A CLASSICAL AND POPULATION GENETIC DESCRIPTION OF TWO NEW SIBLING SPECIES OF *AEDES (OCHLEROTATUS) INCREPITUS* DYAR

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ABSTRACT. The distribution of diagnostic allozymes at three loci among 33 populations of *Aedes increpitus* Dyar in the western United States revealed the existence of three genetically distinct population groups currently considered to be members of this taxon. On the basis of these biochemical characters and an analysis of morphological characters in larvae, pupae and adults, two new sibling species are described: *Ae. clivis* and *Ae. washinoi*. A description of biochemical characters of adults, and morphological characters of adults, pupae and larvae for each new species is presented. *Aedes increpitus* is redescribed on the basis of material from several western U.S. states. A discussion of the genetic structure of populations within the complex is presented, with special attention to the structure of populations as they occur in sympatry. Hybridization between *Ae. increpitus* and *Ae. washinoi* occurring in nature is described and the significance of interspecific hybridization as it relates to the taxonomic status of species is discussed.

INTRODUCTION

In the western United States the *Aedes stimulans* group of the subgenus *Ochlerotatus* Lynch Arribalzaga is represented by seven species: *Ae. aloponotum* Dyar, *Ae. excrucians* (Walker), *Ae. fitchii* (Felt and Young), *Ae. increpitus* Dyar, *Ae. mercurator* Dyar, *Ae. riparius* Dyar and Knab and *Ae. squamiger* (Coquillett) (Eldridge et al. 1986). Most of these species occur in colder parts of the Northern Hemisphere. In the majority of cases, those species whose distributions extend south are restricted to progressively higher elevations. *Aedes increpitus* is unusual in that it is the only species in this group which occurs in both high mountain as well as Pacific coastal environments. Because *Ae. increpitus* occurs in a wide variety of habitats, it serves as an excellent subject to study habitat-associated genetic divergence and speciation. We undertook a study, employing electrophoretically detectable allozyme variability, to determine the levels of genetic divergence among populations of *Ae. increpitus* in a variety of habitats. In addition, we conducted a study of the morphology of all life stages and both sexes.

Analysis of allozyme variability using gel electrophoresis has been widely applied to research on the Culicidae and the literature in this area is voluminous. Isozyme studies have been conducted to achieve a variety of different goals. These include descriptions of the inheritance and genetic linkage relationships of enzyme genes (Narang and Seawright 1982, Matthews and Munstermann 1990, Munstermann 1990), defining the genetic structure of populations (Tabachnick et al. 1979, Bullini and Coluzzi 1982, Wallis et al. 1983, Munstermann 1985, Black et al. 1988), and estimating phylogenetic relationships among established species and species groups (Miles 1978, Hilburn and Rai 1981, Pashley and Rai 1983, Eldridge et al. 1986, Schultz et al. 1986). In the area of taxonomy, allozymes have been used as characters for the identification of "difficult" species and to assemble diagnostic keys (Saul et al. 1977, Miles 1979, Corsaro and Munstermann 1984, Hunt and Coetzee 1986, Munstermann 1988, Narang et

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al. 1989a, Humeres et al. 1990). Recently, studies aimed at describing population genetic structure using allozymes have led to the discovery of new species (Kaiser et al. 1988, Narang et al. 1989b, Green et al. 1990, Lanzaro et al. 1990). White (1985) included allozyme characters in his description of *Anopheles bwambae*, a member of the *An. gambiae* sibling species complex. In this paper we describe the distribution of diagnostic allozymes among populations of *Ae. increpitus* and apply a population genetics approach to the interpretation of these data to provide the basis for recognizing three species in this taxon. We include formal descriptions of two new species which incorporate biochemical (allozyme) characters as well as morphological characters in them.

MATERIALS AND METHODS

Collection sites and techniques. Larval *Ae. increpitus* were collected from 29 sites in California, three sites in Oregon, and one site in Utah (Table 1). Larvae were collected in the field and transported to the laboratory in three-liter plastic containers. In the laboratory, larvae were transferred to plastic trays in the water in which they were collected, with tap water added to a final volume of ca. one liter. Trays were held at 26°C and periodically the larvae were fed a 1.6% bovine liver powder (ICN Biochemicals) suspension in tap water. A subsample of fourth-stage larvae from each collection were removed and placed individually in 25 × 200 mm glass culture tubes. Fourth-stage larval and pupal exuviae were retrieved and mounted on numbered glass microscope slides. Half of the emerged adults from this subsample were mounted on paper points on insect pins and half were frozen at −70°C for electrophoresis. Pinned and frozen adults were labeled in association with their larval and pupal exuviae. Pinned specimens with their associated exuviae have been deposited in the Bohart Museum, University of California, Davis as vouchers. Frozen adult voucher specimens remain stored in the laboratory of one of us (BFE). The remaining larvae from each sample were mass-reared to the adult stage, held for 48 h, examined to confirm the previous larval identification of *Ae. increpitus* and frozen at −70°C for electrophoresis.

Electrophoresis procedures. Standard procedures for horizontal starch gel electrophoresis were followed (Harris and Hopkins 1976, Steiner and Joslyn 1979) using 12.5% (wt/vol) Connaught starch. Two buffer systems were used: Ayala-C (Ayala and Powell 1972) and C-buffer (Clayton and Tretiak 1972). Whole mosquitoes were homogenized in 30 μl of distilled water. The three enzyme systems considered in this paper (including Enzyme Commission number and buffer system used in gel and electrode chambers) were aconitase (ACON, 4.2.1.3, C-buffer); diaphorase (DIA, 1.6.4.3, C-buffer); and lactate dehydrogenase (LDH, 1.1.1.27, Ayala-c buffer). The identification of loci and designation of alleles were based on banding phenotypes being consistent with those previously described in mosquitoes and other insects (Harris and Hopkins 1976, Lanzaro 1986, Pasteur et al. 1988, Richardson et al. 1986). To ensure identity of alleles among populations, samples representing each population were tested concurrently on the same gel in combination with all other populations. In addition, individuals from a newly developed laboratory strain of *Ae. aegypti* (Linnaeus), designated the ROCK/DAVIS strain, were used as controls on all gels. This strain is fixed for single alleles at all of the enzyme loci included in this study (Lanzaro, unpublished). Nomenclature for allozymes is based on electrophoretic mobilities relative to the *Ae. aegypti* standard. Allozymes are designated as the ratio of the distance that a particular allele migrated through the gel to the distance that the *Ae. aegypti* standard migrated, multiplied by 100. For example, if the product of a particular allele migrated 20 mm from the point of origin and the *Ae. aegypti* standard migrated 40 mm that allele would be designated as (20/40) × 100 = 50. These are referred to in the text as either "allele 50" or in superscript following the abbreviation for that locus.

Table 1. Collection data for 33 populations of *Aedes increpitus* complex mosquitoes analyzed for isozyme frequencies.

Collection site	Elev. (m)	Date	Species
Fremont, Alameda Co., CA	0	II-15-89	*Ae. washinoi*
Hope Valley, Alpine Co., CA	2,100	V-2-90	*Ae. increpitus*
Cabbage Patch, Calaveras Co., CA	1,900	V-30-90	*Ae. increpitus*
Sun River, Deschutes Co., OR	1,274	III-31-89	*Ae. increpitus*
Twin Bridges, El Dorado Co., CA	2,100	IV-12-89	*Ae. clivis*
Shaver Lake, Fresno Co., CA	1,700	V-3-89	*Ae. clivis*
Eureka, Humboldt Co., CA	0	III-1-89	*Ae. washinoi*
North Lake, Inyo Co., CA	2,765	V-6-89	*Ae. increpitus*
Klamath Falls, Klamath Co., OR	1,311	III-31-89	*Ae. increpitus*
Honey Lake, Lassen Co., CA	1,215	III-21-88	*Ae. washinoi*
San Rafael, Marin Co., CA	0	I-6-89	*Ae. washinoi*
Lake City, Modoc Co., CA	1,380	IV-17-90	*Ae. increpitus*
Mono Village, Mono Co., CA	2,200	V-8-90	*Ae. increpitus*
Tioga Pass, Mono Co., CA	2,250	V-8-90	*Ae. increpitus*
Prunedale, Monterey Co., CA	50	I-24-89	*Ae. washinoi*
Weber Canyon, Morgan Co., UT	1,544	IV-4-89	*Ae. increpitus*
Portland, Multnomah Co., CA	0	III-30-89	*Ae. washinoi*
Chicago Park, Nevada Co., CA	750	III-6-90	*Ae. washinoi* and *Ae. clivis*
Nevada City, Nevada Co., CA	720	III-28-90	*Ae. washinoi* and *Ae. clivis*
Graeagle, Plumas Co., CA	1,237	IV-11-90	*Ae. clivis* and *Ae. increpitus*
Quincy, Plumas Co., CA	1,100	IV-11-90	*Ae. clivis* and *Ae. increpitus*
San Luis Obispo, San Luis Obispo Co., CA	0	I-26-89	*Ae. washinoi*
Isla Vista, Santa Barbara Co., CA	0	I-26-90	*Ae. washinoi*
Redding, Shasta Co., CA	160	III-21-89	*Ae. washinoi*
Lassen State Park, Lassen Co., CA	2,000	V-22-89	*Ae. increpitus-Ae. washinoi* hybrid
Pondosa, Shasta Co., CA	1,250	IV-21-90	*Ae. clivis*
Etna, Siskiyou Co., CA	1,120	IV-14-90	*Ae. clivis*
Montague, Siskiyou Co., CA	1,300	IV-15-90	*Ae. clivis*
Pinecrest, Tuolumne Co., CA	1,750	V-10-90	*Ae. clivis*
Union Creek, Trinity Co., CA	1,300	V-24-89	*Ae. clivis*
Packers Creek, Trinity Co., CA	1,400	V-24-89	*Ae. clivis*
Hot Springs, Tulare Co., CA	1,900	V-2-89	*Ae. clivis*
Summit Trail, Tulare Co., CA	2,000	V-2-89	*Ae. clivis*

Morphological studies. Adult males and females, with associated pupal and larval exuviae were examined from among voucher specimens prepared as described above. Slide-mounted whole larvae present in the collections of the Bohart Entomological Museum were also examined. Descriptions of adults were prepared after examination of at least 10 males and 10 females. At least 10 larvae or larval exuviae and 10 pupal exuviae were examined for the purpose of counting setal branches. For each species, material from at least four different geographic areas was examined. The holotypes and several paratypes of each of the new species described have been deposited in the National Museum of Natural History (NMNH), Smithsonian Institution, Washington, DC.

Data analysis. Analysis of allozyme frequency data was performed using the BIOSYS-1 computer program of Swofford and Selander (1981).

TAXONOMIC TREATMENT

Aedes (*Ochlerotatus*) *increpitus* Dyar, 1916

Diagnosis. Resembles *Ae. clivis* and *Ae. washinoi* in all life stages. Branching of seta 5-C of larva variable, ranging from single to 4-branched; usual condition is double. Both adult sexes have an allozyme for the enzyme diaphorase (Enzyme Commission number 1.6.4.3), designated DIA^{84}, which has a lower electrophoretic mobility than the allozyme DIA^{97}, shared by *Ae. clivis* and *Ae. washinoi*.

Female. *General*: Integument dark reddish brown, darkest on head and abdomen, lightest on thoracic pleura. *Head*: Occiput with many dark brown slightly forked, erect narrow scales, interspersed with forked and unforked creamy white scales. Vertex with forked erect and narrow appressed creamy white scales and a few smoky brown appressed scales. Ocular scales as distinct row along ocular line. Several pale yellow and dark brown curved bristles present on vertex. Gena and postgena almost completely covered with flattened white scales. Pedicel of antenna with a few narrow white scales on inner surface. First flagellomere with patch of white scales on inner surface. Remainder of antenna dark brown, each flagellomere with a few long curved dark brown bristles. Palpus mostly dark-scaled, with patches of white scales at bases of palpomeres 3 and 4, the former forming a fairly distinct band. Proboscis entirely dark-scaled. *Thorax*: Mesonotum integument dark reddish brown, covered with narrow bronze and white scales, the latter as elongate patches laterally and posteriorly, and 2 indistinct longitudinal parallel lines. Dorsal setae all dark brown. Pleura covered with many flattened white scales. No hypostigmal scale-patch. Mesokatepisternum scaled only along posterior margin, scales not extending to ventral angle. Mesomeron with only 2–3 white scales near posterior margin. Mesanepimeron with a few yellowish white bristles on lower part and a thick patch of white scales. Prominent postprocoxal scale-patch present. *Wings*: Mostly dark scaled, but with some scattered white scales along length of costa and subcosta. Distinct patch of white scales at base of costa. *Legs*: Coxae covered with white scales on anterior surfaces. Femora mostly white-scaled, but with scattered dark scales, giving salt and pepper appearance. Tibiae similarly marked, but with some surfaces almost entirely white-scaled. White scales at femoral-tibial joint resulting in a "knee spot." Tarsi with broad basal white bands of scales on most tarsomeres, usually occupying from 0.15–0.50 of tarsomere. Pale band may be missing from hindtarsomere 5. Tarsal claws bifid, slightly curved beyond middle. *Abdomen*: Terga 2–6 with broad basal white bands, extending all across segments; ventral surface nearly all white-scaled, but with some lateral dark spots of scales.

Male. *General*: Markings similar to those of female. Antennae plumose, most segments bearing light brown long silky flagellar setae. Palpus long, first palpomere dark-scaled. Second palpomere with basal band of pale scales and some scattered pale scales on apical portion. Third palpomere with a basal band of pale scales and scattered pale scales apically, on some specimens, may be almost entirely pale-scaled. Apex bearing long silky setae. Fourth palpomere with pale basal band and many long shiny brown setae. Fifth palpomere with a pale basal band that may be indistinct on some specimens.

Male genitalia. Gonocoxite 48 × 16 units including basal lobe. Numerous setae arising from pronounced alvioli. Some setae very stout and long, especially those at apical margin of basal lobe. Apical lobe pronounced and thumb-like, setose. Basal lobe covered with bristles, some longer and stouter than others. Claspette with many setae, 2 of them prominent and arising from conical bases. Claspette filament curved and blade-like, with sharp angle near middle of convex surface, appearing like a meat cleaver. Ninth tergum with 2 truncate lobes, each with 4–9 stout blade-like setae at apex and a single seta at base. Tenth sternum heavily sclerotized along apical margin, 3 setae at flared tip. Phallosome conical, longer than wide.

Pupa. Setal branching as shown in Table 2.

Table 2. Setal branching of pupae of *Aedes* (*Ochlerotatus*) *increpitus* (n = 9).

Seta no.	Cephalothorax CT	Abdominal segments I	II	III	IV
0	—	—	1	1	1
1	1–2(1)	11–20(16)	1–9(6)	2–4(4)	2–4(3)
2	1–2(2)	1–3(2)	1	1	1
3	1	1–3(3)	2–4(2)	1–3(2)	1–4(3)
4	1–2(2)	1–6(2)	1–4(3)	1–4(2)	1–2(2)
5	1–2(1)	1–4(1)	1–5(1)	1–5(2)	2
6	1–2(1)	1	1	1–2(1)	1–3(1)
7	1–3(2)	1–2(1)	1–2(1)	1–4(3)	1–2(2)
8	2–4(4)	—	—	1–3(3)	1–3(1)
9	1–2(2)	1	1–2(1)	1	1
10	4–6(6)	—	1	1–3(1)	1–2(1)
11	1–2(2)	—	—	1	1
12	1–5(2)	—	—	—	—
13	—	—	—	—	—
14	—	—	—	1	—

Seta no.	Abdominal segments V	VI	VII	VIII	Paddle P
0	1	1	1	1	—
1	1–4(2)	1–4(2)	1–2(1)	—	—
2	1–2(1)	1–2(1)	1	—	1
3	1–3(1)	1–3(1)	1–2(1)	—	—
4	1–3(2)	1–3(1)	1–2(1)	—	—
5	1–2(2)	1–2(2)	1–2(1)	—	—
6	1–2(1)	1–2(1)	1–4(1)	—	—
7	1–4(2)	1–2(1)	1–2(1)	—	—
8	1–2(1)	1–2(1)	1–3(3)	—	—
9	1	1	1–3(2)	4–7(6)	—
10	1–2(1)	1	1–2(1)	—	—
11	1	1	1–2(1)	—	—
12	—	—	—	—	—
13	—	—	—	—	—
14	—	1	1	—	—

Larva. Setal branching as shown in Table 3.

Type data. Lectotype (Stone and Knight 1956), adult male, bearing following labels: (1, uppermost), "FB25"; (2), "Fallen Leaf Lake Tahoe Cal June 3 1916"; (3), "Type No 20350 U.S.N.M."; (4), "HG Dyar Coll"; (5), "Aedes increpitus Type o/Dyar"; (6), "Lectotype Stone & Knight, 1956." Also present in the NMNH collection is a series of pinned adults collected by Dyar at Fallen Leaf Lake between June 1 and June 24, 1916. A few of these adults have batch-associated larval and pupal exuviae mounted on slides.

Material examined. In addition to the type series, specimens were examined from the following localities: Bridgeport, Mono County, California; Bishop, Inyo County, California; Weber Canyon, Morgan County, Utah; Alturas, Modoc County, California; Susanville, Modoc County, California; Livingston, Park County, Montana; Missoula, Missoula County, Montana; Bridger Canyon, Gallatin County, Montana; Manhatten, Gallatin County, Montana.

Aedes (Ochlerotatus) clivis, new species

Diagnosis. This species is a sibling species of both *Ae. increpitus* Dyar and *Ae. washinoi* n. sp. and therefore resembles these species very closely in all life stages. Adults of both

Table 3. Setal branching of fourth-stage larvae of *Aedes* (*Orchlerotatus*) *increpitus* (n = 20).

Seta no.	Head	Thorax			Abdomen		
	C	P	M	T	I	II	III
0	—	4–13(6)	—	—	—	1	1
1	1	2–3(2)	1	1–5(3)	1–9(4)	2–6(3)	1–7(4)
2	—	1–2(1)	1–2(1)	1–3(2)	1–2(1)	1	1
3	1	2–3(2)	1	1–4(4)	1–4(3)	1–2(2)	1
4	1–4(2)	1	1–4(2)	4–7(5)	4–9(5)	4–9(6)	1–6(2)
5	1–4(2)	2–4(2)	1	1–2(1)	3–7(5)	2–5(3)	2–3(2)
6	1–3(1)	1	1–7(5)	1	2–3(2)	2	2–3(2)
7	4–7(6)	3	1–2(1)	7–12(10)	1–2(2)	1–3(2)	1–5(2)
8	1	1–3(1)	8–10(8)	3–7(5)	1–3(1)	1–3(2)	1
9	1	1	4–12(9)	5–11(6)	1–3(1)	1–2(1)	1
10	1–2(1)	1–2(1)	1	1–2(1)	1	1	1
11	3–5(5)	1–5(2)	1–5(3)	1–2(1)	1–2(1)	1–2(1)	1–3(1)
12	2–5(3)	1–2(1)	1	1–2(1)	1	1	1–2(2)
13	1	—	4–13(7)	6–13(6)	1–4(1)	1–6(4)	2–3(2)
14	1–2(1)	2–3(2)	—	—	—	—	—
15	1–3(3)	—	—	—	—	—	—

Seta no.	Abdomen						Siphon
	IV	V	VI	VII	VIII	X	S
0	1	1	1	1	1	—	—
1	1–3(2)	1–3(2)	3–5(3)	1–3(2)	1–7(5)	1	4–5(5)
2	1	1	1	1–2(1)	1–4(1)	6–15(8)	1
3	1–2(2)	1–2(1)	1–3(1)	1–2(2)	7–10(7)	1	—
4	1–4(2)	1–4(3)	1–4(2)	1–3(1)	1	—	—
5	1–4(3)	1–4(3)	2–3(2)	2–5(3)	4–8(4)	—	—
6	2	2	1–2(2)	1–6(4)	—	—	1
7	1–5(4)	1–5(4)	1–4(2)	1–2(1)	—	—	1–2(1)
8	1–2(1)	1	1–4(2)	1–6(5)	—	—	1–3(1)
9	1	1	1	1–6(4)	—	—	1
10	1	1	1	1	—	—	—
11	1–3(1)	1–2(1)	1–2(1)	1–4(1)	—	—	—
12	1–2(1)	1	1	1–3(1)	—	—	—
13	2–3(3)	2–4(3)	6–8(6)	1–4(2)	—	—	—
14	1	1	1	—	1–3(1)	—	—
15	—	—	—	—	—	—	—

sexes are characterized by having an allozyme for the enzyme lactate dehydrogenase (Enzyme Commission number 1.1.1.27), designated LDH^{88}, with a higher electrophoretic mobility than either *Ae. increpitus* or *Ae. washinoi* (Fig. 1). Most specimens can be identified as 4th-stage larvae by seta 5-C being 3-branched. Approximately 75% of all specimens examined had this condition, whereas no *Ae. washinoi* specimens examined had this condition, and only 25% of the *Ae. increpitus* specimens examined had as many as 3 branches (Fig. 2).

Female, male, and male genitalia. Indistinguishable from *Ae. increpitus*.

Pupa. Setal branching as shown in Table 4.

Larva. Setal branching as shown in Table 5. Seta 5-C usually 3-branched on at least one side, rarely 2-branched, never single.

Type data. Holotype, female, accession number 91157-G01-I18, with associated pupal and 4th-stage larval exuviae. Collected at Strawberry Lake, Tuolumne County, California, VI-6–1991, by Michael Gurnee and Steven Schutz. Deposited with NMNH. Paratypes, 17 females with associated 4th larval

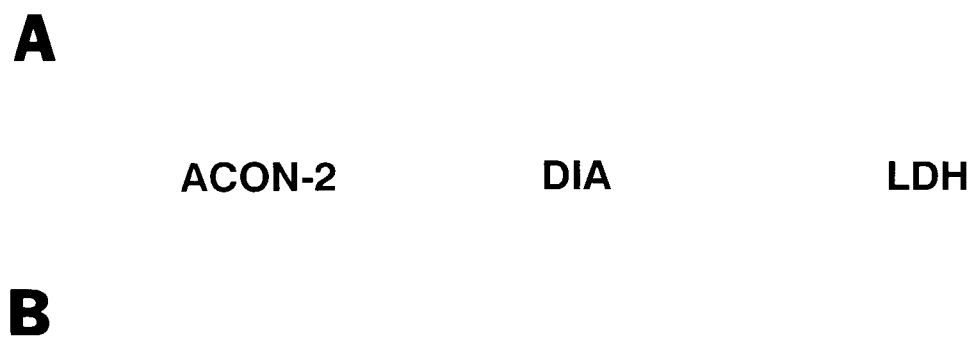

Fig. 1A, Gels illustrating the phenotypes of diagnostic loci for species in the *Aedes increpitus* complex. **ACON-2**: I = *Ae. increpitus*=+; II = *Ae. clivis*; III = *Ae. washinoi*. **DIA**: I = *Ae. clivis*; II = *Ae. washinoi*; III = *Ae. increpitus*. **LDH**: I = *Ae. clivis*; II = *Ae. increpitus*; III = *Ae. washinoi*. B, Gels illustrating heterozygote phenotypes for each locus. Arrowheads indicate individual heterozygotes.

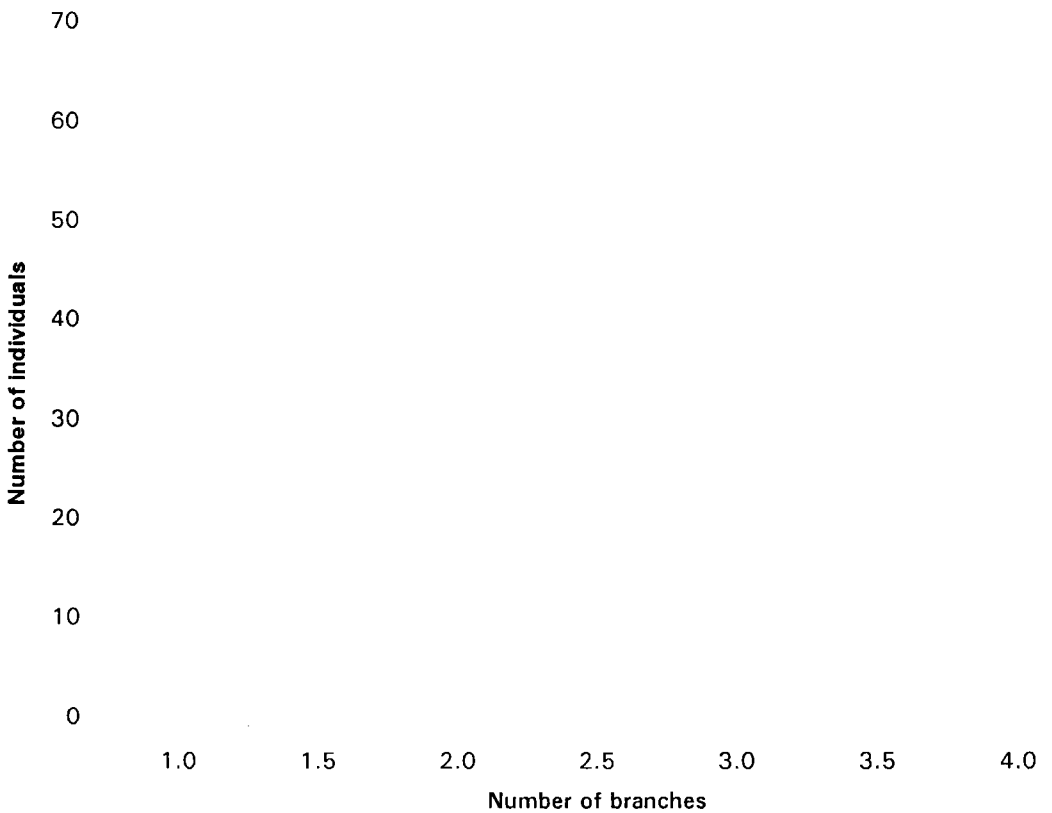

Fig. 2. Frequency diagram showing numbers of individual 4th-stage larvae with branching patterns for seta 5-C indicated. Fractional numbers represent asymmetric pattern. Populations sampled described in text.

Table 4. Setal branching of pupae of *Aedes* (*Ochlerotatus*) *clivis* (n = 10).

Seta no.	Cephalothorax CT	Abdominal segments I	II	III	IV
0	—	—	1	1	1
1	1–2(1)	9–12(12)	2–6(6)	2–4(3)	1–4(2)
2	1–2(1)	1–2(1)	1	1	1
3	1–2(1)	1–3(2)	2–3(3)	1–3(2)	1–4(3)
4	1–2(1)	1–3(2)	1–3(3)	1–3(2)	1–2(1)
5	1–2(1)	1–3(2)	1–3(2)	1–3(3)	1–2(2)
6	1–2(1)	1–2(1)	1	1–2(1)	1–2(1)
7	1–3(1)	1–3(1)	1–2(1)	1–2(2)	1–2(1)
8	1–2(2)	—	—	1–2(2)	1–2(1)
9	3–6(6)	1	1	1	1
10	1–2(2)	1	1	1	1–2(1)
11	1–3(2)	—	—	1	1
12	—	—	—	—	—
13	—	—	—	—	—
14	—	—	—	1	1

Seta no.	Abdominal segments V	VI	VII	VIII	Paddle P
0	1	1	1	1	—
1	1–2(1)	1–2(1)	1	—	1
2	1	1	1	—	—
3	1–3(1)	1–2(1)	1–2(1)	—	—
4	1–3(2)	1–2(2)	1–2(1)	1	—
5	1–2(2)	1–3(2)	1–2(2)	—	—
6	1–2(1)	1	1–2(2)	—	—
7	1–3(1)	1	1–2(1)	—	—
8	1–2(2)	1–2(1)	1–2(2)	—	—
9	1	1	2–3(3)	2–9(7)	—
10	1	1	1	—	—
11	1	1	1	—	—
12	—	—	—	—	—
13	—	—	—	—	—
14	1	1	1	1	—

and pupal exuviae from same collection. Adults of 2 females (91157-G01-I13, I14) were analyzed electrophoretically and confirmed to belong to this species. Voucher larval and pupal exuviae from these tests are deposited with the Bohart Museum of Entomology, University of California, Davis along with 10 female paratypes (91057-G01-I08, -I09, -I10, -I11, -I12, -I15, -I16, -I17, -I19, -I20) with associated exuviae. In addition to holotype, 7 female paratypes with associated exuviae (91057-G01-I01, -02, -I03, -I04, -I05, -I06, -I07) deposited with NMNH.

Material examined. In addition to the type series, specimens were examined from the following localities: Summit Trail, Tulare County, California; Shaver Lake, Fresno County, California; Union Creek, Trinity County, California; Montague, Siskiyou County, California; Pondosa, Shasta County, California.

Distribution. This species has the most restricted distribution of the three species in the *Ae. increpitus* complex. Larvae were collected from elevations ranging from 720 to 2,000 m throughout the Klamath, Cascade and Sierra Nevada mountain ranges in California. All collections were from the state of California. Its eastward distribution seems to be delimited by the Modoc Plateau to the north and the crest of the Sierra Nevada to the south. All collections made east of these geological

Table 5. Setal branching of fourth-stage larvae of *Aedes* (*Ochlerotatus*) *clivis* (n = 8).

Seta no.	Head	Thorax			Abdomen		
	C	P	M	T	I	II	III
0	—	3–5(4)	—	—	—	—	—
1	1	1–4(1)	1	2–4(2)	3–8(8)	2–6(4)	3–6(3)
2	—	1	1–3(2)	1	1	1	1
3	1	1–3(1)	1	2–4(2)	1–2(1)	1	1
4	1–4(2)	1–2(1)	1–2(1)	2–6(3)	2–4(4)	2–4(3)	1–2(2)
5	2–3(3)	2–3(2)	1	1–2(1)	4–5(4)	3–4(3)	2–3(3)
6	1–2(1)	1–2(1)	7–11(7)	1	2–4(2)	2–4(2)	2
7	5–10(8)	1–4(3)	1	9–16(11)	1–2(2)	2–4(2)	1–4(2)
8	1–2(1)	1–2(2)	8–12(10)	2–5(3)	2	1–3(2)	1–2(1)
9	1–3(2)	1	8–14(10)	9–13(12)	1–3(3)	1	1
10	1–2(1)	1	1	1	1	1	1
11	1–5(1)	1	—	2	1–2(1)	1	1
12	1–3(3)	1	1	1	1	1	2
13	1	—	4–6(4)	4	1–2(2)	3–5(4)	2–3(3)
14	1–2(1)	2–3(2)	4–8(4)	—	—	1	—
15	2–4(3)	—	—	—	—	1	1

Seta no.	Abdomen						Siphon
	IV	V	VI	VII	VIII	X	S
0	—	—	—	—	—	—	—
1	2–6(2)	3–5(3)	1–2(1)	1	7	1–6(1)	6–7(6)
2	1	1	1	1	1	3–20(13)	1
3	1–2(1)	1–2(1)	1–2(1)	1	6–12(7)	—	—
4	2–4(2)	1–3(2)	2–3(2)	1	1	5–15(1)	—
5	2–4(4)	2–4(2)	2	2	3–6(5)	—	—
6	2–3(2)	2–3(2)	2	2–3(2)	—	—	1
7	2–4(2)	2–3(3)	1–2(1)	1	—	—	1
8	1–2(1)	1–2(1)	1–2(1)	5	—	—	1–5(3)
9	1	1–2(2)	1	3	—	—	1
10	1–2(1)	1–2(1)	1	1	—	—	—
11	1	1	1	1	—	—	—
12	2	2	1	1	—	—	—
13	2	2–3(2)	6–10(10)	1	—	—	—
14	—	—	—	—	—	—	—
15	1	—	—	—	—	—	—

features were composed exclusively of *Ae. increpitus*.

Etymology. The species name is descriptive, taken from the Latin adjective *clivis*, which means sloping or inclining, in this case referring to the mountainous habitat in which this species occurs. The name agrees in gender with the name *Aedes*, derived from the Greek adjective αηδηζ, meaning distasteful or disagreeable.

Aedes (Ochlerotatus) washinoi, new species

Diagnosis. All life stages of this species very closely resemble both *Ae. increpitus* and *Ae. clivis* n. sp. Adult males and females are characterized by having an allozyme for the enzyme aconitase (Enzyme Commission number: 4.2.1.3), designated ACON-2^{96}, with a higher electrophoretic mobility than either *Ae. increpitus* or *Ae. clivis* (Fig. 1). Likewise, adult males and females have an allozyme for lactate dehydrogenase (E.C. number: 1.1.1.27), LDH^{72}, with a lower electrophoretic mobility than either *Ae. increpitus* or *Ae. clivis* (Fig. 1). Expression of allozyme phenotypes is identical in adult males and females. Seta 5-C of 4th-stage larva single or 2-branched, more frequently the latter (Fig. 2).

Table 6. Setal branching of pupae of *Aedes* (*Ochlerotatus*) *washinoi* (n = 11).

Seta no.	Cephalothorax CT	Abdominal segments I	II	III	IV
0	—	—	1	1	1
1	1–2(1)	8–14(10)	5–10(6)	1–4(3)	1–2(1)
2	1–2(1)	1–2(2)	1	1	1
3	1	1–2(2)	2–3(2)	1–3(2)	2–4(3)
4	1–2(2)	2–4(3)	1–3(2)	2–4(3)	1–3(2)
5	1–3(2)	1–3(2)	2–4(3)	2–4(3)	2
6	1–2(1)	1–2(1)	1	1–3(1)	1–2(2)
7	1–3(1)	1–2(1)	1	1–2(2)	1–2(1)
8	1–3(3)	—	—	1–3(1)	1–2(1)
9	1–2(1)	1–2(1)	1	1	1
10	2–5(5)	—	1–2(1)	1–2(1)	1
11	1–4(2)	—	—	1	1
12	1–3(2)	—	—	—	—
13	—	—	—	—	—
14	—	—	—	1	1

Seta no.	Abdominal segments V	VI	VII	VIII	Paddle P
0	1	1	1	1	—
1	1–2(1)	1–4(1)	1–2(1)	—	1
2	1–2(1)	1	1	—	—
3	1–2(2)	1–2(1)	1–2(1)	—	—
4	1–5(2)	1–2(1)	1–2(1)	1	—
5	2	1–2(2)	1–2(1)	—	—
6	1–2(1)	1–2(1)	1–3(2)	—	—
7	1–3(3)	1–2(1)	1–2(1)	—	—
8	1–2(1)	1–2(2)	1–3(2)	—	—
9	1	1	2–3(3)	4–8(6)	—
10	1	1	1	—	—
11	1	1	1	—	—
12	—	—	—	—	—
13	—	—	—	—	—
14	1	1	1	—	—

Female, male, and male genitalia. Indistinguishable from *Ae. increpitus*.

Pupa. Setal branching as shown in Table 6.

Larva. Setal branching as shown in Table 7.

Type data. Holotype, male, accession number 92031-G01-I09, with associated pupal and 4th-stage larval exuviae. Collected Union City, Alameda County, California, I-31-1992 by Glenn E. Conner. Deposited with NMNH. Paratypes, 11 males and 4 females with associated 4th larval and pupal exuviae from same collection. Adults of 4 males (92031-G01-I02, -I06, -I07, -I15) were analyzed electrophoretically and confirmed to belong to this species. Voucher larval and pupal exuviae from these tests are deposited with the Bohart Museum of Entomology, University of California, Davis, along with 2 male and 2 female paratypes (92031-G01-I04, -I05, -I13, -I14) with associated exuviae. In addition to holotype, 5 male and 2 female paratypes (92031-G01-I01, -I03, -I08, -I10, -I11, -I12, -I16) with associated exuviae deposited with NMNH.

Material examined. In addition to the type series, specimens were examined from the following localities: Fremont, Alameda County, California; Walnut Creek, Alameda County, California; Moraga, Contra Costa County, California; Jersey Island, Contra Costa County, California; San Mateo, San Mateo County, California; Moss Landing,

Table 7. Setal branching of fourth-stage larvae of *Aedes* (*Ochlerotatus*) *washinoi* (n = 10).

Seta no.	Head	Thorax			Abdomen		
	C	P	M	T	I	II	III
0	—	3–4(4)	—	—	1	1	1
1	1	1	1	1–2(2)	3–5(4)	2–4(3)	3–5(3)
2	—	1–2(1)	1–2(2)	1–2(1)	1–3(1)	1–4(1)	1
3	1	1–3(2)	1–2(1)	1–4(3)	2	1–2(2)	1
4	1–2(1)	1	1–2(2)	2–5(3)	2–6(2)	1–3(2)	1–3(1)
5	1–2(1)	1–2(2)	1	1	2–5(4)	4	1–4(4)
6	1	1	5–9(7)	1	1–2(2)	2	1–2(2)
7	6–11(8)	2–3(2)	1	9–12(11)	1	1–3(2)	2–4(4)
8	1	1–2(1)	5–11(7)	2–3(2)	—	1–3(2)	1
9	1	1	6–11(8)	6–9(7)	1–3(2)	1–4(1)	1
10	1–2(1)	1	1	1	1	1	1
11	3–5(5)	1–3(1)	2	1	2–3(2)	1	1
12	2–5(5)	1	1	1	1	1	1
13	1	—	3–6(4)	3–5(4)	2–5(3)	5–6(5)	1–2(1)
14	1	2	3–5(4)	3	—	—	—
15	2–5(3)	—	—	—	—	—	—

Seta no.	Abdomen						Siphon
	IV	V	VI	VII	VIII	X	S
0	1	1	1	1	—	—	—
1	3–5(3)	3–5(3)	2–3(3)	—	1	1	5–7(6)
2	1	1	1	—	1	5–10(8)	1
3	1–2(1)	1	1–3(1)	—	6–10(8)	1	—
4	1–3(2)	3	1–3(2)	—	1	8–16(11)	—
5	1–3(1)	2	—	—	3–5(4)	—	—
6	2	2	1–2(2)	—	—	—	1
7	2–3(3)	1–4(4)	1–3(2)	—	—	—	—
8	1	1	2–3(2)	—	—	—	1–4(3)
9	1–2(1)	1	1	—	—	—	1
10	1	1	1–2(1)	—	—	—	—
11	1	1–2(1)	1–2(1)	—	—	—	—
12	1–2(1)	1–2(1)	1	—	—	—	—
13	1–2(1)	1–2(2)	3–5(5)	—	—	—	—
14	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—

Monterey County, California; Sonoma, Sonoma County, California.

Distribution. Larvae of this species have been collected at low elevations (< 100 m) throughout the Pacific Coastal Plain from Portland, Oregon south to Santa Barbara, California. Its range extends east, where larvae were collected at mid-elevations (< 1,300 m) in the Great Basin (Honey Lake), and from the coast across the Central Valley of California into the lower Sierra Nevada (elevations < 800 m, Nevada City and Chicago Park).

Etymology. This species is named in honor of our friend and colleague, Dr. Robert K. Washino, in recognition of his outstanding contributions to the study of mosquito biology.

RESULTS AND DISCUSSION

Description of biochemical characters. There are two loci coding for the enzyme aconitase in mosquitoes. The locus considered here is designated ACON-2. The product of this locus migrates cathodally when electrophoresed under the stated conditions. The phenotype of the enzyme product of the ACON-2 locus consisted of a single, slow

(ACON-2^{85}) or fast (ACON-2^{96}) migrating band in the homozygote, and a double-banded phenotype in the heterozygote (Fig. 1A). The two bands in the heterozygote migrated to a position corresponding to the positions of the single bands of slow or fast migrating homozygotes (Fig. 1B). We observed two loci coding for the enzyme diaphorase in adults of the *Ae. increpitus* complex. Both loci migrated anodally under the stated conditions. The locus considered here is designated DIA-2 and migrated slower relative to the other diaphorase locus. The phenotype of the enzyme product of the DIA-2 locus presented as slow (DIA-2^{84}) or fast (DIA-2^{97}) migrating single bands in homozygotes (Fig. 1A). Typically, the heterozygote appeared as a broad, smeary band, which covered an area on the gel equivalent to the distance between the fast and slow bands as they appeared in homozygotes (Fig. 1B). On exceptional gels the heterozygote could be seen to consist of three bands, in a pattern consistent with a protein having a dimeric molecular structure. Dimeric structure for diaphorase has been previously reported (Richardson et al. 1986). There was one locus coding for the enzyme lactate dehydrogenase (LDH) in the material we studied. The product of this locus resulted in anodally migrating bands with three distinct mobilities (LDH^{72}, LDH^{85}, and LDH^{88}). The homozygotes produced single-banded phenotypes (Fig. 1A). The heterozygote produced a multiple-banded phenotype, which normally had a "smeary" appearance. Resolution of the heterozygote phenotype was improved by extending the amount of time during electrophoresis. This allowed the visualization of a five-banded phenotype in the heterozygote (Fig. 1B). This phenotype is typical for protein molecules with a tetrameric structure. Green et al. (1990) also reported a tetrameric structure for LDH in *An. minimus* Theobald.

Distribution of biochemical characters among populations. Allele frequencies were calculated under the assumption that each sample site represented a single randomly mating population. Three genetically distinct population groups were observed. We recognize these groups as being three distinct species: *Ae. increpitus*, *Ae. washinoi* and *Ae. clivis*. The decision on which of the three species should bear the name "*increpitus*" was based on the location of the type locality for this species. The type locality for *Ae. increpitus* was given by Dyar (1916) as a site at Fallen Leaf Lake near Lake Tahoe in El Dorado County, California. Although the present study does not include a collection from the type locality, material was analyzed from Hope Valley in Alpine County which is located 18 km from Fallen Leaf Lake. Furthermore, the type locality lies on the eastern slope of the Sierra Nevada. Of seven collections made on the east slope, all were the same species based on the criteria described in this paper. Therefore, we retain the name *Ae. increpitus* for this species. The location of populations sampled of each species is given in Table 1. The geographical locations indicate the coherent spatial distribution of alleles among populations of each species. *Aedes washinoi* is characterized as carrying the 96 allele at the ACON-2 locus, the 97 allele at the DIA-2 locus and, the 72 allele at the LDH locus. *Aedes clivis* is characterized as carrying the 85 allele at the ACON-2 locus, the 97 allele at the DIA-2 locus, and the 88 allele at the LDH locus. *Aedes increpitus* carries the 85 allele at the ACON-2 locus, the 84 allele at the DIA-2 locus, and the 85 allele at the LDH locus. Mean gene frequencies characterizing each species are presented in Table 8.

The diagnostic value of alleles at the three loci are given in Table 9, and were calculated using the method of Ayala and Powell (1972). The values listed represent the probability of making a correct diagnosis based on the genotype at each of the diagnostic loci. There are at least two diagnostic loci for each pairwise comparison and the LDH locus can be used to distinguish all three species. The diagnostic values all exceed 99%.

Distribution of morphological characters. An analysis of setal patterns and other morphological characteristics of samples of the three species revealed significant differences only in the branching of larval seta 5-C (= upper head hair of authors). Figure 2 depicts

Table 8. Frequencies of alleles at three diagnostic loci in three species of the *Aedes increpitus* species complex.

	ACON-2			DIA			LDH			
Enzyme	n	96	85	n	97	84	n	72	88	85
Species										
Ae. washinoi	549	0.993	0.007	528	1.000	0.000	448	0.961	0.039	0.000
Ae. clivis	381	0.000	1.000	410	0.997	0.003	348	0.001	0.999	0.000
Ae. increpitus	331	0.004	0.996	344	0.028	0.962	326	0.024	0.000	0.976

Table 9. Diagnostic values* of three loci for distinguishing species in the *Aedes increpitus* species complex.

	Enzyme loci		
Comparison	ACON-2	DIA	LDH
Ae. washinoi vs. *Ae. clivis*	0.999	†	0.998
Ae. washinoi vs. *Ae. increpitus*	0.996	0.999	0.999
Ae. clivis vs. *Ae. increpitus*	†	0.996	0.999

* Calculated after Ayala and Powell (1972). Locus considered diagnostic when the probability of assigning an individual to the correct species is 99% or higher.
† Not diagnostic.

the distribution of branching of these setae based on examination of many specimens from each species. *Aedes clivis* can be separated in nearly all instances from *Ae. washinoi* and *Ae. increpitus* based on the significantly greater number of branches for seta 5-C. Analysis of data for 5-C indicated that the mean number of branches differs significantly for all three species (Fisher's LSD comparison, $P < 0.001$).

Genetic structure of populations. The initial assumption that each site represented a single, randomly mating population was tested by calculating goodness of fit to Hardy-Weinberg equilibrium for each locus. Highly significant deviations from Hardy-Weinberg equilibrium were observed in four populations (Table 10). Chi-square values for ACON-2 indicated a significant deficiency of heterozygotes at two sites, for DIA-2 a significant deficiency of heterozygotes was observed in two populations and all four populations showed a deficiency of heterozygotes for the LDH locus (Table 10). The allelic composition of populations at two sites in Nevada County suggests that they, in fact, represent two, reproductively isolated populations in sympatry, one of *Ae. washinoi* and the other *Ae. clivis*. The two Plumas County sites represent *Ae. clivis* and *Ae. increpitus* in sympatry. In each case the deficiency of heterozygotes represents a "Wahlund effect" resulting when sympatric populations, with limited genetic interchange, are sampled as single populations. These results demonstrate that there is very little or no gene flow between *Ae. clivis* and either *Ae. increpitus* or *Ae. washinoi*, even when these species occur in sympatry. Therefore, highly efficient reproductive barriers must exist which prevent effective hybridization of *Ae. clivis* with either of its sibling species. This observation provides the strongest evidence for the recognition of *Ae. clivis* as a valid species. The distribution of genotypic frequencies in the remaining 25 sites studied fit Hardy-Weinberg expectations.

Extensive hybridization between *Ae. increpitus* and *Ae. washinoi* was observed in a contact zone at a site in Shasta County, California (Lassen State Park). A collection at this site revealed hybrid genotypes consisting of species-specific alleles at the three loci considered here, suggesting that gene introgression occurs in this area. We use the term "hybrid" here, and in the subsequent discussion, to refer to the array of recombinant genotypes

Table 10. Goodness of fit to Hardy-Weinberg expectations for heterozygote frequencies at the ACON-2, DIA and LDH loci in populations of *Aedes increpitus* sibling species occurring in sympatry.

Species and Location	Heterozygotes											
	ACON-2				DIA				LDH			
	N	Obs	Exp	χ^2	N	Obs	Exp	χ^2	N	Obs	Exp	χ^2
Ae. washinoi/Ae. clivis Chicago Park, Calif.	66	0	31.76	67.10	—	—	—	—	68	3	33.65	57.29
Ae. washinoi/Ae. clivis Nevada City, CA	118	2	44.94	109.25	—	—	—	—	144	9	53.68	100.95
Ae. increpitus/Ae. clivis Graeagle, Calif.	—	—	—	—	108	0	53.34	109.04	107	0	53.09	110.05
Ae. increpitus/Ae. clivis Quincy, Calif.	—	—	—	—	50	0	9.09	55.06	50	0	9.09	55.06

containing genes from both of the parental forms (Barton and Hewitt 1985) and which are recognizable as such by electrophoretic analysis. Of 83 individuals collected from the hybrid zone and analyzed electrophoretically, only 14 (17%) possessed multilocus genotype arrays consistent with their being F_1 hybrids and only 2 individuals had genotypic arrays consistent with their being parental forms. Therefore, the majority of individuals must represent the progeny of hybrid parents or back-crossing of hybrids to parental forms. A lack of parental and F_1 genotypes is expected in a hybrid zone with long-term random mating (Barton and Hewitt 1985, Hewitt 1989). These results suggest that there is little, if any, reduced viability in F_1 hybrids and indicates a very close genetic relationship between these two species. An analysis of goodness of fit to Hardy-Weinberg equilibrium in this population reveals that the genotypic frequencies at the three polymorphic loci conform to expectations (Table 11). This suggests that mating is random, that there are no pre-mating reproductive barriers and that there is no strong selection against hybrid genotypes, that is, there are no strong post-mating barriers.

The taxonomic status of *Ae. clivis* is clear: this species carries two allozyme characters which unambiguously separate it from *Ae. washinoi*, and three allozymes which are diagnostic relative to *Ae. increpitus*. In addition, this species possess morphological characters in the larvae which are either diagnostic or statistically significant in differentiating it from the other two species. More importantly, where *Ae. clivis* occurs in sympatry with the other two species, it maintains its integrity by mating barriers which prevent gene introgression.

The case of *Ae. washinoi* is more problematic. There is currently no consensus regarding the taxonomic status of hybridizing forms. Barton and Hewitt (1985) consider two populations to be biological species only when reproductive isolation is complete, and therefore no viable hybrids can be formed. The species concept most widely accepted is the so-called biological species concept of Mayr (1963). Although this definition of species involves reproductive isolation, Bigelow (1965), and later Mayr (1982), suggested that the gene pools of different species need not be completely isolated, so long as they "remain 'reproductively isolated' in the sense that they do not fuse into a single population" (Mayr 1982, p. 285). The results of this study indicate that the latter situation seems to apply to northern California populations of *Ae. washinoi* and *Ae. increpitus*. Populations which are genetically "pure" *Ae. washinoi* persist, both to the east and west of the hybrid population, and pure *Ae. increpitus* populations are found both to the northeast and southeast. Significant barriers to gene introgression must exist which maintain the genetic integrity of the parental forms outside of a narrow hybridizing zone, suggesting that

Table 11. Goodness of fit to Hardy-Weinberg expectations for heterozygote frequencies at the ACON-2, DIA and LDH loci in a *Ae. washinoi*/*Ae. increpitus* hybrid population.

Heterozygotes	N	Observed	Expected	χ^2
ACON-2	73	38	35.59	0.34
DIA	72	40	33.21	3.63
LDH	113	54	44.27	5.54

these taxa are following independent evolutionary paths, and should therefore, be considered distinct species.

In conclusion, we present data which demonstrate that the taxon currently known as *Ae. increpitus* does not represent a single species, but rather exists as a complex of at least three distinct sibling species. Each of the species in the complex possesses characters which are diagnostic for each. The spatial distribution of these characters in nature is consistent with a reasonable distribution of populations within and among three distinct species. The occurrence of pairs of species in sympatry with no evidence of gene introgression has been demonstrated for two cases. Finally, hybridization in nature is reported for two of the species, but the hybrid zone appears to be spatially restricted, since it is bounded by populations of both parental forms which maintain their specific characteristics.

The increasing use of sophisticated molecular and biochemical techniques in systematic and population genetics studies will undoubtedly result in an increase in the recognition of hybridizing forms. The occurrence of this phenomenon underscores problems with current concepts of species, but should result in a refinement of these concepts in the future. In the meantime, taxonomic decisions involving such cases will have to be made based on a sound analysis of available data and interpreted within the framework of current, albeit imperfect, evolutionary theory.

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