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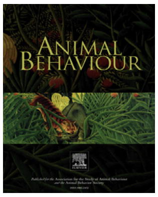

Behavioural barriers to reproduction may evolve faster than sexual morphology among populations of a dung fly (Sepsidae)

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Reproductive traits often evolve rapidly, and some suggest that behavioural traits, in particular, can diverge faster than morphology, resulting in sexual isolation between populations/species. An earlier study of a Neotropical dung fly, *Archisepsis diversiformis* (Diptera: Sepsidae), reported anecdotally that two central American populations that were approximately 500 km apart (Costa Rica and Panama) differed in male courtship behaviour despite being morphologically similar. Here, I present results of an in-depth study designed (1) to test whether these two populations show qualitative and/or quantitative differences in mating behaviour and morphology, (2) to test whether individuals from either population show some degree of reproductive isolation and (3) to characterize population variation in a particularly fast evolving mitochondrial gene fragment, *cytochrome oxidase c subunit I* (*COI*), to estimate genetic differences between the two populations. Despite similarities in overall courtship, I identified behaviours that were clearly population specific, and report that both populations showed strong premating isolation in one-on-one crosses. However, after extended exposure in mass-container group crosses, individuals did produce adult F1 offspring, suggesting that isolation is incomplete. Surprisingly, morphometric analyses indicated that these two populations differed significantly in sexually monomorphic adult wing shape but differed only moderately in sexually dimorphic male forefemur shape, and not at all in male genital clasper shape. Finally, both populations were genetically similar, forming a single, monophyletic cluster with low uncorrected pairwise distances (*COI* threshold <1 %), suggesting that marked behavioural differences between both populations have evolved quickly but without complete isolation.

The evolution of sexual isolation plays a crucial role in the origin and maintenance of genetic and phenotypic differences among species and is a pivotal event in the evolution of biological species (Dieckmann & Doebeli, 1999; Dobzhansky & Mayr, 1944). Ongoing processes of incipient speciation among populations/species often involve isolating mechanisms during different phases of sexual interactions that can restrict gene flow (Coyne & Orr, 2004; Panhuis, Butlin, Zuk, & Tregenza, 2001). Strong pre- and/or postcopulatory sexual selection on reproductive traits, in particular, can subsequently result in rapid phenotypic divergence between groups (Groning & Hochkirch, 2008). Behaviour involving male–female interactions can be especially important as premating barriers, often including chemical or olfactory signals, tactile and physical stimulation, complex acoustic songs and calls, and also visual cues based on elaborate noncontact courtship repertoires (Boul, Funk, Darst, Cannatella, & Ryan, 2007; Curé, Mathevon, Mundry, & Aubin, 2012; Pröhl, Hagemann, Karsch, & Höbel, 2007). These behavioural traits are often essential for mate recognition, and some suggest that they can be evolutionarily labile and diverge faster than morphology (Boul et al., 2007; Mendelson & Shaw, 2005; Podos & Warren, 2007; Williams & Mendelson, 2010).

In insects, various comparative studies suggest that reproductive behaviour can evolve rapidly, resulting in the generation and maintenance of species boundaries among close relatives (Gleason & Ritchie, 1998; Luan, De Barro, Ruan, & Liu, 2013; Puniamoorthy, Ismail, Tan, & Meier, 2009; Vedenina, Kulygina, & Panyutin, 2007). For example, in certain *Drosophila* flies and *Gryllus* crickets, sister species can differ significantly in acoustic traits (Fitzpatrick & Gray, 2001; Gray, 2004; Klappert, Mazzi, Hoikkala, & Ritchie, 2007; Shaw & Herlihy, 2000; Snook, Robertson, Crudgington, & Ritchie, 2005) and in some *Gerris* water striders, *Staminodeus* flower weevils and *Phytalmia* antlered flies, recently diverged taxa can vary strongly in visual and/or tactile behaviour (Arnqvist & Rowe, 2002; Franz, 2003; Schutze, Yeates, Graham, &

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Dodson, 2007). Of particular interest are studies that report distinct population-level variation in the presence and/or preference of behavioural cues among populations of widespread species. For instance, in the dung fly *Sepsis punctum*, North American populations from the east and west coast vary in the intensity of premating visual displays, which are completely absent in European populations (Puniamoorthy, Blanckenhorn, & Schäfer, 2012). In *Drosophila pseudoobscura*, recently sampled populations from Bogota showed significant differences in courtship latency and even sexual isolation from North American populations (Kim et al., 2012). Hence, it is likely that behavioural divergence involving male–female interactions could restrict gene flow, resulting in incipient speciation. As such, studies taking into account variation in different reproductive traits, especially among populations of widespread species, are important for understanding the mechanisms involved in establishing sexual isolation (Klappert et al., 2007; Peretti & Córdoba-Aguilar, 2007).

Complex sexual ornaments are particularly common in insects, and such secondary traits can diverge rapidly because of sexual selection, often with consequences for speciation (Arnqvist & Thornhill, 1998; Eberhard, 1985, 1996). Flies belonging to the family Sepsidae, for instance, can vary drastically in body size and, in addition to highly divergent male genitalia (Bowsher, Ang, Ferderer, & Meier, 2013; Eberhard, 2001a, 2001b, 2001c), male forelegs are secondarily modified with spines, bristles and/or protrusions, which are species specific, often serving as primary characters for taxonomic delineations (Pont & Meier, 2002; Puniamoorthy, Su, & Meier, 2008). Production of such elaborate traits is associated with developmental costs, whereby males trade off reduced growth against morphological complexity (Blanckenhorn, Chew, Ang, Puniamoorthy, & Meier, n.d.). In the Neotropical species *Archisepsis diversiformis* (Ozerov, 1993), as in most sepsids, males mount females by using their modified forelegs to clasp the female's wing bases (Eberhard, 2002). This particularly widespread fly ranges from Mexico, the Caribbean islands to most parts of central and south America (Ozerov, 2005). Males can be found in high densities waiting for females at dung pats, and often perform leg displays during copulation, the latter of which typically last 20–25 min (Eberhard & Huber, 1998). Interestingly, more than a decade ago, Eberhard (2002) noted possible differences in male mating behaviour between two *A. diversiformis* populations, one from Costa Rica (~1000 m above sea level, Central Valley near San José) and another from Panama (~20 m above sea level, Barro Colorado Island in Lake Gatun). He indicated that both populations were at least superficially similar in the morphology of male forelegs and genital claspers, but neither behavioural nor morphological differences were measured quantitatively (Eberhard, 2001c, 2002; Eberhard & Huber, 1998). In this study, I revisit this, to test whether these two populations could be in the early stages of diversification. Namely, I (1) document differences in mating behaviour and morphology, (2) test for reproductive isolation and (3) characterize genetic differences in a particularly fast evolving mitochondrial gene fragment, *cytochrome oxidase c subunit I* (*COI*) between these two populations of *A. diversiformis*.

METHODS

Sampling and Fly Cultures

Individuals were collected from the sites reported in the earlier study (Eberhard, 2002), namely San Antonio de Escazu, San Jose, Costa Rica (CR: 9°53′24″N, 84°8′18″W; on cattle dung), and on Barro Colorado Island in Panama (PAN: 9°9′0″N, 79°50′59″W; on monkey droppings). Multiple females from each population were set up individually with cattle dung (as per Puniamoorthy et al., 2008), and the emerging offspring were identified to species and used to establish laboratory cultures (Ang et al., 2013; sepsidnet-rmbr.nus.edu.sg). Rearing and behavioural experiments were conducted within a 3-month period at the Escuela de Biologia, Universidad de Costa Rica (August–October 2010). Parental cultures were housed and maintained under ambient room conditions in four to six replicate containers per population (ca. 26 °C; 60% humidity) and supplied regularly with fresh cow dung and concentrated sugar water. Field-collected males were stored in absolute ethanol for molecular analyses (later performed at the Institute of Molecular Life Sciences, University of Zurich).

Body Size and Development Time

Females from parental lines were allowed to oviposit for 2–3 h in cow dung and the offspring were reared in a climate chamber, standardized at 24 °C, 60% humidity and a 14:10 h light:dark cycle. Development time was recorded as time from oviposition to ecolsion. Flies were individually sexed within 24 h of emergence, and males and females were maintained as virgins in separate containers. A random subset of adults was frozen for the morphometric study and head width was measured as a standard index of body size (Puniamoorthy et al., 2012) using a Leica MS 5 microscope (Leica Microsystems, Switzerland).

Mating Experiments

Mating trials were carried out approximately 4 days after separation by introducing one male and one female into a small petri dish. All interactions were videorecorded using a SONY hand-held video camcorder with a 20× magnification lens upon the introduction of both individuals and ended after a successful copulation or after 30–45 min. All videotapes were digitized using the editing software iMovies (Apple Computer, Inc., CA, U.S.A.) and analysed to detail behavioural elements.

Within-population pairings

Virgin males and females were used to establish mating profiles for each population based on a target number of 10–15 successful copulations per population. The interactions were scored qualitatively (i.e. different types of courtship elements were categorized based on behavioural character descriptions previously defined for sepsid flies; Puniamoorthy et al., 2009; Tan, Ng, & Meier, 2011). New behaviours were coded as new states, and video clips of individual behaviours are available online on YouTube (see Results, Table 1; character matrix and descriptions in Supplementary Material, Table S1).

Between-population pairings

One-on-one trials. Reciprocal mating crosses were conducted between both populations (female CR × male P; female P × male CR). All focal females were first introduced to a male from a ‘foreign’ population for a maximum of 1 h and, after a refractory period of 2–3 h, the females were introduced to a male from their same ‘local’ population. Behavioural interactions of all crossing experiments were also videorecorded and analysed as described earlier.

Group trials. In addition to the one-on-one tests, four replicate group trials with five virgin males and five virgin females of the two different populations were set up, assuring prolonged exposure to only ‘foreign’ population mating partners. Each group container was provided with fresh dung and sugar water. All parental adults were killed after 2 weeks, and the dung was left in the containers and maintained in climate chambers under standardized