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Title; First report of the molecular characterization of the endosymbiont *Candidatus portiera* from cotton whiteflies collected from Pakistan

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Abstract: *Candidatus portiera* is an obligate primary endosymbiont harboured by white flies including the cotton whitefly *Bemisia tabaci.* A survey of *C. portiera* endosymbionts was conducted by using polymerase chain reaction with universal primers for *16SrDNA* within Pakistani whitefly population collected from different cotton growing areas of the Punjab further analysed by cloning of the PCR products, RFLP analysis, finally sequences were obtained from commercial labs and phylogenetic analysis were done of all the detected *C. portiera* clone. This is the first report regarding the identification of *C. protiera* from the Pakistan. The *C. portiera* was detected almost in all samples of whitefly from 16 different location of Punjab Pakistan This study aims to contribute to the understanding of the Primary endosymbionts, their host specificity and their diversity across the world.
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Key words; Whitefly, primary endosymbiont, 16S rDNA, *Candidatus portiera*

Introduction

Endosymbionts are universal in nature and responsible for the evolution of the insects for millions of years (Baumann 2005). Primarily insect endosymbionts can be grouped into two primary and secondary endosymbionts (Werren 1997). Endosymbiont have been stabilized within host by fulfilling their nutritional requirement for their physiological functions and metabolism. From DNA and protein metabolism to glycolysis, from lipid biosynthesis to cell processes; there are several functions in which these endosymbionts are involved, ultimately reaching epic level of vertical transmission to next progeny (Ahmed et al. 2010) Endosymbionts are grouped into primary endosymbionts (P-endosymbionts) and secondary endosymbionts (S endosymbionts) (Baumann and Baumann 2005). P endosymbionts are known to be present in all host individuals, vertically transmitted and provide essential nutrients to the hosts. They have coevolved with insects for a long time and have formed intimate relationships with their hosts (Baumann and Baumann 2005; Skowronski et al. 2010). On the other hand, the S-endosymbionts are facultative and both vertically and horizontally transmitted (Bing et al. 2013; Thao and Baumann 2004; Thao et al. 2000; Thao et al. 2002). Unlike P-endosymbionts, S-endosymbionts make contributions to the insect hosts as well as play negative roles in the survival of hosts. For example, S-endosymbionts, such as *Rickettsia*, *Hamiltonella*, *Wolbachia*, *Regiella*, *Serratia*, can provide nutrients (Bing et al. 2013; Thao and Baumann 2004; Thao et al. 2000; Thao et al. 2002) , improve tolerance to heat stress (Gauthier et al. 2015; Enders et al. 2015; Cayetano and Vorburger 2013; Donati et al. 2013; Fan and Wernegreen 2013; Mavrianos et al. 2013; Nachappa et al. 2012; Keshavmurthy et al. 2012; Weston et al. 2012; Ratzka et al. 2011; Littman et al. 2010; Rosic et al. 2010; Vaisman et al. 2009; Braeken et al. 2008; Wilcox et al. 2003; Roger et al. 1998; Morioka and Ishikawa 1992). At the same time, S-endosymbionts such as *Wolbachia*, *Arsenophonus*, *Cardinium* and *Rickettsia* are likely to be parasitic rather than beneficial to the insect hosts (Bing et al. 2013; Thao and Baumann 2004; Thao et al. 2000; Thao et al. 2002). They manipulate insects' reproduction by forcing asexuality, killing males, feminizing genetic males, and inducing cytoplasmic incompatibility (CI) together with parthenogenesis, with apparent selfish effect of assisting the spread of their infections into host populations (Werren 1997; Werren et al. 2008; Clark et al. 2008).

C. portiera is an obligate primary endosymbiont harboured by *Bemisia tabaci*, it is

localized in specialized cells known as 'Bacteriocytes" (Baumann and Baumann 2005) . *C. portiera* is thought to be associated with white flies for approximately 180 million years. It is known to have been associated with carotenoid biochemical pathway of whitefly thus providing essential nutrient to its host (Sloan and Moran 2012). C. *portiera* exhibits bacterial homologues of the fungal carotenoid biosynthesis genes in whiteflies. Therefore, related lineages of sap-feeding insects appear to have convergent acquired the same functional trait by distinct evolutionary mechanisms (Sloan and Moran 2012) .

B. tabaci (family: Aleyrodidae) is small sap sucking insect commonly known as sweet potato or cotton whitefly (Ahmed et al. 2010) . It is responsible for reducing plant vigour by directly feeding on the phloem and also by transmitting Geminiviruses (Bedford et al. 1994; Perefarres et al. 2012) while *B. tabaci* is species complex, comprising of 24 indistinguishable species known as biotypes (De Barro et al. 2011). The most invasive biotypes are Q and B which are involved in economic losses worth of millions annually (Khasdan et al. 2005). These biotypes differ on basis of biochemical polymorphism, host range insecticide resistance and transmission competency (Ahmed et al. 2013; Ahmed et al. 2010). Although endosymbionts can potentially induce population differentiation. We investigate the *C. Portiera* community in *B. tabaci* from Pakistan. This work would further help to explore bacterial endosymbiont-host association at a small evolutionary scale and the role of these communities in the evolution of *B. tabaci* species complex.

Material and methods

Sampling and DNA extraction

The whiteflies, *B. tabaci* samples were collected from different districts of Punjab (Pakistan) from cotton fields in the year 2012. Collection details are summarized in table 1 (location and host). Samples were stored in ethanol (95%) and kept at -20 ºC before DNA extraction. De Barro and Driver method was used for total genomic DNA extraction from whitefly individuals (De Barro et al. 2011).

Amplification of 16S-rRNA gene

DNA extracted from *B. tabaci* was verified by agarose gel electrophoresis. Primers used for verification of *C. porteia* targeted 16S-rRNA gene, the sequence for specific forward primer 16S-rRNA gene was F, 5′- TGCAAGTCGAGCGGCATCAT -3′ while for reverse primer, it was R 5′-AAAGTTCCCGCCTTATGCGT -3 (Gueguen et al. 2010) . PCR was performed by hot start method by using reaction mixture of 25 µL containing 5 µL of template DNA, 2.5 µL of 10X PCR buffer, 0.25µL of *Taq* DNA polymerase, 2.5 µL of $MgCl_2$ (25 mM), 2.5 µL of dNTPs (2mM each), 0.5 µL for each primer, and 12.25 µL of double distilled H_2O to make up the reaction volume. Optimized PCR profile was; initial denaturing at 94°C for 3 min; followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; while final extension was done at 72°C for 10 min. Amplicons were analysed by 1.0% agarose gel electrophoresis and visualized by using UV transilluminator.

Cloning and Sequencing the 16S-rRNA gene

The amplicons were ligated into pTZ57R/T cloning vector (Fermentas). The recombinant vectors were transformed into *E. coli* DH5α using heat-shock method (42 ºC, for 2 min). The transformants were cultured on LB-plates supplemented with Ampicillin, IPTG and X-gal for selection of positive clones basedon blue-white selection. The white colonies were transferred to culture tubes, plasmids were purified using plasmid extraction kit (Fermentas). The positive clones were confirmed via restriction analyses using *Eco*R1 and *Pst*1 enzymes. Positive clones were sequenced by Macrogen Korea.

Phylogenetic analysis

Primary analysis of clones for assembling and analysing was done with the aid of Laser gene Software (DNAStar Inc., Madison, WI, USA) then and submitted to GenBank. Phylogenetic dedrograms were obtained by using MEGA 6, and alignment was done by MUSCLE v. 3.7.

Results

The PCR assay based investigation using the *C. portiera* specific universal primers yielded amplification for the endosymbiont. It is revealed that C. *portiera* is present among the *B. tabaci* population as all the samples from different locations yielded amplifications. The 16S-rRNA gene was successfully amplified from samples produced an amplicon of an average size of 1070bp. The clones obtained from amplified samples were named as A12 (Acce. No LN717258), A13 (Acce. No LN717259), A14 (Acce. No LN717260). The sequence analyses revealed these clones to show 95-99% similarities with the BLAST results of NCBI. However Clones identified in this study showed higher (99.2%) (give quantitative number) similarities with the sequences from Indian and Chinese samples (Pan et al. 2012; Chu et al. 2006).

Present results have clearly established that *B. tabaci* population were infected with *C. portiera* in Vehari and Mianwali (Table 2). The computational analyses were carried out for the sequenced 16S-rRNA genes using various software. A total of 30 aligned sequences of C. *portiera* for 16S-rRNA gene belonging

to whitefly were included in the analyses (Figure 1). Evolutionary history was concluded by using the Maximum Parsimony method. The consensus tree constructed from 5 most parsimonious trees is given (figure 1). Branches corresponding to partitions reproduced in less than 64% trees are collapsed. The consistency index is 0.949045 (0.714286), the retention index is 0.836735 (0.836735), and the composite index is 0.794099 (0.597668) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei M 2000) with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 30 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 904 positions in the final dataset. Evolutionary analyses were conducted using MEGA6. The analyses involved 30 nucleotide sequences. Codon positions were included. All positions containing gaps and missing data were eliminated. There were a total of 904 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

It is clearly evident from the phylogenetic tree that the clones A12, A13, A14 forms a separate clade with Indian and Chinese clones (Pan et al. 2012; Chu et al. 2006) These clones have about 95-99.2 % similarity (Table 1).

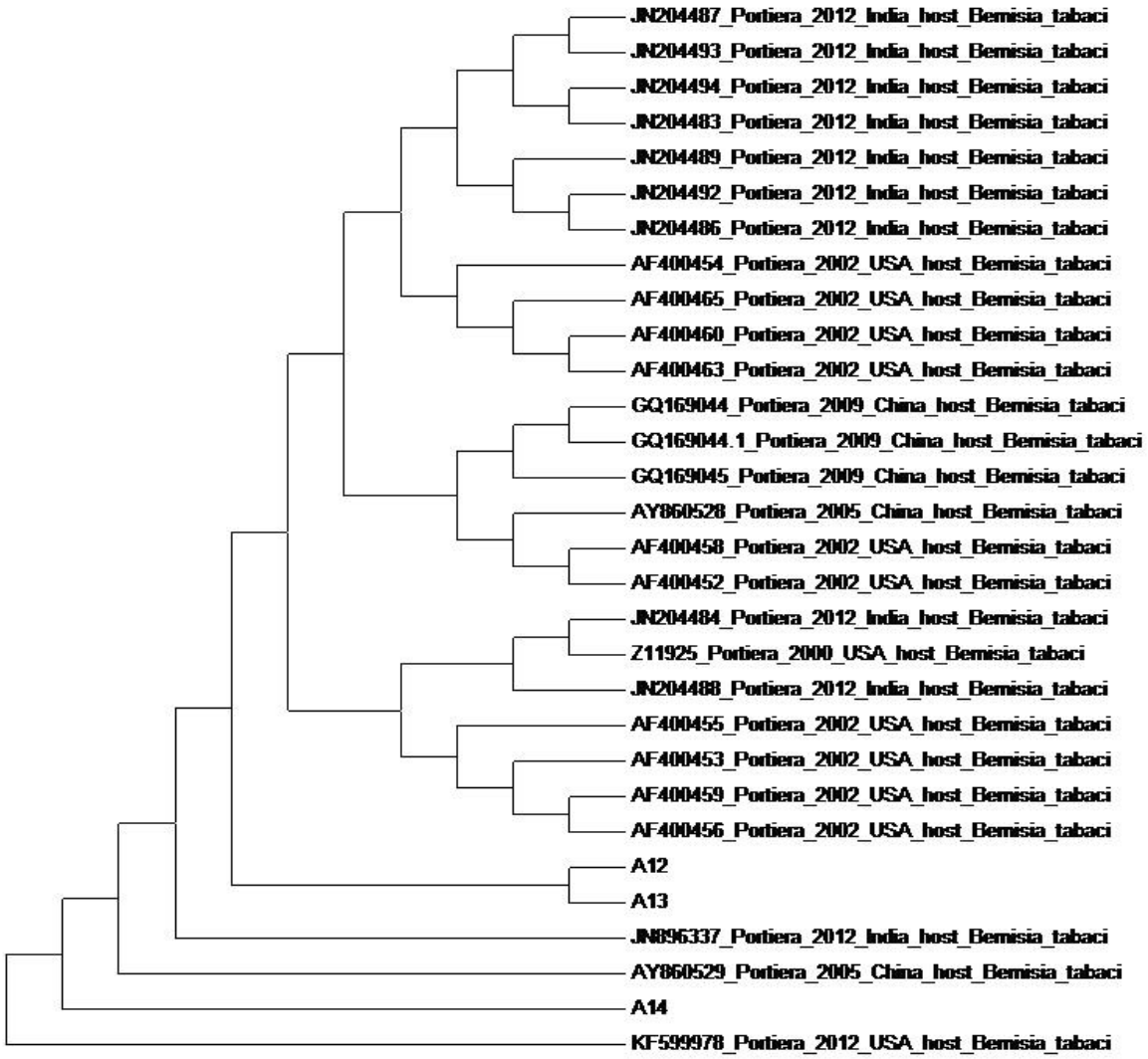

Figure 1. Maximum Parsimony analysis of *C. porteira* 16s rDNA sequence isolated from *B. tabaci*. The evolutionary history was inferred using the Maximum Parsimony method. The consensus tree inferred from 5 most parsimonious trees is shown. There were a total of 904 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Table 1. Characterization of *C. portiera* 16s rDNA sequence isolated from *B. tabaci*

Clone	LOCATION	Size bps	Highest homology with Gene Bank sequence	% homology
A12	**Vehari**	**1099**	AF400459_Portiera_2002_USA_host_Bemisia_tabaci	**99.2**
A13	**Vehari**	**1079**	AF400459_Portiera_2002_USA_host_Bemisia_tabaci	**98.3**
A14	**Mianwali**	**1092**	JN204488_Portiera_2012_India_host_Bemisia_tabaci	**99.1**

Discussion

C. Portiera aleyrodidarum is obligate, primary endosymbiotic bacterium of whiteflies, including the sweet potato whitefly *Bemisia tabaci*, and provides essential nutrients to its host (Santos-Garcia et al. 2014; Kuechler et al. 2013; Jiang et al. 2012; Ito et al. 2012; Kuchler et al. 2010; de Souza et al. 2009; Brelsfoard et al. 2008; Huang and Gogarten 2007; Baumann et al. 2002). Complete genome of this bacterium from the B and Q biotypes of *B. tabaci* Was also sequenced (Jiang et al. 2012). *B. tabaci* samples collected from different cotton growing regions in Punjab were positive for prevalence of this primary endosymbiont. Previous studies have revealed that PCR amplification of the 16S rRNA gene, a highly conserved region within the bacterial genome, is helpful in the detection and identification of the prokaryotes (Mariani and Tuan 1998). For the same reason 16S specific primers were used in this study for detection of the C. *portiera* in whitefly populations.

The phylogenetic analyses suggest high degree of genetic similarity among the C. *portiera's* clones of *B. tabaci* from Pakistan and further suggest high degree of genetic ancestral lineage among these clones. These results suggest a definite but undefined pattern of genetic lineages. In addition all the white fly population tested in this study harboured the endosymbiont suggesting that C. *portiera* is evenly distributed among the whitefly population in different cotton growing regions.

Additionally P-endosymbionts were phylogenetically analysed to distinguish their evolutionary relationships. Results of screening with laboratory populations may partially reflect the situation in the field hence they provide a useful basis to explore the association of other symbionts with the *B. tabaci*, the diversity as well and the role of these symbionts in the whitefly species composites in the field.

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