Phylogenetic status of Asian rice gall midge, *Orseolia oryzae* in the Cecidomyiidae family

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ABSTRACT

The Asian rice gall midge, *Orseolia oryzae* is one of the major pests which seriously infests the rice of several Asian countries and it causes major economic loss. The formation of silvery white hollow tube gall (silver shoot) is the major symptom of the rice gall midge infestation. Different biotypes or populations of rice gall midge were observed after the wide use of gall midge resistant rice varieties and the chemical control of the gal midge is difficult, because the larvae are safe inside the gall. In this study, we have developed a DNA barcode and determined the phylogenetic status of Asian rice gall midge, *O. oryzae* in Cecidomyiidae family. Among the species used in this study *Feltiella acarivora* is the nearest relative of *O. oryzae* and *Resseliella yagoi* is the distant relative. The mitochondrial DNA of the *O. oryzae* also showed clear bias to AT and it showed close similarity mean observed AT composition of the insect mtDNA. This study also revealed that the nucleotides in the each codon position of *O. oryzae* have variation in the strong and less constrained position of codons with other species of Cecidomyiidae family.

Keywords: cytochrome oxidase; *O. oryzae*; phylogeny; Cecidomyiidae

INTRODUCTION

The Asian rice gall midge, *Orseolia oryzae* seriously infests the rice of several Asian countries (Bentur *et al*., 2003). This major pest causes 10–100% crop losses in India (Siddiq, 1991). Based on the studies from eastern and southern India, Krishnaiah (2004) reported that the Asian rice gall midge infestation causes an annual yield loss of about 4,77,000 tons of grains, that leads to the economic loss of 80 million US$. Taxonomically *Orseolia oryzae* is classified under the family Cecidomyiidae and the subfamily Cecidomyiinae. In India, the Asian rice gall midge is reported from all states except few (Bentur *et al*., 1992). The formation of silvery white hollow tube gall (silver shoot) is the major symptom of the rice gall midge infestation. The affected tillers fail to produce panicle and that causes the yield loss.

The adult of rice gall midge are nocturnal and possess wing with a wingspan of 3.5–4 mm in females and 3–3.5 mm in males. The rice gall midge

females have bright red, stout abdomen and males have dark abdomen. The oviposition starts after few hours of copulation and they can lay up to 100–200 eggs either singly or in group of 3–4 near the base of the plant, on the ligules or in their vicinity on the leaf blade or on the leaf sheath. The eggs are tubular, shiny white with pinkish, red, or yellow shading, and will turn to shining amber before hatching. The larvae will form after 3–4 days of incubation. The freshly hatched larvae are about 1mm in size and they creep down the leaf sheath until they reach the growing point of the tillers. The larvae reach the interior of the bud and start feeding inside the developing buds that lead to the tubular gall formation which resembles onion leaf. The larvae feed until the pupation and the pupation will occur near the base after 15–20 days of larval period. After 2–8 days of pupal period an adult gall midge will form (Figure 1). The chemical control of the gal midge is difficult, because the larvae are safe inside the gall. Planting the resistant varieties of rice is effective method to prevent this pest (Pathak and Khan, 1994).

Different biotypes or populations of rice gall midge were observed after the wide use of gall midge resistant rice varieties (Roy *et al*., 1971; Chatterji *et al*., 1975; Bentur *et al*., 1987; Nair and Devi, 1994; Srinivas *et al*., 1994; Singh, 1996). Many studies reported the emergence of new virulent populations of rice gall midge from different locations in India (Bentur *et al*., 2003). Seven biotypes of rice gall midge were reported from India (Vijaya Lakshmi *et al*., 2006; Himabindu *et al*., 2010). The genetic studies of the different gall midge populations can help us to understand the evolutionary process, which leads to the formation of virulence in insect population against the gall midge resistant rice varieties. SCAR/RAPD and RFLP markers have been used to study the biodiversity of the gall midge populations (Behura *et al.,* 1999; Katiyar, 2000). Microsatellite based analysis of rice gall midge using hybridization capture approach revealed that 15 loci were hyper variable and showed polymorphism among different biotypes (Bentur *et al*., 2011). The transcriptome analysis of rice gall midge showed the different response of insects during the compatible and incompatible interactions (Sinha *et al*., 2012). Here we report the phylogenetic status of Asian rice gall midge, *O. oryzae* in Cecidomyiidae family.

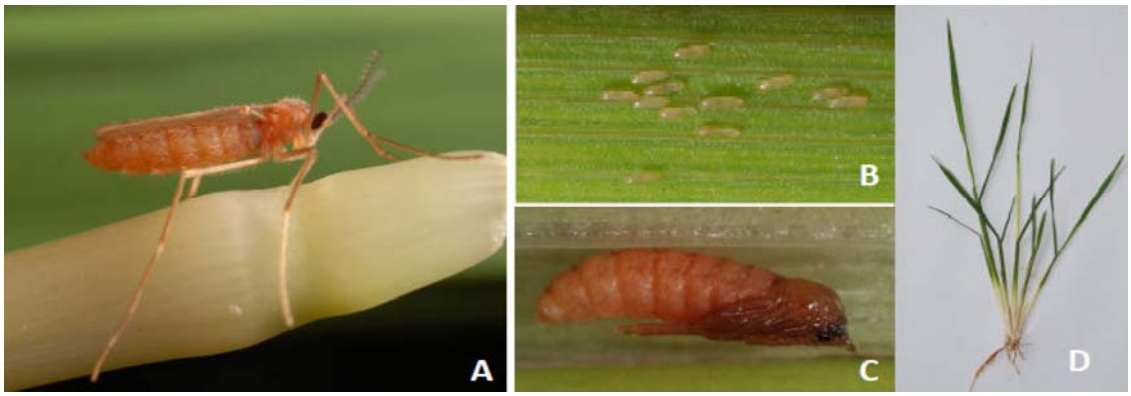

Figure 1 Various stages of Asian rice gall midge, *Orseolia oryzae*. A: Adult insect; B. Eggs; C. Pupa; D. Gall midge infested paddy.

MATERIALS AND METHODS

Ten adult *O. oryzae* were collected from the paddy field of Kerala and *Parapoynx stagnalis* collected from paddy field used as outgroup in the phylogeny analysis. The genomic DNA was extracted using GeNei Ultrapure Mammalian Genomic DNA Prep Kit (Bangalore GeNei, Bangalore) as per the Manufacturer's instruction. The partial gene sequence of *COI* of *O. oryzae* was PCR amplified using the forward primer with DNA sequence 5′ CATTGGAGATGACCAAATTTATAATG 3′ and the reverse primer with DNA sequence of 5′ TAAACTTCAGGGTGAC CAAAAAATCA 3′ (Folmer *et al.,* 1994). The PCR reaction mixture consisted of 2 nanogram of genomic DNA in 1 µl, 1 µl each forward and reverse primers at a concentration of 10 µM, 2.5 µl of dNTPs (2 mM), 2.5 µl 10X reaction buffer, 0.20 µl Taq polymerase (5 U/µl) and 16.8 µl H_2O. The PCR temperature profile consisted of 95 °C/3 minutes as initial denaturation and followed by 45 cycles of 95 °C/10 seconds, 50 °C/45 seconds, 72 °C/45 seconds and with a final extension of 72 °C for 3 minutes. The PCR amplified product was column purified using Mo Bio UltraClean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) as per the manufacturer's instructions. The purified product was sequenced with forward and reverse primers using the Sanger's sequencing method at SciGenom Labs, Cochin.

The forward and reverse sequences were assembled by using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2) after removing the forward and reverse primer sequences and the consensus was taken for the analysis. The phylogeny was inferred using the Neighbor-joining method by MEGA5 software

RESULTS AND DISCUSSION

The PCR amplification of partial mitochondrial *COI* gene of *O. oryzae* yielded a single product with about 600 bp in size. The sequence obtained after removing the primers used for PCR amplification was submitted to GenBank (Accession No. KC506565).

The comparative *COI* sequence analysis of *O. oryzae* with some other species of Cecidomyiidae family indicates the presence of unique nucleotides in the different positions of *O. oryzae*. The nucleotide 'A' in the 20, 21,99 and 140, nucleotide 'T' in the 179, 237 and 407 and nucleotide 'C' in the 176, 293 and 344th position is the unique in the *COI* sequence of *O. oryzae* compared to that many species of Cecidomyiidae family. The uniqueness in the 'G' nucleotide is not found in *COI* sequence of *O. oryzae* compared to other species of Cecidomyiidae family.

The nucleotide compositional analysis revealed the AT bias in the total nucleotide composition with following proportion: T= 43.20%, A= 33.50, C= 13.60% and G= 9.70%. In the total nucleotide composition of *COI* sequence *O. oryzae* shows very little variation (maximum +2.5 or -2.5) compared with other species of Cecidomyiidae family (Table 1). The overall frequency distributions of nucleotides at the 1st, 2nd, and 3rd codon positions were as follows: A = 11.70%, 50.70%, and 38.20%; C = 26.20%, 4.90%, and 9.7%; G = 12.4%, 0%, and 16.7%; and T = 50.0%, 44.0%, and 35.0%, respectively. Other species of Cecidomyiidae family showed variation in overall frequency distributions of nucleotides at the 1st, 2nd, and 3rd codon positions as follows: A = 0 to 0.7%, -9% to 4.2, and 0.7 to 2.8%; C = 0 to 1.5%, -3.5 to 0.7 and 0.7 to 4.9%; G = 0 to 0.1%, 0 to 1.4%, and 0.7 to 2.7%; and T = -1 to -2%, -3 to 12% and -5 to 1%.

The evolutionary divergence of *O. oryzae* in Cecidomyiidae family is given in table 2. *O. oryzae* showed less divergence (10.88%) with *F. acarivora*

Table 1 Composition of nucleotides and composition of nucleotides in each codon position in the *COI* sequence of *Orseolia oryzae* and some other species of Cecidomyiidae family. The GenBank accession numbers are given in parenthesis.

Name of species	T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
Orseolia oryzae (India)	43.2	13.6	33.5	9.7	50	26.2	11.7	12.4	44	4.9	50.7	.0	35	9.7	38.2	16.7
Feltiella acarivora (AB699002)	43.0	13.6	32.6	10.9	49	26.9	11.7	12.4	46	2.8	50.7	.7	34	11.1	35.4	19.4
Feltiella acarivora (AB699001)	43.0	13.6	32.6	10.9	49	26.9	11.7	12.4	46	2.8	50.7	.7	34	11.1	35.4	19.4
Feltiella acarivora (AB699000)	43.0	13.6	32.6	10.9	49	26.9	11.7	12.4	46	2.8	50.7	.7	34	11.1	35.4	19.4
Sitodiplosis mosellana (JQ609303)	42.7	13.9	32.3	11.1	48	27.6	11.7	12.4	47	1.4	50.7	1.4	33	12.5	34.7	19.4
Resseliella soya (AB506020)	45.0	13.9	30.5	10.6	49	26.2	12.4	12.4	54	2.1	42.4	1.4	32	13.2	36.8	18.1
Resseliella soya (AB506022)	44.8	14.1	30.5	10.6	49	26.2	12.4	12.4	53	2.8	42.4	1.4	32	13.2	36.8	18.1
Resseliella soya (AB506021)	44.8	14.1	30.5	10.6	49	26.2	12.4	12.4	53	2.8	42.4	1.4	32	13.2	36.8	18.1
Asteromyia carbonifera (DQ241909)	41.8	14.1	33.5	10.6	48	27.6	11.7	12.4	42	4.2	54.2	.0	35	10.4	34.7	19.4
Pitydiplosis puerariae (AB614592)	45.7	14.3	30.0	9.9	49	26.9	11.7	12.4	56	2.8	41.7	.0	33	13.2	36.8	17.4
Pitydiplosis puerariae (AB614593)	45.7	14.3	30.0	9.9	49	26.9	11.7	12.4	56	2.8	41.7	.0	33	13.2	36.8	17.4
Asteromyia carbonifera (DQ241891)	41.8	13.9	33.9	10.4	48	27.6	11.7	12.4	42	3.5	54.9	.0	35	10.4	35.4	18.8
Asteromyia carbonifera (DQ241894)	42.3	13.6	33.7	10.4	49	26.9	11.7	12.4	42	3.5	54.2	.7	36	10.4	35.4	18.1
Contarinia sp.(AB597020)	42.3	14.5	33.3	9.9	49	26.9	11.7	12.4	48	2.8	49.3	.0	30	13.9	38.9	17.4
Contarinia sp.(AB597023)	43.6	14.3	31.9	10.2	49	26.9	11.7	12.4	51	2.1	46.5	.7	31	13.9	37.5	17.4
Contarinia sp.(AB597022)	43.6	14.3	31.9	10.2	49	26.9	11.7	12.4	51	2.1	46.5	.7	31	13.9	37.5	17.4
Resseliella yagoi (AB506003)	43.6	15.2	30.3	10.9	48	27.6	11.7	12.4	51	3.5	44.4	.7	31	14.6	34.7	19.4
Pitydiplosis puerariae (AB614576)	45.7	14.3	29.8	10.2	49	26.9	11.7	12.4	56	2.8	41.7	.0	33	13.2	36.1	18.1
Parapoynx stagnalis	34.3	16.4	35.3	14.0	46	25.7	13.2	15.4	28	9.6	60.3	2.2	29	14.0	32.4	24.3
Avg.	43.2	14.2	32.0	10.6	49	27.0	11.8	12.5	48	3.2	48.3	.5	33	12.4	36.1	18.6

Table 2 Percentage of genetic divergence of *Orseolia oryzae* isolated from Kerala, India with some other species of Cecidomyiidae family. The GenBank accession numbers are given in parenthesis.

Name of Species	% Divergence
Feltiella acarivora (AB699002)	10.88%
Feltiella acarivora (AB699001)	10.88%
Feltiella acarivora (AB699000)	10.88%
Asteromyia carbonifera (DQ241909)	10.90%
Asteromyia carbonifera (EU439909)	10.90%
Asteromyia carbonifera (DQ241891)	10.94%
Sitodiplosis mosellana (JQ609303)	11.16%
Asteromyia carbonifera (EU439883)	11.23%
Asteromyia carbonifera (DQ241894)	11.24%
Asteromyia carbonifera (EU439910)	11.25%
Resseliella soya (AB506020)	12.21%
Resseliella soya (AB506022)	12.53%
Resseliella soya (AB506021)	12.53%
Contarinia sp. (AB597020)	12.97%
Contarinia sp. (AB597019)	12.97%
Pitydiplosis puerariae (AB614592)	13.89%
Pitydiplosis puerariae (AB614593)	13.89%
Pitydiplosis puerariae (AB614580)	13.91%
Pitydiplosis puerariae (AB614576)	14.19%
Pitydiplosis puerariae (AB614563)	14.19%
Contarinia sp. (AB597023)	14.23%
Contarinia sp. (AB597022)	14.23%
Resseliella yagoi (AB506003)	14.83%
Resseliella yagoi (AB506002)	14.83%

and highest divergence with *R. yagoi* among the species of Cecidomyiidae family analyzed in this study. The members of Cecidomyiidae family used in this study showed 11.60 overall mean divergences in the *COI* sequence. The phylogeny tree depicted using NJ method (Figure 2) clearly revealed the evolutionary position of *O. oryzae* in the Cecidomyiidae family. Among the species used in this study *F. acarivora* is the nearest relative of *O. oryzae* and *R. yagoi* is the distant relative.

The *COI* sequence analysis clearly revealed the genetic structure and phylogenetic status of *O. oryzae.* The presence of specific nucleotides in specific locations of *COI* sequence differentiateed the *O. oryzae* from other species of Cecidomyiidae family. Like all other insect *COI* sequence, the *COI* sequence of *O. oryzae* also showed clear bias toward AT, which led to AT rich genome (Lessinger *et al*., 2000). The AT composition of the *O. oryzae* is very close to the mean observed AT composition (77%) of Mt DNA of insects (Stewart and Beckenbach, 2005). The ATcomposition bias with low G in the sense and C in the template strand may have arisen through directional mutational pressure (Jermiin *et al*., 1994).

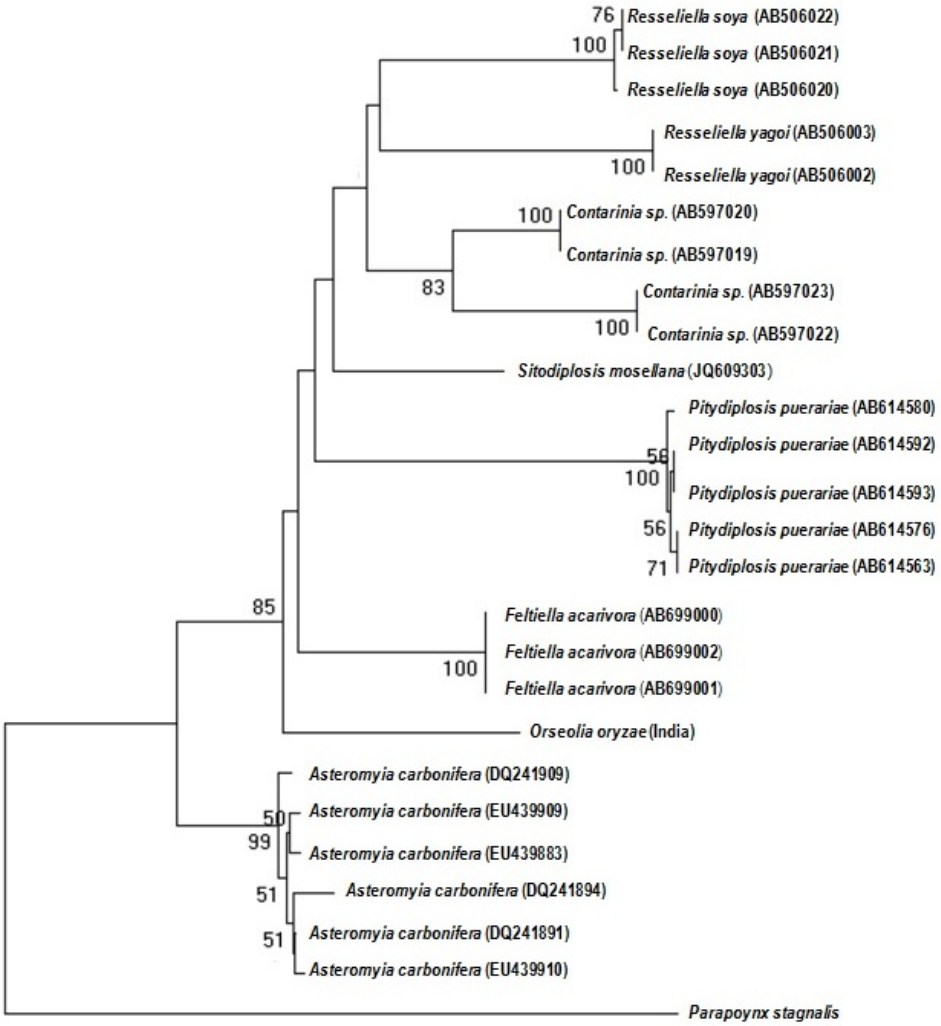

Figure 2 Phylogenetic relationship of *Orseolia oryzae*. The evolutionary history was inferred by Neighbor-Joining method using mitochondrial COI gene sequence. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

The analysis of nucleotides in each codon position revealed the high AT composition in the second codon position (94.70%) compared to the third (73.20%) and first position (61.70%). The high content of the T in the second codon position may be related to the preference for non-polar and hydrophobic amino acids in the membrane associated proteins (Yang *et al*., 2012).

In the nucleotide triplet code, there is strong compulsion in the nucleotide changes in all second codon position and many first codon positions. Due to the degenerative character of the triplet code many third codon position and some first codon positions are less constraint. The variations in the strong constraint positions lead to the variations in the amino acids sequence. But the variations in the less constraint position will not affect (silent) the phenotype and these less constrained codon positions evolved at high rate (Nei, 1987; Irwin *et al*., 1991). In the mitochondrial genes the silent codon positions are freer to vary due to the expanded codon recognition, allowing a single tRNA molecule to decode as many as four codons (Gray, 1989). In this study, we have found that related species showed less nucleotide substitution in the strong constrained codon positions. The *O. oryzae* showed variations in the strong and less constrained positions with other species of Cecidomyiidae family. Therefore, it showed variation in the phenotypic characters also.

The gall midge in the Cecidomyiidae family was the most diverse group of gall forming insects and they are found in all geographical regions (Gagne 1994; Gagne 2004). They showed diversity in the host plant, shape of galls and location of galls in the plants. The species of gall midges are highly specific (Carneiro *et al*., 2009) but the genera promote high richness within the group (Araújo, 2011). The genetic divergence analysis of the *O. oryzae* in the Cecidomyiidae family also supports the morphological diversity within the family Cecidomyiidae. The Cecidomyiidae family showed less divergence within the species. Different species of Cecidomyiidae family showed high divergence between the species and they also showed diversity in the phenotypic characters like host plant, gall shape and location of gall in the host plant.

The *COI* sequence analysis clearly depicts the genetic structure and evolutionary status of the *O. oryzae*. This study concluded that the mitochondrial DNA (mtDNA) of the *O. oryzae* also showed clear bias toward AT and it showed close similarity with mean AT composition of the insect mtDNA. The nucleotides in the each codon position of *O. oryzae* have variation in the strong and less constrained position of codon with other species of Cecidomyiidae family. These variations lead to the phenotypic variations. The *COI* sequence of *O. oryzae* isolated in this study has considerable variation with all other species of Cecidomyiidae family; therefore it can be used as a barcode for the identification and genetic characterization of *O. oryzae*.

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