TESTING THE OUT-OF-FLORIDA HYPOTHESIS ON THE ORIGIN OF CHEATING IN THE YUCCA–YUCCA MOTH MUTUALISM

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Abstract.—Mutualistic interactions can be exploited by cheaters that take the rewards offered by mutualists without providing services in return. The evolution of cheater species from mutualist ancestors is thought to be possible under particular ecological conditions. Here we provide a test of the first explicit model of the transition from mutualism to antagonism. We used the obligate pollination mutualism between yuccas and yucca moths to examine the origins of a nonpollinating cheater moth, *Tegeticula intermedia*, and its pollinating sister species, *T. cassandra*. Based on geographic distribution and ecological factors affecting the pollinators, previous research had indicated that the cheaters evolved in Florida as a result of sympatry of *T. cassandra* and another pollinator species. We used mitochondrial DNA (mtDNA) sequences and amplified fragment length polymorphism (AFLP) data to investigate the phylogeographic history of the pollinator-cheater sister pair and to test whether the cheaters arose in Florida. Contrary to predictions, phylogenetic and population genetic analyses suggested that the cheaters evolved in the western United States and subsequently spread eastward. Western populations of cheaters had the most ancestral haplotypes and the highest genetic diversity, and there was also significant genetic structure associated with a geographic split between eastern and western populations. In comparison, there was evidence for weak genetic structure between northern and southern pollinator populations, suggesting a long history in Florida. The western origin of the cheaters indicated that the pollinators have more recently become restricted to the southeastern United States. This was supported by AFLP analyses that indicated that the pollinators were more closely related to the western cheaters than they were to geographically proximate cheaters in the east. Shared mtDNA between pollinators and eastern cheaters suggested hybridization, possibly in a secondary contact zone. The results negate the out-of-Florida hypothesis and reveal instead a long, complex, and disparate history for the pollinator-cheater sister pair.

Key words.—Cheater, exploitation, hybridization, incomplete lineage sorting, phylogeography, pollination mutualism.

Cheating in mutualistic interactions can occur when mutualists or other members of the community opportunistically exploit mutualisms (Addicott and Tyre 1995; Denison 2000; Maloof and Inouye 2000; Irwin et al. 2001; Anderson and Midgley 2002; Bshary and Grutter 2002). In a few cases, cheaters have been identified as species that have evolved from a mutualist ancestor (e.g., Dafni 1984; Pellmyr et al. 1996; West et al. 1996; Després and Jaeger 1999). A lingering problem has been to explain this distinctive case of how cheaters evolve from mutualists (Bronstein 2001). Historically, cheating was thought to cause breakdown of the mutualism, perhaps leading to reciprocal extinction of mutualist and cheater lineages (Trivers 1971; Axelrod and Hamilton 1981; Bull and Rice 1991; Doebeli and Knowlton 1998; Schwartz and Hoeksema 1998).

Recent models of mutualism, however, demonstrate that there are circumstances that make possible the evolution and subsequent persistence of cheaters with mutualists (Law et al. 2001; Ferrière et al. 2002). These models suggest that mutualists and cheaters can coexist, and furthermore, that divergent selection could drive sympatric speciation, resulting in the evolution of mutualist and cheater sister species. Although there is yet no evidence for sympatric speciation in mutualist lineages, coexistence of mutualist species may be important in the evolution of cheating in several interactions (Compton et al. 1991; Pellmyr et al. 1996). Specifically for the case where two mutualist species coexist, the evolution of cheating in one species would not necessarily cause the breakdown of the mutualism because the second mutualist species would also be present. The overlap of two mutualist species, then, may facilitate the shift to cheating in one of the mutualist lineages.

This scenario of coexisting mutualists has been proposed as the catalyst driving the evolution of cheater species in the obligate pollination mutualism between yuccas and yucca moths, where two separate species of cheaters have been identified (Pellmyr et al. 1996; Pellmyr and Leebens-Mack 2000). Yucca moths feed only on yucca seeds, and yuccas are pollinated only by yucca moths (Riley 1892; Powell 1992; Pellmyr 2003). Cheater yucca moths lay their eggs into developing fruit and have lost the specialized mouthparts that pollinator moths use to actively deposit pollen. Emerging later in the season than pollinators, cheaters are dependent on the actions of the pollinators for their reproduction. The nonpollinating cheater moth, *Tegeticula intermedia*, lives throughout the eastern two-thirds of the United States (Fig. 1) and appears to have recently split from its pollinating sister species, *T. cassandra*, that is endemic to the extreme southeastern United States (Pellmyr and Leebens-Mack 2000). The cheater moth has a broad host range that includes *Yucca filamentosa*, *Y. glauca*, *Y. constricta*, and *Y. baileyi*. In Florida and southeastern Georgia, *T. intermedia* and *T. cassandra* coexist on *Y. filamentosa* with another closely related pollinator, *T. yuccasella*. Thus, sympatry of the two pollinator species on populations of *Y. filamentosa* in the southeastern

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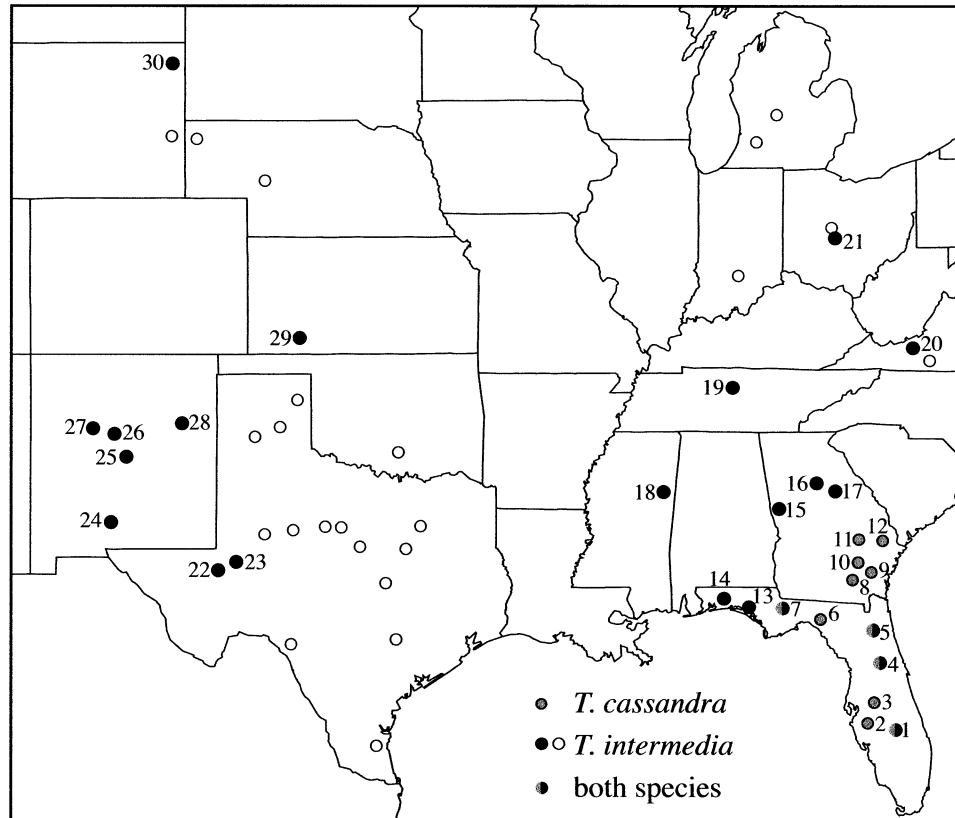
FIG. 1. Site locations for *Tegeticula intermedia* and *T. cassandra* in the United States. Black circles show the locations of collection sites of *T. intermedia* included in this study and open circles show known localities. Gray circles show the locations of *T. cassandra* collection sites and the extant range of *T. cassandra*. Black and gray circles indicate locations where both *T. intermedia* and *T. cassandra* were collected. The numbers correspond to the site information presented in Table 1.

United States fits the description of the proposed ecological setting that could lead to the evolution of cheating in the *T. intermedia–T. cassandra* lineage (Fig. 2).

The purpose of this paper is to test this explicit model of the transition from mutualism to antagonism. This study represents the first genetic test of the conditions leading to the evolution of cheating in mutualisms, and will be followed by an ecological analysis of the life-history changes during the shift to antagonism. Based on the current geographic distribution of the three moth species, cheaters are predicted to have originated in the southeastern United States if the coexistence of *T. cassandra* and *T. yuccasella* facilitated the shift to cheating. The recent split between *T. intermedia* and *T. cassandra* offers an opportunity to test an out-of-Florida origin for the cheaters and allows for a comparison to the historical biogeography of the region (Fig. 2). We take a phylogeographic approach using mitochondrial DNA (mtDNA) sequences and amplified fragment length polymorphism (AFLP) data to examine the origins of the cheater *T. intermedia*. First, we address whether the cheaters arose in the southeastern United States and subsequently moved northward and westward as predicted by Pellmyr and Lee-bens-Mack (2000). Second, we examine whether historical biogeography has played a significant role in determining the genetic structure of *T. intermedia* and *T. cassandra* populations. Finally, we ask whether shared mitochondrial haplo-

types discovered during the course of this study result from incomplete lineage sorting or hybridization. This paper provides the first test of the out-of-Florida hypothesis using a battery of population genetic and phylogeographic tools.

MATERIALS AND METHODS

DNA Isolation and Sequencing

Moths were collected and stored either at −80°C or in 95% ethanol at 4°C. Samples of *T. intermedia* were collected from five populations of *Y. glauca*, one population of *Y. elata*, three populations of *Y. baileyi*, and 13 populations of *Y. filamentosa*. The locations of *T. intermedia* form two distinct clusters divided by the Mississippi River. These clusters will be referred to as eastern and western populations of *T. intermedia*. *Tegeticula cassandra* were collected from 12 populations of *Y. filamentosa* (Table 1, Fig. 1). Samples of *T. yuccasella* from three *Y. filamentosa* sites in Florida (Eglin Air Force Base, Apalachicola Bluffs Preserve, and Gold Head Branch State Park), one *Y. glauca* site in New Mexico (Cuervo), and one *Y. elata* site in Texas (Hueco) were also included as outgroups. DNA was extracted from a total of 397 individuals using Isoquick Nucleic Acid Extraction Kits (Orca Research Inc, Bothell, WA). Before extraction, the head, wings, and genitalia of adults were removed and kept as vouchers. For
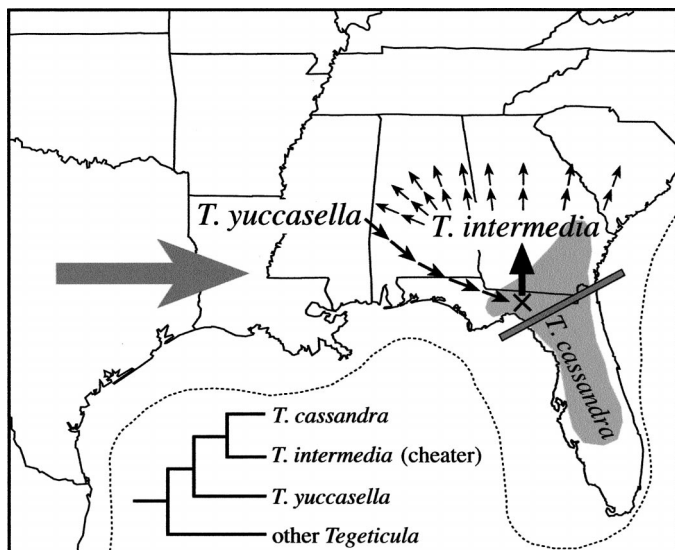
FIG. 2. The out-of-Florida hypothesis on the evolution of cheating as proposed by Pellmyr and Leebens-Mack (2000) and the historical biogeography of the southeastern United States. The pollinator *Tegeticula yuccasella* was predicted to have come into recent contact with *T. cassandra* (range indicated by gray area) in Florida or southern Georgia. As a consequence of the overlap of the two pollinator species, *T. intermedia* arose (thick black arrow) and subsequently spread northward and westward (small black arrows). The historical biogeography of the southeastern United States is well known for the Pliocene and Pleistocene. The presence of continuous habitat during times of low sea level allowed many species to extend their ranges. This surge of immigrants along the Gulf Coast Corridor is indicated by the large gray arrow and the dotted line shows an example Pleistocene coastline. By the mid-Pleistocene, Florida's western affiliations had weakened, and many species were separated from their western relatives. Moreover, during periods of high sea level, such as those during the early Pliocene, there may have been a seaway across parts of northern Florida (Webb 1990) that served as a barrier to gene flow for many species (gray bar across northern Florida).

larval specimens, the entire individual was ground during DNA extraction.

We sequenced 779 bp of the mitochondrial cytochrome oxidase I (COI) gene for 145 *T. intermedia* individuals and for 252 *T. cassandra* individuals. This region corresponded to positions 1461–2302 of the *Drosophila yakuba* COI gene (Clary and Wolstenholme 1985). Fifty-two of these moths were sequenced previously (Pellmyr et al. 1996; Pellmyr and Leebens-Mack 1999). Five *T. yuccasella* were included as outgroup taxa based on the analyses of Pellmyr and Leebens-Mack (1999). MtDNA sequences were determined using previously described methods for automated DNA sequencing on an ABI 377 sequencer (Applied Biosystems, Foster City, CA; Segraves and Pellmyr 2001). Polymerase chain reaction (PCR) amplification was conducted in 30-μL reaction volumes containing 1× PCR buffer (Promega, Madison, WI), 2.5 mM magnesium chloride, 0.2 mM dNTPs, 0.25 μM each primer, 1 unit *Taq* polymerase, and approximately 10 ng DNA template for 35 cycles at 95°C for 60 sec, 52°C for 60 sec, 72°C for 90 sec. PCR products were cleaned using Qiagen PCR purification columns (Qiagen, Inc., Valencia, CA). Dye terminator reactions were carried out following the Big Dye protocol (Applied Biosystems) with the exception that one-

quarter reactions were conducted with the addition of a Tris buffer (1 M Tris-acid, 1 M magnesium chloride, pH 9.0). Dye terminator reactions were cycled at one cycle at 96°C for 2 min, 25 cycles at 96°C for 30 sec, 50°C for 30 sec, and 60°C for 4 min. The resulting products were cleaned using Centri-sep Sephadex columns (Princeton Separations, Adelphia, NJ). Samples were separated on a 5% Long Ranger acrylamide gel (BMA Products, Rockland, ME), and electrophoresis followed the 36E–1200 run module for 7 h. Both forward and reverse strands were sequenced for each individual, edited, and then combined into a single consensus sequence using Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI).

Phylogenetic Analyses

The mtDNA sequences were readily aligned by eye. Aligned sequences were examined using phylogenetic analyses implemented in PAUP* 4.0b10 (Swofford 2002). To simplify analyses, individuals within species groups bearing identical sequence haplotypes were condensed into a single haplotype designation. The model of sequence evolution was selected using hierarchical likelihood-ratio tests involving an initial neighbor-joining search with LogDet distances to identify a tree (Huelsenbeck and Crandall 1997; Sullivan et al. 1997). This model of evolution was then used in a subsequent tree search under maximum likelihood to find the best tree. One hundred bootstrap replicates were generated to estimate support for the resulting tree topology. We also constructed a haplotype network for *T. intermedia* using TCS 1.13 (Clement et al. 2000). Networks may more accurately represent the relationships among closely related haplotypes because they can contend with the presence of ancestral haplotypes. Haplotypes that occur in the interior of a network tend to represent ancestral haplotypes because they are more likely to produce mutational derivatives (Donnelly and Tavaré 1986). The presence of many closed loops in the haplotype networks precluded the use of nested clade analysis (Templeton et al. 1995), which may provide less information when there is limited phylogenetic signal (Althoff and Pellmyr 2002). Thus, demographic analyses were used to examine population genetic structure.

Demographic Analyses

To further examine the population structure and demographic history of pollinator and cheater populations, we used several population genetic approaches implemented in Arlequin version 2.0 (Schneider et al. 2000). For the following analyses, we used the Tamura measure of haplotype divergence (Tamura 1992) to correct for unequal base frequencies and Ti:Tv rates, and the estimated gamma from the maximum-likelihood analysis. Analysis of molecular variance (AMOVA) was used to examine population structure associated with the predicted biogeographic patterns for both species. For *T. intermedia*, structure was examined at two levels: between populations in the eastern and western United States and between northern and southern populations within the eastern United States. For *T. cassandra*, this analysis examined structure between populations north and south of the proposed contact zone in Florida.

TABLE 1. Site localities, sample sizes, and haplotype distribution for the molecular analysis of *Tegeticula intermedia* and *T. cassandra*. Site numbers correspond to the numbers presented in Figure 1. Haplotype designations correspond to those presented in Figures 3 and 4. *Tegeticula cassandra* haplotypes that were identical to *T. intermedia* are indicated in bold with an asterisk, *T. cassandra* haplotypes that were more similar to *T. intermedia* are underlined, and *T. cassandra* haplotypes that were basal to the *T. intermedia* clade are indicated in bold italics.

Site no.	Location	Latitude	Longitude	*T. intermedia*		*T. cassandra*	
				N	Haplotypes	N	Haplotypes
1	Archbold Biological Station, Lake Placid, FL	27°14′00″N	81°24′00″W	20	UA	23	E, <u>G</u>, K, M, *N*, <u>Q</u>, W, X, KA
2	Lake Manatee State Park, FL	27°28′42″N	82°20′08″W	0	—	20	E, K, M, Q, AM
3	Inverness, FL	28°59′00″N	82°25′00″W	0	—	26	A, E, <u>G</u>, J, M, Q, *AP*, <u>IA</u>, <u>CN</u>, TA
4	Ocala National Forest, FL	29°08′00″N	81°31′00″W	1	UA	30	A, E, <u>G</u>, I, J, M, Q, <u>W</u>, <u>V</u>, AK, AL, A<u>T</u>, NA
5	Gold Head Branch State Park, FL	29°49′55″N	81°57′11″W	1	UA	20	A, <u>G</u>, I, J, M, P, Q, T, U, *V*, JA
6	Perry, FL	30°07′02″N	83°34′55″W	0	—	22	A, *C*, I, M, *N*, P, U, BA, WA, XA, YA, ZZ
7	Apalachicola Bluffs Preserve, Bristol, FL	30°34′08″N	84°56′52″W	21	AA, UA	20	A, *L*, M, P, S, Z, BA, *CA*, DA, EA, *UA**
8	Douglas, GA	31°31′35″N	82°44′50″W	0	—	5	<u>F</u>, *L*, P, CL
9	Jesup, GA	31°42′28″N	81°44′33″W	0	—	21	A, D, <u>F</u>, *L*, M, Q, <u>R</u>, S, Z, DA, <u>RA</u>
10	Baxley, GA	31°46′41″N	82°20′55″W	0	—	33	A, <u>F</u>, *L*, M, *N*, P, S, CI, CT, GA, HA, ZZ
11	5 km N Oak Park, GA	32°21′29″N	82°18′53″W	0	—	20	A, <u>F</u>, *L*, M, *N*, P, S, Z, LA
12	Statesboro, GA	32°26′55″N	81°47′00″W	0	—	12	<u>F</u>, *L*, M, *N*, P, *AF**
13	Destin, FL	30°23′36″N	86°29′45″W	6	OA, PA, UA	0	—
14	Eglin Air Force Base, FL	30°43′15″N	86°44′18″W	19	AF, AO, UA	0	—
15	Camp Meeting Rock, GA	33°18′19″N	85°07′30″W	8	Y, AF, UA	0	—
16	Walnut Grove, GA	33°44′33″N	83°51′09″W	3	AF	0	—
17	Union Point, GA	33°36′56″N	83°04′29″W	1	AF	0	—
18	Columbus, MS	33°29′44″N	88°25′38″W	4	AF	0	—
19	Vine, TN	36°01′52″N	86°21′28″W	6	AF, FA	0	—
20	Goldbond, VA	37°22′48″N	80°30′40″W	4	AB, AF	0	—
21	Georgesville, OH	39°53′27″N	83°13′19″W	4	AF, UA	0	—
22	Royalty, TX	31°22′20″N	102°52′00″W	5	AD, AE, AI	0	—
23	11 km SW Odessa, TX	31°50′44″N	102°17′51″W	4	AI, AV, BD, O	0	—
24	White Sands National Monument, NM	34°46′00″N	106°20′00″W	1	CS	0	—
25	Punta de Agua, NM	34°36′00″N	106°17′00″W	5	AI, AQ, AW, AX, CV	0	—
26	Los Lunas, NM	34°48′22″N	106°43′58″W	5	AI, CW, CX, CY, CZ	0	—
27	Correo, NM	34°57′18″N	107°11′03″W	14	AE, AH, AI, AQ, AY, AZ, BD, CC, CD, DH	0	—
28	Cuervo, NM	35°01′52″N	104°24′29″W	5	AE, AI	0	—
29	Fowler, KS	37°23′08″N	100°11′43″W	6	AG, AH, AI, DF, MA	0	—
30	Sundance, WY	44°24′23″N	104°22′31″W	2	AF	0	—

Mismatch distributions were independently generated for *T. intermedia* and *T. cassandra* and compared against the model of sudden population expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992). A unimodal distribution indicates that there has been demographic change (e.g., population expansion after a bottleneck) in the population under consideration, whereas a multimodal distribution suggests demographic equilibrium. We used DnaSP 4.00 (Rozas et al.

2003) to calculate Tajima's D test of selective neutrality to test for demographic change (Tajima 1989a,b). A significantly negative Tajima's D is expected under a model of sudden population expansion and a positive value is expected when populations are subdivided or bottlenecked. In addition, nucleotide diversity (π) and haplotype diversity ($\hat{H}$) were calculated using Arlequin, and nucleotide diversity (θ) was calculated using DnaSP. The estimates of nucleotide diversity

and subsequent calculations of effective population size from π were not substantially influenced by the presence of shared mtDNA haplotypes found during the course of the study (data not shown). We have not included analyses of isolation by distance because sample sizes were too small in several instances, particularly for cheaters in the western and northeastern United States.

Hybridization Versus Ancestral Polymorphism

Because some *T. cassandra* individuals shared mtDNA haplotypes with *T. intermedia*, we needed a means to distinguish between incomplete lineage sorting and hybridization. To do this, we used the program MDIV (Nielsen and Wakeley 2001) to estimate migration rate (*M*) under a finite-sites model. This program uses a Markov chain Monte Carlo (MCMC) method to jointly estimate demographic parameters following a coalescent process. This approach is specifically designed to distinguish between hybridization (migration) and incomplete lineage sorting for two closely related populations or species. Each MCMC run was conducted with 2×10^6 to 3×10^6 cycles of the Markov chain with a burn-in time of 500,000 cycles. Convergence of the chains was assessed by running five chains with different seeds and starting values of M_{max}, the maximum value of the migration rate scaled to the effective population size, and T_{max}, the maximum value of the divergence time scaled to the effective population size ($M_{max} = 6$–10, $T_{max} = 6$–10). The output of MDIV provides an estimate of *M,* the migration rate scaled to its effective population size. Significance of migration rates was determined by comparing the posterior probability of the mode of the distribution (an estimate of *M* based on the posterior probability) to the posterior probability of no migration and by conducting likelihood ratio tests (LRTs) comparing the likelihoods of the estimate of *M* and *M* = 0.

Divergence Time Estimation

MDIV was also used to determine estimates of nucleotide diversity, θ (twice the effective population size times the mutation rate), and the scaled divergence time, *T* (divergence time divided by the effective population size), to calculate divergence time between *T. intermedia* and *T. cassandra*, and between two emerging disjunct groups within *T. intermedia*. Divergence time, T_{DIV} was calculated using the equation:

$$T_{DIV} = \frac{T\theta}{2\mu}, \tag{1}$$

where *T* and θ were estimated in MDIV. The mutation rate, μ, was calculated from the clock estimates of Pellmyr and Leebens-Mack (1999) and estimates of sequence divergence derived from five individuals taken from each of the species in the *T. yuccasella* species complex (D. Althoff, K. Segraves, J. Leebens-Mack, O. Pellmyr, unpubl. ms.). Prior to calculations of μ, sequence divergence was corrected using the HKY85+I model of evolution. We used the age estimate of 3.2 million years for the radiation of the *T. yuccasella* species complex (Pellmyr and Leebens-Mack 1999) and determined the maximum sequence divergence within the species complex. The mutation rate was calculated as ($d/2t$)gL, where *d* is sequence divergence (substitutions per site), *t* is divergence

time, *g* is generation time (one year), and *L* is the length of the sequence. This calculation yielded an estimate of $\mu =$ 0.635 percent sequence divergence per million years. This value of mutation rate is somewhat lower than the commonly used estimate of 2.3% per million years (Brower 1994). Convergence of the chains was assessed following the techniques outlined in the previous section. The presence of shared mtDNA haplotypes between the species did not substantially influence the estimates of divergence time (data not shown).

Amplified Fragment Length Polymorphism Analyses

AFLPs were also used to assess population structure in 91 of the samples included in the mtDNA analysis plus five samples of *T. yuccasella*. Details of the procedures are presented in the supporting online documentation (available at http://dx.doi.org/10.1554/03-489.1.s1). Presence/absence of fragments was assessed visually using the GeneScan software version 3.1.2 (Applied Biosystems). In total, 308 fragments were scored for 30 *T. cassandra* individuals and 61 *T. intermedia* and compared in a phylogenetic analysis. DistAFLP (Mougel et al. 2002) was used to calculate the Jaccard index between all pairwise combinations of individuals. This distance matrix was used to conduct a minimum evolution search in PAUP* with *T. yuccasella* included as an outgroup. The presence/absence of fragments was also used to conduct a second PAUP* minimum evolution search with Nei-Li distances.

To make direct comparisons between the mtDNA analyses and the AFLPs, we also used AMOVA to test for population structure associated with biogeography. The Jaccard index was used as the distance matrix, and genetic structure was examined following the same groupings used in the mtDNA analyses (see above). Finally, we estimated genetic diversity (Ht and Hw) for each species and population grouping using the software AFLPSurv (Vekemans et al. 2002) following the methods of Lynch and Milligan (1994).

RESULTS

Phylogenetic Patterns

The mtDNA sequencing revealed 83 haplotypes among the 397 individuals sequenced, and there were no indels inferred among the 779 bp examined (GenBank accession numbers AY563474–AY563506, AY568227–AY568276). Thirty-three haplotypes were found in *T. intermedia* and 50 in *T. cassandra* (see online documentation). Corrected sequence divergence ranged from 0.001 to 0.034 substitutions per site among *T. cassandra* haplotypes (average 0.010), 0.001 to 0.012 substitutions per site among *T. intermedia* haplotypes (average 0.006), and 0 to 0.030 substitutions per site between the species (average 0.0158). The likelihood ratio tests failed to reject the HKY85+G+I model as the simplest model fitting the data. The proportion of invariable sites was 0.77, Ti:Tv ratio was 9.25, gamma was 0.67, and base frequencies were AT biased (A = 0.32, C = 0.13, G = 0.15, T = 0.40). The maximum-likelihood analysis resulted in a single tree that largely reflected species designations (−ln L 1882.19, Fig. 3). There was strong support for the ingroup, but only moderate support for a limited subset of branches within the ingroup. *Tegeticula cassandra* was polyphyletic, with some
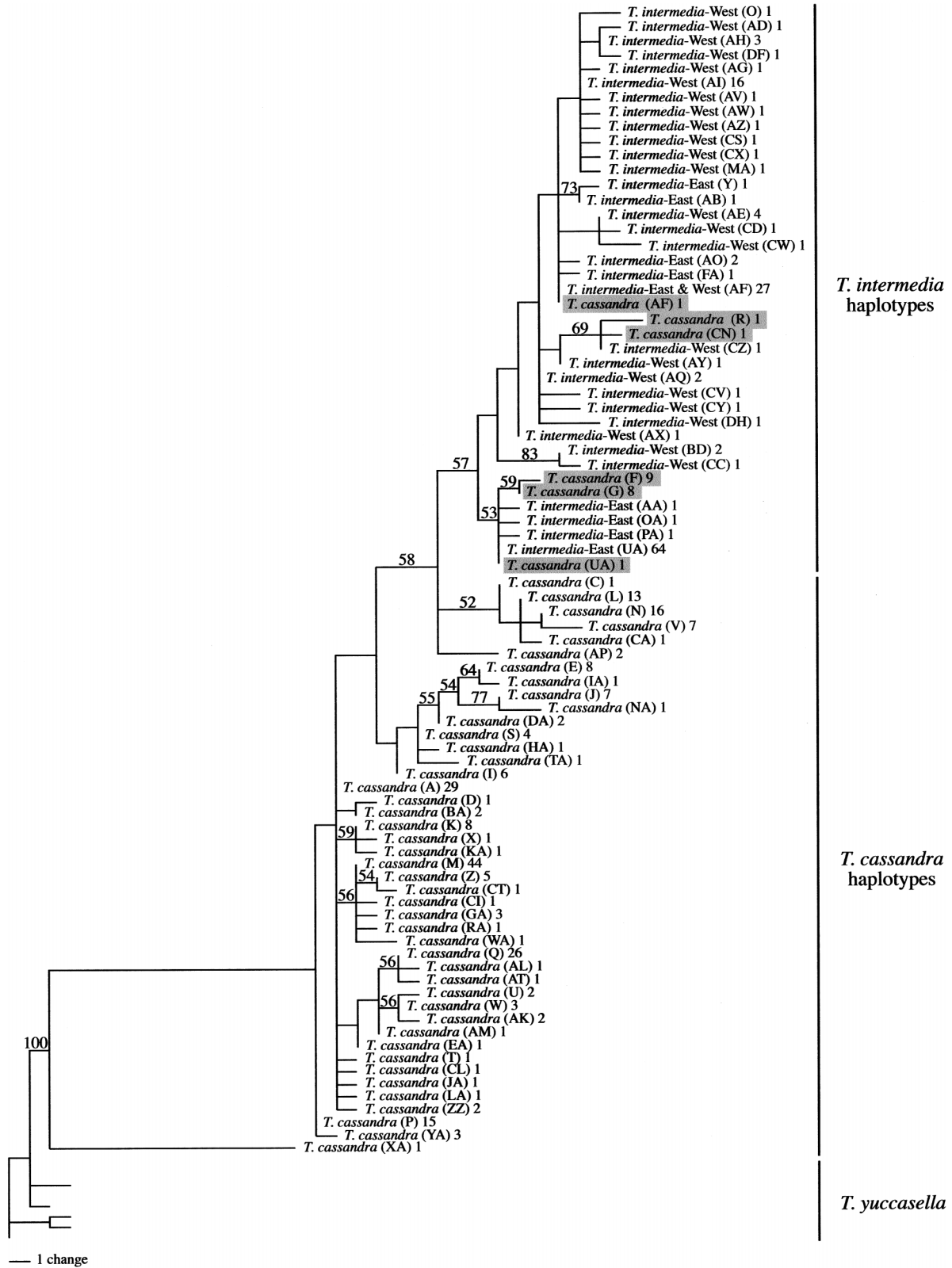
FIG. 3. Maximum-likelihood tree for *Tegeticula intermedia* and *T. cassandra* haplotypes. The two major groups and the outgroup are indicated with vertical bars to the right of the tree. The shaded haplotypes are *T. cassandra* haplotypes that are more closely related to *T. intermedia*. Letters and numbers following the species name correspond to the haplotype designation and the number of individuals with that haplotype. Numbers above branches are bootstrap values greater than 50%.
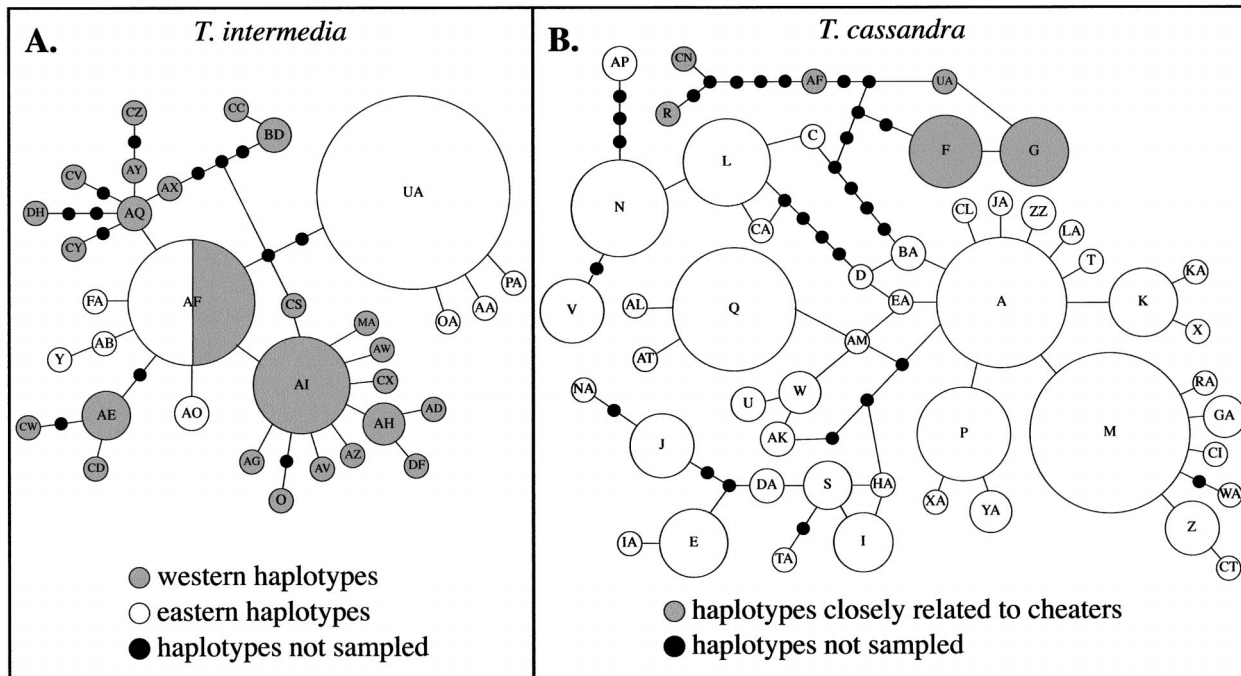
FIG. 4. (A) Mitochondrial DNA haplotype network for *Tegeticula intermedia*. Gray circles indicate haplotypes found exclusively in the western United States, and white circles are haplotypes found exclusively in the eastern United States. The gray and white circle represents haplotype AF that was found in both eastern and western populations. (B) Mitochondrial DNA haplotype network for *T. cassandra*. Gray circles indicate haplotypes that clustered with *T. intermedia*. The connections between the *T. intermedia* and *T. cassandra* networks occur at haplotypes AF and UA. For both networks, black circles indicate the position of haplotypes not sampled. The area of the circle indicates the frequency of the haplotype. Each branch in the diagram represents a single step, irrespective of its length.

haplotypes appearing more closely related to *T. intermedia*. In two instances, *T. cassandra* shared identical haplotypes with cheaters (the eastern *T. intermedia* haplotypes, AF and UA), there were four other haplotypes more closely related to *T. intermedia* (haplotypes F, G, R, and CN), and several other haplotypes that were basal to the *T. intermedia* clade (haplotypes C, N, L, V, CA, and AP). Of the 252 *T. cassandra* examined, 21 individuals had haplotypes nested in the *T. intermedia* clade.

Haplotypes UA, AF, and AI were the most frequently found haplotypes in *T. intermedia*. As expected based on the prevalence of these haplotypes, AF and AI were placed in interior positions of the haplotype network relative to other haplotypes (Fig. 4A). Of the 33 haplotypes found in *T. intermedia*, 13 were only a single step from either AF or AI. Although UA was also common, this haplotype was near the tip of the network and had few connections. The interior positions of haplotypes AF and AI in combination with their relatively high frequency suggest that these are the most ancestral haplotypes in *T. intermedia*. Haplotype AI was found exclusively in the western United States, and AF was commonly found in the eastern United States and shared by a single individual from the west (see Table 1). For *T. cassandra*, haplotypes A, M, and Q were the most common and were generally found throughout the range (Table 1). Of these haplotypes, A had the most connections, was a single step from 10 other haplotypes, and was in the interior of the network (Fig. 4B). The abundance of closed loops in the *T. cassandra* network reflects weak phylogenetic signal.

Demographic Patterns: Mitochondrial DNA Data

AMOVA detected significant genetic structure in both *T. intermedia* and *T. cassandra* (Table 2). There was strong genetic structure associated with the division between eastern and western *T. intermedia*. About 43% of the molecular variance was explained by this geographic subdivision. There was no structure associated with the proposed biogeographic split between northern and southern Florida for *T. intermedia*; however, within *T. cassandra*, there was weak genetic structure associated with this secondary zone of contact. This subdivision explained about 6.5% of the molecular variance within *T. cassandra*, whereas more than 90% was within populations. Both moth species conformed to a model of demographic change (mismatch: *T. intermedia* P = 0.212; *T. cassandra* P = 0.307).

The presence of a significantly negative Tajima's D for *T. intermedia* (Table 3) indicated support for rapid population expansion. The cheater moths had high haplotype diversity and relatively low nucleotide diversity, also suggestive of a bottleneck followed by population expansion (Grant and Bowen 1998). The diversity estimates were highest for populations of *T. intermedia* in the western United States. In the east, populations in the southern biogeographic region were monomorphic for a single haplotype (haplotype UA), and the three study populations in northern Florida were also composed primarily of this haplotype (66%, 84%, or 95% of individuals, respectively, had haplotype UA). As a result, the estimates of effective population size were relatively small

TABLE 2. Analysis of molecular variance (AMOVA) results based on *Tegeticula intermedia* and *T. cassandra* mitochondrial DNA haplotypes. Groupings were based on biogeographic regions. *Tegeticula cassandra* was tested for significant genetic structure associated with the predicted division between northern and southern populations. *Tegeticula intermedia* was examined for structure associated with the division between eastern and western populations. In the eastern United States, *T. intermedia* was tested for significant structure associated with northern and southern populations analogous to the analysis for *T. cassandra*. The negative variance components for *T. intermedia* reflects an absence of genetic structure. ϕ_{ST} summarizes population structure associated with the correlation of haplotypes within individual populations relative to all populations.

AMOVA	*T. intermedia* east/west		*T. intermedia* north/south eastern populations only		*T. cassandra* north/south	
	Variance components	Percent explained	Variance components	Percent explained	Variance components	Percent explained
Among regions	0.795***	43.12	−0.066	−8.31	0.189**	6.49
Among populations within regions	0.434**	23.56	0.620***	78.20	0.084***	2.87
Within populations	0.614***	33.32	0.239***	30.11	2.64***	90.64
ϕ_{ST}	0.670***		0.699***		0.094***	

** $P < 0.001$, *** $P < 0.0001$.

for eastern populations of *T. intermedia*. In contrast, Tajima's *D* was nonsignificant for *T. cassandra*, and the pollinators exhibited high haplotype and nucleotide diversity (Table 3). These patterns are indicative of large, stable populations with long evolutionary histories (Grant and Bowen 1998). The relatively high estimates of effective population size also lend support to this pattern. Although the absolute estimates of effective population size would be influenced by the value of nucleotide diversity (i.e., π or θ) and the mutation rate used to calculate them, the relative values are consistent and should be meaningful for comparative purposes.

Hybridization and Divergence Time

The MCMC analysis indicated that gene flow between *T. intermedia* and *T. cassandra* was significantly greater than zero (LRT $\chi^2 = 16.53$, $P < 0.05$). The estimate of *M* with the greatest posterior probability was 0.64 (Fig. 5). Within *T. intermedia*, however, gene flow between eastern and western populations was not statistically different from zero (LRT $\chi^2 = 0.16$, $P > 0.05$; Fig. 5). Estimates of divergence time were 9.3 million years (range = 5.3–17.5 million years) for the split between *T. intermedia* and *T. cassandra* and 3.4 million years (range = 0.83–5.9 million years) for the split between eastern and western populations of *T. intermedia*.

Amplified Fragment Length Polymorphism Analyses

The two distance methods resulted in similar tree topologies with relatively poor support (see Fig. 6). This analysis indicated that *T. cassandra* and western *T. intermedia* were basal to eastern *T. intermedia*. In general, western *T. intermedia*, eastern *T. intermedia*, and *T. cassandra* formed distinct clades and *T. cassandra* was more closely related to western *T. intermedia* than to the eastern cheaters. Thus, the minimum evolution analysis supports a close relationship between western *T. intermedia* and *T. cassandra*.

The AMOVA on the AFLP data indicated significant genetic structure within *T. intermedia* that corresponded to the split between the eastern and western United States populations (Table 4). This split explained about 7% of the genetic variance within the cheaters. Similar to the mtDNA results, there was no structure within the eastern cheaters. For the pollinators, however, AMOVA indicated no structure between northern and southern populations, whereas the AMOVA on the mtDNA indicated weak but significant structure. The estimates of genetic diversity were similar to the mtDNA data. *Tegeticula cassandra* had the highest diversity, and the eastern cheaters had the lowest diversity (Table 3).

DISCUSSION

Origins of the Cheaters

The overlap of the pollinators *T. yuccasella* and *T. cassandra* has been proposed as the catalyst mediating the evolution of cheating in the *T. intermedia* lineage (Pellmyr and Leebens-Mack 2000). Under this scenario, the overlap of the two pollinators in the southeastern United States spurred the evolution of the cheater *T. intermedia*, and the cheaters subsequently spread northward and westward out of Florida. This particular situation, however, was not supported by the phy-

TABLE 3. Diversity estimates and Tajima's D for *Tegeticula intermedia* and *T. cassandra*. Diversity was examined at the species level and for each of the predicted subgroups based on biogeography. Estimates of haplotype diversity ($\hat{H}$), nucleotide diversity (π and θ), effective population size (N_e), and Tajima's D are based on mitochondrial DNA results. Total genetic diversity (H_t) and average genetic diversity within populations ($H_w \pm 1$ SD) were estimated from the amplified fragment length polymorphism data. Significantly negative Tajima's D are indicated by an asterisk. Effective population size was estimated by equating $2N_e\mu$ to π.

Species and groups	$\hat{H}$	π	θ	N_e	Tajima's D	H_t	H_w
T. intermedia	0.761 ± 0.031	0.004 ± 0.002	0.005 ± 0.004	1130–4400	−1.866*	0.277	0.292 ± 0.014
Western United States	0.882 ± 0.045	0.004 ± 0.002	0.005 ± 0.003	1230–4790	−1.586[ns]	0.307	0.307 ± 0.019
Eastern United States	0.507 ± 0.045	0.002 ± 0.001	0.004 ± 0.002	496–2540	−0.368[ns]	0.226	0.278 ± 0.020
North group	0.584 ± 0.040	0.002 ± 0.001	0.004 ± 0.002	600–2870	−0.368[ns]	0.211	0.280 ± 0.023
South group	0	0	0	0	—	0.264	—
T. cassandra	0.931 ± 0.008	0.007 ± 0.004	0.009 ± 0.004	2660–8710	−1.424[ns]	0.344	0.317 ± 0.023
North group	0.908 ± 0.012	0.007 ± 0.004	0.008 ± 0.004	2370–7880	−1.335[ns]	0.359	0.322 ± 0.037
South group	0.907 ± 0.014	0.007 ± 0.004	0.009 ± 0.004	2730–9000	−1.237[ns]	0.325	0.311 ± 0.027

* $P \leq 0.05$; ns, not significant.

logeographic analysis. Instead, the analysis suggested that the cheaters originated in the western United States rather than in the Southeast. Although the mtDNA tree suggested that the most basal haplotypes were from the eastern United States, these relationships were poorly supported. As a result, we turned to the distribution of haplotypes and genetic diversity to gain a better understanding of the origins of the cheaters. Centers of origin should have higher diversity than peripheral populations that are more recently founded and that have had less time for new haplotypes to accrue (McDonald 1997; reviewed by Hewitt 2000). Haplotype diversity and genetic diversity in *T. intermedia* was highest in the western United States. In fact, western populations had nearly two times the haplotype diversity of eastern populations. Furthermore, the two most interior haplotypes were both found in western populations and lend additional support for a western origin of the *T. intermedia* lineage.

An origin in the western United States nullifies the hypothesis that the cheater *T. intermedia* arose in a contact zone in Florida between the pollinators *T. cassandra* and *T. yuccasella*. A western origin, however, does not contradict the idea of sympatry catalyzing the evolution of cheating. Obviously, a similar ecological scenario could have played out in the west, but no data exist to test this hypothesis. A western origin of *T. intermedia* would also imply that the historical range of ancestral *T. cassandra* once occupied areas outside of its current range in the southeastern United States, fed on western yuccas, and has since become extinct everywhere in its historical range except in the extreme southeastern United States. Both yucca and yucca moth species diversity are much greater in the west and therefore the possibility that two pollinators coexisted is arguably high. As a result, *T. cassandra* may have easily coexisted with *T. yuccasella* or another pollinator species and given rise to the cheater lineage in the west. Without knowledge of the ancestral range of *T. cassandra*, testing this hypothesis will be problematic. *Tegeticula cassandra* lives exclusively in the southeastern United States, and there are no known western populations or closely related pollinators that could be considered ancestral remnants of *T. cassandra*.

The pattern of genetic structure for *T. intermedia* also provides additional clues about the subsequent history of the cheaters. The striking genetic subdivision found between eastern and western cheater populations suggests that these groups of populations have been isolated for a considerable length of time. The AFLP results for *T. intermedia* indicated genetic structure associated with the geographic break between eastern and western populations, and the mtDNA data showed that eastern and western populations of *T. intermedia* harbored nearly unique suites of haplotypes. There was also no evidence for migration, and AMOVA on mitochondrial and nuclear markers indicated that a significant genetic variance was associated with this division. Eastern and western populations of *T. intermedia* were estimated to have diverged about 3 million years ago, which corresponds roughly to predictions based on historical biogeography (Webb 1990).

The low genetic diversity within populations of southeastern *T. intermedia* also suggests that the cheaters have more recently entered the extreme southeastern United States. This is supported by the observations that haplotype diversity
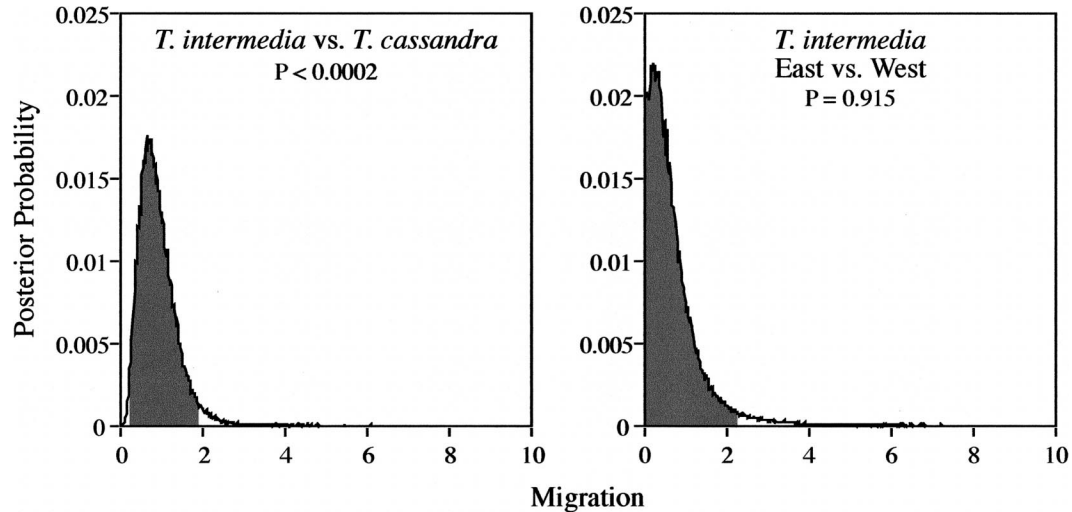
FIG. 5. Posterior distributions of migration (*M*) for comparisons between *Tegeticula intermedia* and *T. cassandra* and between eastern and western groups within *T. intermedia*. The *P*-values indicate the probability that migration is zero, and the shaded areas show the 95% credibility intervals.

drops substantially in populations on the Florida peninsula, that there is only a single mtDNA haplotype in the most southern Florida populations, and that there was no genetic structure associated with a proposed biogeographic split in northern Florida in either the mtDNA or AFLP data. In contrast, *T. cassandra* exhibited weak but significant genetic structure between northern and southern populations in Florida, suggesting perhaps a longer history in the Southeast. One possibility is that *T. intermedia* was present in a refugium east of the Mississippi River, probably along the southeast Atlantic or Gulf Coast where native populations of *Y. filamentosa* existed.

The estimate of the split between *T. intermedia* and *T. cassandra* corresponds roughly with the historical biogeography of the region. Previous estimates of the split between the sister species, however, suggest a more recent divergence time (Pellmyr and Leebens-Mack 1999). Estimates based on a maximum-likelihood approach place the split at 1.26 ± 0.96 million years ago (Pellmyr and Leebens-Mack 1999), whereas the present study estimated a divergence time of approximately 9 million years ago. One possible explanation for the discrepancy between these calculations is that violation of the assumptions underlying the Bayesian analysis has pushed back the divergence time for the current estimate. The Bayesian estimate assumes no population subdivision and constant population size (Nielsen and Wakeley 2001). The data clearly violate these assumptions, thus the Bayesian calculation may overestimate the divergence time (reviewed by Arbogast et al. 2002). The substantial genetic structure within *T. intermedia* associated with the opening and closing of the Gulf Coast Corridor suggests that the divergence of cheaters and pollinators is probably older than the estimate of 1.26 million years, and the divergence time of the species probably lies between the two estimates.

Hybridization and Ancestral Polymorphism

During the course of this study, we discovered several instances of shared mtDNA haplotypes between *T. cassandra*

and *T. intermedia*. The analyses revealed that some *T. cassandra* shared identical haplotypes with *T. intermedia* or had haplotypes that were more closely related to *T. intermedia*. The haplotypes shared between cheaters and pollinators were common haplotypes in eastern populations of cheaters (haplotypes AF and UA), while other haplotypes appeared to be derivatives of these two haplotypes (see Fig. 4B, haplotypes F, G, R, and CN). Two processes may explain this pattern. First, limited hybridization between the pollinators and cheaters may have introduced cheater mtDNA into the *T. cassandra* lineage, and subsequent mutations created additional related haplotypes. Second, the shared haplotypes may be a result of ancestral polymorphism in two closely related lineages that have not had time for lineage sorting to run to completion.

The results suggest that the shared mtDNA haplotypes between *T. cassandra* and *T. intermedia* are likely a result of hybridization. There are several lines of evidence in support of this hypothesis. First, the mtDNA based analysis of migration indicated gene flow between pollinators and eastern cheaters but not between eastern and western populations of cheaters (Fig. 5). The estimates of migration between *T. cassandra* and *T. intermedia* were significantly greater than zero, suggesting that the shared mtDNA haplotypes are a result of hybridization. Second, pollinators only shared haplotypes common to eastern populations of cheaters. One of these haplotypes was exclusively found in the eastern United States, and only a single western individual shared the other haplotype. Third, the demographic analyses are consistent with the hypothesis that the cheaters have recently expanded eastward, possibly bringing them into secondary contact with *T. cassandra*. Both the unimodal mismatch distribution and significantly negative Tajima's *D* indicated that the cheaters have undergone range expansion. The most compelling evidence for a recent expansion of *T. intermedia* into the Southeast is the reduction in mtDNA haplotype diversity. The number of haplotypes per population drops in Florida, and the most southern populations have only a single haplotype.
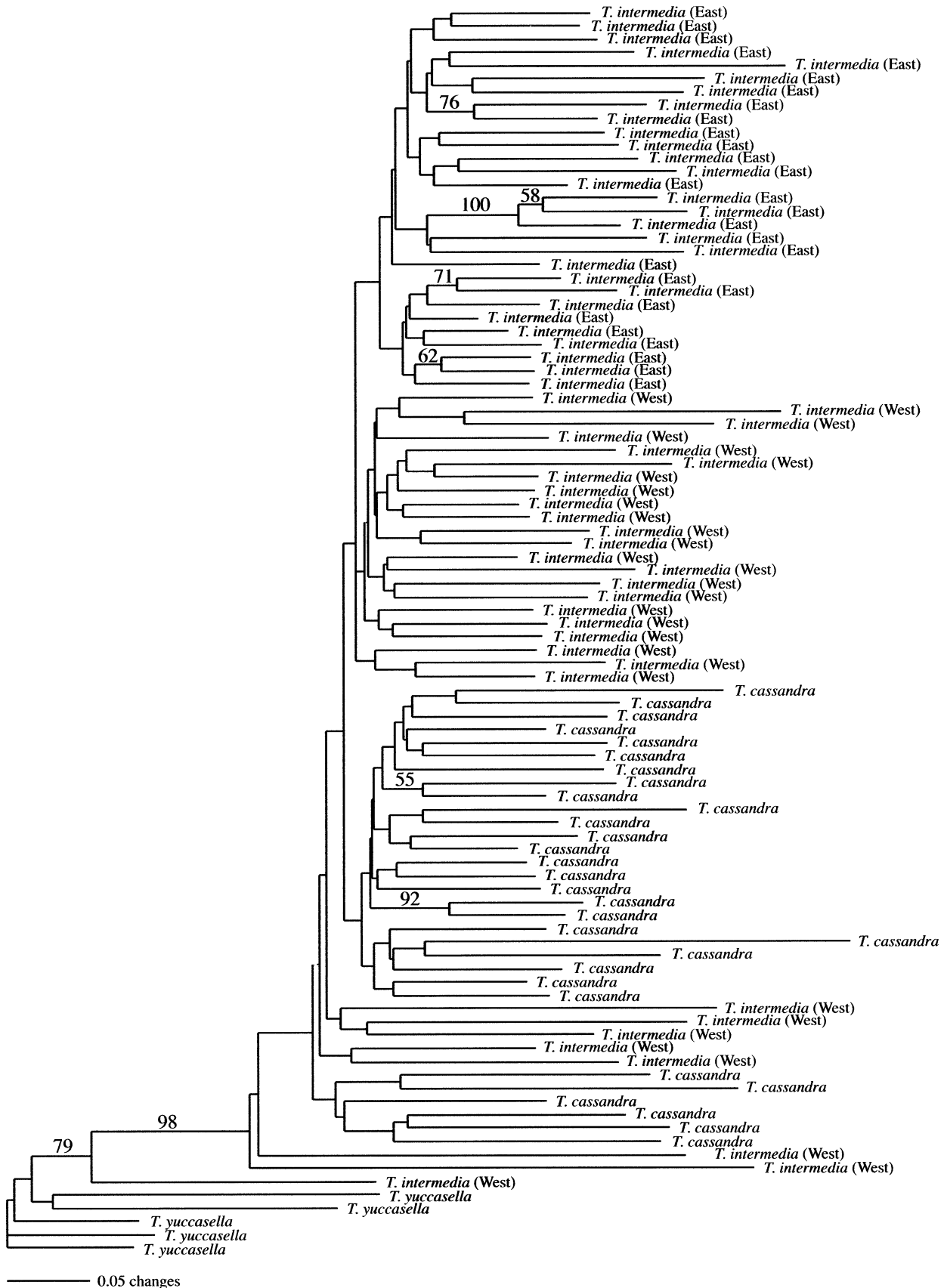
FIG. 6. Phylogram calculated from the Jaccard distance matrix of the amplified fragment length polymorphism data. Some *Tegeticula cassandra* were more similar to *T. intermedia* from the western United States. The numbers over the branches indicate bootstrap support.

TABLE 4. Analysis of molecular variation (AMOVA) results based on *Tegeticula intermedia* and *T. cassandra* amplified fragment length polymorphism (AFLP) data. Groupings follow the same biogeographic regions presented in Table 2.

AMOVA	*T. intermedia* east/west		*T. intermedia* north/south eastern populations only		*T. cassandra* north/south	
	Variance components	Percent explained	Variance components	Percent explained	Variance components	Percent explained
Among regions	0.011***	7.30	0.007	5.79	0.001	0.56
Among populations within regions	0.003	2.29	0.004*	2.99	0.004	2.89
Within populations	0.137**	90.42	0.116*	91.21	0.134*	96.55
ϕ_{ST}	0.096**		0.088*		0.035*	

* $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

A secondary zone of contact between *T. intermedia* and *T. cassandra* could facilitate hybridization, at least initially, as the two species came into sympatry. The southeastern United States has been characterized as a zone of contact between closely related lineages of plants and animals. Remington (1968) deemed the southeast a major ''suture-zone,'' or a region of secondary contact and hybridization between species, and several species in this region have hybridized after secondary contact (Avise and Smith 1974; Philipp et al. 1983; Wooten and Lydeard 1990; Scribner and Avise 1993). Furthermore, hybridization has been documented between *T. intermedia* and the western pollinator *T. elatella*, demonstrating that *T. intermedia* is capable of hybridizing with pollinators under natural conditions (K. Segraves, D. Althoff, and O. Pellmyr, unpubl. ms.).

The AFLP results, however, suggest that genetic drift may also be playing a role. AFLP analysis indicated a well-defined split between eastern and western *T. intermedia*, and the mtDNA based analysis of migration also showed that there was effectively no gene flow between these two groups. Interestingly, the minimum evolution analysis showed that *T. cassandra* was more closely related to western populations of cheaters than they were to eastern populations, despite the fact that *T. cassandra* is closer in geographic proximity to eastern cheaters. These two groups have relatively large effective population sizes ranging on the order of two to 17.5 times larger than the eastern cheaters (Table 3). These large effective population sizes will increase the number of generations required for fixation to occur in these groups and suggests that shared polymorphism is more likely to be found between *T. cassandra* and western *T. intermedia*. The relatively smaller effective population size in the eastern cheaters may decrease the number of generations to fixation and could explain the clearly defined split within *T. intermedia*. These patterns were not apparent in the mtDNA analyses, but this could be explained by the difference in effective population size between nuclear and mitochondrial markers (Kingman 1982). Thus, the differing patterns between mtDNA and AFLP data may be explained by a combination of differences in the rate of drift between the two types of markers, differences in the rate of sorting among the three groups, and hybridization between two closely related species. Genetic drift may explain the AFLP pattern of the close relationship between *T. cassandra* and western *T. intermedia*, whereas hybridization during secondary contact can explain the shared mtDNA haplotypes between the species.

Conclusions

The phylogeographic analysis of mtDNA and nuclear markers suggests a complex history for the origin and subsequent migration of the pollinator-cheater sister pair. The data presented here are most consistent with the following scenario (Fig. 7). The cheater *T. intermedia* likely originated in the western United States, possibly in a zone of sympatry between *T. cassandra* and another pollinator species. *Tegeticula cassandra* historically existed in the western United States, but these populations became extinct after the cheaters diverged from their pollinating ancestor and *T. cassandra* became endemic to the Southeast. The cheaters subsequently
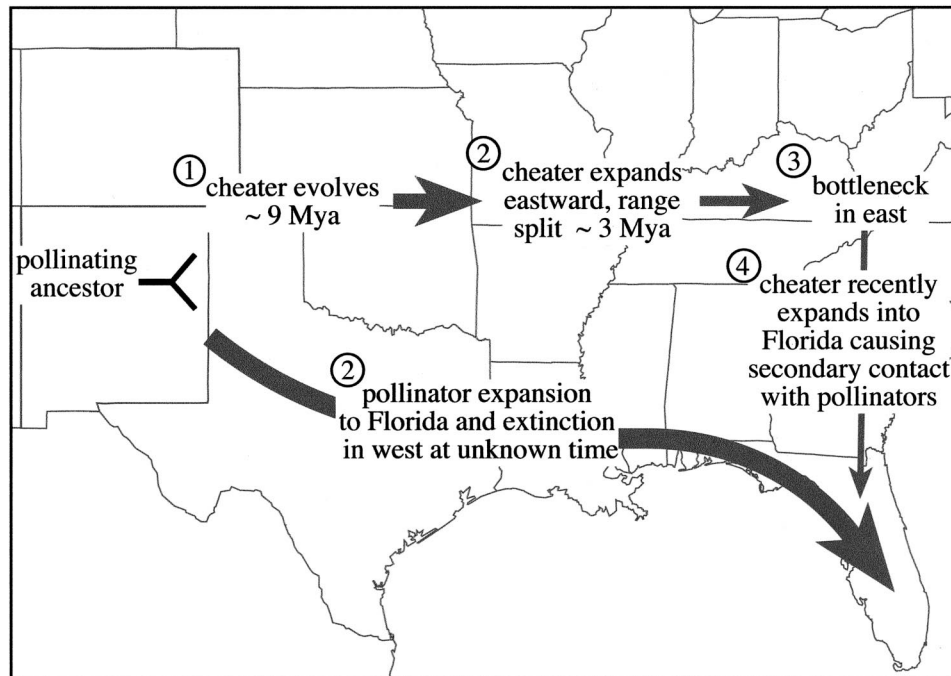
FIG. 7. Revised scenario for the evolution of cheating based on the results from this study. The width of the arrows indicate the decrease in effective population size of the cheaters as they moved eastward. The numbers indicate the relative sequence of events. The precise timing of the pollinator expansion is unknown and may have occurred prior to the evolution of the cheater lineage. Mya, million years ago.

expanded eastward and experienced a population bottleneck. The bottleneck decreased the time required for eastern populations to reach fixation and created the well-defined split between eastern and western populations of *T. intermedia*. More recently, the cheaters moved southward into Florida and came into secondary contact with *T. cassandra*. This secondary contact resulted in limited hybridization between these closely related lineages. The long-term residency in Florida created the biogeographic subdivision observed between northern and southern populations of *T. cassandra*, while the absence of gene flow between eastern and western *T. intermedia* generated substantial genetic structure. The results of this study allow us not only to reject the out-of-Florida hypothesis in a narrow sense, but also unveil disparate histories of the pollinator-cheater sister pair.

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