Wolbachia endosymbiont infection in two Indian butterflies and female-biased sex ratio in the Red Pierrot, *Talicada nyseus*

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The maternally inherited obligate bacteria *Wolbachia* is known to infect various lepidopteran insects. However, so far only a few butterfly species harbouring this bacterium have been thoroughly studied. The current study aims to identify the infection status of these bacteria in some of the commonly found butterfly species in India. A total of nine butterfly species belonging to four different families were screened using PCR with *Wolbachia*-specific *wsp* and *ftsZ* primers. The presence of the *Wolbachia* super group 'B' in the butterflies Red Pierrot, *Talicada nyseus* (Guerin) (Lepidoptera: Lycaenidae) and Blue Mormon, *Papilio polymnestor* Cramer (Papilionidae), is documented for the first time in India. The study also gives an account on the lifetime fecundity and female-biased sex ratio in *T. nyseus*, suggesting a putative role for *Wolbachia* in the observed female-biased sex ratio distortion.

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1. Introduction

The maternally inherited endosymbiotic α–proteobacteria called *Wolbachia* is known to infect 15%–75% of insect species (Werren and Windsor 2000; Jeyaprakash and Hoy 2000; Puttaraju and Madhu 2002; Puttaraju and Prakash 2005a, b; Prakash and Puttaraju 2007). This endosymbiont is known to cause some reproductive abnormalities in its insect hosts, such as cytoplasmic incompatibility (Laven 1967; Breeuwer and Werren 1990; Breeuwer *et al.* 1992; O'Neill *et al.* 1992; Prakash and Puttaraju 2007), parthenogenesis (Stouthamer *et al.* 1990, 1993; Arakaki *et al.* 2001), male killing (Fialho and Stevens 1997, 2000; Hurst *et al.* 1999, 2000) and feminizing of genetic males (Rousset *et al.* 1992; Hiroki *et al.* 2002). *Wolbachia* exist in 11 clades (A to K) referred as super groups, which infect not only wide range of insects but also many non-insect invertebrates. However, the super groups A and B are known to infect the insect kingdom (Werren *et al.* 1995; Prakash and Puttaraju 2007). The association of *Wolbachia* with order Lepidoptera is very well studied, but only a few species of butterflies are known to be infected by *Wolbachia.* It has been shown that the presence of particular clades of *Wolbachia* cause feminization and cytoplasmic incompatibility in the common grass yellow butterfly, *Eurema hecabe* (Hiroki *et al.* 2004). However, recent studies also suggest that some *E. hecabe* infected with a single strain of *Wolbachia* have two distinct reproductive phenotypes (Narita *et al.* 2007). Two male-killing bacteria were reported in *Acraea encedon*, which were shown to belong to the same strain (Jiggins *et al.* 2001), and this particular strain is also known to cause male killing in *Hypolimnas bolina*, indicating that *Wolbachia* might have moved between host species, retaining its phenotype (Dyson *et al.* 2002). Further, the male-killing *Wolbachia* has been reported in the Indo-Pacific populations of *Hypolimnas bolina*, which causes sex ratio distortion compared to the normal sex ratio (Charlat *et al.* 2005). It is also known that apart from *Wolbachia*, some other bacteria like *Spiroplasma* can cause male killing in butterflies by killing the male embryo (O'Neill *et al.* 1997; Hurst and Jiggins 2000; Jiggins *et al.* 2000; Tabata *et al.* 2011).

Keywords. *Papilio polymnestor*; PCR; sex ratio; *Talicada nyseus*; *Wolbachia*

The Red Pierrot, *Talicada nyseus* (Guerin) (Lepidoptera: Lycaenidae), is a small butterfly found in many parts of India and Sri Lanka (Karunaratne *et al.* 2002). It is commonly found around semi-arid plains, degraded patches of evergreen forests and semi-evergreen forest, gardens, hill stations and forests. It is abundantly found near its food plant *Kalanchoe spp.* (Saxifragales: Crassulaceae). In India this butterfly is widely distributed in the peninsular area and is also known to colonize the foothills of the Himalayas due to change in habitat (Singh 2005). The larvae of this species are known to feed on the mesophyll tissues of the leaves of its host plant *Kalanchoe spp.* without disturbing the upper and lower epidermis. An adult butterfly usually feeds on nectar but is also known to feed on lichens (Karunaratne *et al.* 2002). It is further estimated that *T. nyseus* can damage most of its host plant species that are used for ornamental purpose.

During a regular field survey in Bannerghatta Biological Park, Bangalore, it was found that the population of *T. nyseus* was dwindling and males were relatively rare. Reliable data on the fecundity and sex ratio in *T. nyseus* are not available and this study gives an account of female-biased sex ratio in this butterfly species. This kind of sex ratio distortion or female-biased sex ratio has been previously reported in some other butterflies from different families. This female-biased sex ratio is known to be caused by *Wolbachia* in some butterfly species. In *Acraea encedon,* female-biased sex ratio was first reported by Poulton in 1914 and it was thought that this sex ratio distortion was the result of meiotic drive. Later it was proved that the cause for sex ratio distortion was the presence of the maternally inherited bacteria *Wolbachia* (Jiggins *et al.* 1998, 1999). *Eurema hecabe* is known to be infected by different types of *Wolbachia* strains (Hiroki *et al.* 2002, 2004; Narita *et al.* 2007) and shows various types of reproductive anomalies such as cytoplasmic incompatibility, feminization and male killing.

The present study was carried out to identify the infection status of *Wolbachia* and its super group in some of the butterfly hosts that are commonly found in India. Further, the present study also provides information on lifetime fecundity and sex ratio of *T. nyseus* harbouring *Wolbachia* infection under controlled environmental conditions.

2. Materials and Methods

2.1 *Collection of butterflies*

The butterfly species were collected from their natural habitats in the forest areas of Bannerghatta Biological Park, Bangalore, and Jnana Bharathi Campus, Bangalore University. Butterflies were collected using a butterfly collecting net. The collection was done in the morning hours from 8.30 a.m. to 1.00 p.m. The collected species were transferred to the laboratory carefully in butterfly collecting cages, identified and separated with respect to their sex.

2.2 *Rearing of Talicada nyseus*

The adult *T. nyseus* collected were released in one cage with nectar and host plants for mating and increase of population. The other developmental stages such as eggs, larvae and pupae were reared in aerated boxes until they metamorphosed into adult. The emerged adults were released in the same cages for increasing the stock population.

2.3 *Experimental setup*

One virgin female along with two males from the stock population were released separately in a cage along with host and nectar plants for mating and egg laying. For nectar *Lantena* plants were used. A total of 12 individual females ($n=12$) were used for the study in each cage separately. The fecundity was estimated manually by repeatedly counting the number of eggs on the host plants at regular time intervals (twice a day until the female died). The egg-bearing plants were carefully taken out of the cages and kept separately under observation. The hatchability was ascertained by counting hatched eggs on host plants. The larvae were grown to complete metamorphosis on *Kalanchoe* leaves. The pupae were counted and taken out from the plants and reared in aerated boxes. The adults emerged were then examined morphologically to determine their sex ratio.

2.4 *Molecular detection of Wolbachia infection*

The genomic DNA was isolated by two methods *viz* the phenol-chloroform isoamyl alcohol method (Sambrook *et al.* 1989) and the Kit method (Ultraclean Tissue and Cells DNA isolation Kit by MO BIO Laboratories, Inc.). The DNA from nine species of butterflies belonging to four different families was isolated by the phenol-chloroform isoamyl alcohol method for initial screening of *Wolbachia* infection, whereas the DNA from the female *T. nyseus* used for the experiment was isolated by the Kit method. The extracted DNA was quantified by agarose gel (0.8%) electrophoresis.

Molecular diagnosis of *Wolbachia* and its super groups A and B were done by *Wolbachia*-specific PCR assay using specific primers like *wsp* 81f 5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′, *wsp* 691r 5′-AAA AAT TAA ACG CTA CTC CA-3′ (Dyson *et al.* 2002), *ftsZ Adf* 5′-CTC AAG CAC TAG AAA AGT CG-3′ *ftsZ Adr* 5′-TTA GCT CCT TCG CTT ACC TG-3′ (Prakash and Puttaraju 2007) and *ftsZ Bf* 5′- CCG ATG CTC AAG CGT TAG AG-3′ *ftsZ Br* 5′-CCA CTT AAC TCT TTC GTT TG-3′ (Prakash and Puttaraju 2007). PCR amplification was carried out with

Table 1. The PCR results showing the infection status of *Wolbachia* super groups in butterfly species belonging to four different families

Family	Butterfly species	Number of individuals screened	Number of individuals infected with *Wolbachia*	Super group status
Nymphalidae	*Ariadne merione*			
	Danaus genutia			
	Tirumala limniace	30	None	–
	Danaus chrysippus			
	Euploea core			
Papilionidae	*Papilio demoleus*	30	None	–
	Papilio polymnestor	30	30	B
Lycaenidae	*Talicada nyseus*	30	30	B
Pieridae	*Catopsilia pyranthe*	30	None	–

A total of 30 individuals from each species collected from the study area were screened for their *Wolbachia* infection. The results obtained reveals that only two species, *Papilio polymnestor* and *Talicada nyseus* belonging to the family Papilionidae and Lycaenidae respectively, were found to be infected with this endosymbiont. The present infection status in these two butterflies was reported for the first time in India.

Primus 96 Advanced Gradient Thermo cycler and Eppendorf Thermo cycler using 25 µl reaction volume consisting of 12.5 µl of master mix (Fermentas), containing 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.15 mM MgCl2), 0.2 mM dNTPs, 2.5 mM MgCl2 and 0.5 U *Taq* DNA polymerase, 20 pm of 1 µl each of forward and reverse primers, 50 ng of 1 µl of template DNA, 9.5 µl of nuclease-free water to make up 25 µl. The reaction conditions consisted of an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplified PCR products were separated through 1% agarose gel electrophoresis run in 1X TBE (89.2 mM Tris-HCl, 88.9 mM boric acid and 2.0 mM disodium-ethylenediaminetetre-acetic acid) buffer for a length of 5–6 cm at a constant 50 V. A standard molecular weight marker DNA ladder (Low range DNA ruler plus, Bangalore GeNei) was used in each electrophoretic run and the gel was documented with the gel documentation system (Alpha imager EP, JH Bio systems).

A total of 30 individuals from each species were subjected to molecular diagnosis studies to estimate the *Wolbachia* infection status in them (table 1). Since *Wolbachia* is predominantly inherited through maternal cytoplasm, only the females were used for the molecular studies.

3. Results and discussion

Phenotypic studies in *Wolbachia*-infected butterfly species indicate that certain strains of *Wolbachia* do not show the same phenotype (such as male killing) when they are in different hosts (Charlet *et al.* 2005). However, studies by Hiroki *et al.* (2002) suggest that *Wolbachia* in butterflies can also cause feminization. So far few species of butterflies have been studied, and the association of *T. nyseus* and *P. polymnestor* with *Wolbachia* has been documented in the present study.

3.1 *Molecular diagnosis of Wolbachia and its super group*

The major finding of the current study is the presence of female-biased sex ratio in *T. nyseus* that harbour maternally inherited *Wolbachia. Wolbachia* infection is known to cause either feminization or male killing in its butterfly host. A total of nine different butterfly species from four families screened in the present study for *Wolbachia* super group infection by PCR amplification (table 1) revealed that only two, Red Pierrot, *T. nyseus*, and Blue Mormon, *P. polymnestor*, were found to be infected with *Wolbachia*. This is a prime data on the infection status of *Wolbachia* in Indian butterflies. The *Wolbachia* infection present in *T. nyseus* and *P.* polymnestor further revealed that the infected *Wolbachia* belongs to the super group B. The DNA from the female *T. nyseus* used for the experiment ($n=12$) were also subjected to PCR amplification and confirmed the super group B infection in them (figure 1). An attempt has been made to study the sex ratio in detail, but this was successful only in *T. nyseus* and it was not possible to study the same in *P. polymnestor* because of the lack of availability of sufficient numbers of flies in both Bannerughatta Biological Park and the Jnana Bharathi Campus of Bangalore University in order to rear and record the sex ratio.

3.2 *Reproductive Biology of Talicada nyseus infected with Wolbachia*

During the field collection of adults of *T. nyseus*, it was observed that under natural conditions, the sex ratio of this butterfly was female biased. The field collection was done for 15 days in Bannerghatta Biological Park to obtain male

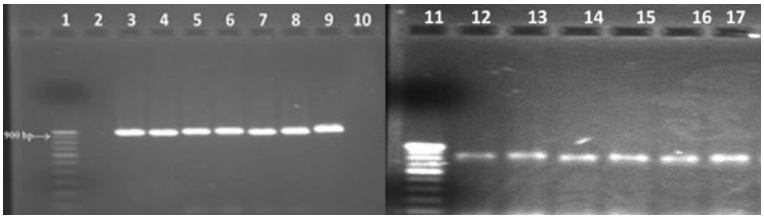

Figure 1. The gel photographs showing the PCR amplification results in all the 12 experimental females. The amplificaton of *ftsZ* B primer sequence demonstrates the presence of *Wolbachia* super group B infection in *T. nyseus*. Lane 1 and 11 is the Low range DNA ruler (1000 bp), Lane 9 and 10 are Positive and negative control for *ftsZ* B primer respectively. Lane 3 to 8 and lane 12 to 17 are the 12 females used for the experiment which shows positive for *Wolbachia* super group B infection.

and female butterflies to maintain stock population in the laboratory. Among randomly collected 323 adult *T. nyseus* from the field, it was found that only 55 were males and the remaining 268 were females. This is in the congruence with the female-biased sex ratio observed in the laboratory-maintained stock population.

The detailed report of fecundity, hatchability and sex ratio of *T. nyseus* under controlled conditions provided interesting results. The lifetime fecundity of *T. nyseus* was observed to range from 4.00±0.00 to 89.9±0.43 (table 2). The eggs laid by each single female were counted repeatedly and mean±SD was calculated to minimize the error. The observed percentage hatchability with respect to fecundity varied from 50.00% to 98.20% under controlled conditions (table 2). Most of the eggs hatched during early hours of the day. The average number of pupal emergence and adult eclosed in all the 12 experimental setup were recorded as 25.66 and 16.83 respectively. The sex ratio was female-biased with more females than males (figure 2). The male:female ratio ranged from 0:2.0 to 1:13.0 (table 2).

The observed fecundity clearly demonstrate that the fecundity is considerably good in *T. nyseus* even though they harbour *Wolbachia*. However, during initial experimentation, we found a low fecundity value but it consistently increased at latter stages of the experiment. These results indicate that the *Wolbachia* infection has no influence on the fecundity of *T. nyseus*. The poor fecundity we found during the initial stage of experiments was perhaps due to the influence of climatic factor on experimental females during the period of experiment or might be due to handling error. The percentage hatching observed in the experimental setup was also good compared to that of fecundity under controlled conditions (table 2). But adult eclosion was less compared to fecundity under controlled conditions (table 2). The sex ratio showed that females dominated over males in a given brood (figure 2). The sex ratio varied and was female biased in *T. nyseus* and was comparable to the data recorded in the wild. Similar female-biased sex ratios were observed in wild *T. nyseus* populations during our field work in the Bannerghatta Biological Park. The result obtained confirms that *T. nyseus*

Table 2. The results obtained from the experimental setup showing fecundity, hatchability and sex ratio of butterfly *T. nyseus*

Individual female number	Fecundity	Hatchability (%)	Formation of pupae	Adults eclosed	Sex ratio (M:F)
1	4.00±0.00	50	2	2	0:2.0
2	6.62±0.51	57.14	4	4	1:1.0
3	17.12±0.99	61.11	7	5	1:2.0
4	43.50±1.69	88.63	20	16	1:5.5
5	67.37±3.06	60.86	26	20	1:8.0
6	53.25±1.48	74.72	25	20	1:5.0
7	47.62±0.74	80.45	21	17	1:6.5
8	17.87±0.35	94.44	16	12	1:9.0
9	62.5±0.51	81.30	32	22	1:6.3
10	75.3±0.08	95.01	41	25	1:11.5
11	89.9±0.43	98.20	56	31	1:5.2
12	86.7±0.32	86.05	58	28	1:13.0

A total of 12 individual virgin females were taken for the experiment. The fecundity was calculated manually by counting eggs on the host plant. The mean ± SD were calculated from the fecundity data obtained from each individual female. The hatchability % was calculated by counting hatched eggs with respect to fecundity. The sex ratio was estimated by counting the number of males and females emerged from each experimental setup.

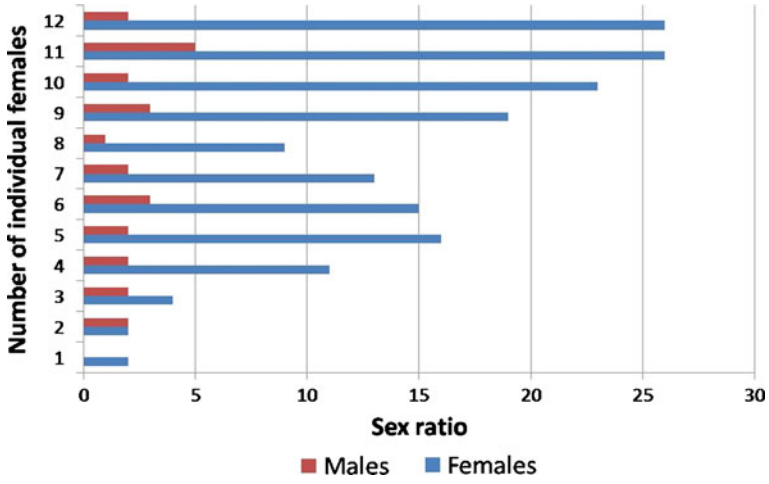

Figure 2. Shows the sex ratio in the offspring of all the 12 female *T. nyseus* used for the experiment. The females are relatively more than that of males in all the experimental setups.

that harbours *Wolbachia* super group B infection has a female-biased sex ratio. A similar observation was also made by earlier workers in other butterfly species that harbour *Wolbachia*. The female-biased sex ratio of the present study was comparable with the male killing (Fialho and Stevens 1997, 2000; Hurst *et al.* 1999, 2000) and feminizing (Rousset *et al.* 1992; Hiroki *et al.* 2002) phenotype caused by *Wolbachia.* However, the sex ratio of *T. nyseus* might be restored if they are cured of the *Wolbachia* infection with some antibiotics, as observed in *Acraea encedon* (Jiggins *et al.* 2001), or it may remain same even after antibiotic treatment, as observed in *Hypolimnas bolina* (Dyson *et al.* 2002). This needs a separate investigation to check the influence of antibiotics on *T. nyseus* harbouring *Wolbachia*.

Based on the data obtained from the current study in comparison with previously available information, we can predict that the sex ratio of *T. nyseus* that harbour *Wolbachia* is distorted in the wild as well as under controlled conditions. This study suggests that the endo-symboint *Wolbachia* may be responsible for sex ratio distortion in this species of butterfly either by causing male killing or feminization. Further investigation is required to confirm and exploit *Wolbachia* for beneficial purpose including increasing the fitness of the female hosts.

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