 5.0 (Tamura et al., 2011) and MP method with 1000 bootstrap replications. The *Roseomonas* spp. from this current study showed more similarity to *R. cervicalis* (JF281737) isolated from marine sponge in India. Bootstrap value ≥65% is shown in the figure. The isolate obtained in this study indicated in shaded rectangle.

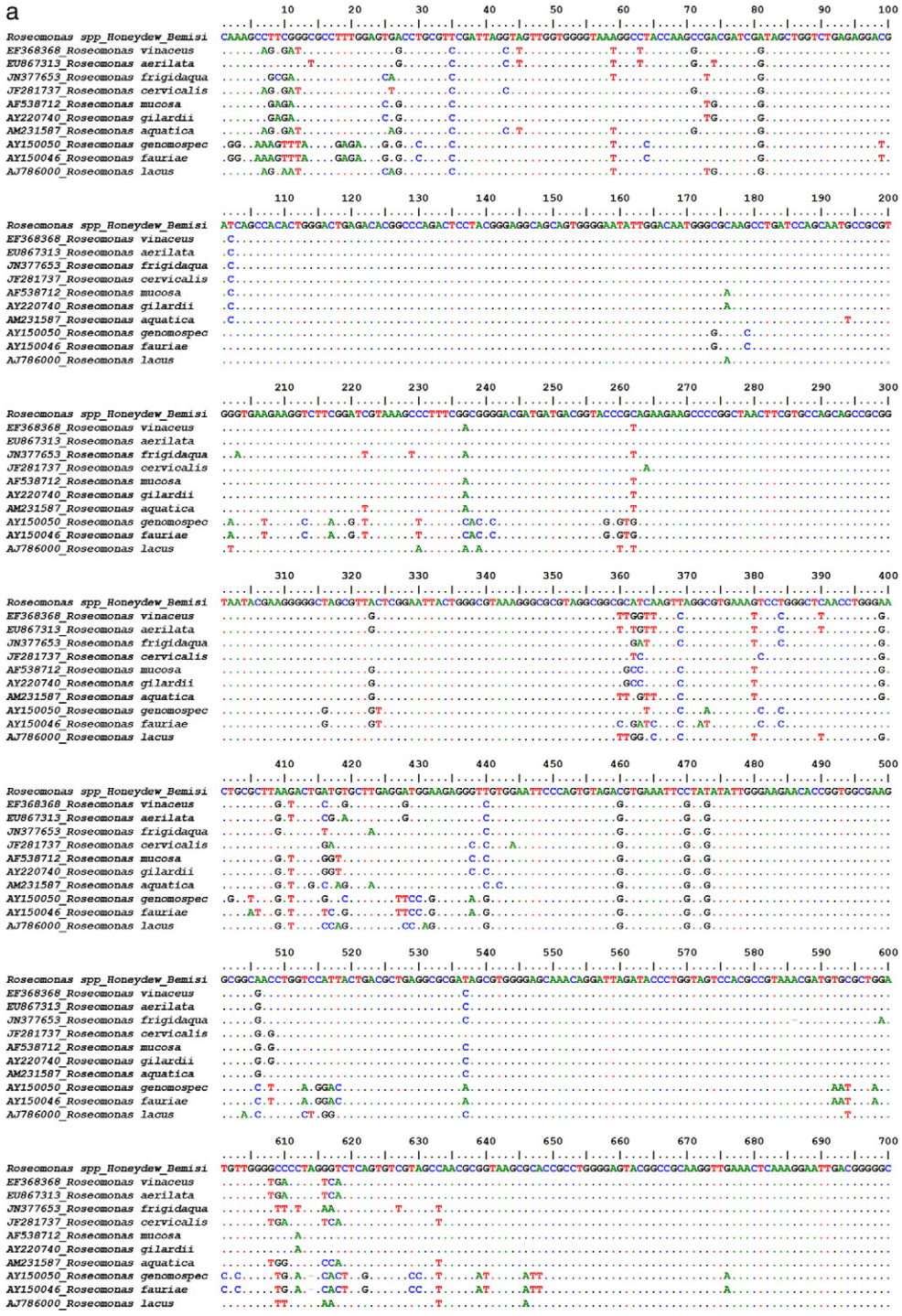


**Fig. 3.** Consensus sequences of 1227 bp from 16S ribosomal DNA (16S rDNA) genes for *Roseomonas* spp. from the present study and those retrieved from NCBI-GenBank. Dots indicate nucleotides identical throughout the species compared.

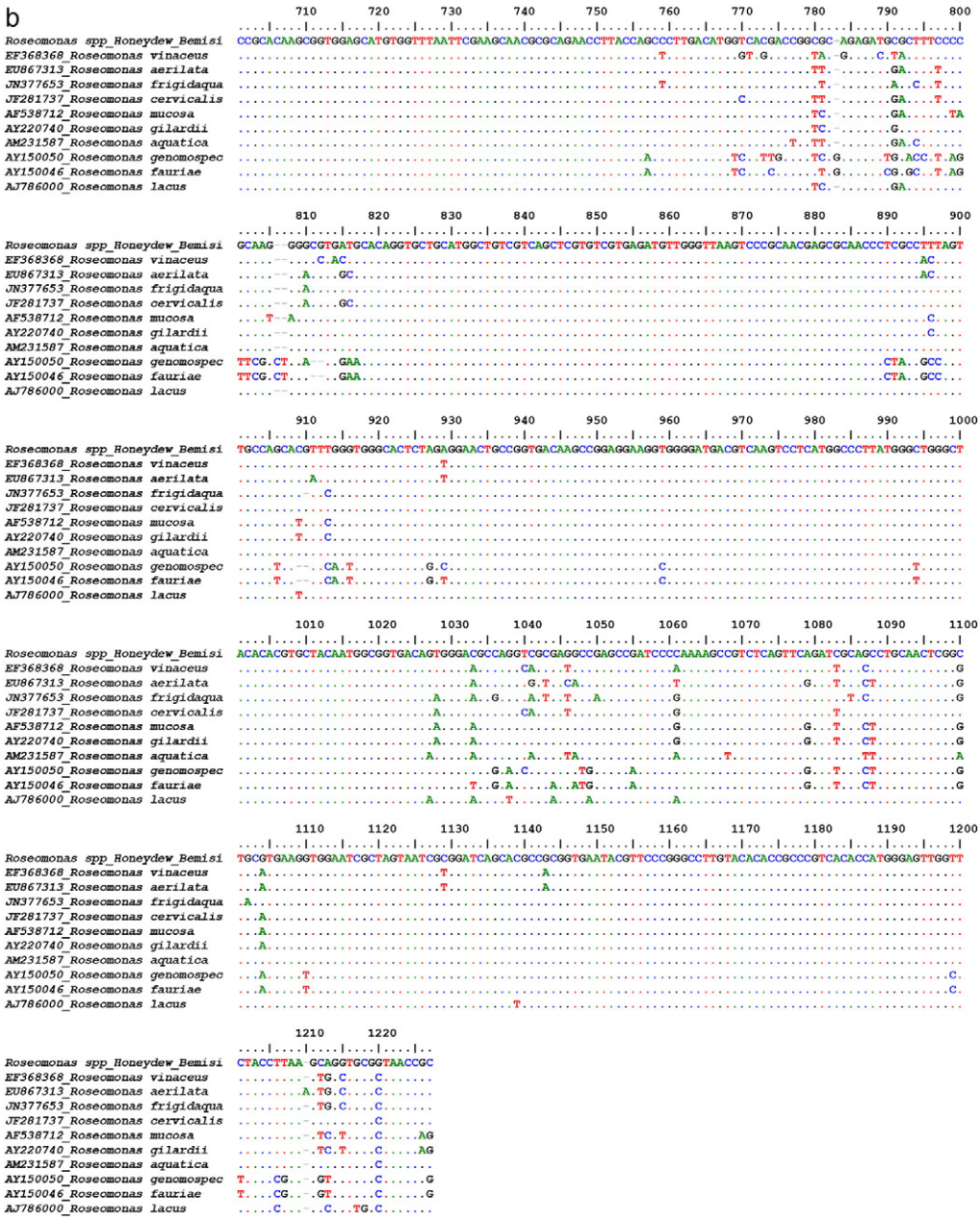


**Fig. 3** (*continued*).

showed that the volatiles released by the honeydew associated bacteria from aphids can be employed to increase the efficacy of natural enemies, which holds a great promise in non-chemical mode of insect pest management. Therefore, in order to fine tune the effectiveness of this approach, we need to enumerate both culturable and non-culturable microbial flora associated with the honeydew of various sap sucking insect pests. This will in turn help in developing non-chemical, eco-friendly pest management approach for various sap sucking pests which are assuming serious proportion presently. Further, it is also

**Table 4**
Roseomonas spp. specific primer details.

| Sl. no. | Primer | Binding site | Primer sequence (5′ → 3′) | Product size (bp) |
|---|---|---|---|---|
| 1 | Roseo For | 361–384 | 5′-GCATCAAGTTAGGCGTGAAAGTCCT-3′ | 699 bp |
| 2 | Roseo Rev | 1035–1060 | 5′-GATCGGCTCGGCCTCGCGACCTGGCG-3′ | |

interesting to identify, synthesize and test the volatiles for non-sap sucking insect pest management programs also as many of them have developed high levels of resistance to a wider class of insecticides. In a nutshell, both *B. niacini* and *B. endophyticus* could be used as an effective candidate as Biological control agents (BCAs) for managing *B. tabaci* in both open and glasshouse conditions. Hence, our results will prompt further studies that may lead to the use of these newly identified bacteria and its associated semiochemicals for biological control against whiteflies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2013.11.002.

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