

Systematics and Modes of Reproductive Isolation in the Holarctic *Drosophila testacea* Species Group (Diptera: Drosophilidae)

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ABSTRACT Reproductive isolation and morphological differences among allopatric populations of the Holarctic species *Drosophila testacea* v. Roser indicate that this taxon is actually a complex of three morphocryptic species: *D. testacea*, from Europe and continental Asia; *D. orientacea*, n.sp., from Japan; and *D. neotestacea*, n.sp., from North America. Diagnostically important morphological variation is presented, along with distributional data for these three species and *D. putrida* Sturtevant, the only other member of the *testacea* species group. Both pre- and postmating barriers to reproduction were observed in various interspecific crosses. Premating isolation is strongly asymmetric between *D. testacea* and *D. neotestacea*. Modes of postmating isolation include lack of sperm transfer and failure of hybrid eggs to hatch. The revised taxonomy of this group should facilitate analyses of interesting aspects of the evolutionary ecology of these species.

KEY WORDS *Drosophila testacea* group, systematics, reproductive isolation

AMONG THE MOST COMMON *Drosophila* inhabiting temperate and boreal forests around the world are mycophagous species that feed, mate, oviposit, and develop as larvae in fresh and decaying mushrooms. Because of the facility with which these flies can be studied, both in the field and the laboratory, they and their communities are becoming model systems for studies in evolutionary ecology (Lacy 1984a, Hanski 1988, Courtney et al. 1990). The following are some general observations on the natural history of mycophagous *Drosophila*.

A major challenge to mycophagous *Drosophila* is being able to find and develop on patchy, ephemeral, chemically diverse resources. The spatial and temporal patchiness of their resources has forced these species to adopt a wide host range, breeding on many of the fleshy fungi they encounter (Bächli & Burla 1967, Kimura et al. 1977, Lacy 1984b). Despite this host generalism, they have evolved an extraordinary level of resistance to the mushroom toxin alpha-amanitin, which is produced in highly toxic quantities by only a small fraction of the fungi which they use (Jaenike et al. 1983). This suggests that chemical barriers of their host fungi may be minor limitations on the fly population sizes.

A number of factors play an important role in the population ecology of these mycophagous species of *Drosophila*. Larval competition for limited food resources, which has been documented in natural populations of these species (Grimaldi & Jaenike 1984), sets an upper bound to population size, but other factors may regulate populations at lower levels. One important such factor is parasitism by the nematode *Howardula aoronymphium*, which has been reported from Europe (Welch 1959), North America (Montague & Jaenike 1985), and Japan (Kimura & Toda 1989). Parasitism by these nematodes greatly reduces the fertility of infected female flies (Jaenike 1992), and rates of parasitism have been shown experimentally to increase at high densities of adult flies (Jaenike & Anderson 1992).

There are ≈27 species of mycophagous *Drosophila* in the Holarctic Region, and only one, *D. testacea*, von Roser is still considered to be Holarctic in its natural distribution (Wheeler 1981). *Drosophila transversa* Fallén was formerly considered to be Holarctic, but it is now recognized that the North American populations comprise two species (*D. falleni* Wheeler and *D. recens* Wheeler), distinct from the Palearctic one (Wheeler 1960). (*Drosophila subquinaria* Spencer was cited as a subspecies of *D. transversa* by Wheeler [1981]. The distribution of *D. subquinaria* is the Pacific Northwest of North America; its relationship to *D. transversa* still requires close study). The uniqueness of *D. testacea* in its apparent widespread Holarctic distribution raises the question of whether all populations

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currently classified as *D. testacea* are in fact conspecific. Our study was intended to clarify the relationships among North American, Eurasian, and Japanese populations of this taxon. Our studies include detailed morphological comparisons and laboratory investigations of pre- and post-mating reproductive isolation among these populations.

Methods and Materials

Morphology. Specimens were borrowed from the following institutions, with help from their respective curators: American Museum of Natural History, New York (AMNH); California Academy of Sciences, San Francisco (P. H. Arnaud, Jr.) (CAS); Canadian National Collection, Ottawa (J. Cumming) (CNC); Hungarian Natural History Museum, Budapest (L. Papp) (HNHM); Institute of Pedology, Vladivostok, Russia (V. Sidorenko); Moscow State University, Russia (A. Ozerov); U.S. National Museum of Natural History, Smithsonian Institution (W. Mathis) (NMNH); Tokyo Science Museum, Japan (T. Okada) (TSM); University of Guelph, Ontario (S. Marshall); Snow Entomological Museum, University of Kansas (G. Byers); University of Zürich Zoological Museum (G. Bächli); Utah State University (W. Hanson); Washington State University (W. Turner).

Measurements were done on dried point-mounted or minuten-pinned specimens at 64 \times using a digital stage micrometer (Boeckler Instruments, Tucson, AZ). Each measurement was taken twice and averaged. Dissection techniques, morphological terminology, and standard measurements and ratios are as given by Grimaldi (1987) and McEvey (1990).

Reproductive Isolation. Modes of reproductive isolation were studied in crosses between strains of flies obtained from Rochester, NY, in 1990; Regensburg, Germany, in 1990; and Sapporo, Japan, in 1991. Genetically heterogeneous strains were established with flies recently collected from natural populations.

Ethological studies were carried out on flies that had developed to sexual maturity at 22°C on our standard medium (Carolina Instant *Drosophila* Medium; Carolina Biological Supply, Burlington, NC, plus a piece of commercial mushroom, *Agaricus bisporus*). The mating experiments themselves were conducted at room temperature (22–24°) on flies 5–7 d old. To study mating behavior, five males from one population and five virgin females from the same or a different population were placed in a shell vial containing instant medium and a piece of *A. bisporus*. All matings were scored for the next 3 h. For each cross type, five replicate vials were set up simultaneously. For any given pair of populations, intra- and interpopulation crosses were conducted at the same time. The data of primary

interest are the total numbers of pairs that copulated during the 3-h period, the time that elapsed until the first pair began copulation in each vial, and the copulation duration of all mating pairs. Mean time until copulation for all mating pairs is not reported because these means are substantially inflated by one or a few initially unreceptive females that mated late in the 3-h period.

Following the mating experiments, five females known to have mated were dissected to determine if any sperm had been transferred. In the European \times Japanese crosses, in which very few females mated, additional crosses were set up for analysis of sperm transfer. The remaining flies were left undisturbed to determine if they could produce any viable offspring. Finally, in those vials in which F₁ offspring emerged, F₁ males and females were crossed to each other and to both parental species to determine if they were fertile, as would be indicated by production of viable F₂ and backcross progeny.

In those crosses that yielded inviable F₁, parental strains were treated with antibiotics to test whether symbiotic microorganisms were involved in hybrid inviability, as they are in some other species of *Drosophila* (e.g., Hoffman et al. 1986, O'Neill and Karr 1990). Larvae were reared at 22°C for four generations on medium containing either tetracycline-HCl or rifampin at concentrations (0.03%) known to cure other *Drosophila* of such symbionts (Hoffman et al. 1986). Then, the parental strains were reciprocally crossed to determine if they could produce viable hybrid offspring. Cytological evidence of symbiotic microorganisms in the North American and European populations was examined using DAPI stain (Karr & Alberts 1986, O'Neill & Karr 1990).

Results

Taxonomy

Drosophila testacea Species Group

Acrodrosophila Duda 1924: 203 (as subgenus of *Drosophila*). Type species: *D. testacea* von Roser.

Description. Yellow to dark bodied *Drosophila* (intraspecifically variable) with a pair of dark brown spots or spots coalesced into an incomplete band on each abdominal tergite (Fig. 1). Defined as a monophyletic group by a pair of enlarged acrostichal setae ("presuturals") on the mesonotum anterior to transverse suture in or close to rows 2 and 5 (of the 6 rows between anterior dorsocentrals); lengths varying from \approx 3–5 times the length of other acrostichals. Presuturals erect and fine, or thicker and more decumbent (Fig. 2). Face, cheeks, proboscis, and

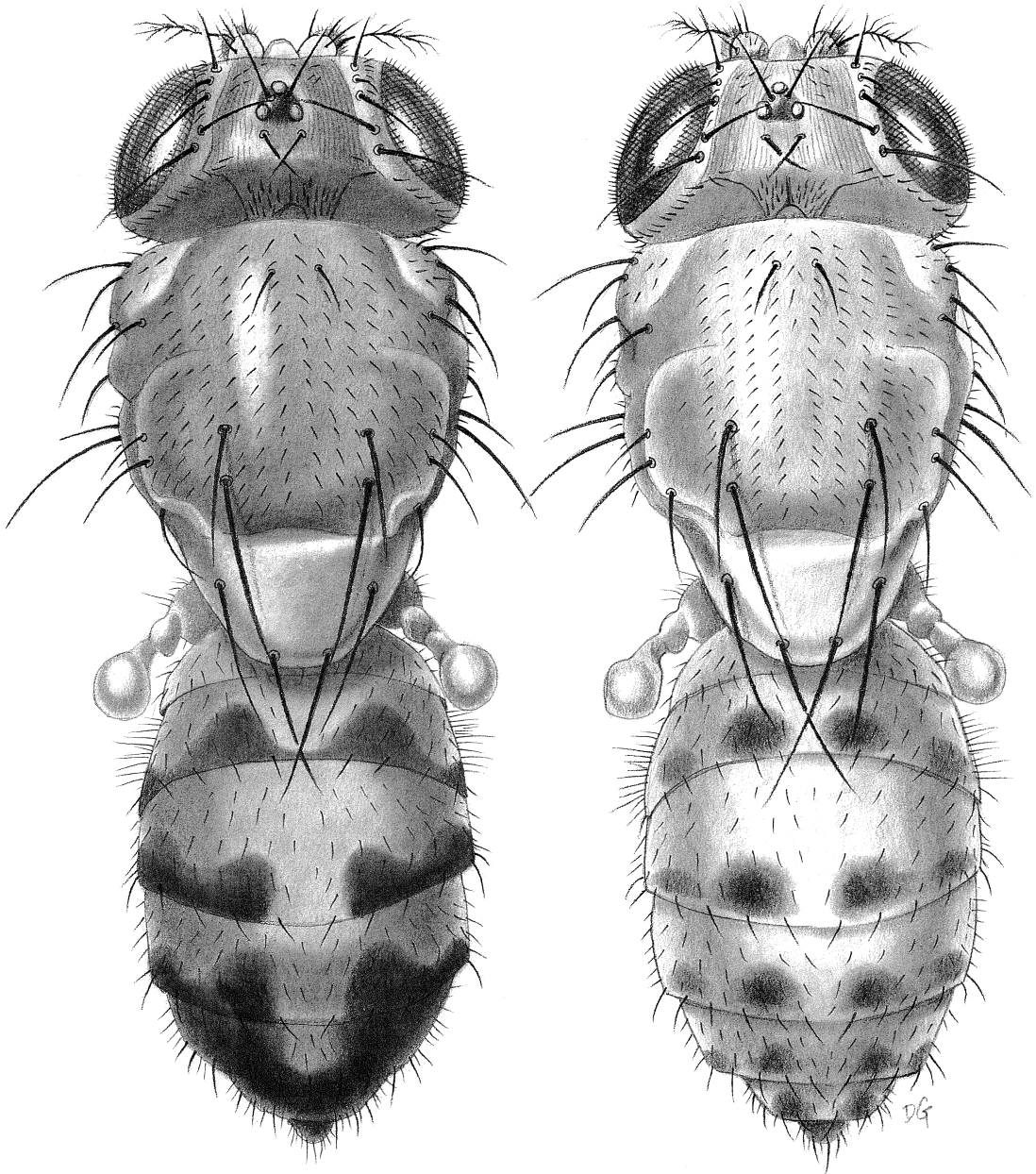


Fig. 1. Dorsal habitus (excluding wings) of *D. neotestacea* showing differences in pattern.

palps yellow. Pedicel yellow, flagellomere I slightly darker. Carina complete; slightly narrower than scape; with flat ridge. Two pairs of vibrissae. Clypeus very shallow. Eyes light red, with dense, stout interfacetal setulae. Posterior reclinate orbital seta slightly longer than proclinate. Anterior reclinate minute, barely larger than fronto-orbital setulae; length about one-quarter that of posterior reclinate, lying midway between ipsilateral proclinate and posterior reclinate. Base of inner vertical seta in line with ipsilateral orbitals. Verticals slightly longer than

posterior reclinate. Ends of inner verticals nearly touching. Outer verticals divergent; slightly posterolateral to inner verticals. Ocellar setae lying on edge of ocellar triangle. Arista with 4–5 dorsal, 2 ventral branches, in addition to small terminal fork (dorsal number intraspecifically variable). Mesonotum and pleura generally unicolorous yellow, sometimes dark brown in colder latitudes, altitudes, and seasons. Anterior dorsocentrals slightly longer than one-half length of posterior dorsocentrals. Distance between bases of ipsilateral dorsocentrals about one-half length

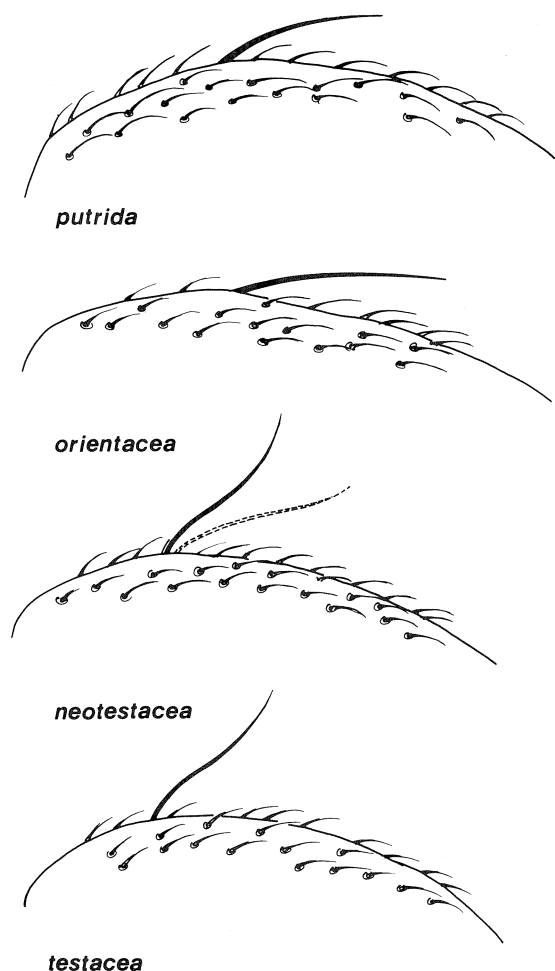


Fig. 2. Lateral view of mesonotum of *D. testacea* group species, showing diagnostic differences in pre-sutural seta.

of anterior dorsocentrals. Postpronotal (humeral) lobe with 2 large setae; 3 notopleural setae; 3 supra-alars, the posterior one largest. Katepisternum with 3 setae; ventral seta largest; middle seta smallest. Six rows acrostichal setulae between anterior dorsocentrals. Legs unicolorous yellow; with dorsopreapical seta on each tibia. Longitudinal rows of cuneiform setulae absent from tibiae-tarsi. Halter yellow; wing hyaline (crossveins sometimes slightly clouded). Cerci (male) yellow to brown.

Discussion. Sabath et al. (1973) found a direct correlation between body color and temperature of larval development in *Drosophila putrida*: larvae raised at cooler temperatures produced darker adults. This effect occurs in all species of the *D. testacea* group because the darkest individuals have routinely been found during the cooler months and the most northerly parts of the distributions.

Research on North American *Drosophila* essentially began with Sturtevant (1916, 1921). He described many species and developed the species group and subgeneric classifications of *Drosophila*, including the *D. testacea* group. Sturtevant was apparently unaware of von Roser's (1840) obscure paper describing several mycophagous *Drosophila* from Europe. Duda (1934-1935, 53) suggested that *D. putrida* Sturtevant was the same species as *D. testacea* v. Roser, basing his decision only on Sturtevant's description. Duda was very close to being right. The holotype specimen of *D. putrida*, in the American Museum of Natural History (examined by D. G.), is externally identical to European specimens of *D. testacea*. Most prominently, the European *D. testacea* and Sturtevant's *D. putrida* type have a pair of long, thin, erect, pre-sutural acrostichal setae, which is unusual in *Drosophila* (so much so that Duda proposed the subgenus *Acrodrosophila* for the 2 species; this subgenus is not recognized today). However, as is shown here, the male genitalia of Sturtevant's "*putrida*" are consistently, albeit subtly, different from *D. testacea*, as are other traits such as mating, thus indicating their status as distinct species.

There would be no problem except that North American drosophilists have been calling *D. putrida* Sturtevant as *D. testacea* von Roser for the past 50 yr. This confusion apparently began at the University of Texas Drosophila Genetics Foundation, perhaps in the 1940s. J. T. Patterson & W. S. Stone (1952) recognized two North American species in the *D. testacea* group, not just the one that Sturtevant had recognized. They properly assumed (but were not exactly correct) that the individuals externally identical to European *D. testacea* were indeed that species; they erred in assuming that the species with short, stout, decumbent presutural setae was *D. putrida*. Up to now, all North Americans have been calling *D. testacea* what we are describing here as *D. neotestacea*, and calling *D. putrida* what has actually been an undescribed species. Strict adherence to the use of types can be totally confusing in situations like this one, where recognition and agreement of species concepts in 50 yr of literature would otherwise be abandoned. The ICZN accepted a proposal to suppress Sturtevant's holotype of *D. putrida* and replace it with a neotype belonging to the currently accepted species concept attached to that name (Grimaldi 1992).

In the present study, only the three species in the *D. testacea* complex (*D. testacea*, *D. neotestacea*, and *D. orientacea*) were measured and compared in detail because they are so subtly different from each other yet are substantially divergent from *D. putrida*. A series of specimens from widely separated areas of the distribution were chosen for measurements. The choice of

Table 1. Morphological differences among species of the *D. testacea* subgroup

Character	<i>Drosophila</i> species		
	<i>testacea</i>	<i>neotestacea</i>	<i>orientacea</i>
Presutural seta length	0.174 (25) 0.150–0.230	0.174 (18) 0.131–0.195	0.200* (17) 0.137–0.240
Cheek depth/eye depth	0.128 (25) 0.091–0.158	0.136 (18) 0.100–0.157	0.126 (17) 0.110–0.144
Length			
Mid-katepisternal seta	0.560 (25)	0.601 (18)	0.655 (17)
Anterior katepisternal	0.414–0.831	0.335–0.822	0.430–0.805
Wing ratios			
C. I.	3.89 (25) 3.34–5.12	3.63* (18) 3.15–4.06	3.98 (17) 3.48–4.65
4-V	1.59 (25) 1.47–1.74	1.57 (18) 1.35–1.74	1.48 (17) 1.37–1.75
Length: aedeagus/aedeagal apodeme	1.11 (4) 0.99–1.41	1.01* (9) 0.90–1.23	1.11 (10) 1.03–1.21
Width: distiphallus/shaft	0.38 (11) 0.35–0.43	0.40 (8) 0.35–0.46	0.33* (10) 0.30–0.36
No. surstylar setae			
Medial	4.5* (6) 4–5	6.0* (12) 4–7	3.5* (9) 3–4
Pegs	11.5 (13) 10–13	13.7* (13) 12–15	11.3 (11) 9–13
Laterals	3.1* (13) 2–4	5.0* (13) 3–6	2.3* (11) 2–3

Asterisks denote means are significantly different ($P < 0.05$) from all others within a row, as determined by ANOVA.

^a Measurements are in millimeters; entries are mean (sample size), and range.

structures to measure was made on the basis of ones traditionally useful for species diagnosis (e.g., ratio of cheek depth/greatest depth of eye; wing ratios such as costal index [C.I.] and 4-V index), as well as structures that showed some variation.

The cheek depth/eye depth ratio, and 4-V Index were not significantly different among any of the three species ($P < 0.05$, pairwise analysis of variance (ANOVA) for unequal sample sizes). Likewise, ratio of the lengths of the mid-katepisternal seta/anterior katepisternal was not significantly different because of the large variance for this character within each sample. Table 1 summarizes the measurement and meristic data; sets of statistically different values are denoted with asterisks. Diagnostically useful values are discussed under each species.

Drosophila testacea von Roser

Drosophila testacea von Roser 1840: 62. Type locality: Württemberg, Germany. Lectotype and paralectotypes (selected by E. B. Basden [1961]): Naturkundemuseum, Stuttgart (Bächli 1990).

Drosophila nigrithorax Strobl 1894: 132 (as variety of *Lordiphosa fenestrarum* Fallén, 1823). Synonymy in Basden (1961, 184).

Drosophila setosa Villeneuve 1921: 160.

Diagnosis. Presutural setae long and fine, slightly curved, always erect (Fig. 2). Costal Index ≈ 3.9 . Length of aedeagus/length of aedeagal

apodeme 0.99:1.41 (mean, 1.11). Distiphallus with flat apical margin and acute corners on margin (versus rounded corners in *D. orientacea* and *D. neotestacea*) (Fig. 3). Surstylus (clasper) with 4–5 (mean, 4.5) fine medial setae, 10–13 (mean, 11.5) pegs, and 3 (sometimes 2 or 4) fine lateral setae. Male genitalia are best separated from its most similar species, the Japanese *D. orientacea*, on basis of width of distiphallus shaft: ratio of greatest width of distiphallus/shaft width is 0.35:0.43 in *D. testacea*, 0.30:0.36 in *D. orientacea*. Also, the middle seta on the katepisternum of *D. testacea* is usually shorter than in *D. orientacea* (Table 2), but this is not an entirely consistent character.

Bächli (1990) provided a very detailed redescription of this species, based on the type series. Bächli omitted critical details of the distiphallus; these are provided in the diagnosis above.

Material Examined. GERMANY: Bavaria, Regensburg, VI-90, J. Jaenike. HUNGARY: Börzsöny hg., Magyarok, 21-VII-79, Bejza & Papp; Szokoya, 18-VII-81, L. Papp. Kiskunsági N. P., Fülöphaza homokbuckás, VI-6-78. D. Draskovits; Mäyagyüd: hüvos, neowes volgy, 17-VII-76, L. Papp. Mátra: Mátrazentimre, 30-IX-79, Mihályi. Ujszentmargita, Hortobágy N. P., Margitai erdő, 29-VIII-75. Visegrád, 19-VIII-70, ex *Lactarius piperatus*. MONGOLIA: Bulgan aimak, Namna ul Gebirge, 23 km NW von Somon, Chutag, 1150 m, 21-VII-68, Z. Kaszab (2 ♀♀). SWITZERLAND: Heitersberg AG, 20–29-VII-84, H. Jungen leg., G. Bächli. USSR: Soviet Far East: Sikhote-Alin' Ra., Ussuri-region, Ussurian

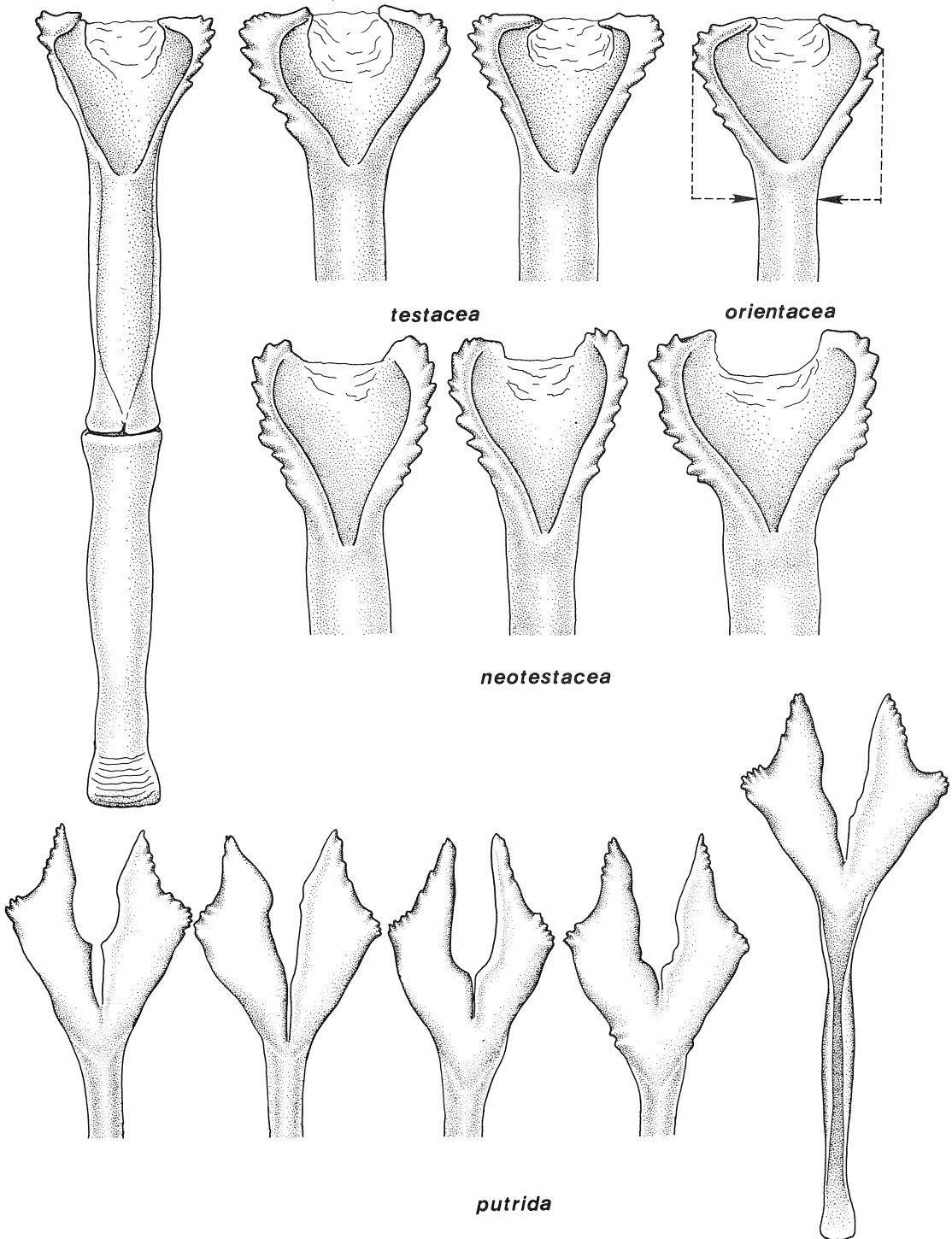


Fig. 3. Distiphallus of *D. testacea* group species. The entire aedeagus and aedeagal apodeme is shown for *D. testacea*, and the entire aedeagus for *D. putrida*.

Table 2. Modes of reproductive isolation among species of the *D. testacea* subgroup

Cross type ^a		Before mating	After mating			
♂	♀	No. pairs mating in 3 h	Sperm transfer	F ₁ egg hatch	F ₁ fertility	
					♂♂	♀♀
Interspecific						
N	T	20/25	Yes	No	—	—
T	N	0/25	—	—	—	—
T	O	1/25	Yes	Yes	Fertile	Fertile
O	T	3/25	Yes	Yes	Fertile	Fertile
N	O	23/25	Yes	No	—	—
O	N	21/25	No	—	—	—
Intraspecific						
T	T	42/49	Yes	Yes	Fertile	Fertile
N	N	47/50	Yes	Yes	Fertile	Fertile
O	O	24/25	Yes	Yes	Fertile	Fertile

^a N, *D. neotestacea*; O, *D. orientacea*; T, *D. testacea*.

Preserve, near Vladivostok, 29-IX-86, Sidorenko (2 M); 15 km SW Barabash–Lewada, 6-VII-86, Sidorenko; Kunashir I., 5 km. S. Lagunnoe Labek, 15-VIII-89, Sidorenko; Kosmomolsky Preserve, 50 km Upper mouth of Gorin Riv. 1-VIII-90, V. Sidorenko. Primorsky kraj, 40 km. SE Ussurijsk, "on rotten meat," A. Ozerov; Kedrovia Pad Nature Preserve, 18-VII-84, A. Shatalkin (1 M). Izmaylovo: Moscow, 18-V-68, V. Belyaeva.

Other Locality Information. Bächli & Pité (1982) provided very extensive bibliographic information on this species, including records of its distribution. The species occurs throughout Europe north to Norway, Sweden, and Finland; west to Great Britain and the Iberian Peninsula; south to Italy, Turkey, and Iran; east to South and North Korea. On the Asian continent, *D. testacea* occurs throughout Russia, extending north to at least Moscow and Lake Baykal. Bächli & Pité (1982) also mentioned Parshad, India. They recorded all distribution records from Japan, which is a species described here as new. *D. testacea* is unrecorded from Burma, Pakistan, and Afghanistan.

New records are presented under Material Examined, and records published since the bibliography of Bächli & Pité (1982) are as follows. BULGARIA: Begovica Basin, Pirin Mtns., P. Lauterer, VII (1 F) (Máca 1987). GERMANY: Edersee (Hesse), Nieder–Werbe, 12–17-VIII, Bächli (162 specimens) (Bächli et al. 1985). NORWAY: Hardangervidda, ZM Bergen, Hurum, 17-VI-85, Tofte; TRI: Malselv, Rosta (Bächli 1986). ROMANIA: Navodari, VIII (1 F) (Maca 1987). USSR: Maritime Territory: 15 km. SW Valentin, Glazkovka, 15–18-VIII-86, A. Ozerov (17 M, 19 F) (Gornostayev 1989). Ukraine, Podolia Region, Manus, Kanev Nat. Reserve, "reared from *Russula* sp. and *Phallus impudicus*" collected VIII, M. Delikatnyj (13 M, 17 F) (Máca 1987). YUGOSLAVIA: Ohrid, e. border Lake Ohrid, IX-6–8-79 (19 specimens); Kupari, near Dubrovnik, IX-16-79 (1 specimen); Porec,

24–27-VII-79 (1 specimen) (Bächli & Kekic 1983).

Gerhard Bächli kindly provided numerous additional, unpublished records, which complete the distribution map in Fig. 4.

***Drosophila neotestacea* Grimaldi, James & Jaenike, new species**

Drosophila testacea of American authors (see text).

Diagnosis. Presutural setae long, thin, slightly sinuate; usually erect, but sometimes barely so (Fig. 2). Costal index ≈ 3.6 (versus 3.9–4.0 for Old World species). Postocellar setae cruciate for about one-third their length. Distinguished best from Palearctic *D. testacea* and Japanese *D. orientacea* by male genitalia: ratio of length of aedeagus/length of aedeagal apodeme less than in other 2 species (mean, 1.01 versus 1.11, respectively); surstylus with more setae (mean of 5 fine laterals versus 2–4 in other species), and more pegs (mean of 13.7 versus 9–13 in other 2 species) (see Table 2). Distiphallus with conspicuously rounded apical corners (Fig. 3).

Holotype. Male, point-mounted. NEW YORK: Orange Co., Cornwall, Black Rock Forest Preserve, 12-IX-90, Grimaldi & Stark, reared from fungus specimen no. 148 (*Ramaria* sp.). Paratypes: 13 ♀♀, 6 ♂♂ with same label data as holotype. Holotype and paratypes in AMNH.

Etymology: Neo, referring to new and Nearctic.

Material Examined: CANADA: BRITISH COLUMBIA: Trinity Valley, 22-VI-37, H. Leéch (1 F). MANITOBA: Selkirk, VII-52, H. L. Carson (1 M). NOVA SCOTIA: Cape Breton Highlands N. P., Lone Shielding, 18–21-VII-83, flight intercept trap, D. & J. Bright (4 M, 3 F). ONTARIO: Arthur, 15–17-VII-83, S. A. Marshall, mushroom traps (2 M, 1 F); Brockville, 6-VIII-1903, W. Metcalfe (1 F); Constance Bay, 30-VIII-80, K. Barber

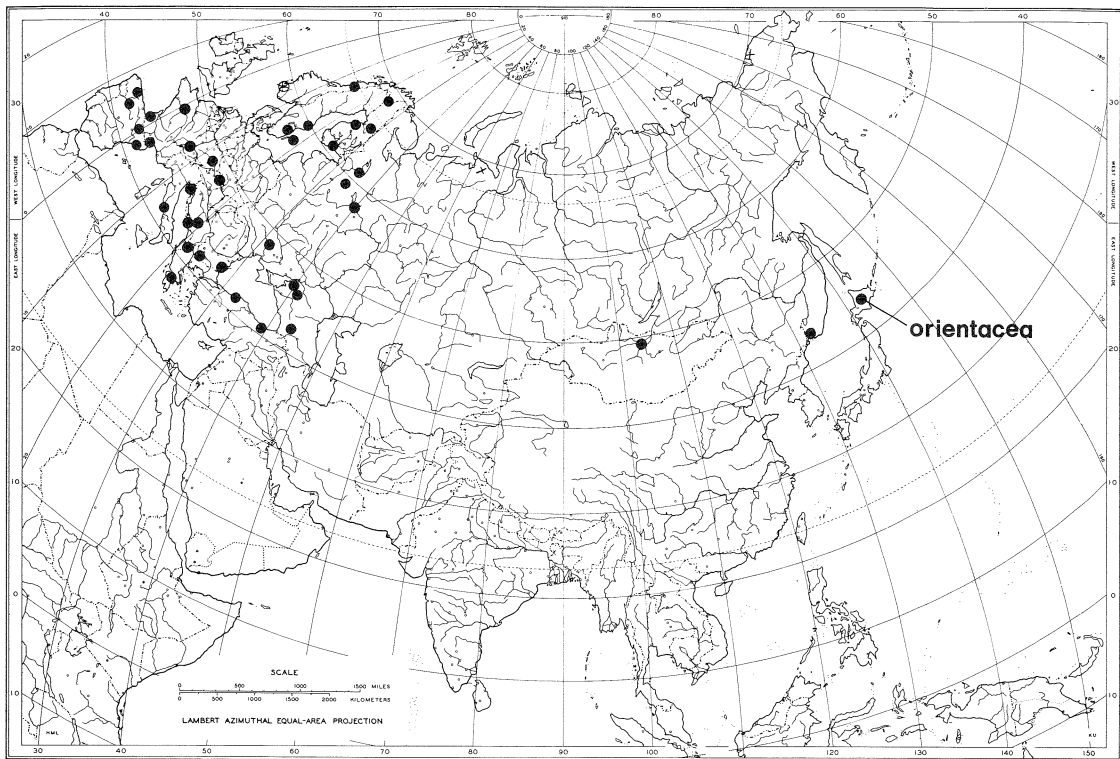


Fig. 4. Distribution map of Palearctic *D. testacea* group species.

(1 M); Guelph, Univ. Guelph Arboretum, VII-10-13-83, "mushroom-baited pitfall trap," B. V. Brown. Maynooth, 30-VIII-52, J. F. McAlpine, "from rotting fungi" (7 M, 3 F); Ottawa, 8-VIII-23, C. H. Curran (2 M), 19-X-51, J. F. McAlpine (1 M, 2 F); 27-X-56, J. R. Vockeroth (2 M); Trenton, 28-VIII-1902, 5-VIII-'08, Evans (1 M, 4 F). QUÉBEC: Bolton Pass, Knowlton, 800 ft., 5-VI-63, J. R. Vockeroth (3 F); Mt. Orford, 1200 ft., 5-VI-63, J. R. Vockeroth (1 M); Norway Bay, 26-VIII-38, G. E. Shewell (2 M, 2 F). YUKON TERRITORY: Dawson City, 13-VII-85, S. A. Marshall, on Boletus, aspen hydrocut (3 M, 9 F); Moose Cr. Campground, aspen mushroom, 3-VII-85, S. Marshall (1 M, 1 F); Deapster Hwy., Tombstone Mtn. Camp 3-VII-85, S. Marshall (1 M).

UNITED STATES: ALASKA: Anchorage, D. D. Miller, 1957 (1 F). ARKANSAS: Sawmill Cr., White Spruce Bog, 18 mi. S. Delta Jct., 15-VII-85, S. Marshall (1 M). CALIFORNIA: 5 mi. W. Willow Creek (county?), VII-51, M. R. Wheeler, W. B. Heed; near Weott (county?), VII-51, M. R. Wheeler, W. B. Heed (1 M). IDAHO: Adams Co., New Meadows, VII-47, M. R. Wheeler (1 M, 3 F). MAINE: Hancock Co., Mount Desert Island, 29-V-3-VI-82, J. Jaenike, VIII-48, M. R. Wheeler (1 M). MASSACHUSETTS: Barnstable Co., Woods Hole, VIII-20-14, A. H. Sturtevant. MICHIGAN: Crawford Co.,

V-3-59, R. & K. Dreisbach (1 F). Ingham Co., East Lansing, 13-VI-71, D. D. Wilder (1 M). Luce Co.: VIII-30-52, R. R. Dreisbach (1 F). Midland Co., IX-15-45, R. R. Dreisbach. MINNESOTA: Beltrami Co., Bemidji, VII-47, M. R. Wheeler & F. D. Cowan, (1 M). Itasca St. Park, VII-52, H. L. Carson (1 M). MONTANA: Glacier Co., Glacier National Park, VII-47, M. R. Wheeler (2 F). (county?) 1.5 mi. N. Fish Creek Camp, VII-47, M. R. Wheeler, F. D. Cowan. NEW JERSEY: Bergen Co., Ridgewood, IV-87, "bred from *Symplocarpus spadice*," A. Soll (1 M). NEW YORK: Broome Co., Chenango Valley State Park, 1-25-VIII-82, D. Grimaldi, "bred from *Russula* and *Cortinarius* mushrooms" (3 M, 4 F). Tompkins Co., Trumansburg, VI-7-84, D. Grimaldi, "reared from *Pleurotus sapidus*" (1 M). NORTH CAROLINA: Macon Co., Highlands, 22-26-IV-88, 3,500 ft., D. Grimaldi; VIII-62, "bred, fungus" D. D. Miller. Swain Co., Clingman's Dome, Great Smoky Mountains, VII-18-22-82, J. Jaenike. Smokemont Camp, VI-50, T. C. Hsu (5). TENNESSEE: Great Smoky Mountains Nat. Park, VII-11-53, H. D. Stalker (1); Alum Cave Bluffs VII-18-22-82, J. Jaenike. VERMONT: Orleans Co., East Charleston, Mad Brook Farm, VII-15-25-82, D. Grimaldi.

Other Locality Information. Patterson & Stone (1952) and Patterson & Wagner (1943) presented distribution maps of this species and *D. putrida*.

They did not examine any Canadian portions of the distributions but did discover differences in the distribution of the two species. A more complete distribution map is given in Fig. 5. *D. neotestacea* is restricted to Canada, Alaska, and the northern half of the United States. Its southernmost range is North Carolina, where it occurs at higher altitudes in the Appalachians (especially during summer months). Patterson & Stone (1952) recorded specimens from western Nebraska, northwestern Montana, and northern Idaho. To the western portion of the distribution we add records here from northern California and British Columbia. It may occur at higher altitudes in the Rocky Mountains of Wyoming and Colorado. The northern limits of the distribution are poorly known. *D. putrida* is restricted to eastern North America and extends to more southerly latitudes (Fig. 6).

***Drosophila orientacea* Grimaldi, James & Jaenike, new species**

Drosophila testacea of Japanese authors (see text).

Diagnosis. Extremely similar to the true European *D. testacea* but differing in the following subtle characteristics. Male genitalia with usually 2 setae (rarely 3, as in *D. testacea*) on lateral surface of surstylus. Width of distiphallus shaft (relative to width of distiphallus) thinner than in *D. testacea* (Fig. 3; Table 1). Presutural setae slightly thicker and more decumbent than in *D. testacea* (Fig. 2). Length of midkatepisternal seta (relative to anterior katepisternal seta) slightly longer than in *D. testacea* (Table 1). Reproductive premating isolation occurs between the strains from Hokkaido and from Germany.

Holotype. Male, from isofemale line lab cultured individuals derived from JAPAN: Hokkaido, Sapporo, collected July 1990 by M. T. Kimura. Deposited in the Tokyo Science Museum. Paratypes (from same culture) in the American Museum of Natural History and the Tokyo Science Museum.

Etymology. *Orient*, referring to its eastern and Japanese distribution.

Other Material Examined. JAPAN: Hokkaido, Sounkyo, Mt. Daisetsu, 17-VIII-53, T. Okada (4M, 3F).

Other Locality Information. Bächli & Pité (1982) listed 51 references on the distribution of *D. testacea* in Japan. Although we examined specimens only from Hokkaido, which are here designated as the new species *D. orientacea*, it is quite possible that all the work on *D. "testacea"* in Japan are referable to *D. orientacea*. More extensive comparisons need to be made between individuals among various localities in Japan and with the proximal mainland.

***Drosophila putrida* Sturtevant**

Drosophila putrida Sturtevant 1916: 339. Neotype: male, American Museum of Natural History (designated by D. Grimaldi [1992]). Type locality: Pompton Plains, NJ.

Drosophila pseudomelanica Sturtevant 1916: 333 (synonymy designated by Wheeler [1981]).

Diagnosis. The most distinctive species in the *D. testacea* group. Easily and reliably separated from sympatric *D. neotestacea* on basis of thicker, shorter, decumbent presutural setae (Fig. 2). Male genitalia distinctive: distiphallus very flat with deep median cleft producing 2 apically pointed lobes, with slightly serrate lateral knob on each. Shape of median cleft quite variable (Fig. 3).

Material Examined. CANADA: ONTARIO, Guelph, Univ. of Guelph Arboretum, VII-10-13-83, "in mushroom-baited pitfall traps," B. V. Brown (1M, F); Wainfleet Bog, 8 km. S. Welland, 22-28-IX-87, D28, pt2-1962, R. Sterling; Oliver Bog, 3 km. S. Salt, 19-25-VI-87, D. Blades; Grand Bend, Pinery P. P., 12-IX-81, K. Barber.

UNITED STATES: ALABAMA: Kushla (county?), IV-15, A. H. Sturtevant (1 F). ARKANSAS: Johnson Co., Haw Creek, 8-VI-91, swept, J. E. Swann (1 F). Logan Co., Ozark Nat. Forest, Magazine Mtn., mushroom-baited pans, 23-V-8-VI-91, J. Swann (3 M). FLORIDA: Highlands Co., Lake Placid, Archbold Biol. Sta, 1-XI-89, "emerged from *Clitocybe* and *Boletus* spp," M. Deyrup. GEORGIA: Liberty Co., St. Catherines Island, 11-20-IV-88, "banana-baited trap," D. Grimaldi (5 M, 4 F). ILLINOIS: Flat Rock (county?), 1915 (no month), "fungus," A. H. Sturtevant. MAINE: Hancock Co., Mount Desert Island, 29-V-3-VI-82, J. Jaenike; VIII-48, M. R. Wheeler, T. Hsu. MICHIGAN: Ingham Co., East Lansing, 13-VI-71, D. D. Wilder. MISSISSIPPI: Warren Co., 2-VI-50, H. D. Stalker (1 M). MISSOURI: Webster Gr., IX-50, H. D. Stalker. NEW JERSEY: Bergen Co., Ridgewood, IV-78, A. Soll, "bred from *Symlocarpus* [skunk cabbage] spadices." Morris Co., Pompton Plains (type locality), X-19-80, D. Grimaldi, "caught in banana trap." NEW YORK: North Greece (county?), I-V-36, A. W. Post. Broome Co., Chenango Valley St. Pk., VIII-82, "reared from *Cortinarius* and *Russula* mushrooms," IV-15-26-82, D. Grimaldi; Erie Co., Buffalo, VI-27-'08, Van Duzee (1 F); Gowanda, VI-7-12, Van Duzee (1 F); Niagara Co., Niagara Falls, 7-26-14 (1 F), 10-15-11 (1 M), Van Duzee. Tompkins Co., S. Lansing, 29-VII-86, W. L. Brown. Trumansburg, VI-7-84, "reared from mushroom, *Pleurotus sapidus*," D. Grimaldi. NORTH CAROLINA: Black Mountains (county?), VI (no year), A. H. Sturtevant; Graham Co., Robbinsville, 9-VI-76, G. E. Bohart; Jackson Co., Cherokee, 5-VI-76, G. E. Bohart; Macon

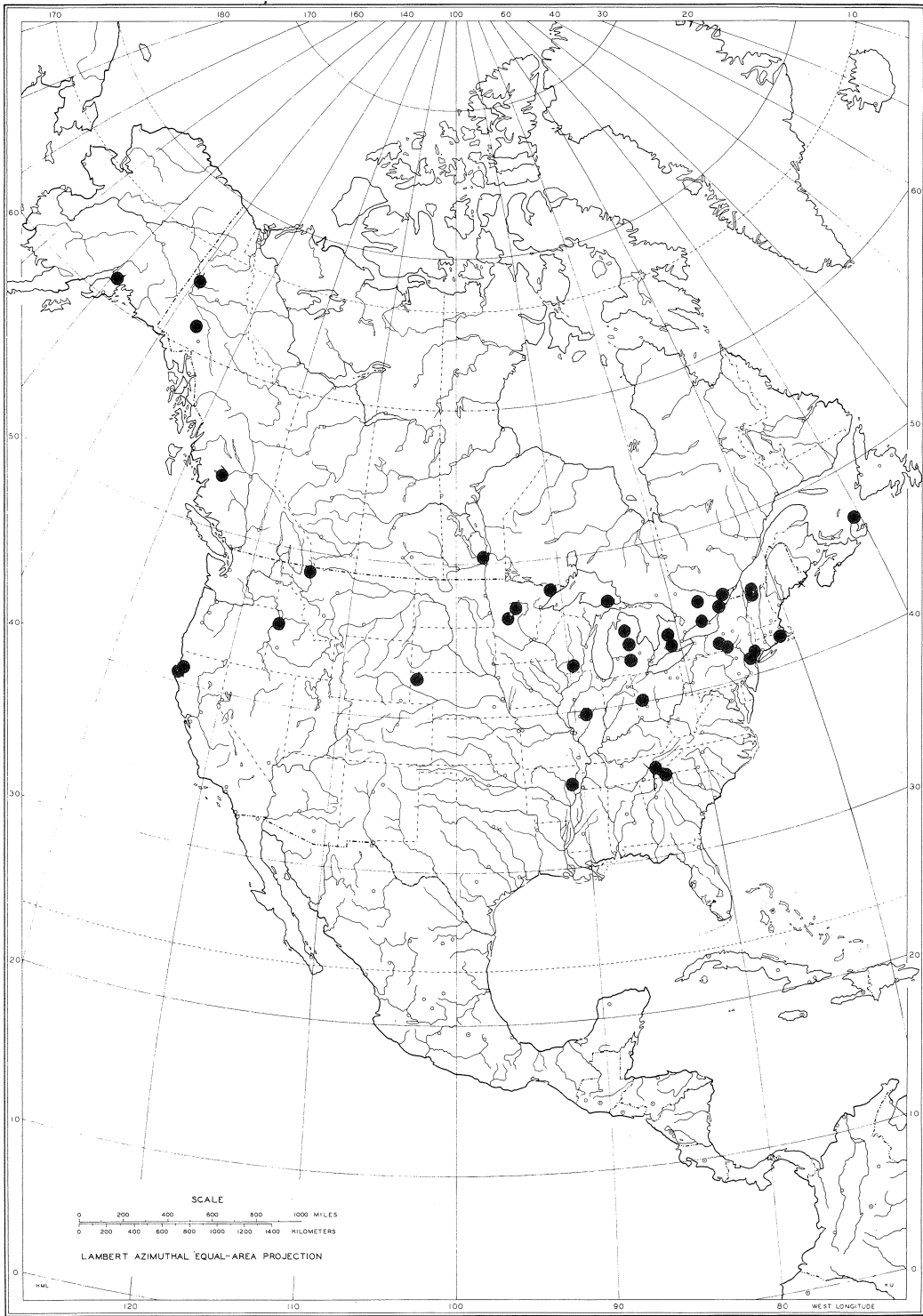


Fig. 5. Distribution map of *D. neotestacea*.

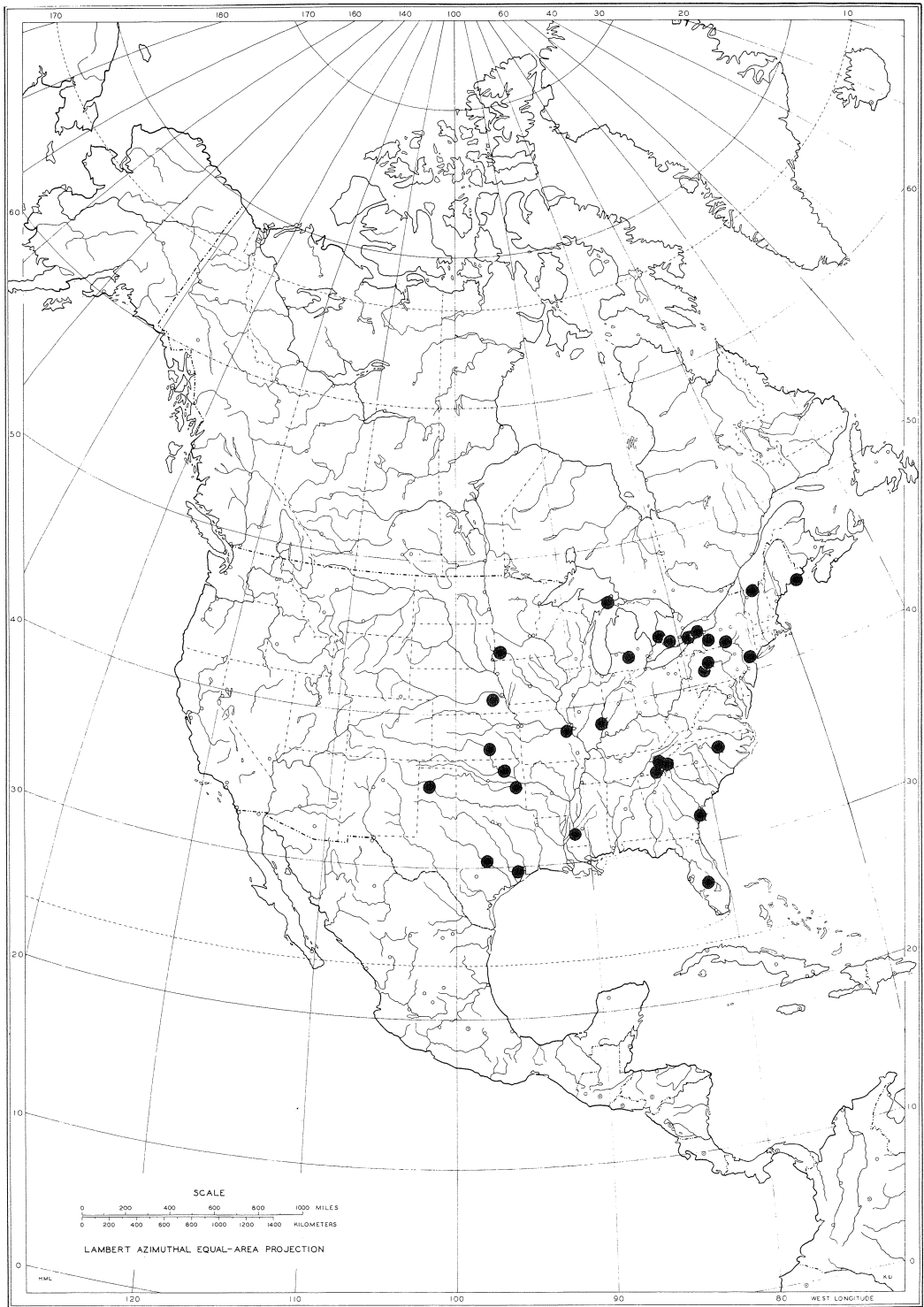


Fig. 6. Distribution map of *D. putrida*.

Co., Highlands, 22-26-IV-88, 3,500 ft., D. Grimaldi; Swain Co., Great Smoky Mountains, Metcalf, VII-16-82, "bred from *Amanita bisporigera*" (1 M). Wake Co., Raleigh VIII-3-82, J. Jaenike, "reared from *Amanita [chlorinosoma?]*". PENNSYLVANIA: Centre Co., Pine Grove Mills, 5-1-72, D. D. Wilder (1 M); State College, 5-VII-81, A. L. Norrbom, "at sap flow on *Quercus alba*" (1 F). TENNESSEE: Elkmont (County^p), VII-20-82, "reared from mushroom, *Suillus pictus*," J. Jaenike (1 M); Alum Cave Bluffs VII-18-22-82, J. Jaenike. TEXAS: Harris Co., Houston, White Oak Bayou, VI-20-83, J. Jaenike. Travis Co., Austin, III-49, M. R. Wheeler. VERMONT: Orleans Co., East Charleston, VII-15-25-82, D. Grimaldi.

Other Locality Information. Patterson & Stone (1952) provided a distribution map of this species. Their records, and the ones given here, indicate that the species ranges from southern Ontario and Quebec to the middle of Florida and Texas and along the Gulf Coast. Patterson & Stone (1952) indicate westernmost records of *D. putrida* from eastern Nebraska and Kansas, throughout Oklahoma, and to western Texas. We did not examine specimens from these parts of the western edge of the range. Patterson & Stone (1952) suggested that the distribution of *D. putrida* closely matches that of the eastern deciduous forests. *D. putrida* appears not to be as cold-tolerant as other species of mycophagous *Drosophila*. In a comparison of larval development times at 15, 21, and 25°C, Grimaldi (1985) found *D. putrida* larval development to be shortest and larvae larger at 21°C, but *D. recens* Wheeler and *D. falleni* Wheeler developed equally well at 15 and 21°C (*D. testacea* was not studied). *D. recens* and *D. falleni* have distributions that encompass *D. putrida*.

Discussion. Male genitalia of the type specimen of *D. pseudomelanica* were dissected, which corroborates Wheeler's (1981) synonymy of that species with *D. putrida*. The type (in the NMNH) has the following labels: Dead Run, Fairfax Co., Virginia, 4.14.14/R. C. Shannon, Coll./Type no. 50006 U.S.N.M. Sturtevant (1916) made no mention of the pair of enlarged presutural acrostichal setulae in this specimen, which are admittedly very decumbent and difficult to see. Both *D. pseudomelanica* and *D. putrida* were described in the same paper, and the former name even has page precedence. However, there is nothing to be gained in supplanting the commonly used name *D. putrida* with one which has never been used.

Reproductive Isolation

Flies mated readily, transferred sperm, and produced viable F₁ in all intraspecific crosses (Table 2). The three species are isolated by a variety of pre- and postmating mechanisms. Very

Table 3. Details of mating behavior within and among species of the *D. testacea* subgroup

Cross ^a		Time to copulation, min (first mating pair/vial)			Copulation duration, min		
♂	♀	\bar{x}	SD	n	\bar{x}	SD	n
Interspecific							
N	T	16.9	5.5	5	11.3	1.4	19
T	N	—	—	—	—	—	—
T	O	48	—	1	20	—	1
O	T	33.5	21.9	2	12.6	7.4	3
N	O	2.2	0.8	5	10.9	3.6	23
O	N	3.2	0.4	5	20.3	12.3	20
Intraspecific							
T	T	7.8	4.7	10	21.2	4.2	37
N	N	2.2	1.2	10	12.5	3.7	46
O	O	3.0	2.9	5	14.8	3.4	21

^a N, *D. neotestacea*; O, *D. orientacea*; T, *D. testacea*.

few flies of *D. testacea* and *D. orientacea* mated in either reciprocal cross (Table 2), and those that mated did so much later than in intraspecific crosses (Table 3). This indicates strong but incomplete ethological isolation. Those females that did mate were able to produce viable and fertile F₁ hybrid progeny.

Strong (absolute in our tests) asymmetric ethological isolation was apparent in crosses between *D. testacea* and *D. neotestacea*; females of *D. testacea* readily accepted males of *D. neotestacea*, but none of the *D. neotestacea* females mated with males of *D. testacea* (Tables 2 and 3). In the former cross, sperm were transferred but no F₁ eggs hatched, indicative of complete isolation after mating.

Little ethological isolation was seen in crosses between *D. neotestacea* and *D. orientacea*; nearly every female mated with a heterospecific male within the 3-h observation period (Table 2), and times to first copulation in a vial were similar for crosses within and between species (Table 3). However, postmating isolation was complete in these crosses. In crosses between *D. neotestacea* males and *D. orientacea* females, sperm were transferred, but no hybrid eggs hatched. In the reciprocal cross, no sperm were transferred.

Mean durations of copulation varied among species. Matings within *D. neotestacea* and *D. orientacea* were similar in duration, but copulation duration was substantially longer in *D. testacea* (Table 3). In interspecific matings, copulation duration was similar to that of the male's species.

Antibiotic treatment failed to cure incompatibility between *D. testacea* and *D. neotestacea*. After four generations of treatment, no viable offspring were produced in crosses between these species. Furthermore, DAPI staining failed to reveal the presence of any cytoplasmic microorganisms.

Discussion

Systematics. Some basic phylogenetic relationships in the *D. testacea* group are very obvious. On the basis of male genitalia, *testacea*, *orientacea*, and *neotestacea* form a monophyletic group, the *D. testacea* subgroup. The male genitalia of *D. putrida* are strikingly different from those of the *D. testacea* subgroup and, in fact, bear close resemblance to those of some species in the *D. quinaria* group. Because of the similar flattened, flanged distiphallus in *D. putrida* and some *D. quinaria* group species, it seems very possible that the *D. testacea* and *D. quinaria* groups are sister groups. The *D. quinaria* group is defined in part by three filaments on the egg; the *D. testacea* group (including *D. putrida*) retains the plesiomorphic state of four egg filaments. The erect, thin presutural setae in *D. neotestacea* and *D. testacea* suggests they are sister species, but the male genitalia of *D. testacea* are most similar to those of *D. orientacea*. Furthermore, R1 elements (retrotransposons within ribosomal genes) are more similar in sequence between *D. testacea* and *D. orientacea* (T. H. Eickbush, University of Rochester, personal communication). The three species in the *D. testacea* subgroup may have an unresolvable, trichotomous relationship.

Reproductive Isolation. Although the three species of the *D. testacea* subgroup are extremely similar morphologically, they are reproductively very strongly isolated by a variety of mechanisms, including ethological barriers, lack of sperm transfer, and hybrid inviability.

Ethological isolation was found to be strong, but not complete, in both directions in crosses between *D. testacea* and *D. orientacea*. In contrast, *D. neotestacea* and *D. orientacea* mated as readily with each other as with members of their own species. Finally, ethological isolation was found to be strongly asymmetric between *D. testacea* and *D. neotestacea*; females of *D. testacea* readily accepted males of *D. neotestacea*, but *D. neotestacea* females uniformly rejected *D. testacea* males. One possible cause of this asymmetry involves noticeably different courtship behavior by the males of these two species. Males of *D. neotestacea* exhibit a "normal" repertoire of courtship elements, including regular wing flicking (see Spieth 1974). In contrast, males of *D. testacea* almost never employ discernible wing flicking while courting a female. Because many *Drosophila*, including *D. neotestacea*, *D. orientacea*, and *D. putrida*, do employ wing-flicking, the lack of this courtship element is almost surely a derived state in *D. testacea*. If wing-flicking is required to stimulate females of *D. neotestacea* but has no influence on *D. testacea* females, this could account for the asymmetrical ethological isolation between these species.

Another type of asymmetrical isolation concerned sperm transfer. Males of *D. neotestacea* successfully inseminated *D. orientacea* females with which they had mated, but in the reciprocal cross, *D. orientacea* males failed to transfer sperm to female *D. neotestacea*. Copulation was not interrupted in the latter crosses; in fact, these copulations lasted somewhat longer on average than any other crosses involving these two species (Table 3). Given the almost identical structures of the male genitalia of these species (Fig. 3), the lack of sperm transfer suggests that something other than genitalic fit must play a key role in copulation. Perhaps movement of the aedeagus or surstyli ("claspers") or both perform an internal or subtle courtship. The great variation in copulation duration in crosses between *D. orientacea* males and *D. neotestacea* females (Table 3), ranging from 1–63 min, suggests a breakdown of the normal process of copulation. In interspecific matings between all other species pairs, mating was always accompanied by successful sperm transfer.

The final mode of reproductive isolation was hybrid inviability, which was found in crosses between male *D. neotestacea* and females of either *D. testacea* or *D. orientacea*. It is possible in these cases that failure of sperm to fertilize heterospecific eggs, rather than hybrid inviability per se, resulted in the absence of F₁ offspring. The species pair *D. testacea*–*D. orientacea* exhibited no postmating isolation in either reciprocal cross, at least through the F₁; both were viable and fertile for both sexes.

It is interesting to note that pre- and postmating isolation do not evolve in parallel in this set of species. Specifically, *D. neotestacea* and *D. orientacea* mate freely with each other, but they fail to produce any viable hybrid offspring. In contrast, *D. testacea* and *D. orientacea* are strongly isolated behaviorally, but in the few cases where they do mate, they produce viable and fertile F₁ of both sexes. These results support the conclusion of Coyne & Orr (1989) that neither pre- nor postmating isolation consistently evolves faster in allopatric populations of *Drosophila*.

Postmating isolation between *D. testacea* and *D. neotestacea* does not appear to result from the presence of symbiotic microorganisms because antibiotic treatment failed to eliminate incompatibility, and DAPI staining provided no cytological evidence for the presence of such microbes in these studies. Thus, postmating isolation between them is probably because of nuclear genes.

Elsewhere, we have reported that populations of the North American species *D. neotestacea* harbor at considerable frequencies an X-linked factor called "sex ratio" that causes male carriers to sire only daughters; this results in substantially female-biased sex ratios at the population

level (James & Jaenike 1990). Whether or not the "sex ratio" factor(s) occurs in *D. testacea* or *D. orientacea* remains to be determined, although we have not yet found it in our samples. Absence of "sex ratio" in these other species would suggest that this condition has arisen very recently in *D. neotestacea*. Clarification of phylogenetic relationships and of even more basic aspects such as species limits will allow evolutionary analyses of this and other patterns of interspecific variation.

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