Characterization of two non-native invasive bark beetles, *Scolytus schevyrewi* and *Scolytus multistriatus* (Coleoptera: Curculionidae: Scolytinae)

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Abstract—*Scolytus schevyrewi* Semenov, the banded elm bark beetle, and *S. multistriatus* Marsham, the smaller European elm bark beetle, are morphologically similar. Reliance on adult external morphological characters for identification can be problematic because of wide within-species variability and the need for good-quality specimens. The inability to identify developmental stages can also hamper early-detection programs. Using two character identification systems, genitalic (aedeagus) morphology, and DNA markers (random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)) to distinguish *S. schevyrewi* from *S. multistriatus*, we examined specimens from geographically distinct populations of both species collected from infested host trees or semiochemical-baited funnel traps. We found that aedeagus morphology can be used to identify the two species. The use of two oligonucleotide primers in the RAPD-PCR analysis yielded distinct DNA banding patterns for the two species. Species identification using RAPD-PCR analysis was validated by a blind test and used to make species identifications of larval specimens. These tools improve the ability to differentiate between *S. schevyrewi* and *S. multistriatus* at immature and adult stages, and could be developed and used for other scolytines as well.

Résumé—*Scolytus schevyrewi* Semenov, le scolyte asiatique de l'orme, et *S. multistriatus* Marsham, le petit scolyte européen de l'orme, se ressemblent morphologiquement. L'utilisation des seuls caractères externes des adultes pour l'identification pose un problème, car il y a un fort degré de variabilité au sein de chacune des espèces et il est nécessaire d'obtenir des spécimens de bonne qualité. L'impossibilité d'identifier les stades immatures peut aussi nuire aux programmes de détection hâtive. En utilisant deux systèmes de caractères diagnostiques, la morphologie génitale (édéage) et des marqueurs ADN (ADN polymorphe amplifié aléatoirement en chaîne par polymérase (RAPD-PCR)), pour distinguer *S. schevyrewi* et *S. multistriatus*, nous avons examiné des spécimens provenant de populations géographiquement distinctes des deux espèces et récoltés sur des arbres hôtes infestés ou dans des pièges appâtés de produits sémiochimiques. Nous

trouvons que la morphologie de l'édéage permet d'identifier les deux espèces. L'utilisation de deux sondes d'oligonucléotides dans l'analyse RAPD-PCR produit des patrons de bandes ADN distincts chez les deux espèces. Nous avons validé l'identification par l'analyse RAPD-PCR dans un test aveugle; nous l'avons aussi appliquée à l'identification spécifique de larves. Ces outils permettent de mieux différencier *S. schevyrewi* de *S. multistriatus* aux stades immatures et adulte; il serait possible d'en mettre au point et d'en utiliser de semblables pour d'autres scolytinés.

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Introduction

Scolytus schevyrewi Semenov (Coleoptera: Curculionidae: Scolytinae), the banded elm bark beetle, recently introduced into the United States of America, is morphologically similar to another non-native invasive species that is well established in North America, *Scolytus multistriatus* (Marsham), the smaller European elm bark beetle (Fig. 1). The two species utilize the same preferred hosts (species of *Ulmus* L. (Ulmaceae)) and have been found in the same host tree (Negron *et al.* 2005; P.L. Johnson, unpublished data). *Scolytus schevyrewi* has been known to attack other hosts such as species of *Salix* L. and *Populus* L. (Salicaceae), *Prunus* L. (Rosaceae), *Elaeagnus* L. (Elaeagnaceae), *Alnus* Mill. (Betulaceae), and *Quercus* L. (Fagaceae) (Wang 1992; Allen and Humble 2002; North American Plant Protection Organization (July 2003)). *Scolytus multistriatus* has attacked ornamental cypresses (*Cupressus* L. (Cupressaceae)) in Australia (Neuman 1987). The known morphological differences between *S. schevyrewi* and *S. multistriatus* include the general size, shape, and color of the adults, and the size, shape, and location of the apical spine on the underside of the abdomen (LaBonte *et al.* 2003).

Native to western Europe, the Middle East, and northern Africa (Bellows *et al.* 1998), *S. multistriatus* was first recorded in North America in Boston, Massachusetts, in 1909 (Chapman 1910) and subsequently spread west across the United States of America and into southern Canada (Baker 1972). *Scolytus multistriatus* is a primary vector of the Dutch elm disease pathogen *Ophiostoma novo-ulmi* Brasier in the United States of America and Canada (Baker 1972; Furniss and Carolin 1977). The native range of *S. schevyrewi* includes Asian Russia, Mongolia, Turkmenistan, Uzbekistan, Tajikistan, Kazakhstan, southern Kyrgyzstan, Korea, and most of China (Wang 1992). *Scolytus schevyrewi* was first identified in North America in 2003 even though it had been collected from Denver, Colorado in 1994 (Negron *et al.* 2005) and again from New Mexico in 1997 (LaBonte *et al.* 2003). By 2004, established populations of *S. schevyrewi* were found in 21 states across the United States of America (Negron *et al.* 2005). Harris (2004) found that up to 96% of *S. schevyrewi* collected from American elm bolts were infected with Dutch elm disease. *Scolytus schevyrewi* is a potential vector of Dutch elm disease (Seybold and Lee 2004). The similar morphology and preferred hosts of *S. multistriatus* and *S. schevyrewi* are the most likely reasons why *S. schevyrewi* was not identified until several years after it became established in the United States of America (Liu and Haack 2003).

The negative impacts of non-native invasive scolytines such as *S. multistriatus* and *S. scolytus* F. (Weber 1990; Haack and Cavey 2000; Allen and Humble 2002) and the establishment of *S. schevyrewi* years before its detection underscore the need to develop additional tools to distinguish between these species in particular and other morphologically similar non-native scolytines at all life stages (Haack 2006) in general. The purpose of this study was to examine alternative techniques and develop tools to characterize *S. schevyrewi* that could be used to distinguish it more easily from *S. multistriatus* and other scolytines, particularly at early life stages and in different conditions. The study included morphometric quantification of the male aedeagus (external genitalia) and examination of diagnostic molecular markers using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). Male genitalic morphology has been used qualitatively to identify bark beetle species (*e.g.*, Sharp and Muir 1912; Cerezke 1964; Vite *et al.* 1974). Molecular genetic markers identified through RAPD-PCR have also been used to differentiate variation within and between species, including non-native, invasive insects. For example, the genetic relatedness of populations

Fig. 1. Adult *Scolytus* spp. from populations in La Grande, Oregon. (*a*) *Scolytus schevyrewi*. (*b*) *Scolytus multistriatus.* Photographs by Patricia L. Johnson.
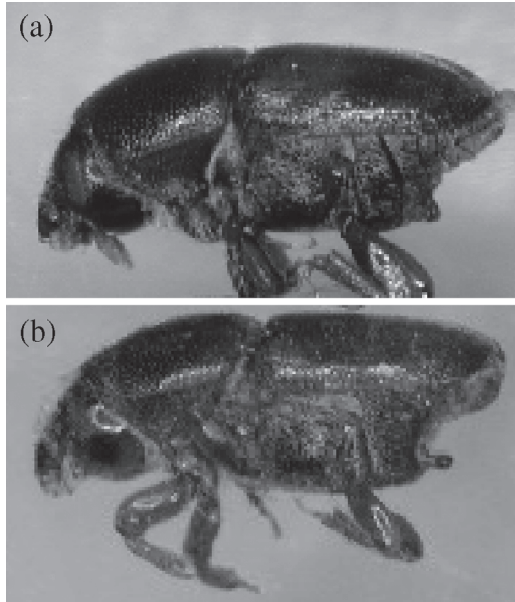
of the non-native bark beetle *Tomicus piniperda* (L.) (Curculionidae: Scolytinae) was traced using RAPD-PCR by Carter *et al.* (1996), who determined that United States populations resulted from multiple introductions. Similarly, differences between Asian and European genotypes of gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), were characterized using RAPD-PCR (Garner and Slavecek 1996; Schreiber *et al.*1997). Hidayat *et al.* (1996) showed that two sibling species of grain weevils, *Sitophilus oryzae* (L.) and *S. zeamais* (Motschulsky) (Curculionidae: Dryophthorinae), could be distinguished not only by variations in their genitalic morphology but also by RAPD-PCR genetic markers. Cane *et al.* (1990) and Stauffer and Zuber (1998) used external morphometrics and molecular genetics to differentiate between species of *Ips* De Geer (Curculionidae: Scolytinae). We expected to find, therefore, that *S. schevyrewi* and *S. multistriatus* each exhibit unique genitalic and molecular characters.

Materials and methods

Sample collections

Samples for this study were collected from five states across the continental United States of America: *S. schevyrewi* from Colorado, Oregon,

and Utah, and *S. multistriatus* from Kansas, Maryland, and Oregon. Specimens of each species were reared from American elm (*Ulmus americana* (L.) (Ulmaceae)) limbs or collected from semiochemical-baited Lindgren funnel traps. Identification of all specimens was confirmed by experts using current external morphological criteria (La Bonte *et al.* 2003). Samples used for morphometrics were from all five states, with five populations (*n* = 10 per population) for each species. Samples of *S. schevyrewi* were reared from elm limbs obtained from Lakewood (2004) and Golden (2005), Colorado (CO1 (39°42′N, 105°06′W) and CO2 (39°78′N, 105°22′W)), and La Grande (2005), Oregon (OR3 (45°20′N, 118°06′W)), and trap-collected from Salt Lake City (2005) and Tremonton (2006), Utah (UT1 (40°45′N, 111°52′W) and UT2 (41°73′N, 112°19′W)). Samples of *S. multistriatus* populations were obtained from trap collections in 2005 and 2006 from Crawford County, Kansas (KS1 and KS2 (37°30′N, 94°51′W)); Prince George County (2006), Maryland (MD (38°84′N, 76°83′W)); and La Grande, Oregon (OR1), plus a population reared from elm limbs from La Grande (2005), Oregon (OR3). *Scolytus schevyrewi* used for analysis of molecular genetics were collected from Lakewood, Colorado (CO1), La Grande, Oregon (OR3), and Salt Lake City, Utah (UT1). Samples of *S. multistriatus* for analysis of molecular genetics were collected from Crawford County, Kansas (KS2), La Grande, Oregon (OR1), and Elgin, Oregon (OR2 (45°34′N, 117°58′W)). Specimens from all populations except those obtained from Maryland and Kansas in 2005 (all specimens of which were used in the experimental analysis) are presently stored at the Forestry and Range Sciences Laboratory, USDA Forest Service Pacific Northwest Research Station, La Grande, Oregon; upon completion of this work voucher specimens will be submitted to Oregon State Arthropod Collection at Oregon State University in Corvallis.

Genitalic morphology

Extraction and measurement of the aedeagus

The aedeagus, the intromittent organ of the male insect, is a sclerotized tube at the distal part of the phallus (Torre-Bueno 1962). Two to five aedeagi of each species were dissected using a technique modified from Sharp and Muir (1912) and examined for variation in morphology. A

Fig. 2. Locations (arrows) where measurements on the aedeagi of *Scolytus schevyrewi* (left) and *S. multistriatus* (right) were made.

0.1 mm
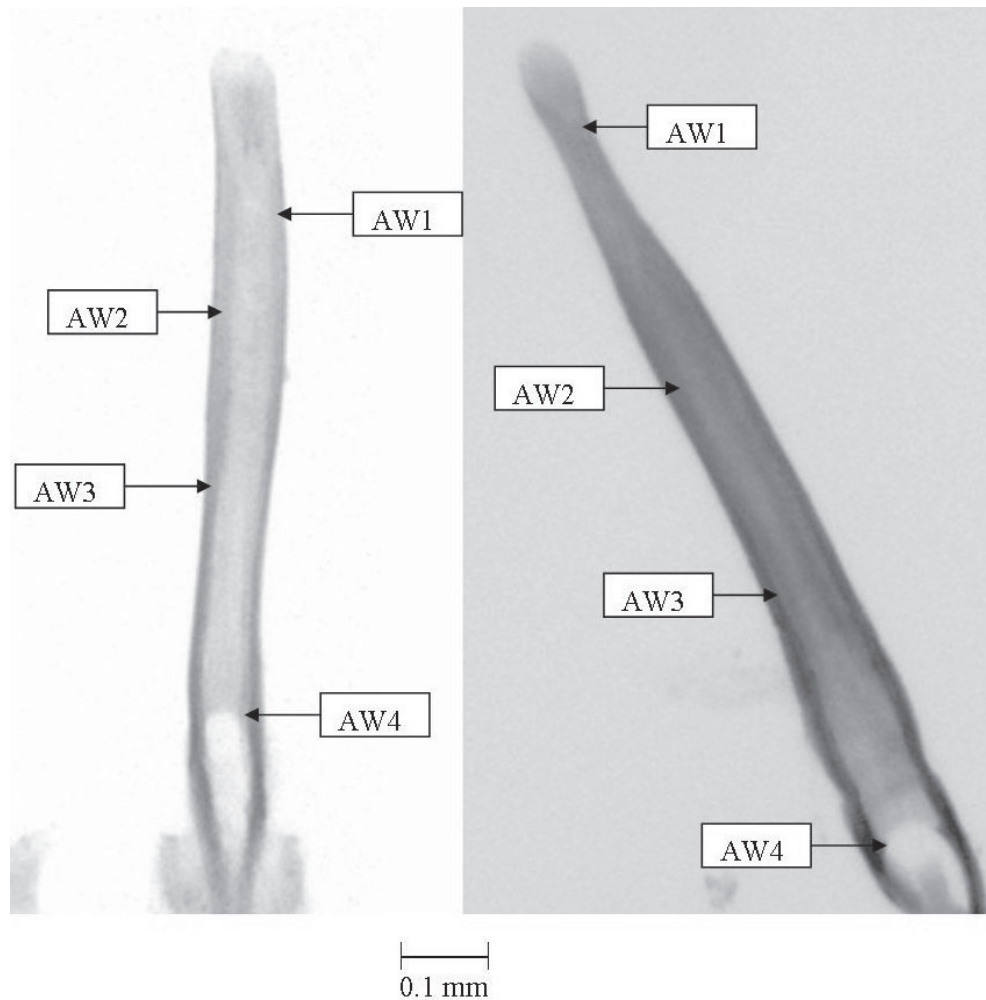
comparison of shape and size was made to identify characteristics that differed between species. A number of measurements were made to quantify observed differences and establish a protocol for this diagnostic technique. Then, for each species, 50 aedeagus samples from populations from three states across the United States of America were analyzed (*S. schevyrewi* from CO1, CO2, OR1, UT1, and UT2; *S. multistriatus* from KS1, KS2, MD, OR1, and OR3). Five measurements were taken: aedeagus length (AL) and four width measurements at specific locations on the aedeagus (AW1, AW2, AW3, and AW4) (Fig. 2). AL was measured from the tip of the apex of the aedeagus to the base of its lobes. AW1 was recorded at the apical end below

the opening at the narrowest part of the aedeagus (Fig. 1). For both species, AW2 and AW3 were recorded at 38% and 69%, respectively, of the distance between the tip of the apex and the basal edge of the membrane between the lobes of the aedeagus (Fig. 2). Because the lobes varied in length, they were not included when measuring AW2 and AW3 for either species. For both species, AW4 was recorded at the basal edge of the membrane between the lobes (Fig. 2). Seventy percent ethanol was used to preserve and (or) moisten the aedeagi as they were being measured. All genitalic-morphology measurements were obtained at a magnification of 80× using a binocular dissecting scope and ocular micrometer.

Data analysis

Aedeagus lengths and widths were not normally distributed and could not be normalized using transformation, therefore we chose to use nonparametric procedures in all analyses of these data. We determined the statistical significance of differences in mean length and widths between the species by means of Wilcoxon's two-sample test using PROC NPAR1WAY in SAS/STAT software (SAS Institute Inc. 2002).

We used a nonparametric nearest neighbor discriminant analysis procedure to evaluate the usefulness of the aedeagus measurements in identifying unclassified insects among the taxa studied. The discriminant model was constructed using all five aedeagus traits with equal weighting in all cases and PROC DISCRIM (METHOD = NPAR, $k = 5$; SAS Institute Inc. 2002). We evaluated model accuracy using cross-validation. The model was initially constructed and analyzed using data from all 100 samples. Then, to validate its use for identification, the model was reconstructed sequentially with data that excluded 1 of the 10 populations from the analysis, *i.e.*, each new model was based on the remaining 90 insects. Using this model, the 10 individuals from the excluded population were then identified to species and the results were compared with their previously determined identification. This was done for all 10 populations. For all significance tests, $P = 0.05$.

Molecular genetics

DNA extraction

Total genomic DNA was extracted using the Wizard Genomic DNA Kit (Promega, Madison, Wisconsin) and a protocol optimized for bark beetles (J.L. Hayes and J. Rinehart, unpublished data). The extraction protocol was modified for each of the different types of collection and storage methods. With the exception of Utah, where only 10 adult specimens of *S. schevyrewi* were available, a sample comprising 20 adult individuals was taken from each population (*S. schevyrewi* from CO1 and OR3; *S. multistriatus* from KS2, OR1, and OR2). A sample comprising 20 larvae and 8 adults of a species of *Scolytus* Geoffroy (not identifiable to species using current taxonomic criteria) reared at the Forestry and Range Sciences Laboratory was also analyzed. Specimens that had been stored in an ultra-cold freezer (–81 °C) were placed in a 1.5 mL microcentrifuge tube with 150 L of Promega Nuclei Lysis Solution (NLS) and then

ground. Specimens collected in propylene glycol and then stored in ethanol were washed three times with 1× TE buffer before continuing with the above protocol. Specimens collected and stored in isopropanol were placed in graduated concentrations of ethanol until 95% was reached, then washed with insecticidal soap for 5 min or less, rinsed in sterile distilled water, and processed as described above. Dry beetles were ground with a mortar and pestle, then washed with 150 L of NLS and the solution was transferred to a 1.5 mL microcentrifuge tube with 150 L of NLS, using the same pipette tip. This process was repeated with 100 L of NLS and then 200 L of NLS was added to bring the total to 600 L. The samples were then processed as described above. Beetles stored in 95% ethanol were dried for 24 h before being processed as described above.

RAPD-PCR

The PCR reaction for RAPD-PCR had a final volume of 50 L with 25 L of *Taq* PCR reaction mix containing 1.5 mmol $MgCl_2$ (Sigma, St. Louis, Missouri), 2 L of DNA (16 ng/ L), 2 L of primer (20–25 ng/ L), and 21 L of distilled water. The PCR was carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Waltham, Massachusetts) with the following program: initial denaturing step at 95 °C for 5 min, then 45 cycles of the following steps — denaturing at 95 °C for 1 min, annealing 34 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 7 min. A final holding temperature of 4 °C was maintained until the PCR reaction product was visualized on a 1% agarose gel stained with ethidium bromide. Sixteen oligonucleotides (Synthegen, LLC, Houston, Texas) were screened to identify those giving consistent results (Table 1). These 16 primers were chosen because they had been used in other bark beetle studies with reliable results (Carter *et al.* 1996; Ruiz 2005). Five of the 16 RAPD-PCR primers (OPB-01, OPAM-07, OPAM-11, OPM-01, and OPT-05) yielded clear banding patterns and were then used for comparing DNA banding patterns specific to *S. schevyrewi* and *S. multistriatus*. Of these last five RAPD-PCR primers, OPB-1 and OPAM-07 yielded distinct banding patterns for each species and were used for comparing species.

DNA bands were scored by placing the gel photograph under a magnifying light and using a

Table 1. List of oligonucleotides screened for use in RAPD-PCR.

Oligonucleotide	Sequence
OPAM-07	AACCGCGGCA
OPAM-11	AGATGCGCGG
OPAM-13	CACGGCACAA
OPB-01	GTTTCGCTCC
OPB-10	CTGCTGGGAC
OPT-05	GGGTTTGGCA
OPM-01	GTTGGTGGCT
B8	GTCCACACCG
B11	GTAGACCCGT
B17	AGGGAACGAG
B19	ACCCCCGAAG
M13	GGTGGTCAAG
M18	CACCATCCGT
S1	CTACTGCGCT
S2	CCTCTGACTG
S4	CACCCCCTTG

Table 2. Aedeagus measurements (mm; for a description see Fig. 2) for representatives from five populations each of *Scolytus schevyrewi* and *Scolytus multistriatus.*

Measurement location	*Scolytus schevyrewi*	*Scolytus multistriatus*
AW1	0.0610±0.0014*	0.0391±0.0006
AW2	0.0767±0.0022*	0.0642±0.0007
AW3	0.0644±0.0015*	0.0687±0.0008
AW4	0.0796±0.0017*	0.08277±0.0007
AL	0.9858±0.0076*	0.9557±0.0060

*Values are given as the mean ± standard error and differ significantly between species ($P \leq 0.05$), based on Wilcoxon's two-sample test.

straight edge to score bands. The data were analyzed using the absence (0) or presence (1) of each band scored, with analysis of molecular variance (AMOVA) in GenAlEx 6 for species differentiation (Peakall and Smouse 2005).

RAPD-PCR validation and application

A blind test was performed to validate species identified by RAPD-PCR with the primers OPB-01 and OPAM-07. Five specimens of each species were randomly placed in microcentrifuge tubes by an assistant before they were extracted and analyzed. We then attempted to identify these specimens based on the results obtained as described above. In addition, the RAPD-PCR technique using the primers OPB-01 and OPAM-07 was applied to DNA from unidentified larvae ($n = 20$) and specimens that could not be identified to species using existing taxonomic criteria ($n = 8$). The latter specimens were collected from elm limbs containing thousands of emerging adults, approximately 5% of which were *S. schevyrewi*. We subsequently attempted to identify each larva and each unidentified adult specimen to the correct species based on the banding patterns obtained from known specimens.

Results

Genitalic morphology

Aedeagus morphometrics

The aedeagi of *S. schevyrewi* and *S. multistriatus* were generally similar in appearance and,

despite some variation within species, consistent differences between species could be detected. The aedeagus of *S. schevyrewi* is parallel-sided, narrowing toward the apically subtruncated tip, whereas that of *S. multistriatus* is parallel-sided, narrowing and constricted before the tip, which is somewhat spatulate apically and expanded at the basal end (Fig. 2). Differentiation was established by quantifying the characteristics of the aedeagi of both species.

Our discriminant model based on aedeagus length and width was able to correctly classify 99 of the 100 insects to the appropriate species in an initial analysis where all insects were included. In 10 sequential analysis runs where insects of one population were excluded from the model, again only one insect from one population was misclassified (Table 2).

Molecular genetics

RAPD markers

Two primers (OPB-01 and OPAM-07) showed differences in DNA banding pattern between the species. Markers resulting from RAPD-PCR for primers OPB-01 and OPAM-07 are in a representative gel shown in Figure 3. The banding pattern for each species was distinct for both primers. OPB-01 yielded a total of 15 bands ranging from 400 to 2000 base pairs (bp) over all populations of *S. schevyrewi*, whereas *S. multistriatus* had a total of two bands at 900 and 1000 bp over all populations. OPAM-07 produced eight bands for each species, ranging from 400 to 1100 bp for *S. schevyrewi* and from 500 to 2000 bp for *S. multistriatus*. The results from these two primers were used for the AMOVA to apportion the genetic variance between species and populations, and individuals within species and populations (Table 3).

Fig. 3. Banding patterns for *Scolytus multistriatus* (Sm; lanes 2, 3, 9, and 10), *S. schevyrewi* (Ss; lanes 4, 5, 11, and 12), and four of eight individuals of *Scolytus* spp. not identifiable using current taxonomic criteria (?; lanes 6, 7, 13, and 14) with each primer. Lanes 2–7 show banding patterns from OPB-01; lanes 9–14 show banding patterns from OPAM-07, and lanes 1, 8, and 15 indicate molecular weights.
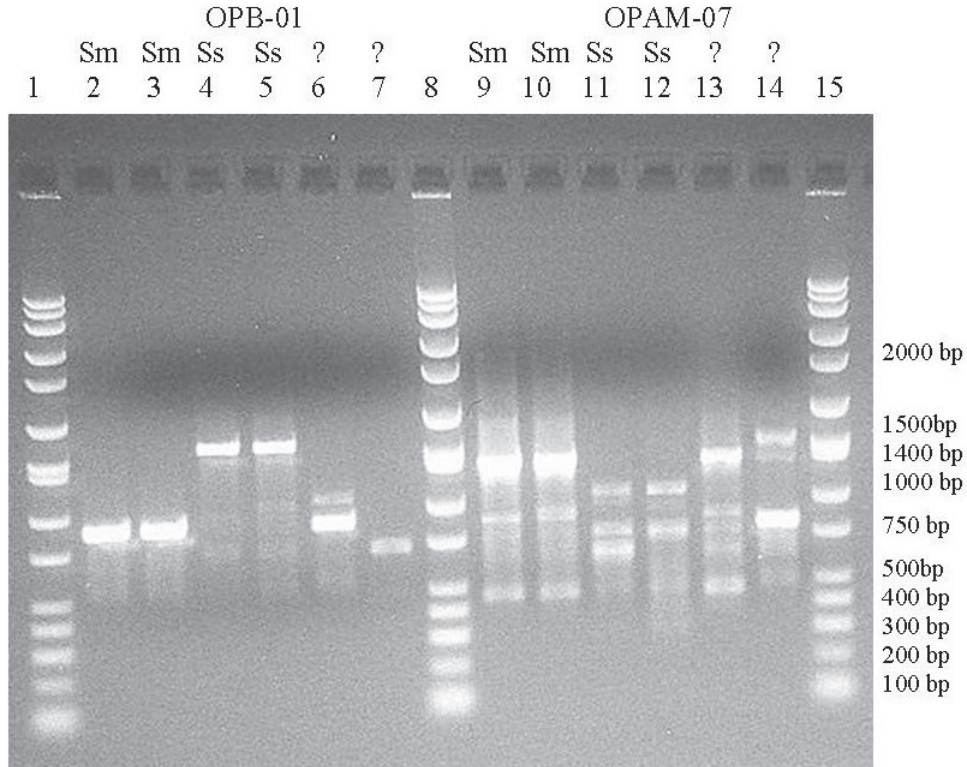
The AMOVA showed that although small, the genetic variance (7%) between species for *S. schevyrewi* and *S. multistriatus* was significant ($P < 0.001$).

Validation and application of RAPD markers

The banding patterns for specimens from the blind validation test were consistent with the previous results obtained for both *S. schevyrewi* and *S. multistriatus* (Fig. 3) and we were able to correctly identify the species of each specimen. We applied the results of the RAPD-PCR to distinguish *S. schevyrewi* from *S. multistriatus* in samples derived from 19 of 20 individual larvae; one sample failed to yield DNA. Of these samples, 18 showed banding patterns consistent with *S. multistriatus* and 1 showed a pattern consistent with *S. schevyrewi* (Fig. 4). Samples from the eight specimens not identifiable to species using current taxonomic criteria yielded banding patterns that appeared to combine those of *S. schevyrewi* and *S. multistriatus* (Fig. 3). When known *S. schevyrewi* and *S. multistriatus* samples were compared with the eight unidentifiable samples by AMOVA, the variance was 7% for *S. multistriatus* but no difference was found for *S. schevyrewi* (Table 3). Individual banding patterns from each of the eight unidentifiable samples were then compared with the bands of the parental populations of *S. schevyrewi* and *S. multistriatus* from Oregon. Comparison of the banding patterns of these eight unidentifiable samples with those of confirmed *S. schevyrewi* and *S. multistriatus* revealed that the unknown specimens yielded markers (six bands) shared by the two species, as well as eight species-specific markers: two found only in *S. multistriatus* samples and six found only in *S. schevyrewi* samples. In addition, six bands were found inconsistently among the eight unidentifiable samples that had not been observed in either *S. schevyrewi* or *S. multistriatus* (Table 4).

Table 3. Analysis of molecular variance of random amplified polymorphic DNA (RAPD) band data for 50 individuals of *Scolytus schevyrewi*, 60 individuals of *S. multistriatus*, and 8 individuals of *Scolytus* sp. not identifiable using current taxonomic criteria.

Source of variation	df	SSD	MSD	Estimated variance	Total percentage	*P*
S. schevyrewi vs. S. multistriatus	1	23.606	23.606	0.129	7	<0.001
Populations per species	4	36.528	9.132	0.209	11	<0.001
Individuals per population	214	347.125	1.622	1.622	82	<0.001
S. schevyrewi populations	2	28.195	14.097	0.382	17	
Individuals per population	97	181.175	1.868	1.868	83	<0.001
S. multistriatus populations	2	8.333	4.167	0.069	5	
Individuals per population	117	165.950	1.418	1.418	95	<0.031
S. schevyrewi vs. Scolytus spp.	1	6.546	6.546	0.00	0	0.999
Populations per species	2	28.195	14.097	0.381	17	<0.001
Individuals per population	112	214.363	1.914	1.914	83	<0.001
S. multistriatus vs. Scolytus spp.	1	5.992	5.992	0.114	7	0.029
Populations per species	2	8.333	4.167	0.066	4	0.022
Individuals per population	132	199.137	1.509	1.509	89	0.006

Note: SSD, the sum of squared deviations; MSD, the mean of squared deviations.

Fig. 4. (*a*) Blind test validating species-specific banding patterns using the oligonucleotide OPAM-07. *Scolytus schevyrewi*: lanes 2, 6, 8, and 11; *S. multistriatus*: lanes 3, 4, 7, and 9. (*b*) Results for larvae with primer OPB-01. Lane 2: *S. schevyrewi*; lanes 3–10: *S. multistriatus*.
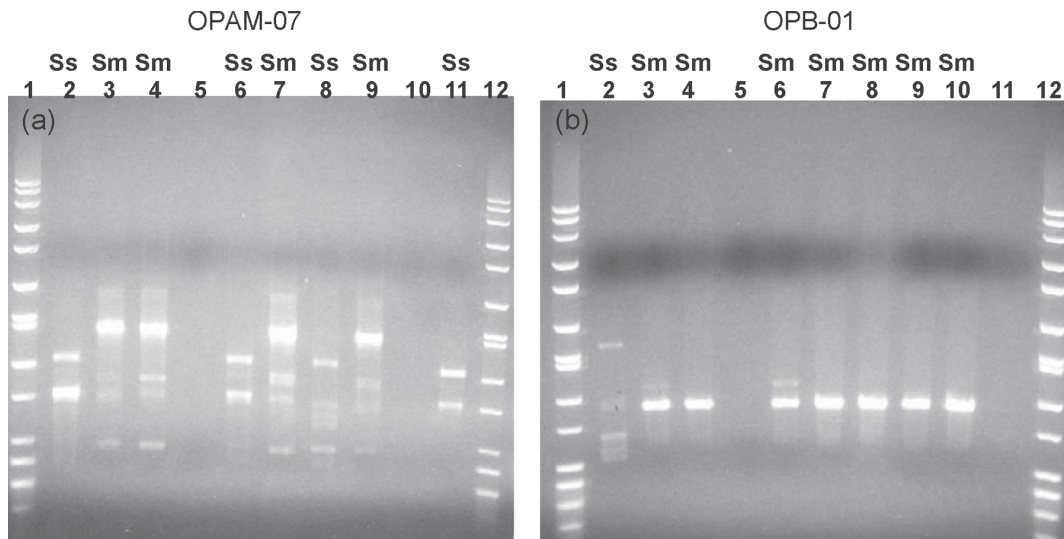
Discussion

Our results show that features of the aedeagus and RAPD-PCR markers can be reliably used to differentiate between *S. schevyrewi* and *S. multistriatus*. We address each of these techniques and findings below.

Aedeagus morphometrics

As expected, the aedeagi of these two species are generally similar in appearance, but differ sufficiently in detailed features to allow species differentiation. Because verbal descriptions can be difficult to apply readily to a given specimen without reference to multiple specimens, and

Table 4. RAPD-PCR species-specific bands of the Oregon populations of *Scolytus schevyrewi* and *S. multistriatus* that are shared in the banding pattern of the eight unidentifiable individuals of *Scolytus* sp.

S. schevyrewi bands (bp)	*S. multistriatus* bands (bp)
1800	1400
1200	800
700	
600	
400	
300	

there is likely to be considerable variation within species, we attempted to develop a means of readily quantifying differences in features. Although our sample was relatively small, the aedeagi of *S. schevyrewi* and *S. multistriatus* proved to be unique to each species across each geographically distinct population used in this study. *Scolytus schevyrewi* and *S. multistriatus* showed some variation in the size of the aedeagus within populations. This may be in proportion to the overall variation in specimen size, a factor for which we did not account but that should be included in future work. In studies of species of Carabidae, Noctuidae, and *Drosophila* Fallen (Diptera: Drosophilidae) (*e.g.*, Garnier *et al.* 2005; Mutanen 2005; Soto 2005), the shape of the male genitalia was quantified by outlining the male genitalia. In studies by Cane *et al.* (1990) and Stauffer and Zuber (1998), external morphology was quantified and molecular genetics used to differentiate various species of *Ips*. To our knowledge the genitalic morphology of no other species of *Scolytus* has been described and (or) quantified using morphometrics within this genus. We anticipate that our quantification technique will allow not only *S. schevyrewi* to be distinguished from *S. multistriatus*, but also either species from other *Scolytus* species, although this remains to be tested. We were unable to obtain specimens from the native ranges of these species for comparison; however, this would be the logical next step in the validation of this technique. If consistent variability were to be detected, this might provide insight into the origins of Nearctic and other populations. It would be particularly interesting to compare the conifer-feeding spruce engraver, *Scolytus piceae*

(Swaine), which is similar in external morphology and keys out in the same couplet as *S. multistriatus* in Wood (1982; *Scolytus* couplet 7(6). The use of our technique is limited to the male adult stage, but can be used for specimens that have been stored dry or in alcohol for years because the aedeagus is sclerotized and does not disintegrate as soft tissue does. A comparative study of other native and nonnative *Scolytus* species using our quantification technique could be useful for identifying nonnative, invasive species of concern, such as *S. scolytus*.

RAPD markers

RAPD-PCR showed a significant difference ($P < 0.001$) in banding patterns between the *S. schevyrewi* and *S. multistriatus* specimens with 2 of the 16 primers screened. The use of more than one primer to distinguish species is important to verify the identity of individual specimens.

In addition to the blind validation test, the use of both primers on larval specimens not only confirmed the species identification of each specimen but also demonstrated that this technique could be used on immature and adult stages.

On the other hand, the use of both primers on the unidentifiable adult specimens yielded patterns containing both unique bands and bands shared by *S. schevyrewi* and *S. multistriatus*. The AMOVA showed no variance between the unidentifiable specimens and *S. schevyrewi*, but there was significant variance between these and *S. multistriatus*. These results suggest that the unidentifiable specimens were *S. schevyrewi* and not *S. multistriatus*. However, the fact that band-by-band analysis showed that these specimens have some unique bands, share some bands with both *S. schevyrewi* and *S. multistriatus*, and share some bands with either one or the other species suggests that the unidentifiable specimens could be hybrids or a distinct but currently unknown species.

Data were obtained using a variety of different specimen-collection and -storage methods and from specimens in varying condition. However, the particular method used to preserve specimens can cause difficulties for DNA extraction. Specimens preserved in ethanol presented the most difficulty for DNA extraction, whereas those frozen immediately after collection yielded the best results and required the simplest extraction procedures. Dry samples that

were ground before NLS was added produced multiple bands, whereas grinding with NLS produced only a single band when the OPT-05 primer was used. The OPT-05 primer produced multiple bands with both *S. multistriatus* and *S. schevyrewi* in DNA extracted from frozen beetles. Carvalho and Vieira (2000) recommended that DNA extraction be done within 1 year of specimen collection to avoid degradation of DNA.

Conclusion

Scolytus schevyrewi and *S. multistriatus* are examples of scolytine species that are difficult to distinguish by traditional taxonomic means, especially at immature stages, for which no taxonomic references appear to exist. Taken together, our results provide two additional diagnostic tools for identifying the two non-native species targeted in this study. The use of more than one tool, whether external or internal morphology or DNA markers, allows greater confidence that scolytine specimens will be accurately identified when collected. However, the use of more than one diagnostic tool may not be possible, given the life stage or condition of the specimens, and only one technique may be employed when there is a need for quick identification. These tools have helped to show that *S. schevyrewi* and *S. multistriatus* not only could occur in the same tree/limb but may also hybridize. The fact that RAPD-PCR of the eight unidentifiable adult specimens produced results similar to the analysis, using the same methods, of identified specimens from other locations suggests that the RAPD-PCR methodology we employed is reliable and repeatable. The markers did not differ substantially in band frequency or band size. If these eight specimens represent a different species, we would have expected to see more unique specimens among the thousands that were reared from elm limbs in the Forestry and Range Sciences Laboratory.

Though beyond the scope of this study, the ecological and morphological similarities between *S. schevyrewi* and *S. multistriatus* raise questions concerning the degree of relatedness of these two species. Not only do they utilize the same host plant species, their mating biology is similar (Wang 1992; Solomon 1995) and, as we have shown, the features of one component of their reproductive morphology are similar. Hidayat *et al.* (1996) showed that two sibling species of grain weevils, *Sitophilus oryzae* and

S. zeamais, were separable not only by minor variations in their reproductive morphology but also by RAPD-PCR genetic markers. Because *S. schevyrewi* and *S. multistriatus* originate from geographically different areas (Wang 1992; Solomon 1995) and thus are subject to different selection pressures, we expected to find, and did find, species-specific molecular genetic markers. The different histories of these two species in North America may have contributed to the differences that we detected, but our samples from multiple sites across the United States of America provided reliably detectable species-specific markers. For our purposes, RAPD-PCR is a particularly useful tool for species differentiation because of its relative simplicity, availability, and low cost.

Given their differences in origin, and that *S. schevyrewi* and *S. multistriatus* can live and reproduce in the same host tree, we hypothesized that quantifiable differences in reproductive morphology exist between these two species and that these differences help to prevent interbreeding. The need for a more thorough examination of the reproductive morphology, not just the aedeagus, of these two species is indicated by our findings. The discovery of possible hybridization suggests that the differences between the two species may not strictly prohibit successful reproduction. The existence of a third unique species is possible, but is less likely than hybridization, because no other evidence of another species has been reported. Moreover, other scolytines that are known to infest elm do not resemble either *S. multistriatus* or *S. schevyrewi*.

With the increasing potential for international transportation of known and unknown insect species and the necessity for detection and eradication or other management measures, the ability to distinguish both native and non-native insects is essential. We have described two tools that, in combination with classic taxonomic procedures, may enhance our ability to identify immature and adult specimens of two non-native bark beetles, *S. schevyrewi* and *S. multistriatus*. Identification via genitalic morphology is limited to adult males but can be used on intact specimens regardless of time spent in storage or method of preservation. RAPD-PCR can be used on specimens in any life stage or condition and that have been preserved in a variety of ways. This set of tools has the potential for use with a wide array of scolytines, both native and non-native, to assist

in detection and eradication efforts aimed at limiting the introduction or spread of invasive species.

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