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Mathew L. Brust
University of Nebraska-Lincoln, mbrust@csc.edu

Erica J. Lindroth
University of Nebraska-Lincoln, elindro1@bigred.unl.edu

W. Wyatt Hoback
University of Nebraska at Kearney, whoback@okstate.edu

Robert Wright
University of Nebraska--Lincoln, rwright2@unl.edu

Kathryn J. Hanford
University of Nebraska, kathy.hanford@unl.edu

See next page for additional authors

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Mathew L. Brust, Erica J. Lindroth, W. Wyatt Hoback, Robert J. Wright, Kathy Hanford and John E. Foster

(MLB, EJL, RJW, JEF; all based Nebraska USA) Department of Entomology, University of Nebraska Lincoln, 202 Entomology Hall, Lincoln, NE 68583-0816. Email: elindro1@bigred.unl.edu, rwright2@unl.edu, jfoster1@unl.edu
(MLB) Present address: Department of Biology, Chadron State College, 1000 Main Street, Chadron, NE 69337. Email: mbrust@csc.edu
(WWH) Department of Biology, University of Nebraska at Kearney, 905 W 25th Street, Kearney, NE 68849. Email: hobackww@unk.edu
(KH) Department of Statistics, University of Nebraska Lincoln, 340 Hardin Hall North, Lincoln, NE 68583-0963. Email: khanford2@unl.edu

Abstract

Melanoplus packardii Scudder was described in 1897. Three additional closely-related species were later described and their status as species has been questioned on numerous occasions. We examined morphology from specimens collected in Nebraska which fit descriptions of three of the four forms and specimens that appeared to be hybrids. We found distinct morphological characters suggesting species status for *M. foedus* and *M. packardii*, but not for *M. foedus fluviatilis*. Examination of aedeagi of these three forms suggests that *M. foedus* and *M. packardii* are each distinct, but that the aedeagi of *M. f. fluviatilis* and *M. f. foedus* cannot be distinguished. Molecular analyses of the three groups did not produce clear separations and suggest gene exchange between these three forms may be ongoing. Together, these data suggest that *M. foedus* and *M. packardii* should be recognized as sibling species, but *M. foedus fluviatilis* is best considered a form of *M. foedus*, typically found in low lying areas.

Key words

Melanoplus packardii, morphometric, genetic, grasshopper, systematics

Introduction

The taxonomic status of members of the *Melanoplus packardii* group (Orthoptera: Acrididae) has been changed numerous times. *Melanoplus packardii packardii* Scudder was described in 1897 (Scudder 1897) and its taxonomic status has not shifted since (Pfadt 2002, Capinera *et al.* 2004). *Melanoplus foedus* Scudder was described in 1897 (Scudder 1897), but was considered a subspecies of *M. packardii* for five years (Blatchley 1920, Hebard 1925). It is currently recognized as a full species (Vickery & Kevan 1985, Pfadt 2002, Capinera *et al.* 2004). *Melanoplus packardii brooksi* Vickery was described from Canada in 1979 (Vickery 1979) and it remains recognized as a subspecies of *M. packardii*. *Melanoplus fluviatilis* Bruner was described as a species in 1897 (Bruner 1897), but without an extensive description. It was formally described in 1920 (Blatchley 1920), but was later placed as a subspecies of *M. foedus* (Hebard 1931). Its taxonomic status has remained unchanged since 1931 (Helfer 1987, Kirk & Bomar 2005). *Melanoplus foedus isleyi* Hebard was described as a subspecies of *M. foedus* and remains as a subspecies under current classification (Hebard 1936a, Capinera *et al.* 2004). Finally, *Melanoplus stonei* Rehn was described in 1904 (Rehn 1904) and has since been recognized as a subspecies of *M. foedus* (Cantrall 1968) or *M. packardii* (Hebard 1928), but is now considered distinct [Hebard 1932 (1931), Vickery & Kevan 1985, Kirk & Bomar 2005].

In summary, the current literature recognizes the following species and subspecies in the *packardii* group: *Melanoplus packardii*, *Melanoplus foedus foedus*, *Melanoplus foedus fluviatilis*, *Melanoplus foedus isleyi*, and *Melanoplus stonei* (Eades & Otte 2009). The ranges of *M. foedus foedus* and *M. foedus fluviatilis* overlap across a large portion of the United States, and yet no apparent hybrids have been mentioned in the literature. In addition, the range of *M. foedus isleyi* appears to overlap the range of *M. foedus fluviatilis* over a smaller area (Eades & Otte 2009).

All forms within this group are very difficult to differentiate because few morphological characters consistently differ between them and even the genitalic differences are few. The cerci of the males of all forms are virtually identical; however, the aedeagus of *M. packardii* differs from that of *M. foedus* (Brooks 1958).

Chapco *et al.* (1999) conducted mitochondrial DNA analyses on several North American *Melanoplus* species and determined that *M. packardii* and *M. foedus* were distinct, but closely related, species. However, genetic analysis of members of the group has also been inconclusive and a later study by Chapco and Litzenberger (2002) determined that *M. packardii* and *M. foedus* had some genetic overlap among individuals. Together, these results suggest that these species either represent a variable single species or they represent a very recent evolutionary radiation with hybridization possible. More detailed studies on the genetic relationships between described species and subspecies within the *M. packardii* group are needed to clarify these relationships.

Collections of large series from this group across Nebraska from 2005 to 2007 show trends that raise questions on the taxonomic status of forms currently recognized as subspecies of *M. foedus* and for *M. packardii*. This study scores morphological traits and uses molecular analyses to test three hypotheses: 1) *Melanoplus foedus* and *M. packardii* are distinct species; 2) *Melanoplus foedus fluviatilis* represents a full species; and 3) local hybridization occurs between *M. packardii* and *M. foedus*.

Methods

Large series of the forms *M. packardii*, *M. foedus foedus*, and *M. foedus fluviatilis* were collected from across Nebraska, as well as from a single site in South Dakota, during the summers of 2005-2007. Specimens were identified to species using a variety of resources, including Bruner (1897), Scudder (1897), Helfer (1987), and Pfadt (2002).

All specimens used for morphological studies were then pinned and placed in the collection at USDA-APHIS in Lincoln, Nebraska. Specimens representing potential hybrids between *M. foedus foedus* and *M. packardii* were grouped with the species or subspecies with

Table 1. Number of specimens of each *Melanoplus* taxon by county of collection examined in morphometric study.

Species	County	Quantity
M. foedus fluviatilis	Buffalo	11
M. foedus fluviatilis	Dawson	78
M. foedus fluviatilis	Furnas	4
M. foedus fluviatilis	Hughes (SD)	2
M. foedus fluviatilis	Kearney	1
M. foedus fluviatilis	Keith	42
M. foedus fluviatilis	Lincoln	12
M. foedus foedus	Arthur	6
M. foedus foedus	Banner	1
M. foedus foedus	Blaine	8
M. foedus foedus	Box Butte	2
M. foedus foedus	Brown	2
M. foedus foedus	Cherry	12
M. foedus foedus	Custer	6
M. foedus foedus	Deuel	1
M. foedus foedus	Dundy	8
M. foedus foedus	Garden	37
M. foedus foedus	Grant	29
M. foedus foedus	Hall	1
M. foedus foedus	Holt	5
M. foedus foedus	Hooker	5
M. foedus foedus	Keith	13
M. foedus foedus	Keya Paha	1
M. foedus foedus	Lincoln	15
M. foedus foedus	Logan	5
M. foedus foedus	Loup	1
M. foedus foedus	McPherson	1
M. foedus foedus	Morrill	53
M. foedus foedus	Red Willow	2
M. foedus foedus	Rock	5
M. foedus foedus	Scotts Bluff	14
M. foedus foedus	Sheridan	19
M. foedus foedus	Sioux	2
M. foedus foedus	Thomas	17
M. packardii	Banner	3
M. packardii	Buffalo	1
M. packardii	Chase	3
M. packardii	Cherry	3
M. packardii	Custer	6
M. packardii	Dundy	3
M. packardii	Franklin	9
M. packardii	Furnas	1
M. packardii	Gage	3
M. packardii	Garden	16
M. packardii	Gosper	1
M. packardii	Greeley	2
M. packardii	Harlan	1
M. packardii	Hayes	2
M. packardii	Hitchcock	2
M. packardii	Howard	4
M. packardii	Keith	31
M. packardii	Knox	4
M. packardii	Loup	4
M. packardii	Morrill	2
M. packardii	Pawnee	16
M. packardii	Scotts Bluff	5
M. packardii	Sheridan	4
M. packardii	Sherman	1
M. packardii	Sioux	6

which they shared the greatest similarity. Genetic examination was then used to determine the identity of these possible hybrids (see below). A total of 151 *M. foedus fluviatilis*, 265 *M. foedus foedus*, and 133 *M. packardii* were examined morphologically (Table 1).

Nonmeasurement data included species determination, date, county, locality, sex, hind tibial color, postocular bar development and predominant color, consistency of dorsal pronotal color (solid or striped), predominant dorsal pronotal color, and secondary pronotal color. Measurement data were obtained for tegmen length (from lowest insertion along lower pronotum to tip), narrowest dorsal distance between eyes, and length of male furculae. Measurements of tegmen length were done using a vernier caliper and estimated to the nearest 0.1 mm, while measurements of the intereye distance and length of furculae were taken with an optical micrometer (Scope: Olympus SZ-STS, 2.5x × 10x = 25x; Micrometer: Olympus AX0001 OB-M, 1/100 mm). *Melanoplus f. fluviatilis* and *M. f. foedus* were separated based on the characters listed in Table 2.

Characters were then analyzed using PROC GLIMMIX (SAS Institute Inc. 2006). Measurement data (tegmen length, distance between eyes, and male furcula length) were analyzed under a normal distribution while categorical variables (tibial color, postocular bar development, and postocular color) were analyzed under a multinomial distribution. Because of distinct differences in size between sexes, males and females were compared separately for measurement variables.

Examination of Aedeagi.—Aedeagi from three males of specimens identified as *M. foedus fluviatilis*, *M. foedus foedus*, and *M. packardii* were examined for structural differences. In each case, the terminal part of the abdomen of three males was severed, intestinal contents removed, and the remaining structure soaked in a solution of KOH for approximately 24 h. After 24 h, these were transferred to 70% ethanol and the aedeagus then removed. Aedeagi were cleaned under a dissecting scope to remove bits of connective tissue. They were placed in 70% ethanol until examination. Photographs of the aedeagi were taken through a dissecting scope, and drawn using a camera lucida. A comparison was made of the general structure of the terminal end of the aedeagus, especially in regard to paramere structure.

Genetic Analyses

DNA Isolation and Quantification.—DNA was isolated from the hind legs of 9 individuals of each form, following a modified Doyle and Doyle (1987) CTAB extraction protocol. Pelleted DNA was suspended in 50 µl 1x TE buffer (10 mM Tris-HCL, 0.1 mM EDTA). The locations, dates of collection, and the number of specimens used in the genetic analyses are presented in Table 3.

AFLP-PCR.—Genetic variation was analyzed using a modified AFLP (amplified fragment length polymorphism) procedure based on Vos *et al.* (1995).

Template preparation.—Approximately 7 µl of 150 ng/µl DNA template was incubated with 0.0625 µl *Eco*RI, 0.125 µl *Mse*I (New England Biolabs), 1.25 µl One-Phor-All buffer (GE Healthcare), 0.125 µl bovine serum albumin (New England Biolabs), and nanopure water for total volume of 12.5 µl at 37°C for 2.5 h in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA). The resulting fragments were then incubated at 25°C for 8 h with a ligation mixture of 0.15 µl T4 DNA ligase, 10x T4 DNA ligase buffer (New England Biolabs, Foster City, CA), 0.5 µl *Eco*RI adapter, 0.5 µl *Mse*I

Table 2. Characters for differentiating *M. foedus fluviatilis* and *M. foedus foedus*.

	M. foedus foedus	*M. foedus fluviatilis*
Hind tibia color	Pinkish red, rarely blue	Pinkish red, purple, or blue
Color of dorsal pronotum	Orange-brown with light stripes laterally	Solid 'fill' with speckled appearance, woody brown
Postocular bars	Lacking or poorly developed, brown to black	Strongly developed, black to rarely brown
Male furcula length	Usually 0.39-0.52 mm, rarely under 0.35 mm	Usually 0.22-0.39 mm, rarely over 0.40 mm
Color inner hind femur	Light yellowish to light orange-brown	Orange to red, may have heavy blackish suffusion, rarely orange-brown

adapter (Operon Technologies), and 3.35 µl nanopure water. A 1:10 dilution was then performed on the ligation product using 1x TE buffer.

Preamplification.—1.25 µl of the ligation mixture was incubated with 10 µl Preamplification Primer Mix II (Invitrogen), 1.25 µl 10x PCR buffer II, 0.75 µl $MgCl_2$, and 0.25 µl Amplitag DNA polymerase (Applied Biosystems). The PCR program consisted of 20 cycles (30 s at 94°C, 1 min at 56°C, 1 min at 72°C). A 1:20 dilution with nanopure water was performed on the product.

Selective amplification.—Reaction volumes containing 4.1 µl nanopure water, 1.2 µl 10x PCR buffer II, 0.72 µl $MgCl_2$, 0.08 µl Amplitag DNA polymerase (Applied Biosystems), 2.0 µl *Mse*I primer (LI-COR), 0.4 µl *Eco*RI IRD-700 labeled primer (LI-COR), and 2.0 µl of the preamplification template were amplified via PCR. Primers were screened and chosen based on the number and clarity of bands produced. The PCR program consisted of one cycle (30 s at 94°C, 30 s at 65°C, 1 min at 72°C), 12 cycles (30 s at 94°C, 1 min at 72°C), and 23 cycles (30 s at 94°C, 30 s at 56°C, 1 min at 72°C). The reaction was stopped by adding 2.5 µl stop solution (LI-COR). The product was then denatured for 1 min at 94°C and stored at -20°C.

Data scoring and analysis.—One-microliter samples were electrophoresed through a KB^{Plus} 6.5% polyacrylamide gel (LI-COR) and the bands detected via infrared florescence, using a laser scanning machine (LI-COR Model 4200S-2). An IRD-700 labeled 50-700 bp size standard was used to estimate fragment size. Sixty-two markers were selected based on clarity, and bands were scored using the program SAGA MX 3.2 (LI-COR). The data were converted to matrix form for further analysis, with a 1 indicating band presence and a 0 indicating absence. Data were analyzed using PAUP 4.0b10 (Swofford 2001). Distance analysis was performed using neighbor joining and minimum evolution, while unweighted maximum parsimony was performed using a heuristic search. Bootstrap analyses of 1000 replicates were performed to assess clade support. *Arphia xanthoptera* (Burmeister) served as an outgroup taxon. One specimen of the outgroup and a total of 34 specimens of the selected taxa were used in the analyses. Two specimens of the selected taxa did not demonstrate markers distinct enough for analysis.

Results

Distribution.—*M. foedus foedus* and *M. foedus fluviatilis* were only found to co-occur at a single site; apparent intergrades were extremely rare and only found at that site. Elsewhere in Nebraska each of these two forms occupied distinct habitats, with *M. f. foedus* occurring in dry

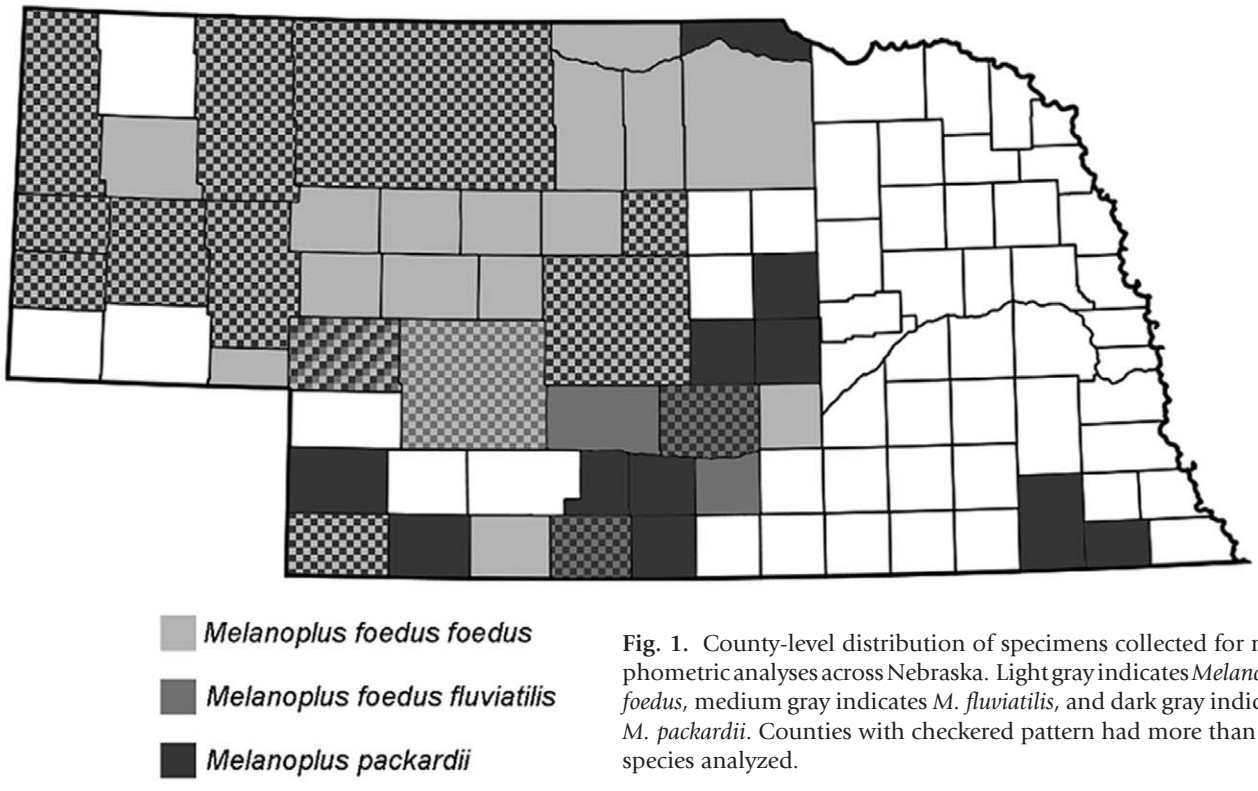

Fig. 1. County-level distribution of specimens collected for morphometric analyses across Nebraska. Light gray indicates *Melanoplus foedus*, medium gray indicates *M. fluviatilis*, and dark gray indicates *M. packardii*. Counties with checkered pattern had more than one species analyzed.

Table 3. Collection information for specimens used in genetic analyses.

Species	State	County	Location	lat N	long W	Date	Quantity
Arphia xanthoptera (outgroup)	Nebraska	Lancaster	Lincoln / Wilderness Park	40.77960	-96.72044	September 20, 2007	1
Melanoplus foedus fluviatilis	Nebraska	Dawson	5 km S of Lexington	40.73792	-99.74183	July 11, 2007	9
Melanoplus foedus foedus	Nebraska	Lincoln	22 km NNW of North Platte	41.32046	-100.86832	July 12, 2007	9
Melanoplus packardii	Nebraska	Pawnee	3 km N of DuBois	40.05749	-96.05039	July 14, 2007	8
M. foedus x packardii?	Nebraska	Morrill	27 km SW of Bridgeport	41.49725	-103.29411	July 24, 2007	4
M. foedus x packardii?	Nebraska	Garden	10 km SSE of Lewellen	41.25720	-102.10593	July 23, 2007	4

sandy uplands and *M. f. fluviatilis* in open sandy woodlands and the upper reaches of sandbars along major rivers (Fig. 1).

Extensive sampling showed *M. foedus* and *M. packardii* co-occur only rarely in Nebraska. However, in some areas of co-occurrence apparent hybrids between *M. foedus* and *M. packardii* were collected fairly commonly, and at these sites, "hybrid" forms tended to predominate over either of the two parent species, making the specie difficult to distinguish. At one site, in Keith County, Nebraska, both species co-occurred in large numbers and no apparent hybrids were observed. This suggests that these two species may be hybridizing in some areas and that the hybridization might be at least partially driven by low abundance of the two forms at these sites.

Morphological examination.—Once separated based on original species descriptions (see Eades and Otte 2009), each of the three forms were found to differ statistically in several characters. All three forms differed significantly in tibial color frequency ($p < 0.0001$). Tibial color of *M. foedus* was nearly always red to pinkish red and that of *M. packardii* was most often blue (Fig. 2). The tibial color of *M. f. fluviatilis* was variable, being red, purple, or blue, or more rarely, pallid (Fig. 2). With the exception of a single specimen from Lake McConaughy, Keith County, Nebraska, all *M. foedus fluviatilis* had the dorsal pronotum solidly colored (usually brown), and this single specimen may be an intergrade with *M. foedus foedus*. All *M. foedus foedus* and *M. packardii* had the dorsal pronotum distinctly striped. While *M. foedus foedus* and *M. packardii* were generally similar in having a striped dorsal pronotum, the color of the dark stripes tended more strongly toward red-brown in specimens identified as *M. foedus foedus* and dark olive-brown in *M. packardii*.

Hind femur and tegmen lengths were similar within sexes for all three forms (Table 4). Both sexes of *M. f. fluviatilis* and *M. packardii* differed from *M. f. foedus* in the minimum distance between the eyes, but were similar to each other (Tables 4 and 5). Males of *Melanoplus f. fluviatilis* and *M. packardii* differed from *M. f. foedus* in the length of the male furculae (Table 5).

Examination of the aedeagi of males showed strong differences in the shape and angle of the basal ring, as well as in the shape and length/width ratio of the parameres among adeagi of *M. packardii* and *M. foedus*. There were no distinct or consistent differences between *M. foedus fluviatilis* and *M. foedus foedus* in aedeagal structure. Although the middle tooth of the inner side of the primary ventral paramere appeared to be slightly more pronounced in *M. f. foedus*, this trait was not consistent (Fig. 3).

Genetic analyses.—A total of 62 characters, both monomorphic and polymorphic, were used in this study. Fifty-two of the 62 characters

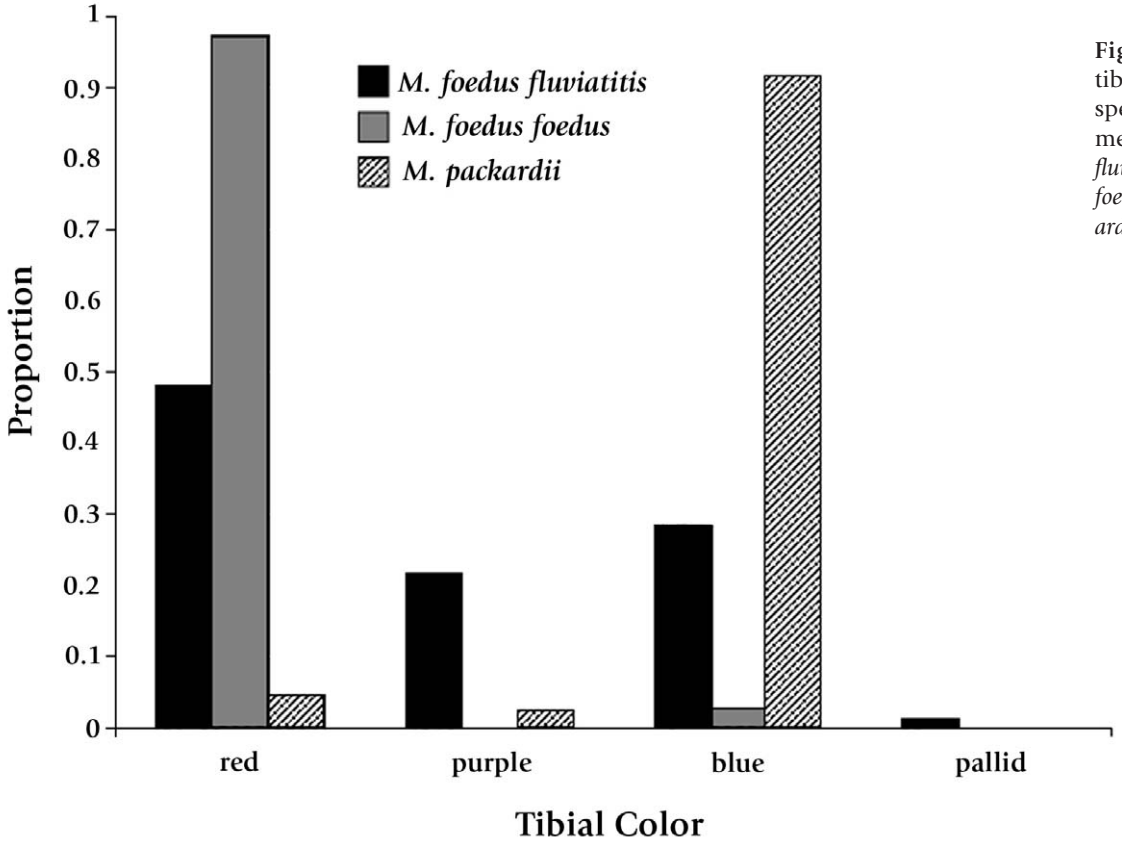

Fig. 2. Proportions hind tibial colors among the 3 species. Number of specimens examined: *M. foedus fluviatilis* (n=150), *M. foedus foedus* (n=264), and *M. packardii* (n=133).

Table 4. Means and standard errors of measurement data (mm) from analyzed specimens.

	Forewing length			Distance between eyes			Furcula length		
Species (by ID)	Sex	Mean	Standard error	Sex	Mean	Standard error	Sex	Mean	Standard error
Melanoplus foedus fluviatilis	female	23.0	1.1	female	0.86	0.08	female	NA	NA
Melanoplus foedus foedus	female	22.9	1.9	female	0.90	0.09	female	NA	NA
Melanoplus packardii	female	23.2	1.4	female	0.85	0.11	female	NA	NA
Melanoplus foedus fluviatilis	male	21.3	1.3	male	0.65	0.06	male	0.34	0.11
Melanoplus foedus foedus	male	21.4	1.4	male	0.73	0.06	male	0.46	0.05
Melanoplus packardii	male	21.9	1.5	male	0.71	0.12	male	0.36	0.06

were parsimony-informative. A large number of polymorphic bands were found both between and within species. Neither distance nor parsimony analysis resolve this species complex (Figs 4, 5), suggesting that there may be frequent hybridization. It is unclear whether each of these forms is a distinct species or if hybridization and resultant introgression occurs. These results are similar to those obtained by Chapco and Litzenberger (2002) when they analyzed numerous species in the genus *Melanoplus* using mitochondrial DNA. These authors were also unable to distinguish between *M. packardii* and *M. foedus*.

Discussion

As anticipated based on descriptions of the forms, *Melanoplus f. foedus*, *M. f. fluviatilis*, and *M. packardii* each display morphometric differences. Compared to *M. f. foedus*, *M. f. fluviatilis* is distinguished by having a solid color on the dorsal pronotum and usually having large, dark postocular bars. The minimum distance between the eyes was significantly different between *M. f. foedus* and *M. f. fluviatilis*, despite the fact that tegmen lengths did not differ. The structure of the aedeagus did not differ between the subspecies, but differed between *M. foedus* and *M. packardii*. Hind tibial color could also be used to differentiate *M. f. foedus* and *M. packardii* in Nebraska approximately 90% of the time. *M. f. fluviatilis* appears to be intermediate in this character, with individuals exhibiting broad variation in color. While *M. stonei* was not analyzed in this study, all specimens collected by the senior author from northwestern Ontario, Canada have red hind tibiae. To further complicate identification within this group, occasional specimens resembling *M. foedus isleyi* occur in Nebraska. This subspecies is differentiated by poorly developed postocular bars and pallid hind tibiae.

Examination of the male aedeagus revealed that in *M. foedus* it is clearly and consistently distinct from that of *M. packardii* in several characteristics. However, the aedeagus of *M. f. fluviatilis* is virtually indistinguishable from that of *M. f. foedus*. The shape and angle of the basal ring as well as the form of the parameres appear to be identical (Fig. 3).

The lack of any distinct clades in the genetic analyses (Figs 4, 5) supports *M. foedus foedus* and *M. foedus fluviatilis* as being the same species and that frequent interbreeding or hybridization occurs between these taxa and *M. packardii*. Our results are similar to those obtained by Chapco and Litzenberger (2002) when they analyzed numerous species in the genus *Melanoplus* using mitochondrial DNA and were unable to distinguish between *M. packardii* and *M. foedus* based on genetic criteria.

Morphological differences in the absence of clear genetic differences suggest several possibilities: 1) *M. foedus* and *M. packardii* are environmentally induced forms of the same species; 2) they are species that were previously separated but have retained the ability to interbreed where populations meet; 3) or they are subspecies in the process of becoming full species. Although our data do not allow resolution of these or other possibilities, the forms in Nebraska occur most often in distinct habitats.

In Nebraska, *M. f. foedus* occurs in upland sandy areas with sparse vegetation and is especially common in the Nebraska Sandhills. *Melanoplus f. fluviatilis* occurs in lowland areas near rivers, especially along the higher areas of sandbars and in open woodland where there is more growth of weedy vegetation. *Melanoplus packardii* is most common in areas with somewhat sparse vegetation on generally heavy soils such as clay or loess. Populations of suspected hybrids between *M. foedus* and *M. packardii* have been found mostly in sparsely vegetated areas on heterogeneous soils, especially in areas where clays are mixed with small stones or gravel. These observations suggest that each form may differ in soil and/or plant preference, but may hybridize where multiple habitats mix.

In areas with heterogeneous soils, apparent hybrids between

Table 5. Species and subspecies comparisons of tegmen length, minimum distance between eyes, and male furcula length. Significant differences (>0.05) are indicated by an asterisk and bold type.

Species Comparison	Sex	Character	p
M. foedus fluviatilis vs M. foedus foedus	female	tegmen length	0.8819
M. foedus fluviatilis vs M. packardii	female	tegmen length	0.2224
M. foedus foedus vs M. packardii	female	tegmen length	0.1199
M. foedus fluviatilis vs M. foedus foedus	male	tegmen length	0.4573
M. foedus fluviatilis vs M. packardii	male	tegmen length	0.1489
M. foedus foedus vs M. packardii	male	tegmen length	0.374
M. foedus fluviatilis vs M. foedus foedus	female	distance between eyes	**<0.0001***
M. foedus fluviatilis vs M. packardii	female	distance between eyes	0.677
M. foedus foedus vs M. packardii	female	distance between eyes	**<0.0001***
M. foedus fluviatilis vs M. foedus foedus	male	distance between eyes	**<0.0001***
M. foedus fluviatilis vs M. packardii	male	distance between eyes	0.0782
M. foedus foedus vs M. packardii	male	distance between eyes	**<0.0001***
M. foedus fluviatilis vs M. foedus foedus	male	furcula length	**<0.0001***
M. foedus fluviatilis vs M. packardii	male	furcula length	0.0908
M. foedus foedus vs M. packardii	male	furcula length	**<0.0001***

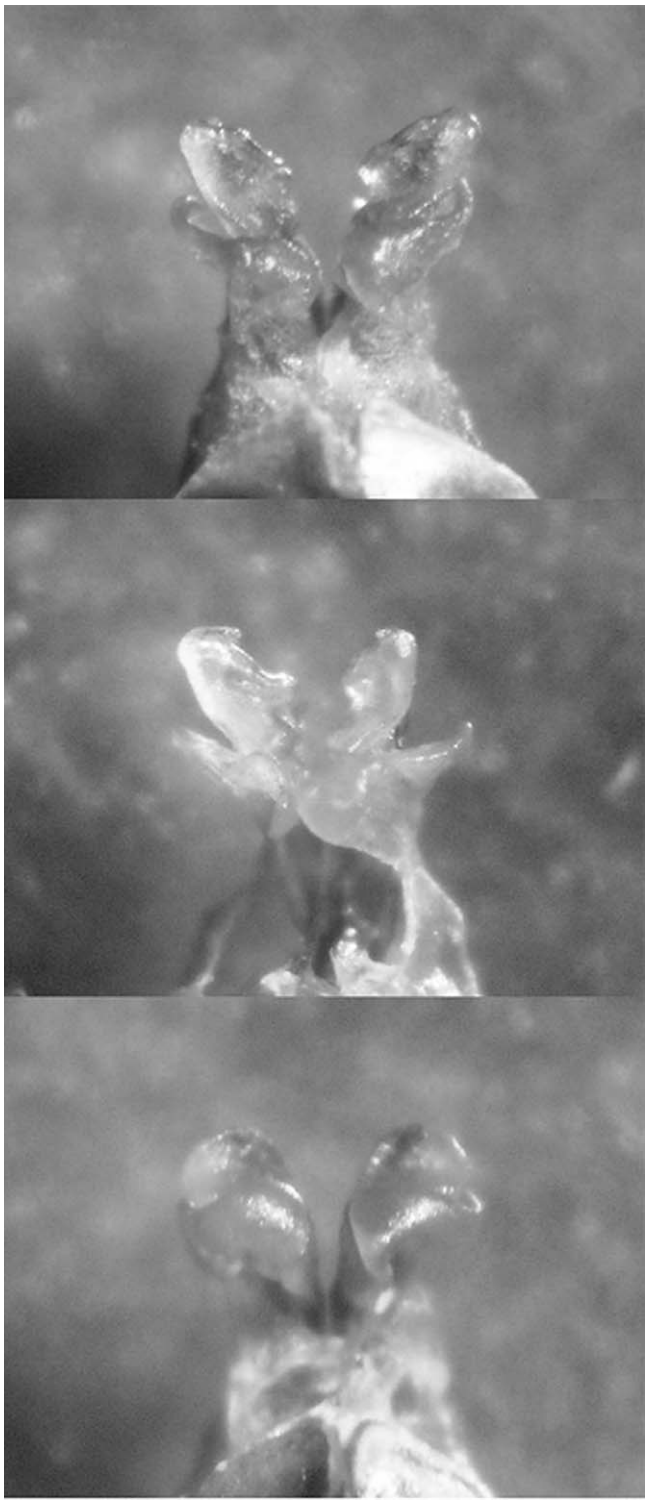

Fig. 3. Dorsal view of parameres and basal ring of aedeagi of *M. foedus fluviatilis* (top), *M. foedus foedus* (middle), and *M. packardii* (bottom).

M. foedus and *M. packardii* tend to predominate over either parent species, but all forms tend to occur at low to moderate densities. At one site in Nebraska (Keith County, *ca* 1.9 km east of Roscoe off Highway 30), both *M. foedus* and *M. packardii* are abundant, but no apparent hybrids were found. At this site, it appears that the forms are capable of differentiating between each other. Together, these observations may lend evidence to male choice driving hybridization, as males of some insect species are known to mate with females of a closely related species if females of the same species are scarce (Platt *et al.* 1978).

The lack of genetic differentiation suggests that *M. foedus* and *M. packardii* are actually one species. However, we feel that differences in aedeagal structure and habitat occurrence should be recognized and that the species designation should remain until additional genetic or behavioral tests can be performed. The lack of resolution in the genetic tests could be the result of occasional hybridization.

While we conclude that *M. packardii* and *M. foedus* are distinct morphologically, including in male genitalia, we suggest that *M. f. fluviatilis* should be synonymized under *M. foedus*. Further research is needed to verify our conclusions, but we suspect that *M. f. fluviatilis* is an environmentally induced variant of *M. foedus* and does not qualify as a valid subspecies based on distribution. Both forms can be found throughout Nebraska in suitable habitat. Thus, one could feasibly consider them ecotypes; however, some of the external traits may be indiscrete or subject to environmental influence (Shelford 1917, Otte and Williams 1972). Such environmental factors have been shown to exert a strong influence on adult coloration in other grasshopper species (Otte & Williams 1972). Furthermore, collections of a related species, *Melanoplus angustipennis* (Dodge), have shown darker individuals more predominant in partially wooded riverine areas compared to in adjacent uplands (although not as dark as *M. foedus fluviatilis*), sometimes with this change noted over a distance of less than 0.2 km (M.L. Brust, unpub.).

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References

Blatchley W.S. 1920. Orthoptera of Northeastern America, with especial reference to the faunas of Indiana and Florida. Nature Publishing Company, Indianapolis, IN.

Brooks A.R. 1958. Acridoidea of southern Alberta, Saskatchewan and Manitoba (Orthoptera). Canadian Entomologist 90: Supplement 9: 1-92.

Bruner L. 1897. The grasshoppers that occur in Nebraska. Annual Report Entomology, Nebraska State Building of Agriculture, 1897. pp. 105-138.

Cantrall I.J. 1968. An annotated list of the Dermaptera, Dictyoptera, Phasmatoptera, and Orthoptera of Michigan. Michigan Entomologist 1: 299-346.

Capinera J.L., Sechrist T.S. 1982. Grasshoppers (Acrididae) of Colorado: identification, biology, and management. Colorado State University Experiment Station Bulletin 584S: 1-161.

Capinera J.L., Scott R.D., Walker T.J. 2004. Field Guide to Grasshoppers, Katydids, and Crickets of the United States. Cornell University Press, Ithaca, NY.

Chapco W., Kuperus W.R., Litzenberger G. 1999. Molecular phylogeny of melanopline grasshoppers (Orthoptera: Acrididae): the genus *Melanoplus*. Annals Entomological Society of America 92: 617-623.

Chapco W., Litzenberger G. 2002. A molecular phylogenetic analysis of the grasshopper genus *Melanoplus* Stål (Orthoptera: Acrididae): an update. Journal of Orthoptera Research 11: 1-9.

Coppock S. Jr. 1962. The grasshoppers of Oklahoma. Oklahoma State University Agriculture Experiment Station Bulletin, Processed Series P-399: 1-141.

Doyle J.J., Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19: 11-15.

Eades D.C., Otte D. Orthoptera Species File Online. Version 2.0/3.5. [accessed 6 June 2009]. http://Orthoptera.SpeciesFile.org.

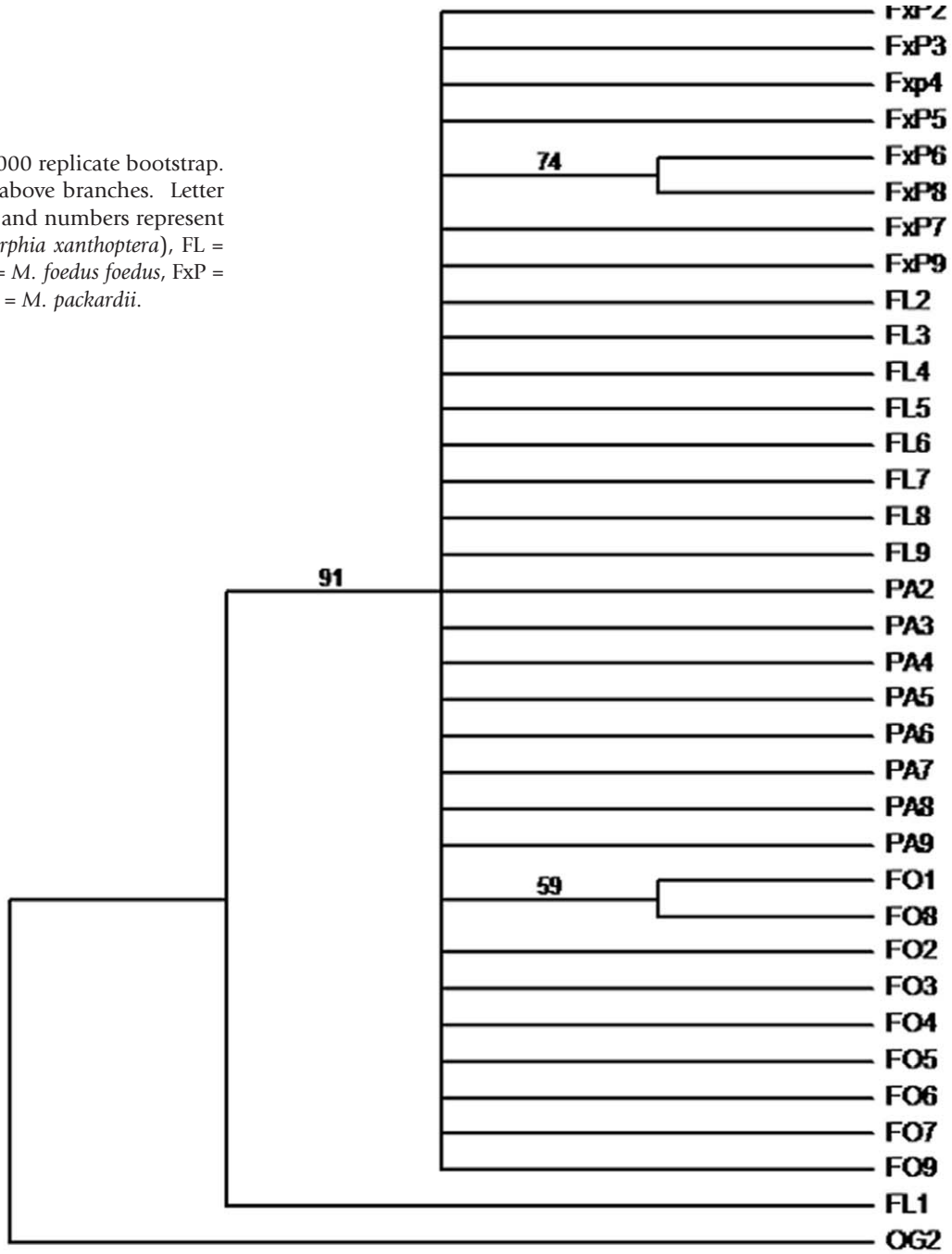

Fig. 4. Neighbor-joining tree. 1000 replicate bootstrap. Bootstrap values (>50) shown above branches. Letter abbreviations represent species and numbers represent individuals: OG = outgroup (*Arphia xanthoptera*), FL = *Melanoplus foedus fluviatilis*, FO = *M. foedus foedus*, FxP = *M. foedus foedus x packardii?*, PA = *M. packardii*.

Froeschner R.C. 1954. The grasshoppers and other Orthoptera of Iowa. Iowa State College of Science 29: 163-354.

Hebard M. 1925. The Orthoptera of South Dakota. Proceedings Academy of Natural Sciences of Philadelphia 77: 35-155.

Hebard M. 1928. The Orthoptera of Montana. Proceedings Academy Natural Sciences of Philadelphia 80: 211-306.

Hebard M. 1931. The Orthoptera of Kansas. Proceedings Academy Natural Sciences of Philadelphi*a* 83: 119-227.

Hebard M. 1932[1931]. The Orthoptera of Minnesota. University of Minnesota Agriculture Experiment Station Bulletin 84: 1-61.

Hebard M. 1934. The Dermaptera and Orthoptera of Illinois. Illinois Natural History Survey Bulletin 20: 125-279.

Hebard M. 1936a. Orthoptera of North Dakota. North Dakota Agriculture Experiment Station Bulletin 284: 1-69.

Hebard M. 1936b. New genera and species of the Melanopli found within the United States and Canada. Transactions American Entomological Society 62: 167-187.

Helfer J.R. 1987. How to Know the Grasshoppers, Crickets, Cockroaches and Their Allies. Dover Publications Inc, New York, NY. 363 pp.

Isley F.B. 1937. Seasonal succession, soil relations, number, and regional distribution of northeastern Texas acridians. Ecological Monographs 7: 317-344.

Kirk K., Bomar C.R. 2005. Guide to the grasshoppers of Wisconsin. Bureau of Integrated Science Services, Wisconsin Department of Natural Resources, Madison, WI. 154 pp.

La Rivers I. 1948. A synopsis of Nevada Orthoptera. American Midland Naturalist 39: 652-720.

Lockwood J.L., McNary T.J., Larsen J.C., and Cole J. 1993. Distribution atlas for grasshoppers and the Mormon cricket in Wyoming 1988-1992. University of Wyoming Agriculture Experiment Station Bulletin B-976. 117 pp.

McDaniel B. 1987. Grasshoppers of South Dakota. South Dakota Agriculture Experiment Station Publication TB 89: 1-163.

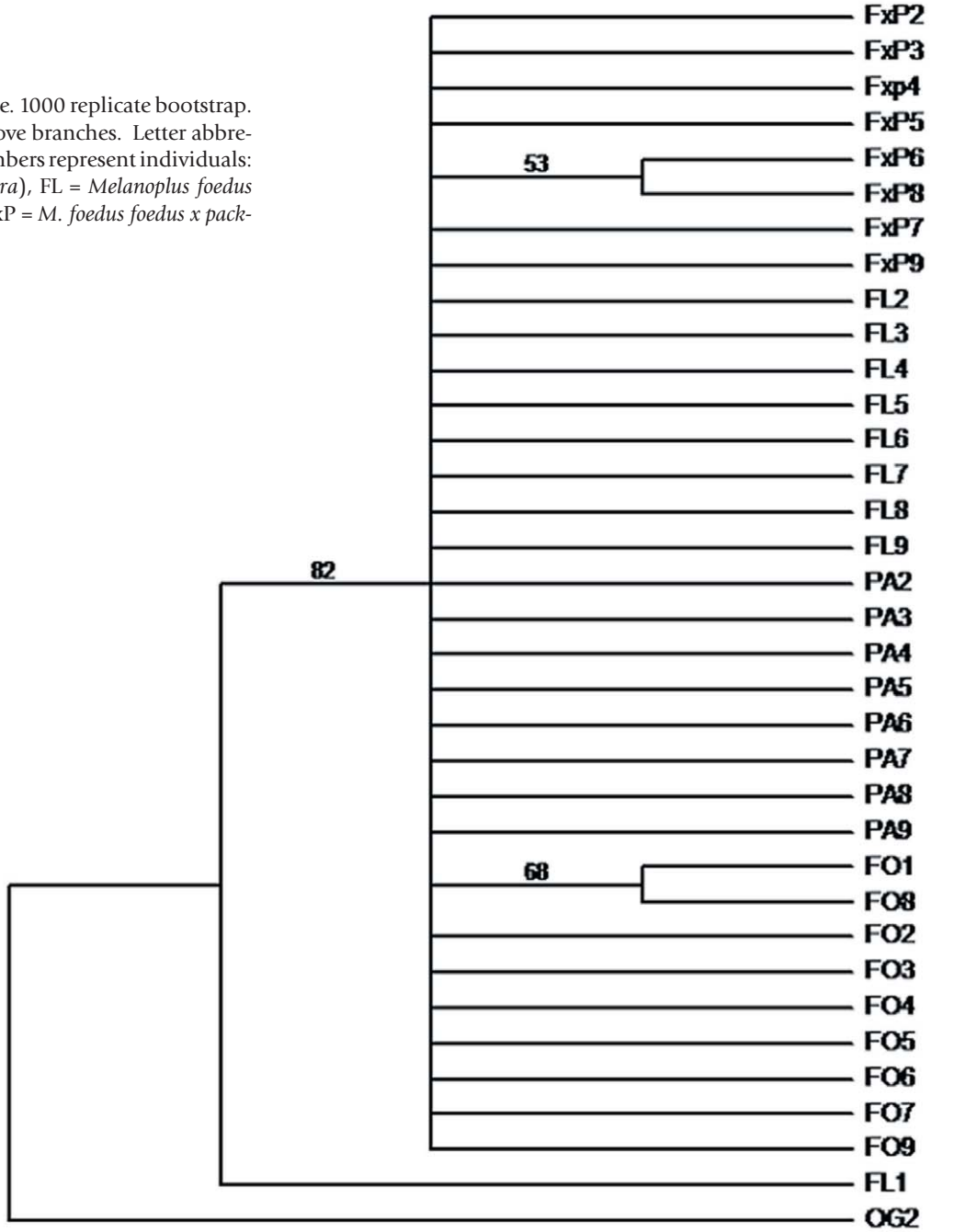

Fig. 5. Unweighted parsimony tree. 1000 replicate bootstrap. Bootstrap values (>50) shown above branches. Letter abbreviations represent species and numbers represent individuals: OG = outgroup (*Arphia xanthoptera*), FL = *Melanoplus foedus fluviatilis*, FO = *M. foedus foedus*, FxP = *M. foedus foedus x packardii?*, PA = *M. packardii*.

Otte D., Williams K. 1972. Environmentally induced color dimorphisms in grasshoppers, *Syrbula admirabilis*, *Dichromorpha viridis*, and *Chortophaga viridifasciata*. Annals Entomological Society of America 65: 1154-1161.

Pfadt R.E. 2002. A field guide to common western grasshoppers, 3rd edition. Wyoming Agricultural Experiment Station Bulletin 912: 1-288.

Platt A.P., Rawson G.W., Balogh G. 1978. Inter-specific hybridization involving *Limenitis archippus* and its congeneric species (Nymphalidae). Journal of the Lepidopterists' Society 32: 239-303.

Rehn J.A.G. 1904. A new *Melanoplus* from New Jersey. Entomological News 15: 85-87.

SAS Institute Inc. 2006. The GLIMMIX Procedure. SAS Institute Inc., Cary, North Carolina, USA.

Schell S., Lockwood J., Schell S., Zimmerman K. Distribution Atlas for Grasshoppers and the Mormon Cricket in Wyoming. http: //www.wygisc.uwyo.edu/grasshopper/ghwywfrm.htm. Accessed 1 December 2007.

Scudder S.H. 1897. Revision of the orthopteran group Melanopli (Acridiidae), with special reference to North American forms. Proceedings Boston Society of Natural History 20: 1-422.

Shelford V.E. 1917. Color and color pattern mechanism of tiger beetles. Biological Monographs 3: 399-432.

Swofford D.I. 2001. PAUP*: phylogenetic analysis using parsimony (*and other methods), ver. 4.0b10. Sinauer, Sunderland, MA.

Vickery V.R., Kevan D.K. 1985. The grasshoppers, crickets and related insects of Canada and adjacent areas. Agriculture Canada. Publication 1777. 918 pp.

Vickery V.R. 1979. Notes on some Canadian Acrididae (Orthoptera). Canadian Entomologist 111: 699-702.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.