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Geographic variation in male courtship acoustics and genetic divergence of populations of the *Cotesia flavipes* species complex

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Abstract

Courtship behaviors of insect populations can vary across the range of a species. Populations exhibiting divergent courtship behavior may indicate genetic divergence or cryptic species. Courtship acoustic signals produced by male wing fanning and genetic structure (using amplified fragment length polymorphisms) were examined for seven allopatric populations of the *Cotesia flavipes* (Hymenoptera: Braconidae) species complex, using four *C. sesamiae* (Cameron) and three *C. flavipes* Cameron populations. Members of this species complex parasitize lepidopteran pests in gramineous crops including sugarcane, maize, and rice. Significant variation was detected in courtship acoustic signals and genetic structure among populations of both species. For *C. sesamiae*, courtship acoustic signals varied more between populations of two biotypes that were collected near an area of sympatry. The two biotypes of *C. sesamiae* were also genetically divergent. For *C. flavipes*, significant differences in acoustic signals and genetic structure occurred among allopatric populations; these differences support the recent designation of one population as a new species. Courtship acoustics play a role in reproductive isolation in this species complex, and are likely used in conjunction with chemical signals. Ecological factors such as host range and host plant use may also influence the divergence of both courtship acoustic signals and genetic structure among populations in the *C. flavipes* complex.

Introduction

Courtship behavior can vary among populations across the geographic range of a species, indicating genetic divergence or reproductive isolation of populations. Few studies have investigated courtship behavior signals in multiple populations of a parasitoid species, or within a species complex (van den Assem & Putters, 1980; Geden et al., 1998). The courtship signals from a species complex of parasitoid wasps might be used to detect genetically divergent populations that are reproductively isolated but have little morphological differentiation (Henry et al., 2002; Lin & Wood, 2002).

The courtship behavior of parasitoid wasps includes chemical, tactile, and acoustic signals (Quicke, 1997). Male parasitoid wasps, including the genus *Cotesia* in the family Braconidae, fan their wings when in close range of females, and this produces low amplitude sounds and substrate vibration signals (van den Assem & Putters, 1980; Sivinski & Webb, 1989; Field & Keller, 1993; Kimani & Overholt, 1995; Joyce et al., 2008, in press). Male wing fanning may function both in chemical communication (Vinson, 1978; Ruther et al., 2000), as well as to produce acoustic signals, which increase mating success (van den Assem & Putters, 1980; Sivinski & Webb, 1989; Field & Keller, 1993; Joyce et al., 2008). Females of *Cotesia marginiventris* (Cresson) are more likely to mate with males when courtship vibration signals are longer in duration and louder in amplitude (Joyce et al., 2008). Parasitoid courtship acoustic signals may be species-specific and involved in reproductive isolation between species, but few studies have been conducted of the relative importance of acoustic signal components.

According to Polaszek & Walker (1991) and Kimani & Overholt (1995), the *Cotesia flavipes* (Hymenoptera:

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Braconidae) species complex consists of three species, *C. flavipes* Cameron, *C. sesamiae* (Cameron), and *C. chilonis* (Matsumura). Recently, a fourth species, *C. nonagriae* (Olliff), was separated taxonomically from *C. flavipes* (Muirhead et al., 2008). These parasitoids have been used in biological control of noctuid and crambid stemborers in more than 40 countries (Polaszek & Walker, 1991; Overholt et al., 1997; Potting et al., 1997). Each of these parasitoid species has a broad, native geographic range, and can be difficult to distinguish based on morphology. *Cotesia sesamiae* is indigenous to Africa, *C. flavipes* is native to the Indo-Australian region, *C. chilonis* is known from Japan and eastern China, while *C. nonagriae* occurs in Australia (Kimani-Njogu & Overholt, 1997; Muirhead et al., 2008). The species of interest in this study, *C. sesamiae* and *C. flavipes*, have allopatric distributions on separate continents.

In addition, *C. sesamiae* has two biotypes with differential ability to develop in *Busseola fusca* Fuller. The virulent biotype of *C. sesamiae* occurs in western and central Kenya, and develops in larvae of two stemboring moths, *B. fusca* and *Sesamiae calamistis* Hampson (Gitau et al., 2006; Figure 1). The avirulent biotype is known from central and eastern Kenya, and develops in *S. calamistis* but not in *B. fusca* (Ngi-Song et al., 1998; Mochiah et al., 2002; Gitau et al., 2006; Figure 1). In central Kenya, both biotypes can occur sympatrically (Dupas et al., 2008). All species in the complex are gregarious, with broods consisting of ca. 40 offspring per host (Wiedenmann et al., 1992; Kimani-Njogu & Overholt, 1997). Male *C. flavipes* emerge before females (Arakaki & Ganaha, 1986) and are expected to exhibit sibling mating and competition for mates. However, observations of emergence and mating on plant leaves found ca. 20% of females mated locally, and there was little evidence of male competition for mates (A Joyce, pers. obs.; Joyce et al., 2009).

Figure 1 Four collection localities for *Cotesia sesamiae* in Kenya. Populations from Kitale and Meru are the virulent biotype (open circles), which develops in *Busseola fusca* and *Sesamiae calamistis*. Populations from Muranga and Mombasa are the avirulent biotype (solid circles), which develops in *S. calamistis* but is encapsulated in *B. fusca*.

Differentiation of species and populations in the *C. flavipes* complex, and determination of reproductive isolation have been investigated using morphological, ecological, behavioral, and molecular methods (Polaszek & Walker, 1991; Kimani-Njogu & Overholt, 1997; Cole et al., 2003; Michel-Salzat & Whitfield, 2004; Muirhead et al., 2006). Prior observations of courtship behavior of *C. sesami*ae and *C. flavipes* found that males wing fan toward females prior to mating (Kimani-Njogu & Overholt, 1997), but courtship acoustic signals were not recorded. Courtship acoustic signals may be species-specific, or populations with divergent courtship behavior could suggest the presence of biotypes or cryptic species in this complex. The species in this study are allopatric in distribution. Allopatric species may not be under selective pressure for courtship signal divergence, whereas sympatric species can be subject to reinforcement, under which selection against hybridizing individuals can result in behavioral divergence (Greenfield, 2002; Coyne & Orr, 2004). However, insect courtship signals have been found to diverge both in allopatric populations as well as in populations of species with sympatric distributions (Bordenstein et al., 2000; Cokl & Virant-Doberlet, 2003; Jang & Gerhardt, 2006; Rodriguez & Cocroft, 2006).

Species designations for *C. flavipes* and *C. sesamiae* are supported by two molecular phylogenetic studies: the first was based on sequence data from two genes, mitochondrial 16S rRNA and NADH 1 dehydrogenase (Smith & Kambhampati, 1999), and the second on four genes, mt16S rDNA, n28s rDNA, NADH 1, and LW Rh (Michel-Salzat & Whitfield, 2004). Also, genetic divergence among populations was detected within both *C. flavipes* and *C. sesamiae* using allozyme data and two mitochondrial genes, 16S rRNA and CO1 (Kimani-Njogu et al., 1998; Muirhead et al., 2006). For *C. flavipes*, 3.0% sequence divergence was found among populations from India/Pakistan and Australia, while *C. sesamiae* formed two genetically distinct groups with 2.5% sequence divergence, one group occurring in west and one in east Kenya which correspond to the virulent and avirulent biotypes (Muirhead et al., 2006).

The first objective of the present study was to determine whether courtship acoustic signals varied among populations of *C. sesamiae* and *C. flavipes*. The acoustic signals were expected to vary more between species (*C. sesamiae* and *C. flavipes*; *C. flavipes* and *C. nonagriae*) than within populations of a species, and also may vary between biotypes of *C. sesamiae* (virulent and avirulent). The second objective was to assess genetic divergence among these same populations using amplified fragment length polymorphisms (AFLPs). Amplified fragment length polymorphisms are used to examine gene flow among populations, and can be used to infer reproductive isolation. Genetic divergence was expected between species and between the two biotypes of *C. sesamiae*. Courtship acoustic signals or genetic markers might detect cryptic species if they exist in this complex. The patterns of acoustic signal divergence among populations are discussed in relation to the patterns of genetic divergence, and in regards to the role of courtship acoustics in reproductive isolation in this species complex.

Materials and methods

Insect collections

Four populations of *C. sesamiae* and three populations of *C. flavipes* were used in this study. Four allopatric populations of *C. sesamiae* were collected in Kenya and shipped as pupae from the International Center for Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya to a quarantine facility at Texas A&M University. Two populations of the virulent *C. sesamiae* biotype were collected in Kitale (1°01′N, 35°0′E) and Meru (0°03′N, 37°39′E) in western and central Kenya, respectively, from *B. fusca* larvae on maize (Figure 1). The other two populations were avirulent and they were collected in Muranga (0°43′S, 37°9′E) and Mombasa (4°3′S, 39°40′E) in central and eastern Kenya, respectively, from *S. calamistis* on maize (Figure 1). Prior to shipment to Texas, the *C. sesamiae* populations from Kitale, Muranga, and Mombasa were reared at ICIPE, Kenya for eight generations, while the Meru population was reared for 20 generations.

Two of the three populations of *C. flavipes* were obtained in areas outside of the species' native distribution, where they had been introduced for biological control. One population was collected in Mombasa, Kenya, reared from larvae of the natural host *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) on maize. *Cotesia flavipes* was previously introduced to Kenya from Northern Pakistan in 1993 (Overholt et al., 1997). This population was reared for one generation at ICIPE, Kenya, prior to shipment to Texas. A second population of *C. flavipes* was obtained from a laboratory colony in Weslaco, Texas. This colony was supplemented several times per year with field-collected *C. flavipes* reared from *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae) on sugarcane (*Saccharum* spp.). *Cotesia flavipes* was introduced into Texas from both India and Pakistan in 1977–1978 for biological control of *D. saccharalis* (Fuchs et al., 1979). Finally, a third *C. flavipes* population was collected from the larvae of *Bathytricha truncata* (Walker) (Lepidoptera: Noctuidae) on sugarcane in Bundaburg (24°51′S, 152°21′E), Australia. In a study conducted concurrently with the present one, the Australian population was separated from *C. flavipes* and designated a new species, *C. nonagriae* (Muirhead et al., 2008, 2010). We refer to this population as the Australian *C. flavipes*. This population was reared for one generation in Australia prior to shipment to Texas A&M University. All parasitoids used in experiments were isolated individually as cocoons in 1 ml glass vials plugged with cotton, so that emerging adults remained virgin. Adult parasitoid wasps were fed by placing a streak of honey inside each vial.

Comparing airborne and substrate courtship components

The airborne sounds and substrate vibrations produced by male wing fanning were compared prior to recording airborne sounds from all populations, to determine whether airborne and substrate patterns were identical. This was necessary because some insects produce identical patterns of airborne sounds and substrate vibrations during courtship (van den Assem & Putters, 1980; Stölting et al., 2002), while others produce airborne sounds with no measurable substrate vibration component (Michelson et al., 1986). The Texas population of *C. flavipes* was used for this comparison because it was readily available.

All wasps used for courtship recordings were <2 days old. Recordings were made at 24 ± 2 °C in an enclosed sound reduction chamber (ca. 1 × 0.75 × 1 m) on a vibration isolation table (TMC™, Model NAF 2000; Peabody, MA, USA). One female and one male wasp were placed in a plastic Petri dish (4 cm in diameter) with an organdy bottom. A laser Doppler vibrometer (Model OFV 353; Polytec, Tustin, CA, USA) (1 mm s^{-1} per volt sensitivity) was positioned above the Petri dish. The beam was focused on reflective tape (4 mm^2) placed onto the organdy bottom of the dish. A condenser microphone (Model C-1000; AKG, Nashville, TN, USA) with a frequency response of 20–20 000 Hz ± 2 dB, was positioned 0.5 cm below the Petri dish arena. The Petri dish arena was supported by resting the outer edges of the dish on two wooden dowels (ca. 2 mm in diameter) clamped to a ring stand, so that the arena was suspended above the microphone and below the laser beam. Both airborne and substrate components were digitized (16 bit, 44 kHz) and recorded

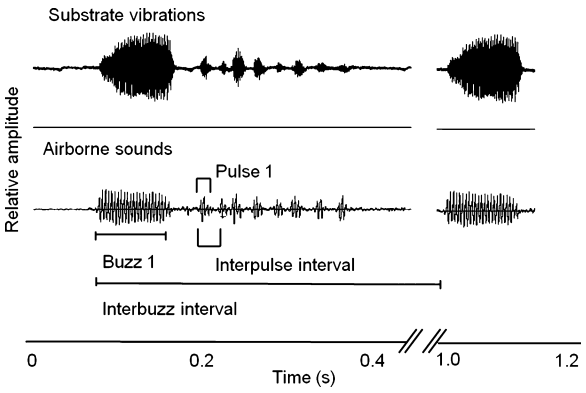

Figure 2 Comparison of substrate vibrations and airborne sound patterns produced during courtship by male wing fanning of *Cotesia flavipes* from Texas. The bottom panel shows the components used for comparison of acoustic parameters among populations. The components compared included the buzz 1, interbuzz interval, pulse 1, and interpulse interval.

simultaneously using Peak software 3.0 (Bias, Petaluma, CA, USA). Ten courting pairs were each recorded for 10 min. Males begin to fan their wings when they are several cm from a female, and continue to fan them intermittently until they attempt to copulate. The first wing fanning bout was used for analyses. The courtship acoustic signals produced by male wing fanning consisted of two parts. The first component was a relatively long buzzing sound (hereafter 'buzz 1'), followed by several pulses of shorter duration (Figure 2). Adobe Audition 2.0 (San Jose, CA, USA) was used to quantify the duration (ms) and fundamental frequency (Hz) of the buzz 1 component (Figure 2). The data for each parameter were compared between airborne and substrate recordings with paired t-tests using SPSS 12.0 (SPSS, 2001). Data are presented here as they are preliminary to the population comparisons in the following section. The airborne courtship sounds and substrate vibrations for males from the *C. flavipes* Texas population had similar mean buzz 1 durations [airborne, 84.25 ± 3.65 ms (mean ± SEM); substrate, 85.25 ± 3.69 ms; $t = 2.0$, d.f. = 9, $P = 0.09$] and frequencies (airborne, 280.50 ± 10.23 Hz; substrate, 284.63 ± 10.42 Hz; $t = 1.9$, d.f. = 9, $P = 0.11$).

Population comparisons of courtship acoustics

The airborne sound and substrate vibration components of the courtship acoustics of *C. flavipes* Texas were found to be similar (see previous section), so subsequent recordings of virgin pairs from each of the seven populations described above were made using only a condenser microphone (described above) to record the airborne component of courtship. Recordings were done at 25 ± 1 °C. Each pair was placed in an arena (described above) and the male wing fanning was recorded for 10 min or less if mating occurred. Recordings were made for 20 *C. sesamiae* pairs from Kitale, 17 from Meru, 20 from Muranga, and 21 from Mombasa. For *C. flavipes*, 19 pairs were recorded from Kenya, 21 pairs from Texas, and 22 from Australia. All males and females were used for only one courtship recording. Each male used was from a different brood. After recordings, the male of each pair was stored in an ultra cold freezer at −80 °C for subsequent AFLP analysis.

The buzz 1 and pulse 1 components of the first wing fanning bout of each courting male were used to measure six parameters: buzz 1 duration, buzz 1 frequency, interbuzz interval (time between the start of buzz 1 and the start of the subsequent buzz), pulse 1 duration, pulse 1 frequency, and interpulse interval for each population. One-way ANOVA was used to compare means for each parameter among different populations within each species. The normality assumptions of ANOVA were not met for buzz 1 duration and interbuzz interval (SPSS, Levene's test), so data were log transformed for analyses. Where significant differences were detected, Tukey's test was used for pairwise comparisons of means (Sokal, 1995; SPSS, 2001).

AFLP markers for *Cotesia sesamiae* and *Cotesia flavipes*

Males from courtship recordings (stored previously at −80 °C), and several additional males from each population, were used for DNA extractions. DNA was extracted from whole males with a QIAGEN© (Valencia, CA, USA) DNeasy extraction kit following their protocol (Qiagen, 2006), but with an incubation at 65 °C for 2 h. The DNA concentration (ng μl^{-1}) for each sample was quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA). Samples were standardized at concentrations of 30 ± 5 ng μl^{-1}, using an Eppendorf Vacufuge Plus (Westbury, NY, USA). For AFLP markers (described below), ca. 150 ng of sample DNA were used per reaction.

Amplified fragment length polymorphisms (Vos et al., 1995; Saunders et al., 2001) were used to obtain genetic fingerprints for each individual parasitoid. Males from the seven populations were randomized on two 96-well plates for AFLP reactions (total males = 154; ca. 22 per population). Each restriction/ligation reaction (well) consisted of the following: 0.05 µl each of *Eco*RI and *Mse*I, 1.1 µl of T4 DNA Ligase Buffer, 1.1 µl of 0.5 M NaCl, 0.55 µl of diluted BSA (bovine serum albumin), 0.03 µl of T4 DNA Ligase, 1.0 µl each of *Eco*RI and *Mse*I adaptor pairs (Applied Biosystems, Foster City, CA, USA), and 0.61 µl of sterile distilled water. The plate with restriction/ligation reactions was held at room temperature overnight (ca. 12 h at 25 °C) to ensure complete digestion (Saunders et al.,

2001). The amplified product was diluted 20-fold using 15 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA.

Pre-selective PCR amplification was performed on an Applied Biosystems thermocycler (GeneAmp® PCR System 9700). Each reaction contained 15 μl of AFLP Preselective Mix (Applied Biosystems), 1 μl of each amplification primer [i.e., *Eco*RI and *Mse*I (Applied Biosystems)], along with 4 μl of the diluted restriction/ligation mixture. The PCR program for pre-selective amplification consisted of an initial warm-up of 95 °C for 1 min followed by 20 cycles at 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 90 s with a final hold at 75 °C for 5 min. The amplified product was diluted 20-fold using 15 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA. Selective amplification was conducted using five primer combinations. For each selective amplification, a reaction consisted of 15 μl of AFLP Platinum Supreme Mix, 1.0 μl of *Eco*RI + 3 (or 2) selective primer, and 1.0 μl of *Mse*I + 3 selective primer. The following five selective primer combinations were used: (1) M-CAT/E-ACT, (2) M-CAA/E-ACT (3) M-CAT/E-AC, (4) M-CAT/E-AG, and (5) M-CAC/E-AG (all Applied Biosystems). The PCR program for selective amplification consisted of an initial warm-up of 95 °C for 1 min, 12 cycles of 95 °C for 20 s, 65 °C for 40 s with a lowering of 0.7 °C per cycle, 72 °C for 90 s, followed by 35 cycles of 95 °C for 20 s, 56 °C for 40 s, 72 °C for 90 s, and finally a hold of 72 °C for 7 min before storing the samples at 4 °C. Prior to capillary electrophoresis, 9 μl of the Genescan™ 400HD ROX™ size standard and 0.5 μl of HiDi™ formamide (Applied Biosystems) were added to 1 μl of the final product of each sample. Sample fragments were separated using automated capillary electrophoresis by the ABI 3100 Automated Capillary DNA sequencer. GeneMapper® version 4.0 (Applied Biosystems) was used to determine presence or absence of fragments. Peaks were examined by eye to ensure the peak detection threshold was at least 1.5 times higher than the mean background level. The peak detection threshold was set for each primer combination, and was typically 100 luminescent units. Each AFLP marker was considered a locus and assumed to have two possible alleles (0 = absent, 1 = present). Bands not present in more than one individual were eliminated (i.e., private alleles) prior to further analyses, as they were not considered informative.

AFLP marker analyses

The SESim method (Medina et al., 2006) was used to determine the number of individuals and markers needed in order to adequately represent the genetic variation of the populations sampled in this study. SESim represents the standard error of the mean similarity index of bootstrapped matrices based on specified number of individuals and markers taken from a sample (Medina et al., 2006). The mean similarity index is calculated using all the similarity values in each of the matrices considered. Matrices are bootstrapped selecting different number of individuals and markers from a molecular marker database created using the study organism collected from the study area of interest. A SESim value <0.05 indicates consistency in the clustering pattern produced by a specific combination of markers and individuals for the studied organism at the geographic scale considered (Medina et al., 2006).

Structure 2.2 software (Pritchard et al., 2000) was used to group individuals with similar genotypes within each species. Structure 2.2 uses a Bayesian algorithm to cluster individuals into K, which is defined as the number of populations in a data set. Parameters used for this analysis include the following: no a priori assignment of individuals to a known population, analysis for haploid insects, a burn-in of 10 000 iterations, an admixture model, and independent loci. The number of potential populations for K was estimated as the number of geographic sampling locations plus 4 (*C. sesamiae*, K = 8; *C. flavipes*, K = 7) as suggested by Pritchard et al. (2000), and each iteration was run 20 times. At the completion of Structure 2.2 runs, ΔK was calculated for each species (Evanno et al., 2005), to determine the most likely number of population clusters (K) for each species. The presence or absence of bands from the AFLP work was used to calculate Nei & Li's (1979) genetic distance between all pairs of populations using the Restdis subprogram in Phylip 3.68.

Results

Population comparisons of courtship acoustics

For *C. sesamiae*, the duration of buzz 1, the interbuzz interval and the buzz 1 frequency differed significantly among populations. The population from Muranga had the shortest buzz 1 duration, Kitale had the longest duration, while Meru and Mombasa were intermediate (ANOVA: $F_{3,74} = 32.08$, $P<0.001$) (Figure 3A). The interbuzz interval was longest in the Meru, shortest in the Muranga, and intermediate in the Kitale and Mombasa populations (ANOVA: $F_{3,74} = 4.97$, $P<0.001$) (Figure 3B), while the buzz 1 frequency was lowest in Meru, highest in Muranga, and intermediate in the Kitale and Mombasa populations (ANOVA: $F_{3,74} = 21.32$, $P<0.001$) (Figure 3C). For all three courtship acoustic parameters, significant differences were detected between the Meru and Muranga populations (Figure 3A–C).

For *C. flavipes*, the duration of buzz 1 differed significantly among populations (ANOVA: $F_{2,61} = 79.67$, $P<0.001$) (Figure 3A). The Australian population had a

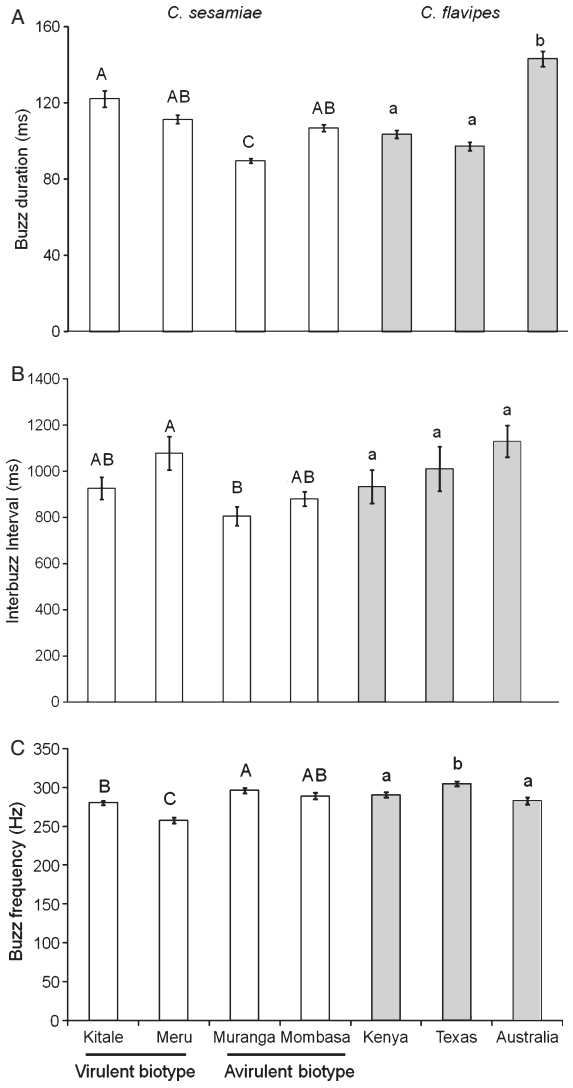

Figure 3 Comparison of the mean (± SEM) (A) buzz 1 duration, (B) interbuzz interval, and (C) buzz frequency of courtship for populations of *Cotesia sesamiae* and *C. flavipes*. Different upper-case letters above columns indicate significant differences within *C. sesamiae*, whereas different lower-case letters above columns indicate significant differences among *C. flavipes* populations (one-way ANOVA, followed by Tukey's test: $P<0.05$).

longer buzz 1 duration than the Kenya or Texas populations, while the duration did not vary between the Kenya and Texas populations (Figure 3A). There were no significant differences in interbuzz intervals among the populations (ANOVA: $F_{2,60} = 5.02$, $P = 0.08$) (Figure 3B). The buzz 1 frequency of the Texas *C. flavipes* population was higher than those of the Kenyan or Australian populations (ANOVA: $F_{2,62} = 11.83$, $P<0.05$) (Figure 3C).

For *C. sesamiae*, the duration of pulse 1, the interpulse interval, and pulse 1 frequency differed significantly among populations. The population from Muranga had a significantly shorter pulse 1 duration than Kitale and Mombasa (ANOVA: $F_{3,73} = 4.25$, $P<0.001$) (Table 1). The pulse 1 interval was shortest in Muranga, longest in Mombasa, and intermediate in the Kitale and Meru populations (ANOVA: $F_{3,72} = 4.61$, $P = 0.005$), while the pulse 1 frequency was lowest in Meru, highest in Muranga, and intermediate in the Kitale and Mombasa populations (ANOVA: $F_{3,73} = 10.95$, $P<0.001$) (Table 1).

For *C. flavipes*, the duration of pulse 1 did not differ among populations (ANOVA: $F_{2,62} = 0.81$, $P = 0.45$), nor did the interpulse interval (ANOVA: $F_{2,60} = 2.46$, $P = 0.09$) (Table 1). The pulse 1 frequency of the Texas *C. flavipes* population was higher than those of the Kenyan or Australian populations (ANOVA: $F_{2,62} = 17.81$, $P<0.05$) (Table 1).

AFLP markers for *Cotesia sesamiae* and *Cotesia flavipes*

The five primer combinations used in this study generated 111 markers for 73 individuals of *C. sesamiae*, and 114 markers for 81 individuals of *C. flavipes*. These numbers of individuals and markers adequately represented the populations involved in this study, producing a SESim value <0.05, which indicates consistency in the clustering pattern produced by a specific combination of markers and individuals for the studied organism at the geographic scale considered (Medina et al., 2006). The *C. sesamiae* individuals sampled in this study grouped into two clusters ($\Delta K = 2$), corresponding to the two biotypes. The first cluster included the populations from Kitale and Meru, and the second cluster contained the populations from Muranga and Mombasa (Figure 4A). The first cluster had 104 polymorphic markers including 12 fixed markers, present in all individuals from these two populations. One marker was specific to Meru and not present in any other population. The second cluster containing the Muranga and Mombasa populations had 93 polymorphic markers and nine fixed markers. Eight markers found in the first cluster (virulent biotype) were not present in the second cluster (avirulent biotype), while four markers found in the avirulent biotype were not present in the virulent biotype.

The *C. flavipes* populations also grouped into two clusters ($\Delta K = 2$) (Figure 4B). The first cluster consisted of the populations from Kenya and Texas, and the second cluster of *C. flavipes* from Australia. The populations from Kenya and Texas had 95 polymorphic loci and 10 fixed loci. A single marker was present in the Kenya population but not in the Texas population. The population from Australia had 92 polymorphic loci and

Table 1 The mean (± SEM) for three courtship acoustic parameters in *Cotesia sesamiae* and *C. flavipes*

Species	Population	Pulse 1 duration (ms)	Interpulse interval (ms)	Pulse 1 frequency (Hz)
C. sesamiae	Kitale	13.85 ± 1.50a	24.10 ± 1.77b	243.81 ± 6.11b
	Meru	11.94 ± 1.04ab	22.76 ± 1.38b	222.34 ± 4.68c
	Muranga	9.35 ± 0.45b	20.20 ± 0.55b	277.75 ± 5.22a
	Mombasa	12.14 ± 0.61a	27.50 ± 2.00a	262.90 ± 7.38ab
C. flavipes	Kenya	12.16 ± 1.26A	27.37 ± 3.45A	250.57 ± 4.17B
	Texas	13.78 ± 1.38A	32.82 ± 3.37A	289.88 ± 6.04A
	Australia	11.77 ± 1.29A	24.14 ± 1.47A	249.61 ± 5.29B

Different lower-case letters indicate significant differences within populations of *C. sesamiae*, whereas different upper-case letters indicate differences in populations of *C. flavipes* (one-way ANOVA, followed by Tukey's test: $P<0.05$).

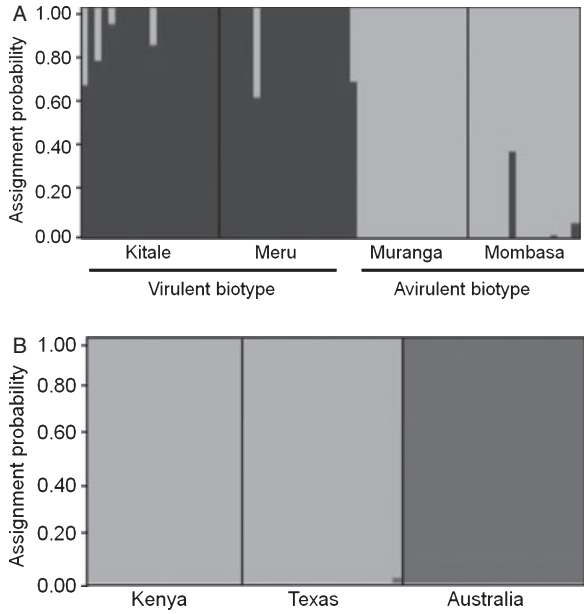

Figure 4 Results from Structure analysis of amplified fragment length polymorphisms (AFLPs) showing clusters of individuals with similar genotypes. Each column represents an individual. The y-axis shows the Bayesian assignment probability, the probability of an individual being assigned to a particular genetic cluster. (A) The x-axis shows four collection localities and two biotypes for *Cotesia sesamiae*. *Cotesia sesamiae* had two genetically divergent populations (K = 2), which corresponded to the two biotypes (virulent, avirulent). Dark grey represents the probability of an individual being assigned to the virulent biotype, whereas light grey represents the probability of being assigned to the avirulent biotype. (B) The x-axis represents three collection localities for *C. flavipes*. The *C. flavipes* collections clustered into two genetically divergent groups, one (light grey) consisting of *C. flavipes* from Kenya and Texas, and the other (dark grey) the Australian population, which is now a new species, *C. nonagriae*.

21 fixed loci. The populations from Kenya and Texas had 13 markers that were not present in the Australia populations, while the Australian population had 10 markers not present in the Kenya or Texas populations of *C. flavipes*.

The Nei-Li genetic distance between the two biotypes of *C. sesamiae* averaged 0.14 (Table 2). The interspecific genetic distances between the *C. sesamiae* and *C. flavipes* populations introduced into Kenya and Texas were in the range of 0.21–0.27, while the *C. sesamiae* populations were even more distant (0.27–0.29) from the Australian *C. flavipes* population. Finally, the *C. flavipes* populations introduced into Kenya and Texas were 0.18 distant from the Australian population (Table 2).

Discussion

Hymenopteran parasitoids are reported to have species-specific acoustic courtship signals (van den Assem & Putters, 1980; Sivinski & Webb, 1989; Joyce et al., in press), but the extent of signal variation among populations within a species has not been studied, nor have these signals been compared among members of a parasitoid species complex. Male wing fanning during courtship has been observed for species in the *C. flavipes* complex, but previous studies did not record or quantify the courtship acoustic signals (Kimani & Overholt, 1995). *Cotesia* spp. produce airborne (near-field) sounds and substrate vibrations, both of which are important in courtship communication (Sivinski & Webb, 1989; Field & Keller, 1993; Joyce et al., 2008). Our simultaneous recordings of airborne sounds and courtship vibrations for *C. flavipes* yielded similar recordings, and we used airborne courtship signals for comparisons among populations.

Courtship acoustic signals varied significantly among populations for both *C. sesamiae* and *C. flavipes*. For *C. sesamiae*, there was a pattern of significant differences in all buzz 1 parameters between the Meru and Muranga populations. The Meru population is the virulent biotype which develops in *B. fusca*, while the Muranga population is avirulent, unable to develop in *B. fusca*. Although the *C. sesamiae* populations from Meru and Muranga are allo-

Table 2 The Nei-Li genetic distance between each pair of the seven populations included of the *Cotesia flavipes* complex

Population	Cs Kitale	Cs Meru	Cs Muranga	Cs Mombasa	Cf Kenya	Cf Texas
Cs Kitale	0	–	–	–	–	–
Cs Meru	0.012	0	–	–	–	–
Cs Muranga	0.140	0.152	0	–	–	–
Cs Mombasa	0.128	0.142	0.014	0	–	–
Cf Kenya	0.228	0.217	0.268	0.250	0	–
Cf Texas	0.218	0.205	0.255	0.231	0.024	0
Cf Australia	0.280	0.277	0.288	0.270	0.175	0.179

Cs = *C. sesamiae*, Cf = *C. flavipes*

patric populations, the Muranga population was collected in an area where the two *C. sesamiae* biotypes can occur sympatrically (i.e., adjacent fields), although they have not been collected in the same field (Dupas et al., 2008).

Previous studies found that intermating between the *C. sesamiae* biotypes in the laboratory results in reduced fitness. A cross between female *C. sesamiae* from Mombasa (avirulent biotype) with *C. sesamiae*-Kitale males (virulent biotype) had eggs encapsulated when ovipositing in *B. fusca* (Ngi-Song et al., 1998). Cross mating in the other direction between *C. sesamiae*-Kitale females and male *C. sesamiae* from Mombasa only produced male offspring in either host, due to male infection with *Wolbachia* (Ngi-Song et al., 1998; Mochiah et al., 2002; Gounou et al., 2008). An additional study examined mating frequencies within and between biotypes (Gounou et al., 2008); ca. 30% of pairings resulted in mating within each biotype, as well as the pairing between virulent males with avirulent females. However, mating frequency was reduced to ca. 20% when virulent females were paired with avirulent males. Thus, strong selection against hybrids is likely if the two biotypes outcross in the field. Infection with microorganisms such as *Wolbachia* can result in reproductive isolation and has been suggested to promote speciation (reviewed in Engelstädter & Hurst, 2009). Cytoplasmic incompatibility between populations can result in a barrier to gene flow and lead to pre-mating isolation and genetic divergence of populations.

The two populations of *C. sesamiae* (Muranga and the Meru) collected closest to where the two biotypes occur sympatrically had significant differences in the buzz 1 durations and interbuzz intervals; however, significant differences were not found for these same courtship parameters between two more distantly allopatric populations from Kitale and Mombasa. Buzz frequencies were significantly different between the Muranga and Meru populations, and also varied among the four *C. sesamiae* populations. The pulse 1 parameters of *C. sesamiae* populations did not show consistent differences with respect to geography. The acoustic courtship signals may have diverged between the biotypes due to reduced intermating between populations (Coyne & Orr, 2004). It is also possible that the differences in the buzz 1 duration of the Meru (virulent) and Muranga (avirulent) populations play a part in reproductive isolation of the two biotypes in the field. However, both these hypotheses remain to be tested.

The difference in courtship buzz durations observed in this study may contribute to the reduced mating frequency of crosses between *C. sesamiae* biotypes observed by Gounou et al. (2008). Few studies of parasitoid wasps have investigated female choice of mating partners (Gu & Dorn, 2003; de Boer et al., 2007; Joyce et al., 2009). Reductions in male courtship buzz durations for the parasitoid *C. marginiventris* reduced mating success (Joyce et al., 2008), demonstrating the importance of the buzz duration and its potential role in female choice. The buzz duration and interbuzz intervals of courtship may similarly influence mating success for parasitoids in the *C. flavipes* species complex. In *Drosophila* species, male courtship songs of *D. persimilis* and *D. pseudoobscura* varied by ca. 30 ms in the interpulse interval of the high rate repetition song. Mating was reduced in the interspecific cross of *D. persimilis* females and *D. pseudoobscura* males, but not in the reverse cross, suggesting that courtship song could be more important for *D. persimils* than for *D. pseudoobscura* (Noor & Aquadro, 1998). *Drosophila* spp. use both acoustic and chemical signals during courtship (Rybak et al., 2002). Reproductive isolation in the *C. flavipes* complex may similarly be mediated by both courtship acoustics and pheromones (Kimani-Njogu & Overholt, 1997), as in other Hymenoptera (Ayasse et al., 2001). Other insects such as lacewings and treehoppers have larger interspecific differences in the duration of courtship song components (ca. 100 ms), but may use primarily vibrational courtship signals and not pheromones (Henry et al., 2002; Rodriguez & Cocroft, 2006).

The Kitale and Meru populations of *C. sesamiae* (virulent biotype) clustered together by AFLP genetic analysis, as did the Muranga and Mombasa populations. The Nei-Li genetic distance between the *C. sesamiae* biotypes averaged 0.14, which is similar to that found here between the two *C. flavipes* populations and the Australian population (0.18), which is now designated a new species, *C. nonagriae* (Muirhead et al., 2008). Sequence divergence of 2.5% was previously found between the two *C. sesamiae* biotypes, comparing one individual per population (Muirhead et al., 2006). Our study used ca. 20 individuals per population and two populations per biotype, and found strong genetic divergence of the two biotypes, which suggests reproductive isolation. It has been suggested that the two biotypes of *C. sesamiae* may be distinct species (Dupas et al., 2008). The limited gene flow between the Meru and Muranga populations that we sampled may also relate to their differential host preferences. According to Gitau (2006), the avirulent *C. sesamiae* biotype is capable of distinguishing between suitable and unsuitable hosts. Behavioral variation in host preference of the two biotypes could also influence their genetic divergence (Gitau, 2006).

For *C. flavipes* populations, the buzz 1 durations of the Kenya and Texas populations were similar, but were significantly shorter than the Australian population, which is now a new species, *C. nonagriae* (Olliff) (Muirhead et al., 2008). Our behavioral and genetic results provide further support for the separation of *C. nonagriae* from *C. flavipes*. The *C. flavipes* and *C. nonagriae* populations used in this study were allopatric in origin, but it is unknown whether or not the ranges of *C. flavipes* and *C. nonagriae* overlap, which could be a selective force for courtship signal divergence. Other factors that might contribute to courtship signal divergence in allopatric populations include adaptation to different environments or host plants (Gleason & Ritchie, 1998; Tregenza, 2002). The role of selection by ecological factors such as host plants in shaping insect acoustic signals has rarely been investigated (Gerhardt & Huber, 2002; McNett & Cocroft, 2008). Perhaps host plant leaf structure could be a selective force on the courtship acoustic signal characteristics of *C. flavipes* such as buzz duration or amplitude.

In this study, the male buzz 1 durations of *C. sesamiae* from Mombasa and male *C. flavipes* from Pakistan were similar (Figure 3A). Previous work by Kimani-Njogu & Overholt (1997) found that mating occurred in a laboratory cross of female *C. sesamiae* – Mombasa and male *C. flavipes* – Pakistan, but not in the reverse cross. The female *C. sesamiae* – Mombasa and male *C. flavipes* from Pakistan cross did not produce female offspring, suggesting post-zygotic isolation of those two species. These two species may not be under selective pressure to differentiate courtship signals, as they have allopatric distributions on separate continents.

We found the *C. flavipes* populations from Kenya and Texas were genetically similar, and both were genetically isolated from *C. nonagriae* from Australia. Previous work found 3% sequence divergence between the Australian population and *C. flavipes* introduced into Kenya (Muirhead et al., 2006). *Cotesia flavipes* was introduced into Kenya from Pakistan in 1993, and into Texas from both India and Pakistan in 1977–1978 (Fuchs et al., 1979; Overholt et al., 1997). The Texas *C. flavipes* population sampled here may have established from the Pakistan introduction, as it is genetically similar to the Kenya population, which was originally from Pakistan. Previous work by Kimani-Njogu et al. (1998) also found that *C. flavipes* from Texas and Pakistan were genetically similar. Our work as well as that of others suggests that molecular markers can be used to detect cryptic species and track introductions of populations (Kankare et al., 2005; Lozier et al., 2009).

In summary, we found differences in courtship acoustic signals and genetic divergence among biotypes of *C. sesamiae* for populations collected near a zone of sympatry for the two biotypes. The differences in acoustic signals and genetic divergence among allopatric populations of *C. flavipes* provide further support for the recent separation of the Australian species, *C. nonagriae*. Lack of divergence of courtship acoustic signals in some groups might be attributed to allopatric distributions or post-zygotic isolation between species. Ecological factors including host range and host plant associations could also influence the divergence of courtship acoustic signals and genetic structure among populations, contributing to reproductive isolation.

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