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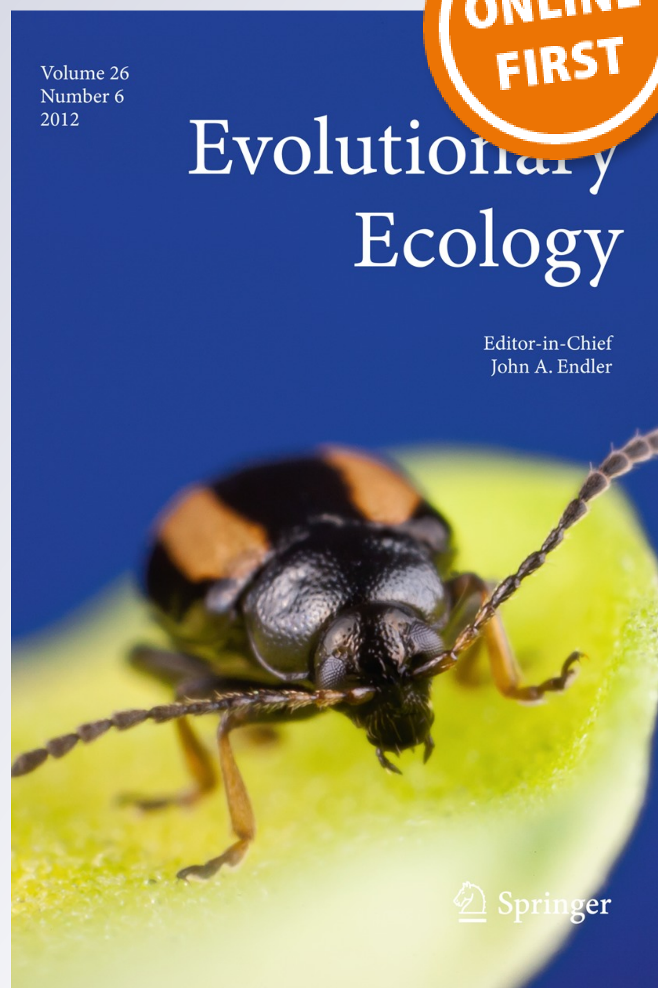

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ORIGINAL PAPER

Genetically differentiated races and speciation-with-gene-flow in the sunflower maggot, *Strauzia longipennis*

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Abstract The ecological interactions parasitic insects have with their hosts may contribute to their prodigious diversity, which is unrivaled among animals. Many insects assumed to be polyphagous generalists have been shown to consist of several differentiated races, each occupying a different host-niche. The sunflower maggot fly, *Strauzia longipennis*, has long been thought to consist of two or more races due to its substantial intra-specific morphological variation. Here, we use nuclear and mitochondrial markers to test the hypothesis that *S. longipennis* is a complex of two or more partially reproductively isolated races. We collected *S. longipennis* flies as pupae from roots of Jerusalem artichoke (*Helianthus tuberosus*) and as adults swept from leaves of mature *H. tuberosus* across the breadth of a field season. Flies were scored for morphological variety (*typica* or *vittigera*), mitochondrial haplotype (A or B) and a panel of 176 AFLP loci. Bayesian clustering and neighbor-joining phylogenetic analyses of AFLP data supported the existence of at least three, possibly four, genetic races of *Strauzia* (clusters I, II, III, and V), as well as a small number of putative interracial hybrids (cluster IV). Clusters I and III each consisted of flies of both morphological varieties and both haplotype groups, while flies in cluster II were all of variety *typica* and all but one was of mitochondrial haplotype B. Flies in cluster II were also collected only as adults on *H. tuberosus* and not among flies reared from pupae collected from *H. tuberosus* roots, suggesting that they use a different plant as their larval host. Mean capture date was significantly different between flies of each genetic race, indicating that partial allochronic isolation may be one contemporary barrier to gene flow between races. Evidence that mitochondrial genomes and morphological traits have moved between lineages implies a model of speciation-with-gene-flow for *S. longipennis* races.

Keywords Reproductive barriers · *Helianthus tuberosus* · Mitochondrial introgression · Incipient species · Speciation continuum

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Introduction

Phytophagous (plant-eating) insects are tremendously diverse—constituting perhaps as much as one third of all animal species (Strong et al. 1984)—and their speciation has been tied to extrinsic reproductive barriers arising during changes in host plant use (Bush 1969; Wood and Keese 1990; Berlocher 1998; Nason et al. 2002; Funk et al. 2011). Many insects find mates on or near the female's oviposition substrate (the host plant), so emergent preferences for novel hosts may often translate into assortative mating and behaviorally isolate different host-associated populations (e.g., Emelianov et al. 2001; Berlocher and Feder 2002). The result is the formation of partially isolated races, presumed to be an early step along the so-called "speciation continuum" (Mallet 2008). Genetically differentiated races within morphologically defined species have also been found to coexist on a single plant species, particularly among taxa that have recently undergone adaptive radiations (Marsteller et al. 2009; Stireman et al. 2012). Further, recent empirical and theoretical work demonstrating that populations can diverge even in the face of effective migration (Nosil 2008; Nosil and Feder 2012), as well as new information about the permeability of genomes to between-lineage gene flow (Turner et al. 1999; Michel et al. 2010; Gompert et al. 2010) both suggest that persistent hybridization between closely related lineages may occur without leading to lineage fusion or breakdown of reproductive barriers. Such studies imply that ecology plays a central role in insect diversity, but many questions remain as to the interplay between gene flow and host plant mediated selection during the early stages of speciation.

The sunflower maggot, *Strauzia longipennis* (Wiedemann) (Diptera: Tephritidae) may be an ideal subject for investigating the dynamics of speciation and gene flow among phytophagous insects because it is thought to consist of multiple genetic races at various stages of divergence (Lisowski 1979; Axen et al. 2010). Most flies in genus *Strauzia* are host-specialists; they oviposit and undergo larval development on a single species of plant. Adult phenology across the genus is variable and corresponds to the time of the year that each species' host plant matures (Stoltzfus 1988). *S. longipennis* is an exception; it has been described as using at least 5 different hosts: three species in genus *Helianthus* (sunflowers) and two other plants in the family Asteraceae, *Smallanthus uvedalia* and *Ageratina altissima* (Foote et al. 1993). Several other genera of Tephritidae are known to have undergone race formation and speciation through host association (Bush 1969; Abrahamson and Weis 1997), so the uniquely broad host range of *S. longipennis* is an indication that it could be a conglomerate of cryptic lineages.

Strauzia longipennis is also uniquely variable in its morphology when compared with congeners, a situation that has caused long-standing taxonomic difficulty (Foote et al. 1993; Steyskal 1986). The fly was initially described as a single species with as many as seven different morphological 'varieties,' for which many characters gradually transition from one variety into another (Loew 1873). Subsequent taxonomic revisions attempted to split *S. longipennis* into two and later into seven species (Lisowski 1985; Steyskal 1986; Stoltzfus 1988), but no characters reliably distinguished any one of the postulated species, so taxonomic authorities have resisted each suggested split (Foote et al. 1993). Currently, as many as six "unofficial" varieties make up the *S. longipennis* complex as defined by Foote et al. (1993). Of these, three morphological varieties have been named as associates of species of *Helianthus* though none has been shown to be exclusive to any one host species. *Strauzia longipennis* var. *longitudinalis* is found only in the Eastern United States. The other two, *S. longipennis* var. *typica* (hereafter '*typica*') and *S. longipennis* var. *vittigera* (hereafter '*vittigera*') co-occur sympatrically in the Midwest and are the focus of

this study. *Vittigera* flies are defined by two symmetrical lines of dark pigment on their dorsal thorax, while *typica* flies have relatively unpigmented thoraxes (Fig. 1). Flies with thoracic pigmentation intermediate between *vittigera* and *typica* are also occasionally collected (Axen et al. 2010).

Attempts to resolve *Strauzia* phylogeny using genetic markers have proved similarly frustrating. A study of electrophoretic variants (allozymes) among flies collected from several different plant hosts and sites in Illinois resolved six distinct clusters defined by fixed or strong allele frequency differences (Lisowski 1979), two of which correspond to the now-named species *S. arculata* and *S. intermedia* (attacking *Helianthus grosseserratus* and *Rudbeckia laciniata*, respectively) (Steyskal 1986). Four clusters, however, overlapped broadly in host plant use and morphology, and electrophoretic intermediates between clusters were found, calling into question the extent of their reproductive isolation (Lisowski 1979). More recently, mitochondrial data collected from adult *Strauzia* captured on *H. tuberosus* revealed two major haplotype groups, leading to the tentative conclusion that two incipient species had been identified (Axen et al. 2010). However, again morphology did not perfectly correspond to haplotype: several different combinations of wing and thoracic characters were common among flies with both haplotypes.

Our goal in this study was to determine whether *S. longipennis* is a complex of two or more partially reproductively isolated races and whether extrinsic barriers to gene flow are associated with reproductive isolation. We had four specific aims. First, we used both mitochondrial and nuclear markers to determine whether *S. longipennis* collected from *H. tuberosus* consists of multiple cryptic species or races, as suggested by Lisowski (1979). Second, we asked whether races differed in their thoracic banding patterns (*vittigera* vs. *typica*). Third, we used collection dates of individual flies to determine whether cryptic lineages were allochronically isolated from one another. Fourth, we compared populations of wild caught adult flies with fly pupae extracted from *H. tuberosus* tissue to determine

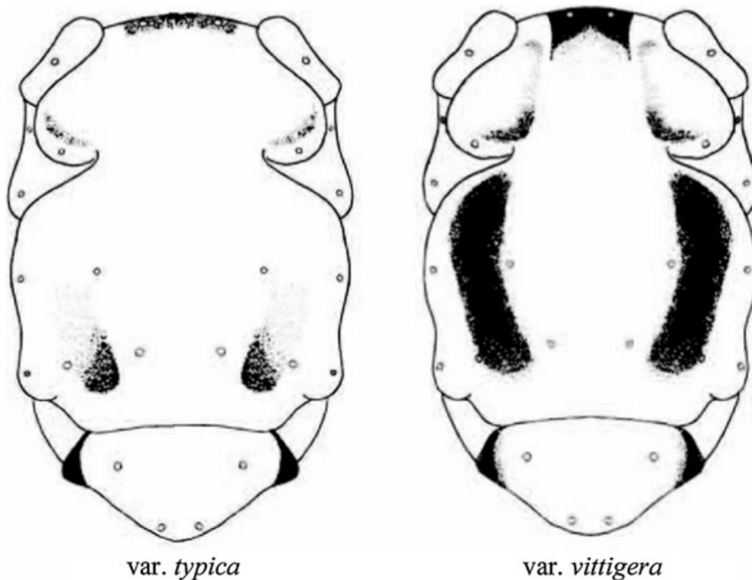

Fig. 1 The two primary thoracic banding patterns found among *S. longipennis* flies: *S. longipennis* variety *typica*, *left*; *S. longipennis* variety *vittigera*, *right*

whether all fly lineages captured as adults on a plant actually use that plant as their larval host. We show that *S. longipennis* is a complex of at least three genetic races at various stages of divergence and that these races are partially allochronically isolated from one another. Neither mitochondrial haplotype group identity nor morphological variety was 100 % diagnostic for distinguishing between individuals from different races, providing support for historical and/or contemporary introgression between races.

Materials and methods

Study organisms

As with many temperate tephritid flies, *Strauzia* have just a single generation per year, emerging as adults in the spring or summer. Adult male *Strauzia* occupy positions on the underside of host plant leaves and aggressively defend territories (Stoltzfus 1988). Females move between plants and successful mating may last anywhere from a few minutes to several hours. Females may mate with multiple males over the course of their lifetime (Westdal and Barrett 1962). Once mated, females then oviposit into the host plant stem; a single lab-reared female can lay as many as 31 eggs in a day (Westdal and Barrett 1962). Larvae feed on the pith of the plant and pupate either in the root crown or in the soil, depending on the fly species (Brink 1922; Stoltzfus 1988). All *Strauzia* overwinter as pupae.

Strauzia longipennis (Wiedemann) pupae were dissected from tubers of *Helianthus tuberosus* growing in Mount Vernon, IA, USA (41°55.358N, 91°25.101W) and Cedar Rapids, IA (41°57.292N, 91°34.733W) in April and May of 2011. Tubers representing plants from the 2010 growing season were extracted from soil and split with a sharp knife to reveal puparia enclosed within. Most plants contained a single *Strauzia* puparium, but two puparia were occasionally found in the same plant. Pupae were held in moist vermiculite at room temperature (approximately 18 °C) until flies eclosed. No exit scars were found on plant stalks, and no pupae were found in soil around plants, supporting a previous observation that *Strauzia* associated with *H. tuberosus* overwinter exclusively in roots and not in the soil (Lisowski 1979).

Adult *Strauzia* flies were caught off of leaves of *Helianthus tuberosus* from May 23 through July 11, 2011 at the same two sites described above and at a third site in Iowa City, IA (41°38.453N, 91°34.138W). All sites were between 14 and 38 km of one another, as multiple independent sampling sites from a relatively small geographic area ensures that genetic differences will be minimally influenced by geographic distance. Two other sunflowers previously recorded as hosts to *S. longipennis*, *Helianthus annuus* and *H. strumosus*, were present at all sites, but were relatively uncommon and flies were not sampled from these plants. Fly collections were attempted every 2 days, for approximately 1–2 h at each site. The date and site of capture, thoracic banding pattern (Fig. 1; *vittigera*, *typica*, or intermediate; see Axen et al. (2010) for details of morphological classification), and sex of each fly was recorded. Flies were frozen and held at −80 °C in 95 % EtOH for use in genetic work.

DNA extraction and mtDNA haplotyping

Whole genomic DNA was extracted from all flies (both those collected as adults and those reared from root-extracted pupae) using DNeasy Blood & Tissue kits (Qiagen Sciences, Maryland, USA). A 648 bp segment of the mitochondrial COI gene was PCR-amplified

using the primers LepF1 & LepR1 (Smith et al. 2008) and the following cycling parameters: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 46 °C for 1 min, and 72 °C for 2 min, with a final extension of 72 °C for 4 min. Reactions were cleaned using Shrimp Alkaline Phosphatase (USB, Swampscott, MA) and Exonuclease I (New England Biolabs, Ipswich, MD) and cycle sequencing was performed in both forward and reverse directions on an ABI 3730 DNA Analyzer using BigDye 3.1 (Applied Biosystems, Foster City, CA) sequencing chemistry. Forward and reverse reads were used to create consensus sequences for each individual and consensi were then automatically aligned using the program SeqMan (DNASTAR, Inc., Madison, WI). TCS 1.21 (Clement et al. 2000) was used to construct a haplotype network using statistical parsimony and a 95 % connection limit.

AFLP genotyping and phylogenetic inferences

Fly DNA extracts were scored for nuclear genomic variation using an AFLP protocol following Egan et al. (2008). Briefly, genomic DNA samples from the same flies as above were digested using the restriction enzymes EcoRI and MseI (New England Biolabs, Ipswich, MA) and site-specific adaptors were ligated onto cut sites. Samples were then PCR-amplified using a pair of preselective primers [Eco + C (5′-GACTGCGTACCAATTCC-3′) and Mse + C (5′-G ATGAGTCCTGAGTAAC-3′)], and then amplified a second time in three independent reactions using a fluorescently labeled forward primer Eco + CAG (5′-GACTGCGTACCA-ATTCCAG-3′) and each of three different reverse primers: Mse + CAA (5′-GATGAG TCCTGAGTAACAA-3′), Mse + CTG (5′-GATGAGTCCTGAGTAACTG-3′) and Mse + CGA (5′-GATGAGTCCTGAGTAACGA-3′). Selective amplification reactions were genotyped on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) and fragment analysis was performed using GeneMarker 2.2.0 (Softgenetics, State College, PA). A panel of loci sized between 100 and 450 bp was first called automatically by GeneMarker and then all loci were inspected visually. Loci with consistently weak signals were dropped from the panel. All individuals were selectively amplified and genotyped twice and individuals with inconsistent signals were not included in the analysis. In total, 176 polymorphic loci were identified and scored for 91 individual flies.

AFLP-SURV (Vekemans 2002) was used to generate 10,000 bootstrapped Nei's D (Lynch and Milligan 1994) distance matrices which were then converted into distance networks using the programs NEIGHBOR and CONSENSE in PHYLIP 3.6 (Felsenstein 1989). Pairwise Fst was also calculated between clusters identified by distance networks using AFLP-SURV and ARLEQUIN 3.5.1.3 (Excoffier and Lischer 2010). Bayesian clustering analysis of AFLP loci was performed using the program STRUCTURE (Pritchard et al. 2000) for each of K = 2–11 with a burn-in of 500,000 and 750,000 Markov Chain Monte Carlo replications under a model of admixture and correlated allele frequencies. Two methods were used to evaluate patterns of genetic structuring resulting from STRUCTURE analyses. First, we used the K at which log-likelihood values (Ln(K)) were at their highest, as in the methods of Falush et al. (2003) and other authors (Michel et al. 2007). Second, we used the statistic ΔK of Evanno et al. (2005), shown in their simulations to produce the most consistent estimate of the true K under conditions of moderate to strong structure. Both ΔK and Ln(K) are ad hoc statistics, each which has been shown to perform well against different types of simulated data (Pritchard et al. 2000; Evanno et al. 2005).

Results

Collections

Almost every tuber inspected in this study contained a *S. longipennis* pupa. A total of 33 flies were reared from 49 pupae dissected from tubers, and 30 of these were variety *vittigera* (19♂, 11♀). The remaining three adult flies, all females, had a morphology intermediate between variety *typica* and variety *vittigera*. No variety *typica* flies were reared from pupal collections. An additional 106 adult flies were caught on or near *H. tuberosus* plants across the duration of the summer. Of these, 35 were variety *vittigera* (25♂, 10♀), 63 were variety *typica* (51♂, 12♀), and 8 were of intermediate morphology (6♂, 2♀). Adult flies of both morphological varieties were collected at all three sites in this study. No other species of *Strauzia* other than *S. longipennis* was reared from or captured on *H. tuberosus*.

mtDNA

Two major haplotype groups were resolved across all flies for which sequence was obtained (n = 98; Fig. 2). These corresponded to the haplotype groups found by Axen et al. (2010). Each haplotype group was comprised of one primary haplotype found in the majority of individual flies in each group, and then a number of less common haplotypes that differed from the primary haplotype by between one and seven base pair substitutions. The two primary haplotypes from each group differed from each another at 18 of 658 bp. One group, hereafter haplotype group A, was composed of 38 flies of *vittigera* morphology (19♂, 19♀), nine of *typica* morphology (3♂, 6♀), and two flies with an intermediate morphology (2♂). The second group, hereafter haplotype group B, was composed of 39 flies of *typica* morphology (21♂, 18♀), eight of *vittigera* morphology (5♂, 3♀), and two flies of intermediate morphology (2♂). Both haplotype groups were obtained from flies at all three collection sites, and geographic location was not significantly associated with haplotype (Fisher's Exact Test; $df = 2$, $P = 0.07$).

AFLP data

Analyses of AFLP data were consistent with the existence of genetic races. The neighbor-joining network resolved three well-supported clusters of flies collectively corresponding to the majority of individuals collected (clusters I, II, and III; Fig. 3). Two additional clusters (clusters IV and V) consisted of five flies and two flies, respectively. Cluster I (n = 40 flies; 24♂, 16♀) was more strongly differentiated from the other clusters: 16 of 176 loci (9.1 %) were amplified exclusively in cluster I (i.e., never in clusters II or III), seven loci (4.0 %) were exclusive to clusters II and III, and four loci (2.3 %) were 'fixed' between cluster I and clusters II and II. Clusters II (25 flies; 13♂, 2♀) and III (19 flies; 11♂, 8♀) were less strongly differentiated from one another: no fixed differences were found at any AFLP loci, but 10 loci were absent in either cluster II or cluster III and found at frequencies >0.15 in the other cluster. Pairwise F_{ST} between clusters I, II, and III ranged from 0.08 to 0.34 (Table 1).

Log likelihood values generated from STRUCTURE analyses of AFLP data best supported K = 2 or 4 populations, depending on the method of evaluation used. The ΔK of Evanno et al. (2005), which performs well in identifying the true number of clusters under

Fig. 2 Parsimony network for a 658 bp segment of mitochondrial cytochrome oxidase I (COI) in 98 *Strauzia longipennis* individuals. Each connection = 1 base substitution. Sizes of polygons correspond to the number of flies having a particular haplotype. Small circles indicate haplotypes found in a single individual fly. *Colors* correspond to morphological IDs: *black* = *S. longipennis* variety *vittigera*, *white* = *S. longipennis* variety *typica*; *gray* = intermediate morphology. *Numbers* inside of shapes designate the number of flies of a particular morphology with that haplotype. "*A*" and "*B*" delineate the two major haplotype groups. The haplotype in group *B* marked with a *asterisks* denotes two individual flies with a unique haplotype that may be distinct from other lineages based on AFLP data (see Fig. 3, cluster V)

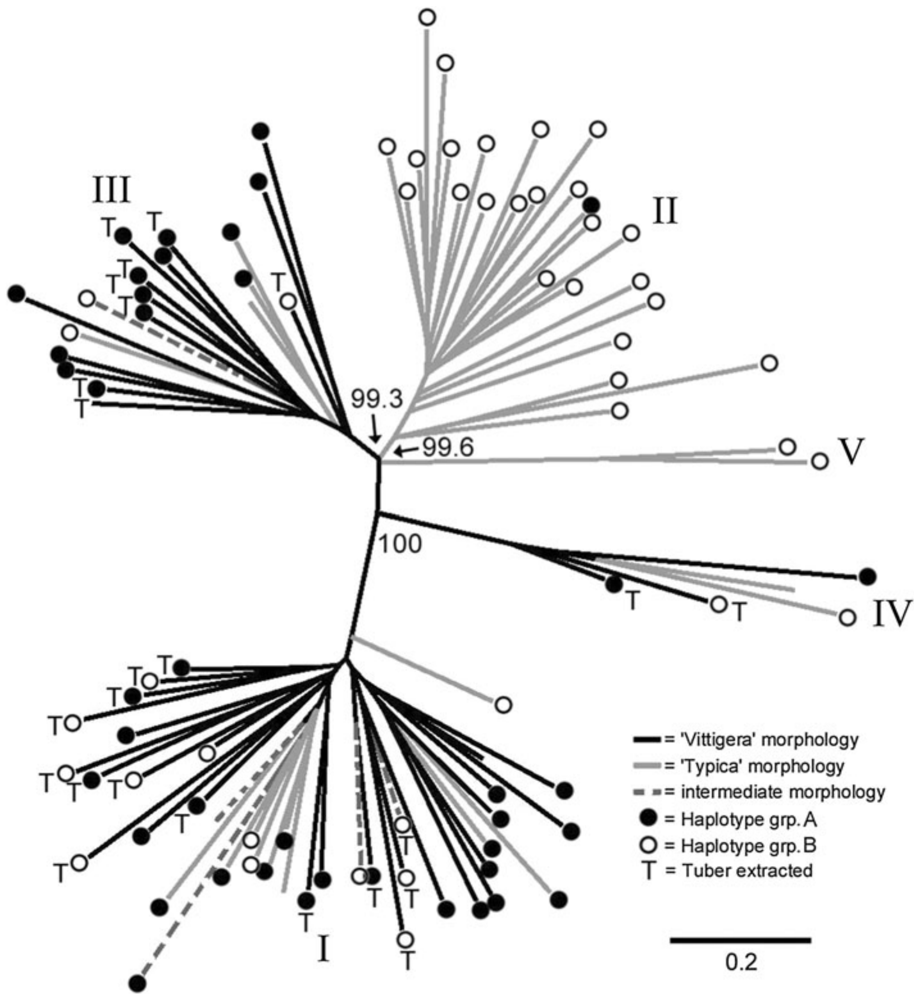

Fig. 3 Neighbor-joining network of Nei genetic distances generated for 91 individual *S. longipennis* flies and based on 176 polymorphic AFLP loci. Branches in *black* denote flies with *vittigera* morphology, *gray lines* denote flies with *typica* morphology, and *dashed lines* denote flies with an intermediate morphology (Fig. 1b). *Closed circles* are flies with a group *A* mitochondrial haplotype, while open circles are flies with a group *B* haplotype (refer to Fig. 2). *Numbers* at branch nodes denote bootstrap support (10,000 bootstraps). "*T*" denotes flies dissected and then reared from tubers of *Helianthus tuberosus*, while flies without a "*T*" were all collected as adults on *H. tuberosus* plants

conditions of moderate to strong genetic structure, showed a modal maximum at a K = 2. For a K = 2 (Fig. 4), flies in cluster I had strong proportional membership with one simulated population (0.990), and flies in clusters II and III to the second population (0.995 and 0.991, respectively). Cluster IV and V flies were of intermediate membership (0.535 and 0.834 proportional assignment to the second population, respectively). Evaluation of unmodified Ln(K) values showed a maximum likelihood value at K = 4, before values decreased and then fluctuated widely, and so we also evaluated this as a potential reflection of "true" genetic structure. For a K = 4, most flies were placed into one of three

Table 1 Pairwise F_{ST} between genetic clusters of *S. longipennis* identified using 176 AFLP markers

Cluster	II	III	IV	V
I	**0.342**	**0.339**	**0.243**	0.218
II		**0.078**	**0.207**	0.087
III			**0.196**	0.107
IV				0.103

Cluster IV flies are putative hybrids between cluster I and clusters II and/or III. Values in boldface are significant at a $P < 0.01$ threshold

populations, corresponding strongly to the clusters I, II, and III resolved by the phylogenetic analysis (Fig. 4). Cluster IV flies were grouped into their own fourth population, while cluster V flies were not strongly allied with any single population.

Patterns of morphology and mitochondrial haplotypes were different between genetic clusters. Cluster I contained flies of both haplotypes (24 Haplotype A, 13 Haplotype B, 3 unscored) and of both morphologies (10 *typica*, 26 *vittigera*, 4 intermediate). No association between haplotype and morphology was found among individual flies in cluster I (Fisher's Exact Test; $df = 1$, $P = 0.08$). Cluster II was composed exclusively of flies of *typica* morphology (n = 25), and all but one fly had the group B haplotype. Conversely, 14 of 19 flies in cluster III were of *vittigera* morphology (4 variety *typica*, 1 intermediate) and 14 of 17 flies scored for mitochondrial sequence had a group A haplotype (3 group B; 2 unscored). As with cluster I, no association between haplotype and morphology was found for cluster III (FET; $df = 1$, $P = 0.12$).

Cluster IV (5 flies; 3♂, 2♀) was rooted at a position intermediate between cluster I and clusters II and III (Fig. 3). Two cluster IV flies had *typica* morphology, and three had *vittigera* morphology. Two of the *vittigera* morphology flies were tuber-extracted, while

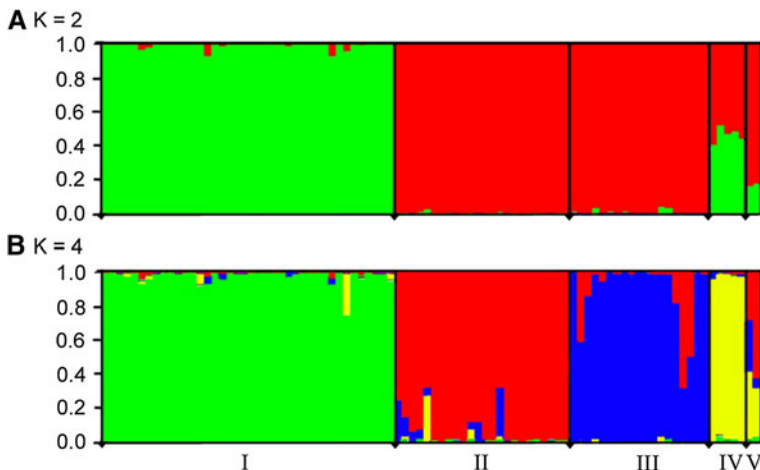

Fig. 4 *Bar* plots of STRUCTURE analyses (admixture with correlated allele frequencies) for 91 individual *S. longipennis* flies assigned to predefined groups according to the five clusters resolved from the NJ distance network (Fig. 3). **a** The ΔK method of Evanno et al. (2005) best supported a K = 2, while **b** methods of Falush et al. (2003), which rely on identifying the value of K with the highest log likelihood value, best supported a K = 4. Both values of K identify structure also resolved by the distance network, and both indicate the existence of cryptic genetic races within *S. longipennis*

the other three flies were captured as adults. Four cluster IV flies were scored for COI, and both haplotypes groups were represented (Fig. 3). Moreover, two of the four AFLP loci with fixed differences between cluster I and clusters II and III were amplified at intermediate frequencies among the cluster IV flies, and cluster IV flies were almost perfectly divided in their population assignment for a K = 2 (Fig. 4). These data point towards cluster IV representing naturally occurring F1 hybrids between cluster I and flies from cluster II and/or III.

Cluster V consisted of just two female flies and was rooted closely to cluster II (Fig. 3). However, we tentatively distinguish it from other clusters due to an intermediate STRUCTURE assignment in both evaluated values of K (Fig. 4), and because both cluster V flies shared a group B haplotype found in no other flies in this study (Fig. 2). Both cluster V flies (2♀) were of variety *typica* morphology and were collected on the final day of the sampling period.

Adult flies from all three major genetic clusters were captured at all three collection sites. Flies reared from *H. tuberosus* tuber-extracted pupae were only from clusters I (8♂, 7♀) and III (5♂, 3♀). No cluster II flies were reared from pupae collected from *H. tuberosus* tubers.

Allochrony between genetically differentiated *S. longipennis*

Although the range of capture dates for the three primary genetic clusters of *S. longipennis* overlapped, mean date of capture in the field was significantly different between clusters (Fig. 5; ANOVA: F = 41.08, $df = 2$, $P < 0.0001$). Cluster III was captured earliest in the year (mean capture date = June 9th), followed by cluster I (June 29th), then cluster II (July 6th). The three putative F1 hybrids (Cluster IV) captured as adults were all caught on the same day (June 30th) at the Cedar Rapids, IA site. Both cluster V flies were collected in Cedar Rapids, IA on the final day of the sampling period (July 11th).

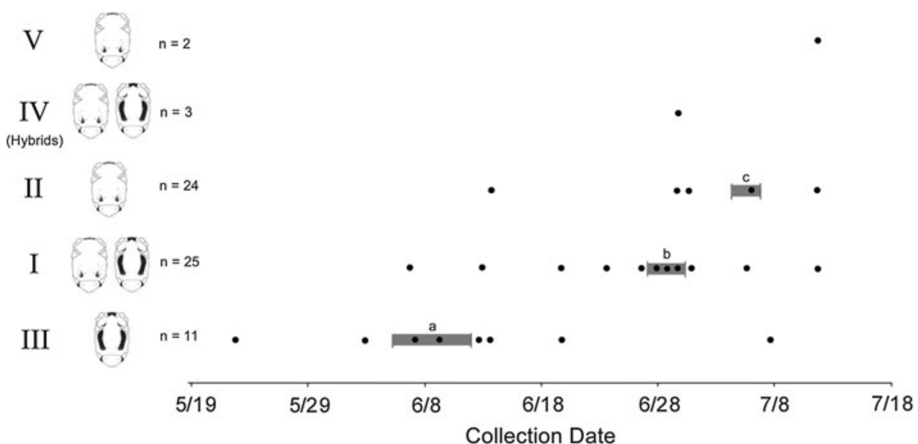

Fig. 5 Field collection dates of adult *S. longipennis* flies from genetic clusters resolved through analyses of 176 AFLP loci (Figs. 3, 4). *Grey bars* denote standard errors around mean collection dates for each cluster. Figures following each category label show the primary thoracic banding patterns found among flies in each cluster. *Lowercase letters* denote significant differences in mean eclosion dates between clusters

Discussion

Strauzia longipennis is a complex of three or more genetically differentiated races

A scan of the *S. longipennis* nuclear genome using AFLPs resolved at least three genetically differentiated clusters of flies (Figs. 3, 4). Cluster I differs most radically from clusters II and III: several fixed differences between these clusters imply that gene flow is greatly reduced between these groups, at least in some regions of the genome. We therefore consider cluster I to be a distinct genetic race. The fixed differences between cluster I flies and other clusters might even warrant elevation to species status, if one were to abide by the genomic cluster species concept (GCSC), which defines species as "…a distinguishable group of individuals that has few or no intermediates when in contact with other such clusters." (Mallet 1995). However, the GCSC in generally criticized for having no set threshold for the tightness of clustering or for the number of differences required to diagnose species (Coyne and Orr 2004) and application of the GCSC to complexes of closely related organisms in various stages of divergence has failed to identify a threshold for species status (e.g., Berlocher 1999), so we will maintain the term 'race' until further evidence warrants a change. We do note that the fixed allelic differences combined with contemporary allochronic isolation (see below) suggest extremely limited effective gene flow into or out of this cluster.

Clusters II and III were more closely related to one another than either was to cluster I, but support for their independence was also strong. No fixed allelic differences were found between AFLP loci in clusters II and III, but several alleles were found at high frequency in one cluster and were entirely absent in the other. All but one cluster II fly (1/25) had a group B mitochondrial haplotype, while cluster III flies had primarily group A haplotypes (14/17). Based on this evidence, we propose that clusters II and III are also genetic races.

Ecological data suggest that a plant other than *H. tuberosus* is likely the primary host for flies in cluster II. None of the *S. longipennis* reared from pupae extracted from tubers of *H. tuberosus* were from cluster II, and adults of cluster II captured on *H. tuberosus* were almost exclusively male (23 of 25). Males in genus *Strauzia* tend to be territorial, staking out leaves and waiting for females to approach (Stoltzfus 1988). That few cluster II females were captured suggests that cluster II males may simply be making errors in host plant choice, while females have more strict fidelity. Alternatively, cluster II flies may oviposit on *H. tuberosus* but not complete development (note that 16 of 49 pupae dissected from tubers did not emerge as adult flies). Additional support for an alternative host for cluster II males comes from taxonomic work associating flies of *typica* morphology with domesticated *H. annuus* (Lisowski 1985; Stoltzfus 1988), a plant species found at all three collections sites. Lisowski's (1979) allozyme study also identified a genetic cluster of flies reared from *H. strumosus* and *H. annuus*, but with no apparent *H. tuberosus* association. Future work should involve sampling of *Strauzia* from other sympatric *Helianthus* species, including *H. annuus*, to determine the natal host for cluster II and to allow for comparative study of host environments.

Putative F1 hybrids were also found in nature. The five flies that comprise Cluster IV were of intermediate assignment for a $K = 2$ in our STRUCTURE analysis, apparent hybrids between cluster I flies and flies in clusters II and III. All AFLP loci fixed between cluster I and clusters II and III were found at intermediate proportions among cluster IV flies, indicating a mixed ancestry. Cluster IV flies also had a mixture of both morphologies and both mtDNA haplotype groups. That fixed differences persist between cluster I and clusters II and III suggests that even though F1 hybrids exist, certain regions of the genome

are robust to introgression, while other regions may be more porous (e.g., Turner et al. 1999; Nosil 2008; Michel et al. 2010). A finer scale, sequence-based marker system may be necessary to evaluate the status of hybrids and their contribution to gene flow in this system (e.g. RAD-tags: Gompert et al. 2010).

The two flies in Cluster V may represent still another reproductively isolated group. Phylogenetic analysis grouped cluster V as sister to cluster II, both clusters share a common morphology, and results from STRUCTURE simulations suggest they may be natural F2 backcross flies. However, there was strong support for the monophyly of these two individuals (Fig. 3), and that the two cluster V flies share a unique haplotype found in no other flies in our study (Fig. 2, asterisk) suggests that they may be distinct from other flies collected. Both cluster V flies were collected on the final day of sampling, and so may represent a fourth, late summer, race that was incompletely sampled in this study.

Genetically differentiated *Strauzia* fly populations are partially allochronically isolated

Significant differences in the date of capture for adult flies in each cluster suggest that allochrony in adult emergence may be a premating reproductive barrier between fly species and races. Flies live an average of 13.5 ± 7.8 (SE) days under ideal lab conditions (n = 16; AAF, unpublished data), so differences of up to 28 days between clusters in their peak incidence in the field depress the propensity for gene flow between demes. Allochronic differentiation is common among phytophagous insects occupying different ecological niches: differences in diapause length and eclosion timing partially or completely isolate races and species of Diptera (Filchak et al. 1999; Joy and Crespi 2007), Hymenoptera (Forbes et al. 2009), and Lepidoptera (Scriber and Ording 2005; Santos et al. 2007), among others (Abbot & Withgott, 2010). Temporal differences are typically correlated with shifts to new hosts with divergent life histories (although see Scriber and Ording 2005). Here, as at least two of three *Strauzia* races are using *H. tuberosus* as their larval host (see below), and as these races occur as adults at different times during the year, we hypothesize that cluster I and cluster III flies colonized *H. tuberosus* independently, and that they currently occupy different temporal niches on the same host plant. Females of cluster III, found earlier in the season, likely oviposit into *H. tuberosus* earlier than cluster I females. As the plant grows and adds tissue during the course of the season, temporal staggering of ovipositon events may allow for both taxa to coexist.

Allochronic differences between genetic races of *S. longipennis* do not necessarily reflect that allochrony was an important pre-mating barrier at the time of initial divergence, nor that it is the only contemporary barrier to gene flow. For instance, many phytophagous insect races also differ in their host discrimination behaviors, which, if mating occurs on or near different hosts, can translate into assortative mating and prezygotic isolation (Funk 1998; Craig et al. 2001; Linn et al. 2003; Matsubayashi et al. 2011). Divergent host performance characters may also isolate demes, wherein feeding or development on a non-natal host plant results in a reduction in fitness (Via 1991; Brazner and Etges 1993; de Jong et al. 2007). Hybrids between insects adapted to different host plants may also be less fit on all hosts than either parental race, a form of extrinsic postzygotic barrier (Dambroski et al. 2005). Finally, intrinsic pre- or postzygotic barriers can isolate taxa, including hybrid sterility and hybrid inviability (Hollocher and Wu 1996), or incompatibility resulting from cytoplasmically inherited endosymbionts (Werren 1997). We must therefore expect that allochronic isolation between *S. longipennis* races is not the only current barrier to gene flow in this system.

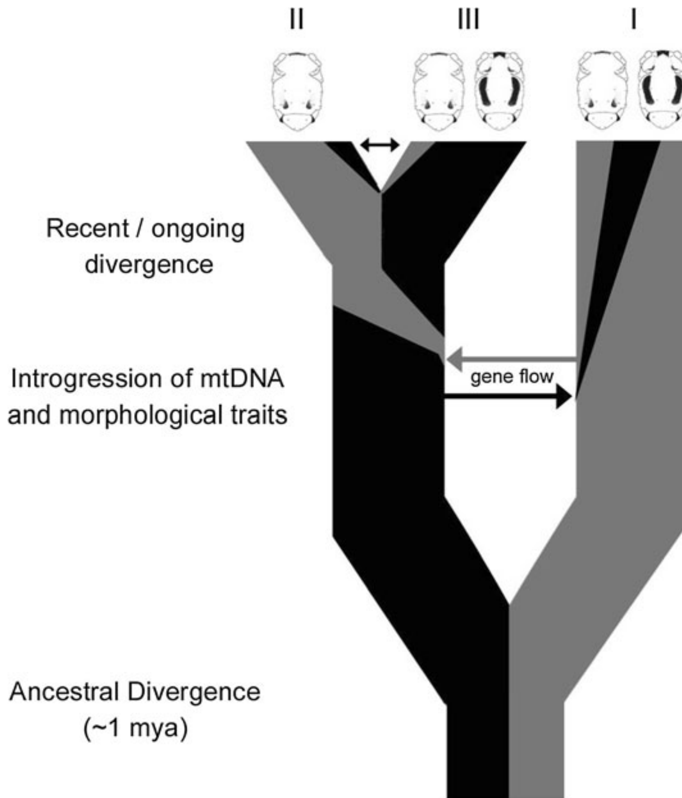

Fig. 6 A hypothesis for speciation and gene flow among three morphologically cryptic races of *Strauzia* collected from *H. tuberosus*. First, after an ancestral split between cluster *I* and the ancestor of clusters *II* and *III*, each lineage may have seized or evolved a unique haplotype and/or morphology. Subsequent gene flow between lineages may have resulted in reciprocal capture of both mitochondrial haplotypes and morphological variation, resulting in their imperfect contemporary relationship with actual species limits. Divergence between clusters *II* and *III* probably represents a more recent repeat of the above: again, two lineages diverge and begin to sort mitochondria and morphology but continue to experience gene flow without lineage fusion

Introgression of mitochondria and morphology: evidence for speciation-with-gene-flow

We propose that the two mitochondrial haplotype groups and the two morphological varieties described by Axen et al. (2010) reflect relatively ancient genetic structure, now obscured due to introgression between lineages. Mitochondrial haplotype groups A and B differ at 18 of the total 658 base pairs, which under the assumption of a standard insect molecular clock, dates their most recent common ancestral sequence to between 0.77 and 1.19 Mya (Brower 1994; Papadopoulou et al. 2010). These do not, however, correspond to differences between contemporary lineages as defined by the more powerful AFLP scan. No genetic cluster identified using nuclear data had just a single haplotype group, and the most divergent lineage, Cluster I, possessed a particularly heterogeneous mixture of both haplotype groups. The most parsimonious explanation for such patterns is that mitochondrial genomes have been captured by non-ancestral lineages (e.g., Llopart et al. 2005). Likewise, the two divergent morphological types (*vittigera* and *typica*) also appear to have

moved between lineages, given that two of the three races contained a mix of both *vittigera* and *typica* morphologies. An alternative explanation might be that morphological variants have evolved in parallel in different lineages, but this seems a more convoluted explanation for these patterns.

Taken together, patterns of genetic differentiation and morphological variation suggest a model of speciation-with-gene-flow for *S. longipennis* races (Fig. 6), wherein movement of genetic material between demes has occurred without a breakdown of genetically differentiated clusters. Speciation-with-gene-flow models are increasingly relevant to emerging animal systems, especially insects, and promise to offer much new information about the genomics of speciation. Such models strongly imply that complete reproductive isolation is not necessary for genetic divergence, and that divergent lineages may experience even relatively high rates of gene flow without consequent lineage fusion (Nosil 2008). To date, such patterns have been elegantly documented in a range of phytophagous insects, including *Anopheles* mosquitoes (Turner et al. 1999; White et al. 2010), *Acyrthosiphon* pea aphids (Via and West 2008), *Neochlamisus* leaf beetles (Funk et al. 2011), and *Rhagoletis* fruit flies (Schwarz et al. 2005; Michel et al. 2010), and early indications are that reduced recombination in some number of genomic regions "protect" important allelic combinations. The study of organisms at various stages along the so-called "speciation continuum" (Hendry et al. 2009), ultimately allows for a better understanding of how reproductive isolating barriers arise and are maintained.

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