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Report

An Olfactory Shift Is Associated with Male Perfume Differentiation and Species Divergence in Orchid Bees

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Summary

Saltational changes may underlie the diversification of pheromone communication systems in insects, which are normally under stabilizing selection favoring high specificity in signals and signal perception [1–4]. In orchid bees (Euglossini), the production of male signals depends on the sense of smell: males collect complex blends of volatiles (perfumes) from their environment [5, 6], which are later emitted as pheromone analogs at mating sites [7]. We analyzed the behavioral and antennal response to perfume components in two male morphotypes of *Euglossa* cf. *viridissima* from Mexico, which differ in the number of mandibular teeth. Tridentate males collected 2-hydroxy-6-nona-1,3-dienylbenzaldehyde (HNDB) as the dominant component of their perfume. In bidentate males, blends were broadly similar but lacked HNDB. Population genetic analysis revealed that tri- and bidentate males belong to two reproductively isolated lineages. Electroantennogram tests (EAG and GC-EAD) showed substantially lower antennal responses to HNDB in bidentate versus tridentate males, revealing for the first time a mechanism by which closely related species acquire different chemical compounds from their habitat. The component-specific differences in perfume perception and collection in males of two sibling species are in agreement with a saltational, olfaction-driven mode of signal perfume evolution. However, the response of females to the diverged signals remains unknown.

Results and Discussion

Orchid bees (Euglossini, Apidae) are solitary to primitively social bees of the Neotropics. The males of all five genera and 200-plus species collect volatile substances from flowers and nonfloral sources; they do not ingest these substances but accumulate them outside their body in hind leg (tibial) pockets [8]. This behavior has given rise to the orchid bee pollination syndrome, where about 700 species of orchids are exclusively pollinated by scent-seeking male orchid bees [9]. Male bees are selective in what they gather, and different species have different preferences for natural and synthetic volatiles. In older males, the accumulated perfume is broadly species specific, with respect to both qualitative composition and relative amounts of compounds [10]. These blends are emitted by the males specifically and exclusively at display sites, which are established in the forest understory for the single purpose of mating [7, 11–14]. Conspecific females have been observed to approach displaying males from downwind, and copulations take place on the male perch [12, 13, 15]. Although female attraction to perfumes has not been demonstrated in bioassays, the perfumes have a likely role in mate recognition.

Communication with exogenous perfumes implies that mutations affecting the olfactory system have the potential to alter male signals, e.g., by increasing or decreasing the males' likelihood of acquiring a certain perfume component. Here we report on component-specific differences in perfume accumulation and antennal perception in two closely related lineages of *Euglossa*.

Morphological and Perfume Variation of *Euglossa viridissima*-like Males in Southern Mexico

E. viridissima Friese [16] is a medium-sized (~12 mm), metallic green orchid bee distributed from Mexico to Costa Rica. Males are characterized by two large tufts of hair on the second sternite and the shape of midtibial hair tufts [17]. Dressler (1978) noted that males are variable with respect to the number (3 or 2) of mandibular teeth [18], with 3 being the typical number. Our investigations broadly confirmed this dichotomy across the Yucatan Peninsula, where the majority of males were tridentate (3D). A variable proportion of males (3% to 70% at different localities) had only two mandibular teeth (2D, Figure 1A). Tests with commercial synthetic attractants carried out at different localities showed that both male morphotypes responded positively to the same compounds; both 3D and 2D males were most strongly attracted to p-dimethoxybenzene, followed by methyl cinnamate and eugenol (Figure 2C).

Notably, there was a conspicuous difference in the composition of volatiles stored in hind leg pockets between 2D and 3D males (Figure 1B). Coupled gas chromatography-mass spectrometry (GC-MS) analyses of hind leg extracts revealed four compounds that were highly characteristic for 3D males but absent in 2D males (HNDB 1 to 4 in Supplemental Data 2 available online). The four compounds furnished almost identical mass spectra, indicating stereoisomers of the same basic structure (see Supplemental Data 1). Column chromatography of a pooled hexane extract of 20 pairs of hind legs yielded HNDB in amounts sufficient for structure elucidation by nuclear magnetic resonance (NMR) spectroscopy, which revealed the target compound to be 2-hydroxy-6-[(1*E*, 3*E*)-nona-1,3-dienyl]benzaldehyde (HNDB 4, Supplemental Data 1). The other mentioned 3D-specific compounds are the three stereoisomers of HNDB 4 (HNDB 1 to 3; see Supplemental Data 1 and 2).

HNDB represented on average 67% of the total amount (sum of integrated ion current) of perfume in 3D males, and differences in the amount of HNDB were primarily responsible for

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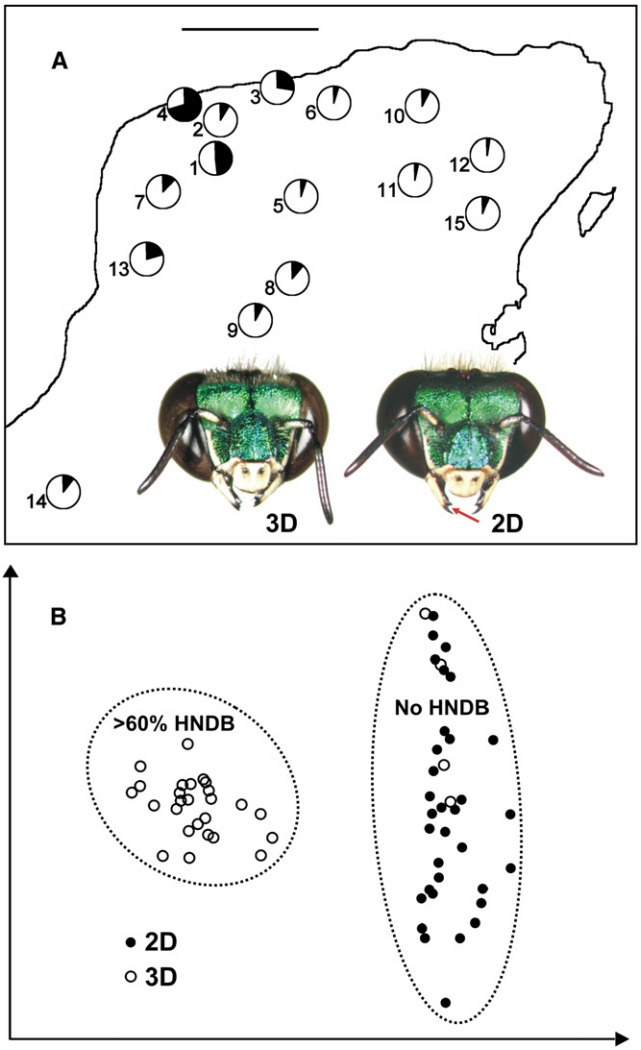

Figure 1. Morphological and Chemical Variability of *Euglossa viridissima*-like Males on the Yucatán Peninsula

(A) Relative abundance of bidentate (2D) and tridentate (3D) males across the Yucatán peninsula. Black segments represent bidentate males. Localities are (1) Xmatkuil, (2) Chablekal, (3) San Crisanto, (4) Chelem, (5) Sotuta, (6) Yalsihón, (7) Maxcanu, (8) Hobonil, (9) Tigre Grande, (10) Tizimin, (11) Valladolid, (12) Nueva Xcan, (13) Hecelchakan, (14) Escarcega, and (15) Coba. A total of 3491 males were morphotyped (at least 73 per site). The scale bar represents 100 km for the map and 4 mm for the bees.

(B) Differences in tibial perfumes between 2D and 3D males as revealed by a multidimensional scaling (MDS) analysis of perfume composition between 63 individual males. Only 3D males contained HNDB. See text for details on the four exceptional tridentate males.

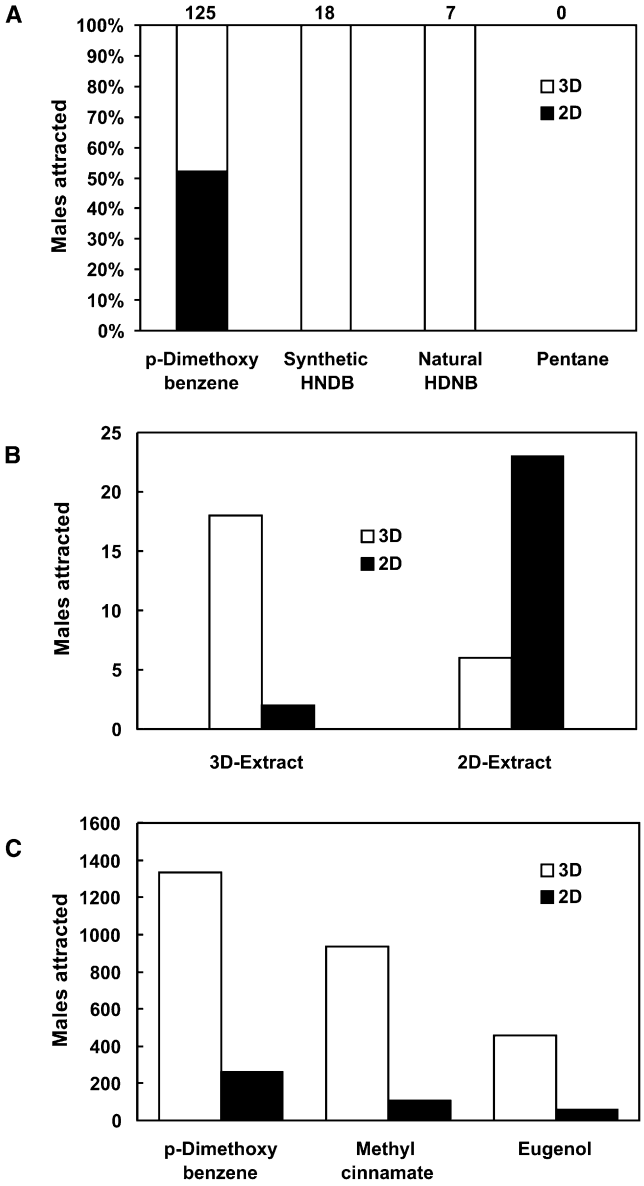

Figure 2. Male Attraction during Bioassays

(A) Attraction of 2D and 3D males to synthetic and natural (isolated) HNDB, contrasted with attraction to p-dimethoxybenzene and solvent controls. All stimuli were presented on the same days at Xmatkuil, where there is a high relative abundance of 2D males (~50% of males).

(B) Attraction of 2D and 3D males to complete hind leg extracts of individual males (2D and 3D) at Xmatkuil.

(C) Attraction of 2D and 3D males to commercial synthetic compounds, p-dimethoxybenzene, methyl cinnamate, and eugenol, pooled across 12 Yucatecan localities (subset of those in Figure 1A, see Experimental Procedures).

the separation of individuals along the horizontal axis in a two-dimensional perfume space (Figure 1B; Spearman Rs = 0.883; $p < 0.0001$). Most of the other perfume components, representing monoterpenes, sesquiterpenes, aromatics, and straight-chain aliphatic acetogenins, were present in variable amounts in both types of males.

The source of HNDB is unknown. Interestingly, we have also found the same four HNDB isomers in perfumes of *Euglossa mixta* from central Panama, suggesting that the source is widespread in Central America (Y.Z. and T.E., unpublished data). Structurally similar compounds occur in phytopathological fungi [19, 20], and fungus-infected substrate (e.g., decaying wood) is a likely candidate for a HNDB source. It should be emphasized that male orchid bees are much less dependent on orchid flowers than is widely believed, and nonfloral substrate such as fungi may have been their original source of perfume [21, 22].

Population Differentiation

We used three microsatellite markers to test for population genetic differentiation among bait-captured males: 2D and

Table 1. Differences in Microsatellite Allele Frequency between 2D and 3D Males at Three Localities in Northern Yucatán, Mexico

Locality (Locus)	p	SE	n (3D)	n (2D)
Ixmatkuil				
ann02	<0.001	0	31	36
ann08	0.078	0.005	36	36
Egc17	0.001	0	36	36
Chablekal				
ann02	<0.001	0	30	18
ann08	0.004	0.001	31	18
Egc17	0.003	0.001	31	18
San Crisanto				
ann02	<0.001	0	32	36
ann08	0.191	0.007	36	36
Egc17	0.014	0.003	36	36

Differences as revealed by exact probability tests [37]. The abbreviations refer to three microsatellite loci ([35], R. Paxton, personal communication).

3D males showed significant differences in allele frequencies at most loci at the three localities (Table 1). This suggests that 2D and 3D males represent two lineages that are reproductively isolated on the Yucatán peninsula, i.e., cryptic sibling species. Notably, females of both lineages established nests in wooden boxes (trap nests) placed around buildings in Xmatkuil. All foundresses had three mandibular teeth, and no differences in morphology were apparent between females that produced 2D sons (n = 13 females) and those that produced 3D sons (n = 3).

The microsatellite analysis indicated that the number of mandibular teeth is not completely fixed in males of the 2D genetic lineage. The four exceptional males in Figure 1B, which had 3 teeth but lacked HNDB, had typical 2D microsatellite haplotypes. Thus, there appear to be rare males of the 2D lineage, which express an additional mandibular tooth but retain 2D-typical perfume preferences.

Behavioral and Antennal Response to HNDB

Synthetic HNDB and HNDB isolated from hind leg extracts of 3D males attracted exclusively 3D males during field bioassays, whereas males of both lineages were attracted in equal numbers to p-dimethoxybenzene at the same time (Figure 2A; significant difference in 2D and 3D relative frequencies between synthetic HNDB and p-dimethoxybenzene, Fisher's exact test, n = 143, $p < 0.0001$). This demonstrates that HNDB is a behaviorally important compound that is actively approached and collected by 3D males only. 2D and 3D males also showed a strong difference in attraction to crude pentane extracts of hind legs of other males that either contained HNDB (3D) or not (2D) (Figure 2B; Fisher's exact test, n = 49, $p < 0.0001$). Presently, HNDB is the only compound we know of that is exclusively attractive to males of one lineage (the 3D males).

An electroantennogram (EAG) is believed to record the sum of potentials from olfactory receptor neurons (ORNs) located all across the antenna, thus reflecting the overall sensitivity of the antenna to tested compounds [23, 24]. In EAG tests, responses of 2D males to synthetic HNDB were significantly smaller than those of 3D males and did not differ from the solvent control. Responses to other compounds varied but were not different between 2D and 3D males (Figure 3A). Differences in the response to HNDB are in agreement with results obtained with gas chromatography coupled to electroantennography

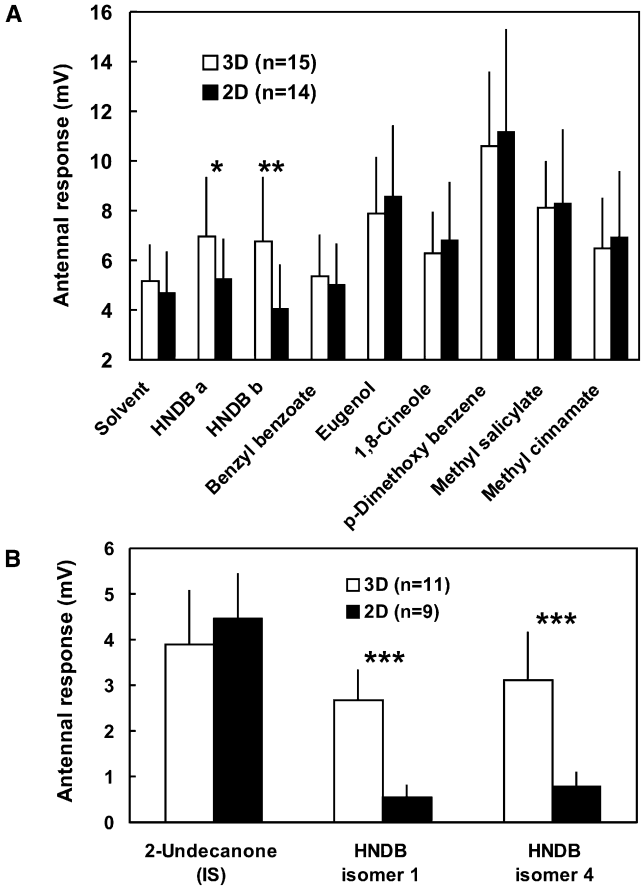

Figure 3. Antennal Response of 2D and 3D Males to HNDB and Reference Stimuli Measured as the Amplitude of Negative Baseline Deflections—Mean and Standard Deviation—during EAG and GC-EAG Tests

(A) EAG: Only HNDB (both stimuli) elicited significantly different responses from antennae of 2D and 3D males (t test: *, $p < 0.05$; **, $p < 0.01$).

(B) GC-EAG: GC peaks of HNDB isomers 1 and 4 elicited strong responses in antennae of 3D males. 2D male antennae showed much weaker (t test: *** $p < 0.0001$) and often marginal responses to the same isomers, but did not differ from 3D male antennae in response to the reference compound, 2-undecanone.

(GC-EAG): antennae of 3D males showed strong baseline deflections in response to eluting peaks of the HNDB isomers 1 and 4, whereas antennal responses of 2D males were marginal (Figure 3B, Supplemental Data 2).

Peripheral Olfaction and Speciation in Orchid Bees

This study has revealed compound-specific differences in antennal perception between males of two closely related lineages of orchid bees. Notably, the observed sensory differences were restricted to the only compound (HNDB) that mediated lineage-specific attraction in bioassays and distinguished male perfume blends in their natural habitat. The component-specific differences in antennal perception reveal for the first time a mechanism by which closely related species acquire different chemical compounds from their habitat. It is tempting to argue that an olfactory shift with respect to HNDB has initiated divergence of 2D and 3D lineages. However, selection or drift after an initial divergence may also have added to the observed olfactory and chemical differentiation.

Lineage-specific differences in antennal sensitivity to HNDB could be based on changes in the binding affinity of a specific

olfactory receptor (OR), or on differential expression of HNDB-receptive ORs or olfactory binding proteins (OBPs) in olfactory receptor neurons. Genomic studies in honeybees and *Drosophila* suggest that families of OR and OBP genes evolve by a birth-and-death mode, with frequent changes in copy numbers as a result of gene duplication, deletion, or pseudogenization [25–27]. Both an increase and a decrease in copy numbers of HNDB-sensitive ORs or OBPs could have mediated differential HNDB collection in 3D and 2D males.

Shifts in signal chemistry as a result of olfactory mutations may also occur in other insects in which pheromone production depends on the sense of smell. For example, male *Bactrocera* fruit flies sequester volatiles from plant sources and release them as courtship signals [28]. In both *Bactrocera* and orchid bees, a shift in male odor acquisition might spread quickly within a population if it is promoted by natural selection, e.g., through reduced search time for males or a reduced risk of falling prey to source-specific predators. Mostly, however, the evolutionary success of an olfactory mutation in males will depend on how females respond to the altered chemical signal. Unfortunately, all previous attemtps to test the behavioral response of females to male perfumes were unsuccessful (see [12, 15]), and no attempt was made here. The lack of data on the female response remains an obstacle for drawing conclusions concerning the mode of signal evolution in orchid bees. However, because male and female perfume preference are probably influenced by overlapping sets of olfactory genes, pleiotropic effects could enhance coevolution between senders and receivers (see [29]). In orchid bees, olfactory pleiotropy in males and females could result in assortative matings within genetic lineages, driving population differentiation by means of divergent sexual selection.

Experimental Procedures

Baiting and Sampling

For assessment of local proportions of 2D and 3D males, baiting with commercially available attractants was done once or twice at 15 localities across the Yucatan peninsula (Figure 1) from October 2006 to April 2008. p-Dimethoxybenzene, eugenol, and methyl cinnamate were used as lures in mesh-covered dispensers that do not allow bees to directly access the bait chemical. The used chemicals are known attractants for *Euglossa viridissima*. In total, 3491 males were captured with hand nets, morphotyped with a hand lens, and released after baiting was finished for the day. In October 2007, males from the localities (1) Xmatkuil, (2) Chablekal, and (3) San Crisanto were collected and processed for perfume analysis (n = 63) and population genetics (n = 193) (see below). For the analysis of chemical preferences (Figure 3C), we used only data from the 12 localities where we had baited twice (excluding localities 2, 14, and 15).

Chemical Analysis of Male Perfumes

Male perfumes were extracted from individual pairs of hind legs in 0.5 ml of hexane containing an internal standard (2-undecanone). GC-MS was carried out with a HP 5890 II GC fitted with a DB-5 column (30 m × 0.25 mm Ø × 0.25 μm film thickness) and a HP 5972 MSD. Injection was splitless, and the oven was programmed from 60°C to 300°C at 10°C/min. A second set of GC-MS analyses was performed with a gas chromatograph series 8000 linked to a Fisons MD800 quadrupole mass spectrometer (Fisons Instruments, Ismaning, Germany) operated at 70 eV. With helium used as the carrier gas, separations were performed with a CP8912 VF-1ms fused silica column (30 m × 0.32 mm Ø × 0.25 μm film thickness) and carried out as follows: after splitless injection at 60°C for 0.5 min, the temperature was kept at 60°C for 5 min and then programmed to 300°C.

Structure assignment of natural compounds was carried out by comparison of analytical data with those of authentic reference samples or by matching spectra and retention indices with those given in the literature [30, 31]. Excluded from the analysis were straight-chain lipids (alkanes, alkenes, alcohols, acetates, diacetates, and wax esters), contained in the bees' labial glands and prominent in head extracts of the studied species [6]. Differences in perfume composition between individuals were calculated as Bray-Curtis distances based on relative compound contributions (% of total ion current) to individual blends. These distances were visualized in two dimensions by nonparametric multidimensional scaling (MDS) with the software Primer v6 [32, 33].

Isolation, Structure Assignment, and Synthesis of HNDB

The isolation of the 3D-specific compounds from pooled hexane extracts (20 pairs of hind legs of 3D males) was carried out by chromatography on 60 Å, 32-63 mesh silica gel (MP Biomedicals, Eschwege, Germany) with a pentane-ethyl acetate gradient starting with pure pentane and stepwise increase of ethyl acetate (1%, 2%, 5%, 10%). NMR spectra of natural products and synthetic samples were recorded with a Bruker Avance 400 (^{1}H: 400.25 MHz, ^{13}C: 100.65 MHz) and a Bruker DRX 500 (^{1}H: 500.13 MHz) spectrometer (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in C_6D_6 with tetramethylsilane ($\delta = 0$) as the internal standard. Mass spectra and details on HNDB synthesis are given in Supplemental Data 1.

Bioassays

Ten milligrams of synthetic HNDB were dissolved in 1 ml of n-pentane (Uvasol, Merck, Germany), and aliquots of 30 μl were applied to filter papers (Whatman 1, 2.5 cm), which were then attached to stems of treelets in Xmatkuil. Repeated presentations were made on three sunny mornings (8:45 to 12:30) in September 2007. Attracted males were captured with hand nets, morphotyped, and retained in vials until the end of the bioassay. On the same days, we also exposed filter papers with HNDB isolated from hind legs (in n-pentane, at the same concentration as synthetic HNDB) and solvent control, as well as mesh-covered dispensers with pure p-dimethoxybenzene to obtain a reference of relative morph abundance at the time in Xmatkuil. In October 2006, we performed similar bioassays with 30 μl aliquots of individual hind leg extracts of 2D and 3D males (n = 6 and 8, respectively) in 1 ml n-pentane. Captures at extracts of 2D and 3D males were pooled for analysis. We later confirmed with GC-MS that all extracts from 3D males contained HNDB, whereas those of 2D males did not.

Electrophysiology

Detailed descriptions of EAG and GC-EAG procedures are given in [34]. For EAG, 5 μg of synthetic HNDB, benzyl benzoate, eugenol, 1,8-cineole, p-dimethoxybenzene, methyl salicylate, and methyl cinnamate (all in n-pentane) were applied to strips of filter paper. The solvent was allowed to evaporate, and air puffs (200 μl) were then directed over the strips into air flowing over the antennal preparation. The HNDB stimulus was given twice for each antenna (HNDB a and b in Figure 3A). For comparison of antennal sensitivity in response to single HNDB isomers, GC-EAG was done with HNDB isolated from hind leg extracts of 3D males. In addition to the four HNDB isomers (in relative concentrations similar to those in 3D male hind leg extracts), the test mix also contained 2-undecanone as reference stimulus (Figure 3B). In addition, GC-EAD was carried out with a complete hind leg extract of a 3D male containing all four HNDB isomers plus a variety of perfume components (see Supplemental Data 2). For both EAG and GC-EAG, the antennal response is given as the amplitude (in mV) of the negative baseline deflection upon stimulus onset.

Microsatellites, PCR, and Population Genetics

One hundred and ninety-three males from three localities (1, 2, and 3) were screened for three polymorphic microsatellite markers (Egc17, accession number EF451841 [35]; ann02, BV728898, and ann08, BV728902 [R. Paxton, personal communication]). DNA was extracted from thoraxes of ethanol-preserved specimens via the protocol given in [36]. Multiplex-PCR reactions were conducted with fluorescent-dye-labeled primers (VIC, 6-FAM and NED; Applied Biosystems). Four microliters of DNA template was used with 12.5 μl HotStar Taq Master Mix (QIAGEN), and the reaction volume was filled up to 25 μl in total with RNase-free water (QIAGEN). PCR reactions were performed in an Eppendorf Mastercycler with the following profile: 95°C for 15 min, then 94°C for 30 s, 52°C for 90 s, and 67°C for 90 s for 22 cycles, and then 67°C for 10 min. Fragment analysis of PCR products was carried out with an ABI Prism 310 Sequencer (PE Applied Biosystems) at the Biologisch-Medizinisches Forschungszentrum in Düsseldorf. For visualization and allele calling, we used the software GENEMARKER V1.71. Exact probability tests of linkage disequilibrium between markers were calculated with GENEPOP v 3.4 [37] and were all nonsignificant within 2D and 3D males. Exact probability tests for genetic differentiation between 2D and 3D males were calculated with the same program.

Supplemental Data

Supplemental Data include details on structure elucidation and synthesis of HNDB and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01418-8.

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