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Evidence for *Wolbachia* in leafhoppers of the genus *Eupteryx* with intersexual morphotypes

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Abstract

Leafhoppers (Hemiptera Cicadellidae Typhlocybinae) of the genus *Eupteryx* are important pests on medical and culinary herbs including sage (*Salvia officinalis* L., Lamiaceae), causing severe economic damage. Individuals of *Eupteryx decemnotata* Rey and *Eupteryx melissae* Curtis show a modified genital morphology at two geographically distant populations in Germany (Bonn and Göttingen). Typical female and male sexual characters are merged. In another species of Typhlocybinae a similar intersexual phenotype, representing feminized males, was explained by *Wolbachia* infection. We investigated *E. decemnotata* and *E. melissae* from both locations for infection by a molecular screening study (PCR) with three *Wolbachia* specific genes (16S rRNA, *ftsZ*, *wsp*). The screening strongly supports *Wolbachia* infections in both host species in Göttingen and in *E. melissae* from Bonn. Phylogenetic analyses of the *ftsZ*, *wsp* and the host-specific *COI* gene indicate a single infection in *E. melissae,* but infection with two different strains in *E. decemnotata* and host-mediated distribution of *Wolbachia*. Further, the data indicate horizontal *Wolbachia* transmission between these leafhopper species. This is the first study demonstrating the presence of *Wolbachia* in *Eupteryx* leafhoppers. Rapid spread of *Wolbachia* in *Eupteryx* populations can potentially threaten sage cultivations if morphologically modified individuals represent feminized males, thereby increasing the reproductive potential of infected populations. We discuss possible implications of *Wolbachia* infection inducing a feminoid phenotype for the population dynamics of leafhopper pests.

Key words: feminization, reproductive parasites, horizontal transmission, molecular screening, plant pest, medical herbs, spice herbs, *Wolbachia* supergroup.

Introduction

Leafhoppers (Hemiptera Cicadellidae Typhlocybinae) of the genus *Eupteryx* are important pests of medical and culinary herbs. Originally native to Mediterranean countries, some of these insects expanded their ranges rapidly in central Europe in the 20th century through commercial trade of their host plants. Species produce up to three generations per year and cause modest to severe damage to cultivated herbs (Dachler and Pelzmann, 1999; Vidano and Arzone, 1976; Nusillard, 2001; Nickel, 2003). Damage of plants by *Eupteryx* results from piercing the leaf parenchyma which causes loss of assimilation tissue and leaf stippling (Pollard, 1968; 1969). Such damages have been reported from cultivation sites in Portugal, Switzerland, Austria, Slovenia, Greece, UK, Germany and the northern USA (Nickel and Holzinger, 2006; Rung *et al*., 2009). Cultivation of sage (*Salvia officinalis* L.) is particularly affected since this plant is of high pharmaceutical value and therefore cultivation is expanding (Hoppe, 2005). So far available pest control strategies are insufficient in both organic and conventional agriculture (Röhricht, 2005; Jung, 2009).

We investigated populations of both *Eupteryx decemnotata* Rey and *Eupteryx melissae* Curtis with a high proportion of individuals with a novel malformation of the female ovipositor. In Italy Negri *et al*. (2006) found that a similar malformation in the leafhopper *Zyginidia pullula* (Boheman) was induced by the reproductive parasite *Wolbachia*.

Wolbachia is a group of intracellular inherited bacteria and well known as agents of various reproductive alterations in its host (Werren, 1997; Stouthamer *et al*., 1999), such as cytoplasmic incompatibility, parthenogenesis, male-killing and feminization of karyotypic males (Hurst *et al*., 1999; Rousset, 2000; Hiroki *et al*., 2002; Hunter *et al*., 2003). *Wolbachia* are transmitted maternally and the modifications on the host enhance vertical transmission within host populations by increasing the frequency of infected individuals (cytoplasmic incompatibility) or by inducing female-biased sex ratios (Hurst *et al*., 1999; Stouthamer *et al*., 1999). *Wolbachia* strains are divided into eight supergroups (A-H) with a wide range of host species including nematodes and arthropods, but are particularly numerous in insects (Werren *et al*., 1995; Zhou *et al*., 1998; Lo *et al*., 2007). Horizontal transmission has been increasingly detected in a variety of species but transmission routes are little understood (Breeuwer and Jacobs, 1996; Werren and Bartos, 2001). Established infections with *Wolbachia* accompanied by altering karyotypic males into functional females (feminization) can strongly accelerate population growth due to higher frequency of reproductive individuals. For detection and characterization of *Wolbachia* infection a common approach is to use multi-locus sequence typing (MLST) introduced by Baldo *et al*. (2006).

In this study, we tested two populations of *E. melissae* (from Bonn and Göttingen, Germany) and one population of *E. decemnotata* (from Göttingen, Germany) positive for *Wolbachia* infection by polymerase chain reaction (PCR) screening, using three *Wolbachia* housekeeping gene loci i.e., 16S rRNA, *ftsZ* and *wsp*. At both locations individuals with modified genital characters had been observed since 2008. In the following we

shall refer to these individuals as "feminoid" since their karyology has not yet been studied. Additionally, the host-specific *COI* gene was sequenced from all investigated individuals. With phylogenetic analyses of all four genes we ask if infection at the two locations was based on single or multiple events. The consequences of a *Wolbachia* infection for modified phenotypes in leafhopper populations with respect to population dynamics and the suspected relevance of *Wolbachia* for the pest management in agriculture will be discussed.

Materials and methods

Sample collection

In summer 2009 leafhoppers were collected from two different populations in Germany, Bonn, [Klein-Altendorf, competence centre for horticulture (Kompetenzzentrum Gartenbau, KoGa)] and Göttingen (historical Botanical Garden); sampling locations are 300 kilometers apart. Insects were collected at three different dates during the vegetation period by sweep-netting. Moving forward with constant speed, fifteen catches were made per sample, using forehand and backhand strokes. Intoxication with ethyl acetate followed immediately, insects were transported to the laboratory and stored in 95% ethanol at 4 °C. *Eupteryx* specimens were determined after Ribaut (1936) under a stereomicroscope. For quantitative analysis individuals were sexed, counted, abundance of *E. melissae* and *E. decemnotata* was determined and genital morphology was examined.

DNA extraction

Total genomic DNA was extracted from single individuals using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol: 'Purification of Total DNA from Animal Tissues (Spin Column Protocol)'. Each insect was homogenized with a pipette tip prior to lysis in 180 µl buffer ATL and 20 µl Proteinase K (600 mAU/ml) at 56 °C for 2-3 hours. After lysis, DNA was washed and eluted in 50 µl buffer AE.

The concentration of extracted DNA was quantified using the nanodrop option of TECAN Infinite M200 NanoQuant Plate (TECAN Trading AG, Männedorf, Switzerland); DNA was stored at −20 °C for not longer than 3 months. Samples with a minimum of 10 ng μl^{-1} DNA were used for molecular analyses.

Screening and sequencing

Individuals were investigated for *Wolbachia* infection by PCR screening with two *Wolbachia* specific genes (*wsp* and *ftsZ)* and a *Wolbachia* specific region of bacterial 16S rRNA gene. Additionally, the *COI* gene of the host was amplified to confirm the presence of host DNA in all samples. For each male, female and feminoid individual all four genes were amplified using the HotStarTaq™ PCR MasterMix (Qiagen, Hilden, Germany); PCR conditions include 12.5 µl HotStarTaq™ (2.5 units of HotStarTaq polymerase, 200 µM of each dNTP, 15 mM $MgCl_2$), 0.5 µl of each primer (50 pM), 3 µl template DNA, 1 µl $MgCl_2$ (25 mM) and depending on the amplified gene 1-2 µl BSA (3%); H_2O was added to a final volume of 25 µl. All PCR conditions included an initial activation step at 94 °C for 15 min and a final elongation step at 72 °C for 10 min.

PCR conditions for *ftsZ* were 34 cycles with 94 °C for 1 min (denaturation), 61.5 °C for 1 min (annealing) and 72 °C for 2 min (elongation) and yielded a 737 bp fragment using the primers ftsZunif (5'-GG(CT) AA(AG) GGT GC(AG) GCA GAA GA-3') and ftsZunir (5'-ATC (AG)AT (AG)CC AGT TGC AAG-3'; Lo *et al*., 2002). For *wsp* conditions were 35 cycles with 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min (elongation), amplifying a 610 bp fragment using the primers wsp_81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC-3') and wsp_691R (5'-AAA AAT TAA ACG CTA CTC CA-3'; Braig *et al*., 1998). For 16S rRNA, 30 cycles with 94 °C for 1 min (denaturation), 52 °C for 1 min (annealing) and 72 °C for 2 min (elongation) generated a 900 bp fragment using the primers 16S_F_V1 (5'-TTG TAG CCT GCT ATG GTA TAA CT-3') and 16S_R_V6 (5'-GAA TAG GTA TGA TTT TCA TGT-3'; O'Neill *et al*., 1992).

The amplification of the 658 bp fragment of *COI* consisted of 35 cycles with 94 °C for 30 s (denaturation), 51 °C for 1 min (annealing) and 72 °C for 1 min (elongation) using the primers HCO2189 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'; Folmer *et al*., 1994).

All PCR runs included a positive control for *Wolbachia* (infected individuals of *Bryobia* sp.). All products were run on 1% agarose gels and visualized by ethidium bromide staining. Positive samples were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and sent for sequencing. Purified PCR products were sequences at the Department of Experimental Phycology and Culture Collection of Algae (Georg August University Göttingen, Germany).

In total, 157 leafhoppers were screened for *Wolbachia* and for the host *COI* gene, 51 individuals of *E. melissae* sampled in Bonn (11 females, 9 males, 31 feminoids), 49 *E. melissae* specimens sampled in Göttingen (24 females, 18 males, 7 feminoids), 25 *E. decemnotata* individuals from Göttingen (12 females, 12 males, 1 feminoid) and 32 *E. decemnotata* sampled in Bonn (12 females, 11 males, 9 feminoids).

Sequence analyses

Sequences were compared with the online databank NCBI using the BLAST option to check if primers specifically amplified the targeted endobacteria. Sequences were edited and ambiguous positions were checked with Sequencher v4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Corrected sequences were assembled in BioEdit v7.0.5. (Hall, 1999) and aligned with ClustalW (Higgins, 1997). Multiple alignment parameters for *COI* and *ftsZ* were 1 (gap opening) and 0.1 (gap extension) and for 16S were 10 (gap opening) and 0.1 (gap extension). For the *wsp* gene multiple alignment parameters for the protein sequences were 15 (gap opening) and 6.6 (gap extension). Due to high variance in the

wsp gene the most similar sequences were first aligned and the deviating sequences Em_GOE_fem_291_c, wsp_*Drosophila* and Em_GOE_fem_289_c were added and aligned consecutively. All alignments were corrected by eye and truncated to the shortest sequence. For phylogenetic analyses 15 individuals from three populations were sequenced (table 1). For *E. melissae* from Bonn 3 females and 3 feminoid individuals were sequenced. Respective numbers for *E. melissae* from Göttingen were 2 females, 1 male and 3 feminoid individuals, and for *E. decemnotata* (Göttingen) 1 female and 2 males. In total, 31 *Wolbachia* specific (16S, *ftsZ*, *wsp*) and 14 host specific (*COI*) PCR products were sequenced and blasted in GenBank to confirm *Wolbachia* infection and host organism identity.

The best fit model of sequence evolution were found with Modeltest (Posada and Crandall, 1998) in PAUP* v4b10 (Swofford, 2002). For phylogenetic analyses Neighbor Joining trees with and without model of sequence evolution were calculated with heuristic search and bootstrapping using branch and bound search with 10,000 replicates in PAUP* v4b10, generating a 50% majority consensus tree. Bayesian analyses were calculated in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) with default settings and with model parameters corresponding to Modeltest parameters. The mcmc chain was run for 1 million generations, saving every 100th generation, burnin was 2500 (25%).

Trees were rooted with the following outgroup taxa: 16S and *ftsZ* datasets included the respective gene sequences of *Wolbachia* isolated from the host *Drosophila sechellia* Tsacas et Bachli (accession numbers: U17059 and U28179); the *wsp* dataset included a sequence of *Wolbachia* isolated from *Drosophila orientacea* Grimaldi, James et Jaenike (accession number: EU126456) and the *COI* dataset included *Eupteryx florida* Ribaut (J. Wilhein, unpublished data) as outgroup taxon. Additionally, Bayesian analyses were run to investigate the phylogenetic origin of *Wolbachia* in *Eupteryx* using 16S and *ftsZ* sequences of supergroups A-F from Czarnetzki and Tebbe (2004). MrBayes were run using default settings and a burnin of 2500.

Results

Field samples

The presence of *E. melissae* and *E. decemnotata* and the frequencies of male, female and feminoid individuals in the genus *Eupteryx* were investigated in the samples from Bonn (figure 1). *E. melissae* was the dominant species in the sampled sage field, representing 42% of all individuals (n = 639, 293 males, 288 females, 55 feminoids, 3 nymphs), whereas *E. decemnotata* was relatively rare, representing only 1.3% (n = 20, males and females represented with 5 individuals each, 8 feminoids, 2 nymphs) of all *Eupteryx* species (n = 1534). Various other *Eupteryx* species were sampled, representing in total 57% (n = 875) of all leafhopper individuals. Sex ratios were nearly equal and males were present in all samples. Feminoid adults were only present in *E. melissae* (n = 55, 8.6%) and *E. decemnotata* (n = 8, 40%). The sex of nymphs remained undetermined.

PCR screening

Wolbachia infection was detected in both species, with higher frequencies in females and feminoids than in males (table 2, figure 2). *Wolbachia* infection was more frequent in *E. melissae* (76%) than in *E. decemnotata* (5.3%). The overall infection level was higher in populations from Göttingen than from Bonn. However, screening results for *Wolbachia* varied and ranged from single amplification band to multiple bands or no results,

Table 1. Summary of genes sequenced from individuals of both study sites and NCBI accession numbers (acc). Individuals positive (+) for at least two *Wolbachia* specific genes (*wsp*, *ftsZ*, 16S) are considered infected with *Wolbachia*. Equivocal sequences were excluded (-), gender abbreviations are f (female), fem (feminoid) and m (male).

Species	Gender	Individuals	Gene and NCBI accession number (acc)							
			16S	acc	*ftsZ*	acc	*wsp*	acc	*COI*	acc
Bonn										
E. melissae	f	15_a	-		+	JN379610	+	JN379623	+	JN379635
E. melissae	f	55_a	-		+	JN379611	+	JN379624	+	JN379636
E. melissae	f	56_a	-		+	JN379612	+	JN379625	+	JN379637
E. melissae	fem	4_c	+	JN379602	+	JN379613	+	JN379626	+	JN379638
E. melissae	fem	81_c	+	JN379603	-		+	JN379618	+	JN379639
E. melissae	fem	82_c	+	JN379604	+	JN379614	+	JN379627	+	JN379640
Göttingen										
E. melissae	f	215_a	+	JN379605	+	JN379615	+	JN379628	+	JN379641
E. melissae	f	216_a	+	JN379606	-		+	JN379629	+	JN379642
E. melissae	m	257_b	-		-		+	JN379619	+	JN379646
E. melissae	fem	289_c	-		+	JN379616	+	JN379630	+	JN379643
E. melissae	fem	290_c	-		-	-	+	JN379631	+	JN379644
E. melissae	fem	291_c	+	JN379607	+	JN379617	+	JN379632	+	JN379645
E. decemnotata	f	305_a	-		-		+	JN379620	+	JN379634
E. decemnotata	m	322_b	-		+	JN379608	+	JN379621	+	JN379633
E. decemnotata	m	324_b	-		+	JN379609	+	JN379622	-	

Table 2. Summary of PCR screening with three *Wolbachia* specific genes. Populations are grouped into sampling location and species; total number of individuals tested, individuals positive for at least two *Wolbachia* specific genes and frequencies (percentages of total) of infected individuals among genders and populations are summarized. Amplification result for 16S, *ftsZ* and *wsp* are listed.

Population	Number of individual tested		Frequency of infected	Genes		
	Total	Positive for *Wolbachia*	individuals (%)	16S	*ftsZ*	*wsp*
Bonn						
E. melissae	51	32	63			
female	11	9	81	2	9	10
male	9	3	33	0	5	3
feminoid	31	20	64	14	20	22
E. decemnotata	32	0	0			
female	12	0	0	0	0	0
male	11	0	0	0	0	0
feminoid	9	0	0	0	0	0
Göttingen						
E. melissae	49	44	90			
female	24	24	100	24	24	24
male	18	13	72	7	17	13
feminoid	7	7	100	6	7	7
E. decemnotata	25	3	12			
female	12	1	8	3	1	1
male	12	2	17	3	2	3
feminoid	1	0	0	0	0	0

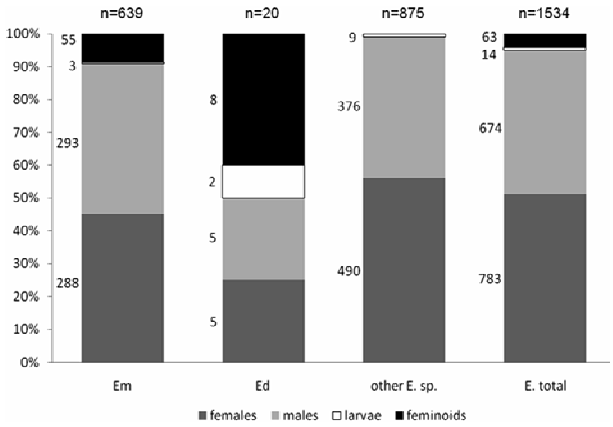

Figure 1. Sex ratio distribution and numbers of females, males and feminoids of the species *E. melissae* (Em), *E. decemnotata* (Ed) and other *Eupteryx* species (other E. sp.) sampled in Bonn. Numbers above columns give the total number of individuals of each species and represents 100%; total number of *Eupteryx* (E. total) is given in the right column. Proportions of females (dark grey), males (light grey), feminoids (black) and nymphs (unknown sex, white) are marked.

screening with additional *Wolbachia* specific primer pairs (Bourtzis *et al*., 1996; Negri *et al*., 2009) and modified PCR-conditions did not improve the screening results. Infection with *Wolbachia* was presumed when at least two genes were amplified unequivocally.

For *E. melissae* 100 individuals were tested for *Wolbachia* and in the Bonn population 63% (9 females, 3 males, 20 feminoids), in the Göttingen population 90% (24 females, 13 males and 7 feminoids) were stated positive for infection.

Of *E. decemnotata* 57 individuals were tested for *Wolbachia.* The population from Bonn was considered uninfected as amplification of the three *Wolbachia* specific genes was ambiguous; the screening with 16S failed completely, in *ftsZ* and *wsp* resulted in multiple bands. Three individuals, one female and two males of Göttingen, were unequivocally positive for *Wolbachia*. The amplification of the *COI* gene was successful and unequivocal in all 157 individuals, indicating that sufficient host DNA was present in all reactions.

Sequences and phylogenetic analysis

BLAST analyses confirmed that all checked 16S rRNA, *ftsZ* and *wsp* sequences were 95-99% identical with *Wolbachia*, and all *COI* sequences were very similar to *COI* sequences of other Cicadellidae in Genbank. All Bayesian and Neighbor-Joining trees were identical or very similar and are available from the corresponding author on request. The 16S rRNA dataset included six sequences of one host species (*E. melissae*) from Bonn and Göttingen. The alignment was gap free, 768 bp long and sequences were very similar. Seven positions were variable among ingroup taxa, the outgroup differed in 15 positions from the ingroup. Similarity of 16S sequences was also reflected in the phylogenetic trees, as *Wolbachia* from both locations separate only slightly and with weak posterior probability and bootstrap support (figure 3A).

Topologies of the phylogenetic trees based on *ftsZ* (10 individuals; figure 3B) and *wsp* (14 individuals; figure 3C) were not entirely congruent and in both phylogenetic trees most sequences of *Wolbachia* separated with weak support, but posterior probabilities and bootstrap supports were higher for clades in the *wsp* based tree.

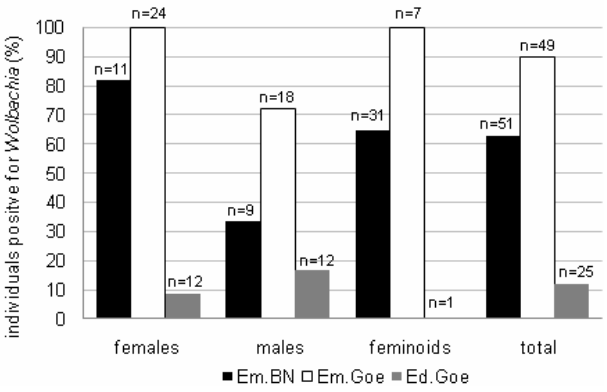

Figure 2. Frequency of *Wolbachia* infection in *E. melissae* and *E. decemnotata* from two populations in Germany (Bonn and Göttingen). Presence of *Wolbachia* was inferred by PCR screening of three *Wolbachia* specific genes (16S rRNA, *ftsZ* and *wsp*) in 157 individuals. Individuals positive for at least two loci were considered infected. Infection frequencies are grouped in gender per population (female, male and feminoid individuals) and the overall infection frequency for each population (total): black bars *E. melissae* (Bonn), white bars *E. melissae* (Göttingen), grey bars *E. decemnotata* (Göttingen); the number of individuals investigated is given above the bars.

However, the *ftsZ* tree consisted of two well supported clusters, one large clade that excluded the sequence isolated from a male individual of *E. decemnotata* (Ed_GOE_m_322_b) from all other sequences, and one that comprised three *Wolbachia* sequences isolated from *E. melissae* from Bonn and Göttingen. *Wolbachia* isolated from female and feminoid individuals of *E. melissae* were never identical.

The tree based on the *wsp* gene comprised three well supported clades that were recovered in all Bayesian and NJ analyses. Similar to the *ftsZ* gene, the sequence isolated from a male individual of *E. decemnotata* (Ed_GOE_m_322_b) was distinct from the remaining individuals and formed an isolated, well supported clade with a sequence isolated from a male of *E. melissae* (Em_GOE_m_257_b). The two other clades included sequences from Bonn and Göttingen of *E. melissae*. *Wolbachia* isolated from three feminoid individuals from Bonn (Em_BN_fem_4c, Em_BN_81_c, Em_BN_82_c) and one female individual from Göttingen (Em_GOE_f_216_a) formed a monophyletic clade. Notably, all sequences in this clade contain a deletion of nine basepairs (5'-GAA AAG GAT-3') between positions 125-133.

The *COI* tree (figure 3D) clearly separated the two species *E. decemnotata* and *E. melissae* in two distinct and well supported clusters. Individuals of *E. melissae*

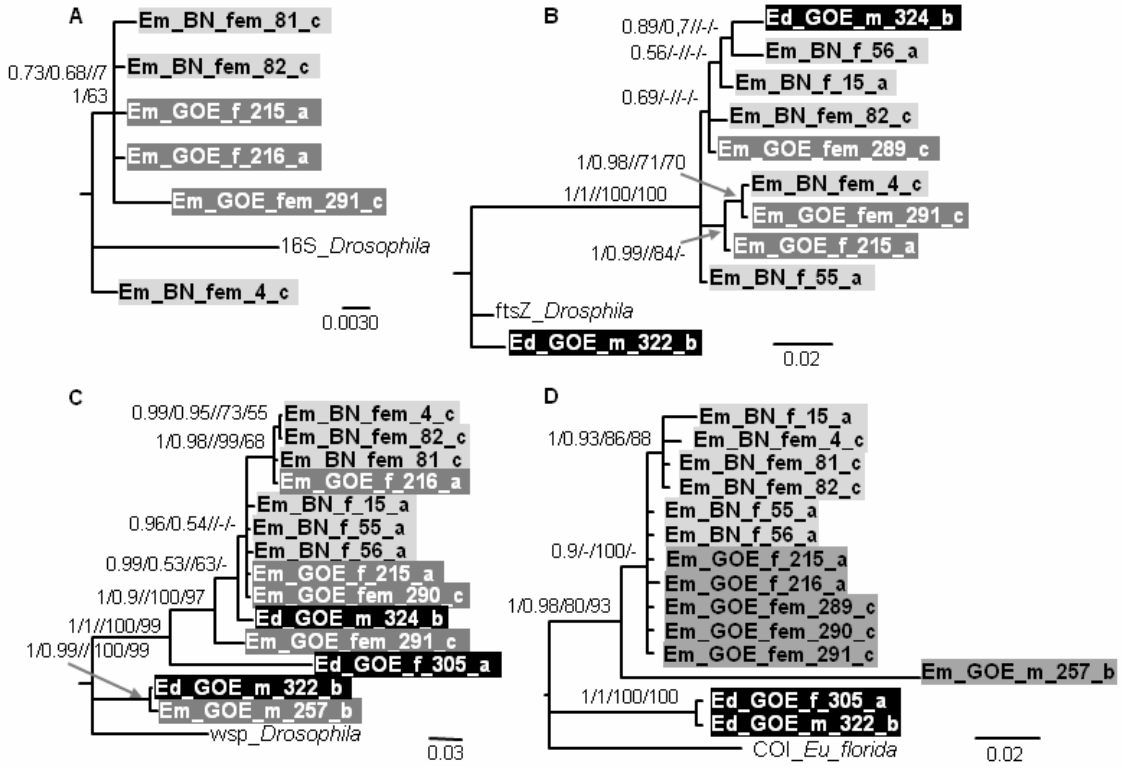

Figure 3. Baysian trees of *Wolbachia* based on partial gene sequences isolated from the two host species *E. melissae* (Em) and *E. decemnotata* (Ed) sampled at the two study sites Bonn (BN) and Göttingen (GOE). Numbers on nodes are posterior probabilities with default/model settings in MrBayes and bootstrap values (10.000 replicates) of the Neighbor-Joining analyses without/with model of sequence evolution, bootstrap supports of <50% are not shown. (A) 16S rRNA gene of *Wolbachia* isolated from six individuals of *E. melissae* from Bonn and Göttingen; (B) *ftsZ* sequences isolated from eight individuals of *E. melissae* from Bonn and Göttingen and two of *E. decemnotata* from Göttingen; (C) *wsp* gene isolated from eleven individuals of *E. melissae* from Bonn and Göttingen and three individuals of *E. decemnotata* from Göttingen; (D) *COI* of the host species isolated from six individuals of *E. melissae* from Bonn and Göttingen and two individuals of *E. decemnotata* from Göttingen. Genders of host individuals are abbreviated as f (female), m (male) and fem (feminoid).

sampled from Bonn and Göttingen were very similar. A monophyletic clade included the individuals Em_BN_f_15_a, Em_BN_fem_4_c, Em_BN_fem_81_c and Em_BN_fem_82_c with high posterior probabilities and good bootstrap support; notably, the three latter individuals in this clade share a nine basepair deletion in the *wsp* gene. The *COI* sequence of individual Ed_GOE_m_324_b had numerous equivocal positions and was excluded from the phylogenetic analysis; however, BLAST results were highly similar with *COI* sequences of Cicadellidae.

In phylogenetic trees including representatives of *Wolbachia* supergroups, *E. melissae* clusters among a strain belonging to supergroup B (16S and *ftsZ*; figure 4), but individuals of *E. decemnotata* cluster among two different strains (only *ftsZ*; figure 5), one of supergroup B (host individual Ed_GOE_m_324_b) and one of supergroup A (host individual Ed_GOE_m_322_b). The backbone of the phylogeny was relatively weak, but terminal branches had high posterior probabilities and supergroup associations are therefore well supported.

Discussion

This study provides the first evidence of *Wolbachia* infection in *Eupteryx* leafhoppers, but with different infection patterns in the two species, *E. decemnotata* and *E. melissae*, and two populations in Germany (Bonn and Göttingen). *Wolbachia* infections commonly cause shifts in the population sex ratio as males might be feminized. This in turn increases the frequency of *Wolbachia* in a population which is inherited maternally.

In this study females and males of the Bonn population were present in all species in an equal ratio, but feminoid specimens were found only in *E. decemnotata* and *E. melissae* with 0.5% and 3.5%, respectively, of all sampled individuals (n = 1534). This is in contrast to many other studies that stated a shift in the sex ratio in the host population induced by *Wolbachia.* However, we may face a very recent infection event and induction of morphological alterations could be related to an infection threshold (Stouthamer *et al*., 1999).

Phylogenetic analyses indicate infection of *Eupteryx* species with different *Wolbachia* strains. For *E. melissae* the phylogenetic trees of the *ftsZ* and *wsp* gene contained well supported clades with host organisms of both sampling locations, suggesting that *E. melissae* was infected only once and that infection spread between sampling locations with its host organism. A deletion of 9 base pairs in the *wsp* gene shared by one individual from Göttingen and three individuals from Bonn further supports a common origin of *Wolbachia* in *E. melissae* of both locations. Phylogenetic analyses based on 16S rRNA and

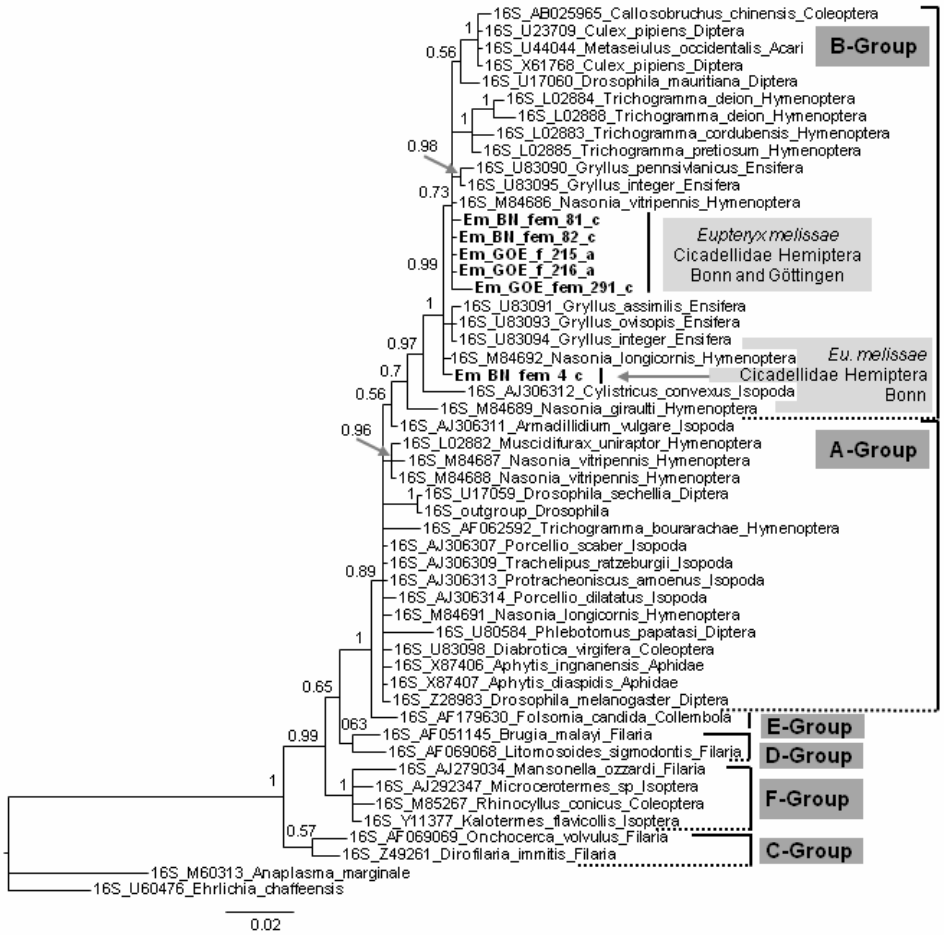

Figure 4. Bayesian tree based on partial 16S rRNA of *Wolbachia* supergroups A-F isolated from different host species after Czarnetski and Tebbe (2004).

ftsZ also confirmed that *E. melissae* was infected by a single strain of supergroup B (figure 4), a strain that is generally common in insects. Migration of individuals between Bonn and Göttingen is possible, either by passive drift in air currents (Koblet-Günthardt, 1975; McKamey, 2002) or anthropogenic introduction, mainly through trade (Nickel and Holzinger, 2006). *E. melissae* is known from Germany since 1910 and from Göttingen since 1994; feminoid individuals were first discovered in 2008 (Nickel and Holzinger, 2006). Higher infection level in the populations at Göttingen and high genetic similarity between *Wolbachia* sequences of both locations suggests a recent common ancestor as well as rapid transfer and spread of *Wolbachia* between both populations by a migrating individual after host populations had established at both sites.

The infection pattern in *E. decemnotata* is more complicated: no sample from the Bonn population could be stated as positive for *Wolbachia* infection, for the population in Göttingen screening and sequencing of the *ftsZ* and *wsp* genes were also difficult as most PCR reactions generated multiple bands or failed completely. Multiple band patterns and poor sequence quality can have two reasons. First, leafhoppers often harbor an assemblage of prokaryotic symbionts that usually provide the host with additional nutrients which are not found in host's diet. Our primers possibly bound unspecifically to prokaryotic DNA other than *Wolbachia*. Second, it is possible that different *Wolbachia* strains are present in one host as found previously in other insects (Fenollar *et al*., 2003; Duron *et al*., 2008). This would explain the presence of numerous SNPs in the relatively fast evolving *ftsZ* and *wsp* genes (Arthofer *et al*., 2009; Schuler *et al*., 2011). Further, two individuals from Göttingen (Ed_GOE_f_305_a, only *wsp* and Ed_GOE_m_322_b, *ftsZ* and *wsp*) were infected with different *Wolbachia* lineages. In contrast, both *ftsZ* and *wsp* sequences of individual Ed_GOE_m_324_b clustered among *E. melissae*, indicating that both species carry the same *Wolbachia* strain, suggesting horizontal transfer of *Wolbachia* between species. The phylogenetic tree based on 16S rRNA including representatives from different supergroups confirmed the finding of two strains in *E. decemnotata*, individual Ed_GOE_m_322_b clusters within supergroup A, whereas individual Ed_GOE_m_324_b carries a strain of supergroup B (figure 5). This is congruent with previous studies showing coexistence of two *Wolbachia* strains in leafhoppers species (Mitsuhashi *et al*., 2002; Shiau *et al*., 2011).

Horizontal transmission among unrelated host species is a widespread phenomenon in *Wolbachia* (Werren *et al*., 1995; O'Neill *et al*., 1997; Vavre *et al*., 1999; Kikuchi and Fukatsu, 2003) and is presumably common in

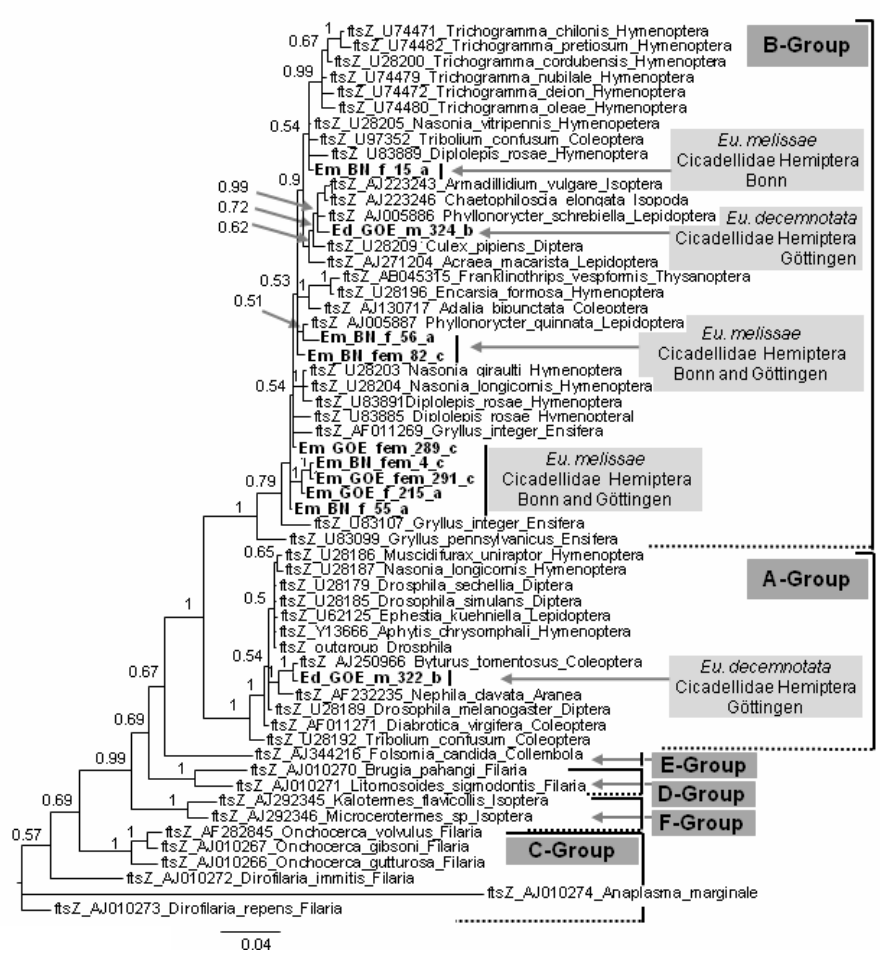

Figure 5. Bayesian tree based on partial *ftsZ* gene of *Wolbachia* supergroups A-F isolated from different host species after Czarnetski and Tebbe (2004).

strains of supergroups A and B (Lo *et al*., 2002). The mechanisms, however, remain unknown (Breeuwer and Jacobs, 1996) but common food resources and parasitoids have been discussed as possible vectors (Mitsuhashi *et al*., 2002; Islam, 2007; Moran, 2008; Stahlhut *et al*., 2010). In some leafhoppers and planthoppers horizontal transmission is proposed since the same *Wolbachia* strains are detected in the host and their parasitoids (Noda *et al*., 2001b) and in two different host species that share the same host plant (Mitsuhashi *et al*., 2002; Noda *et al*., 2001a). In *Eupteryx* leafhoppers the horizontal transmission route *via* the host plant or parasitoids is possible since they often share host plants such as sage and catnip (Nickel, 2003) and parasitoid wasp attacks (notably Dryinidae) are commonly found (Munroe, 1981; Waloff and Jervis, 1987). An indirect infection of leafhoppers by *Wolbachia* infected parasitoids is also possible, but this has not been tested yet and remains unsolved so far. Vertical transmission through interbreeding can be excluded because the two species are morphologically and genetically distinct.

Screening and sequencing results were better for *E. melissae* than for *E. decemnotata*, mostly due to single nucleotide polymorphisms (SNPs) at various sites or strong noise caused by sequencing of unspecific products that interfered with the targeted endobacterium in *E. decemnotata.* Infections with other reproductive parasites are well known in many arthropods (Duron *et al*., 2008), such as Candidatus *Cardinium* (Hunter *et al*., 2003). All samples were screened with a *Cardinium* specific primer pair (CLOf and CLOr1; Weeks and Breeuwer, 2003; data not shown) and results were unequivocally negative. Further studies are needed to understand the dynamics and effects of *Wolbachia* in *E. decemnotata* and *E. melissae*, such as (1) quantification of *Wolbachia*-titer to understand *Wolbachia* infection in males not expressing the modified phenotype, (2) karyotypic visualizations to determine if feminized individuals are genetic males, and (3) localization of *Wolbachia* by electron microscopy and *in-situ* hybridization techniques like FISH.

The fitness and genetic status of feminoid individuals in *E. melissae* and *E. decemnotata* have not been studied yet. However, in the related leafhopper *Z. pullula*, *Wolbachia* induced a transformation of genotypic males into functional females which reproduce. Mating of infected individuals with males was observed but fertility was reduced (Negri *et al*., 2006). If *Wolbachia* infections would have similar effects on feminoid individuals of *Eupteryx* this could be of potential concern to agriculture since available pest management strategies are insufficient to control *Eupteryx* (Jung, 2009) and an increase of the proportion of reproductive individuals in pest populations intensifies damage to host plants. Further, single individuals with aberrant genital morphology of other *Eupteryx* species were recorded since 2009 (H. Nickel, unpublished data), suggesting a rapid spread of *Wolbachia* within the genus and increased risk for herb cultivation. Aside of these concerns, the recent occurrence of *Wolbachia* in *Eupteryx* allows to investigate the infection and transmission rates of *Wolbachia* in a new host species and to study population dynamics in host populations in early stages of infection.

Conclusions

Leafhoppers of the genus *Eupteryx* are important pests on agricultural and medical herbs with fast population growth and high dispersal potential. This study shows that in Germany specimens with intersexual genital morphology (feminoids) are only common in the two species *E. decemnotata* and *E. melissae* but that sex ratios remain equal in populations. Molecular screening indicates that all populations with feminoid individuals are infected with *Wolbachia* and that infections potentially spread fast among populations by host-dispersal and horizontal transmission. Infections appear to be recent and provide a good model system to study host-parasite interactions in natural systems. Regarding the study of Negri *et al*. (2006) who showed that *Wolbachia* infection in the closely related *Z. pullula* turned males with aberrant genital morphology into functional females, this finding in *Eupteryx* have potentially important implications for population dynamics in this genus. As available pest control strategies for the investigated species are insufficient, potential increase of reproductive individuals induced by *Wolbachia* implicates increased damage on crop plants and challenges cultivation of herbs in agriculture. Further, studies on feminoid individuals in *E. decemnotata* and *E. melissae* investigating the karyotype for gender determination and reproductive potential of feminoid individuals and population dynamics will provide insight into host-parasite dynamics and will be of major importance for pest management strategies in agriculture.

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