Molecular and morphological markers for diagnosis of *Sitophilus oryzae* and *S. zeamais* (Coleoptera: Curculionidae)

P. Hidayat*, R.H. ffrench-Constant*, and T.W. Phillips*†

Abstract

Sitophilus oryzae and *Sitophilus zeamais* are considered taxonomically distinct based on subtle differences in genital morphology and presumed differences in body size and grain preferences. Previous findings of successful laboratory hybridisation, genetic similarity of allozymes and chromosomes, and identity of aggregation pheromones raised questions about the validity of *S. oryzae* and *S. zeamais* as valid biological species. We used molecular techniques to test the hypothesis that individuals assigned as *S. oryzae* or *S. zeamais* by morphological criteria represent members of two distinct gene pools, and hence are reproductively isolated species. Weevils were studied from four farms in the U.S. and two recent laboratory cultures from Asia. Putative species were scored on the presence/absence of grooves on the male aedeagus or pointed/rounded lobes of female spiculum ventrale. The polymerase chain reaction was used on the same specimens to analyse randomly amplified polymorphic DNA (RAPD) markers and to selectively amplify regions of mitochondrial DNA for analysis of restriction site polymorphisms with restriction endonucleases. Both methods yielded diagnostic markers that distinguished the two morphotypes, consistent with the presence of reproductively isolated species in sympathy. Other morphological characters (e.g. pronotal punctures) proved unreliable as correlates with genetic markers.

Introduction

Sitophilus oryzae (L.), the rice weevil, and *S. zeamais* Motschulsky, the maize weevil, are very closely related species that are difficult to distinguish. Floyd and Newsom (1959) recognised two distinct species based on size, food preferences, mating incompatibility, and morphology of genitalia. They synonymised *zeamais* under '*oryza*' (not *oryzae*) for the large form, and retained *S. sasakii* (Takahashi) for the smaller form (*S. oryzae sensu stricto*). Kuschel (1978) later confirmed the name of *S. zeamais* for the large form after examining Motschulsky's actual types, and reaffirmed the utility of the male genitalia in distinguishing species. Research in Japan recognises *S. oryzae* and *S. zeamais* as distinct species (Kiritani 1965). Halstead (1963) reviewed proposed morphological characters for distinguishing *S. oryzae* and *S. zeamais* (Kuschel 1961), and concluded that only one gave consistent separations: the male aedeagus of *S. zeamais* possesses two longitudinal grooves on the dorsum of the phallus, while the same surface on *oryzae* males is smooth and evenly convex. Floyd and Newsom (1959) reported characters of the female genitalia that they proposed should be useful for species distinction. Halstead (1963) found the female character highly variable except for one consistency: the apical regions on the lateral lobes of the Y-shaped spiculum ventrale are pointed and tapered in *S. zeamais*, but rounded and blunt in *S. oryzae*. Halstead (1963) assigned identifications to *Sitophilus* specimens in the worldwide collections of his institution and of the British Museum, but no independent measure was invoked with which to test the aedeagal characters against, nor was any mention made of the two morphotypes being found in the same collections. Boudreaux (1969) reported on external characters, most notably the presence (rice weevil) or absence (maize weevil) of a flat median longitudinal puncture-free zone on the dorsal pronotum, that purportedly separated the two species. Boudreaux (1969) developed his study of characters from a single laboratory culture each of *S. zeamais* and *S. oryzae*, but he used his method to discriminate nearly 1800 specimens in the USNM and noted that the two species may occur together in the same accessions.

We questioned the validity of *S. oryzae* and *S. zeamais* as reproductively isolated species based on published reports on controlled mating, genetics, and pheromones. Richards (1944) produced F_1 progeny upon crossing the two species in the laboratory. Smith (1953) examined chromosomes of *Sitophilus* and found that *S. granarius* had a distinct karyotype of 2N=24, while *S. oryzae* and *S. zeamais* had apparently identical karyotypes of 2N=22. Yang et al. (1989) confirmed the 2N=22 karyotype for *S. zeamais* and *S. oryzae*, and also reported interspecific crosses and production of fertile progeny in the laboratory, but only in 3% of the crosses. Beiras and Petitpierre (1981) analysed six allozyme loci in three species of *Sitophilus* and found no diagnostic (e.g., fixed allele) differences between *S. oryzae* and *S. zeamais*. Populations were monomorphic for the same electromorphs at all loci except for esterases, for which there were polymorphisms and frequency differences. All samples analysed by Beiras and Petitpierre (1981) were from laboratory cultures of various origins and none were field-collected in sympatry. Pintureau et al. (1991) studied esterase polymorphism in *S. oryzae*, *S. zeamais*, and *S. granarius*, and hybridised *S. oryzae* and *S. zeamais* (in about 10% of crosses) from broadly different geographic strains. No diagnostic differences were found between the sibling species (*oryzae* and *zeamais*) and interspecific hybrids demonstrated heritability of electrophoretic alleles at one esterase locus. Thus cytological and allozymic studies reveal a very close genetic relationship between *S. oryzae* and *S. zeamais*, and controlled mating studies suggest the potential for hybridisation in the field. Walgenbach et al. (1983) reported that *S. oryzae* and *S. zeamais* use a male-produced pheromone and that the two species were mutually cross-attracted in laboratory bioassays. Chemical elucidation found that *S. oryzae* and *S. zeamais* produced chemically identical pheromones, thus explaining their high level of cross-attraction (reviewed in Burkholder 1990). Use of the same pheromone by *S. oryzae* and *S. zeamais* suggests that there may be no behavioral barrier to individuals of the two species coming together in nature.

* Department of Entomology, University of Wisconsin, Madison, WI USA 53706.
† USDA ARS Stored-Product Insect Research Unit., Madison, WI USA 53706.

The objective of this study is to use molecular markers to determine if weevils identified as either *S. oryzae* or *S. zeamais* based on morphology are genetically distinct. We have centred our study on morphologically distinct specimens collected in sympatry so that the potential for hybridisation would be high. Throne and Cline (1989 1991) documented the seasonal flight activity and occurrence of *S. oryzae* and *S. zeamais* at the same field sites in South Carolina, and made identifications of trapped specimens using Halstead's (1963) genitalic characters. Therefore, weevils possessing morphological characters representative of each species can be collected sympatrically, making tests of the species concept possible via genetic markers.

Materials and Methods

A total of 268 specimens were field collected by us or by cooperators from the following locations: Wuhan, China; W. Java, Indonesia; Frankfort, Kentucky; Bamberg, South Carolina; and Madison, Wisconsin. All U.S. collections were made from in-grain samples or grain/bait bags (Throne and Cline 1991) on farms with grain storage facilities. Collections from Indonesia and China are from unknown habitats, but apparently represented wild populations. Weevils were received in our laboratory alive, subjected to morphological analysis, dissection of genitalia, and then were frozen at –80°C for subsequent DNA extraction.

Morphology

We used the morphological characters described by Halstead (1963) and Boudreaux (1969) in our study of *Sitophilus* field populations. The two 'most reliable' characters presented by Halstead, the presence (*S. zeamais*) or absence (*S. oryzae*) of ridges on the dorsum of the male aedeagus, and the shape of the tips (rounded on *S. oryzae*, pointed on *S. zeamais*) on the Y-shaped sclerite (spiculum ventrale) were easily dissected and scored on our specimens (Figs. 1 and 2). The presence (*S. oryzae*) or absence (*S. zeamais*) of a median longitudinal puncture-free zone on the pronotum (Fig. 3), described by Boudreaux (1969), was scored, as was the number of punctures counted longitudinally along the midline (<20 for *S. oryzae* and >20 for *S. zeamais*). The shape of pronotal punctures (elliptical for *S. oryzae* and circular for *S. zeamais*) required more subjective judgment than other characters, but this was also recorded.

DNA markers

Two methods employing the polymerase chain reaction (PCR) were used in this study to obtain genetic markers. We used the technique of randomly amplified polymorphic DNA (RAPD-PCR; Williams et al. 1990) and screened numerous random primers (10-mers) from the University of British Columbia RAPD Primer Synthesis Project.

We additionally studied restriction fragment-length polymorphisms (RFLP-PCR) on the same individuals used for morphology and RAPD-PCR. In RFLP-PCR a specific sequence is amplified with PCR, digested with a specific restriction endonuclease, then visualised on agarose gel to score fragments and the presence or absence of restriction sites (see Simon et al. 1993). We amplified a mitochondrial DNA (mtDNA) sequence of 1635 bp, spanning the cytochrome oxidase subunits I and II (COI, COII) genes, using primers for conserved regions, 5'-CAACATTTATTTTGATTTTTTGG-3' and 5'-GAGACCATTACTTGCTTTCAGTCATCT-3' (Liu and Beckenbach 1992).

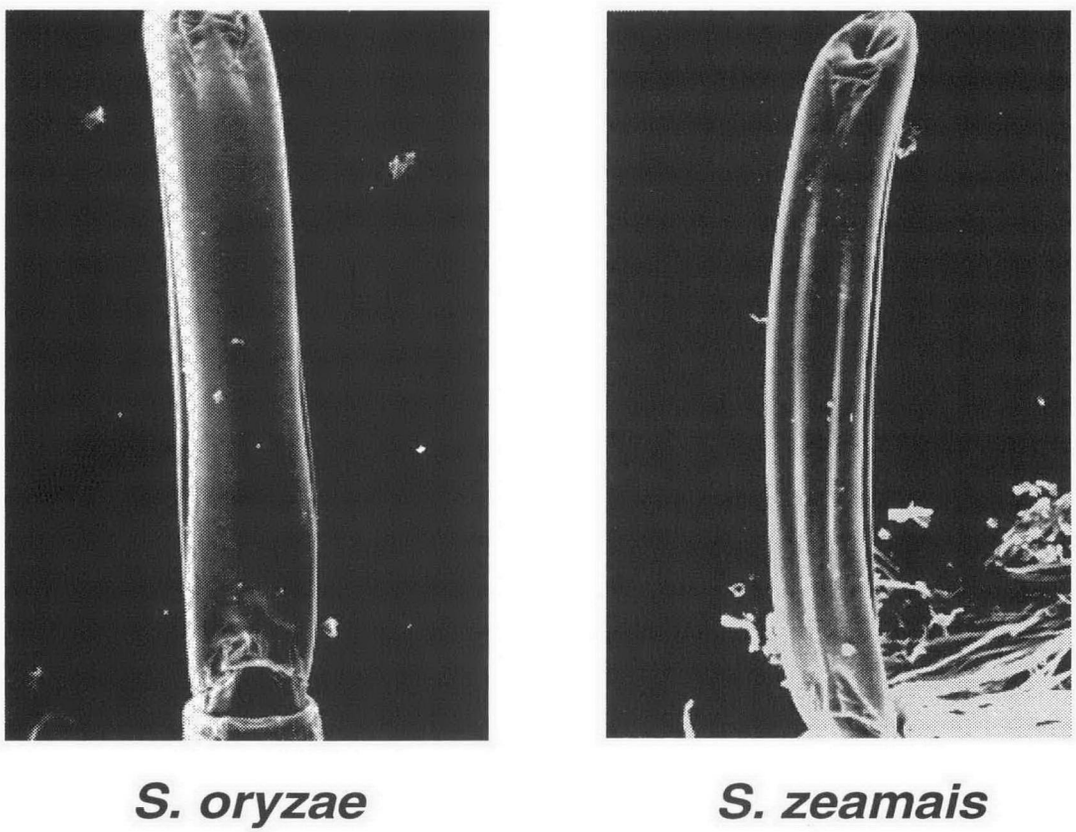

Fig. 1. Scanning electron micrograph (SEM) of dorsal surface of phallus (male aedeagus) in *S. oryzae* (left) and *S. zeamais* (right); note longitudinal grooves on *S. zeamais*.

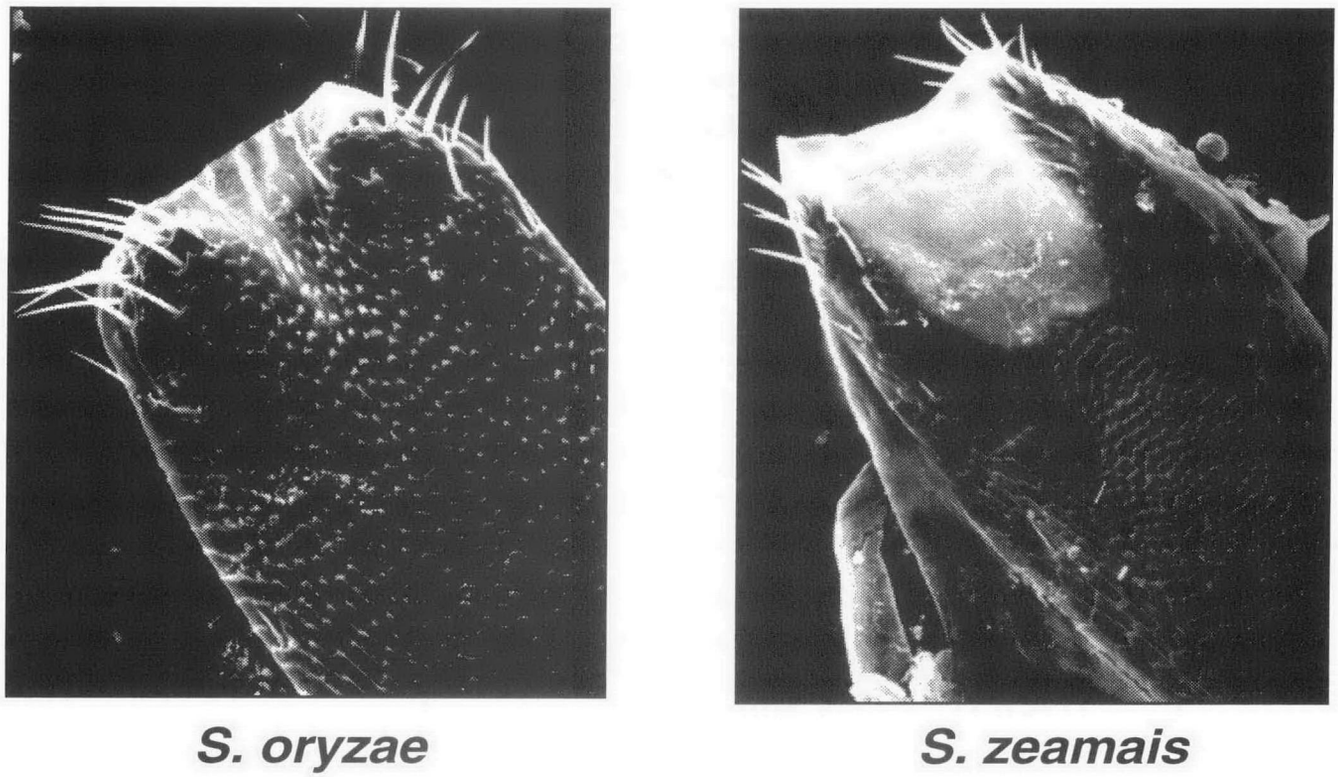

Fig. 2. Y-shaped sclerite of invaginated 8th sternite (spiculum ventrale) from female *S. oryzae* and *S. zeamais*; note shape and thickness of distal processes.

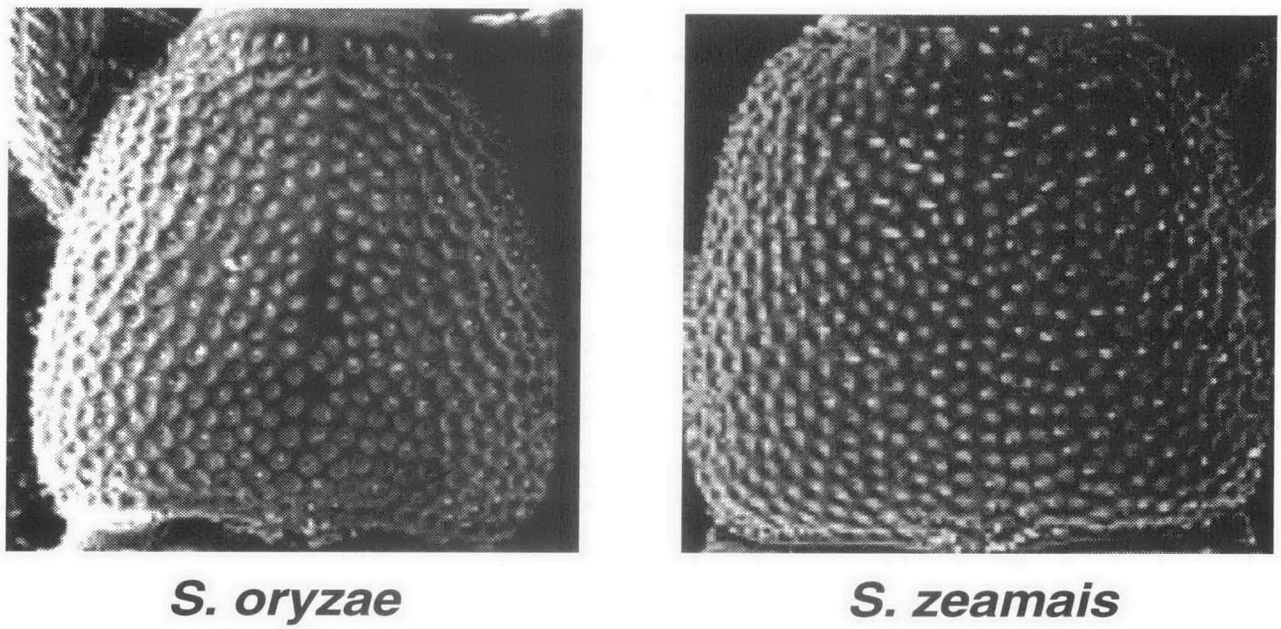

Fig. 3. Morphology of pronotal punctures used for diagnosis (Boudreaux 1969) of *S. oryzae* and *S. zeamais*; note puncture-free zone near mid-line of pronotum in *S. oryzae* and larger number of punctures in *S. zeamais*.

Results and Discussion

We found that most field collections contained a mixture of morphotypes. Additionally, we found that a determination to species made with one character did not always match a determination made with another character (Fig. 4). Thus morphological characters were not correlated with each other and specimens could not be classified with certainty.

RAPD-PCR revealed bands consistently present in one group of weevils and absent in another. When field-collected weevils from Bamberg, South Carolina were subjected to RAPD-PCR with the UBC431 primer, all individuals possessing genitalic morphology of *S. zeamais* (grooved dorsal aedeagus in males, pointed y-shaped sclerite of females) revealed a characteristic RAPD fingerprint, while those with the *S. oryzae* genitalic morphology (smooth aedeagus, rounded tips of y-shaped sclerite) had a different RAPD pattern (Fig. 5). All weevils with *S. oryzae* morphology consistently showed a band at about 1400 bp that was absent in weevils with S. *zeamais* morphology. Other bands predomi-

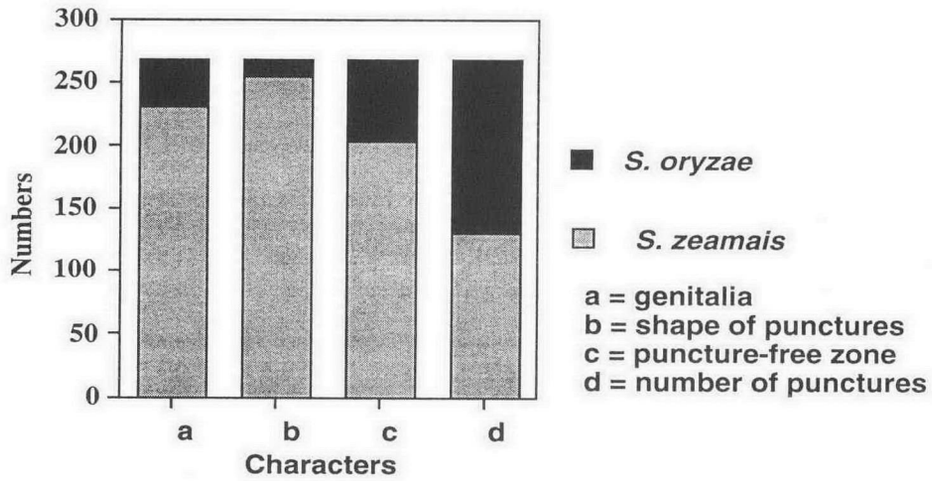

Fig. 4. Distribution of species determinations made on 268 specimens using four different morphological criteria: a = genitalia, b = shape of punctures, c = puncture-free zone, d = number of punctures. *Sitophilus oryzae* ■; *Sitophilus zeamais* ▩.

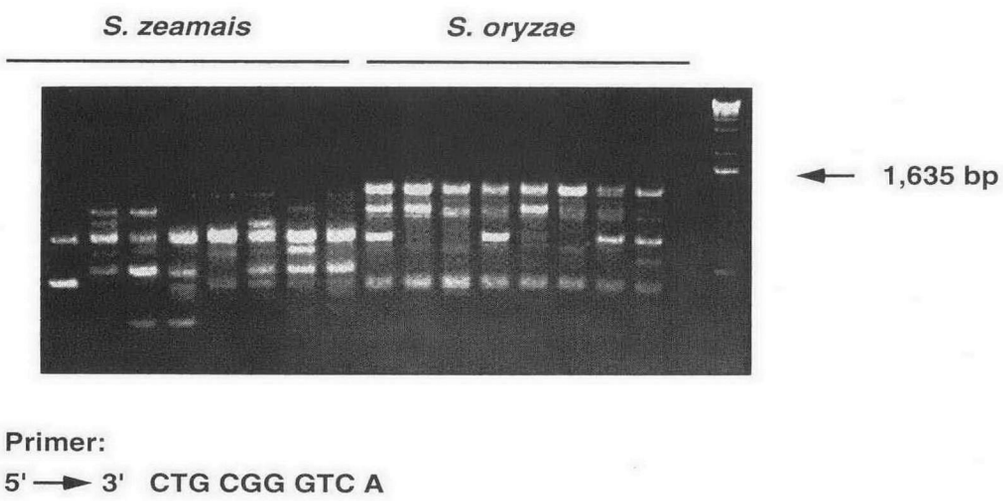

Fig. 5. Agarose gel with RAPD-PCR products from *Sitophilus* weevils with primer UBC-431. Individuals are grouped according to their possession of genital morphology (aedeagus of males, y-shaped sclerite of females) of *S. oryzae* or *S. zeamais*.

nated in one morphotype over the other, but the 1400 kb band was consistently associated with weevils having *S. zeamais* genital morphology at this South Carolina site and in all other collections surveyed in this study.

RFLP-PCR analyses of mtDNA in all specimens so far have yielded a 1635 bp product prior to digestion with restriction enzymes. Four- and five-base restriction endonucleases were surveyed for their ability to cleave this product. The enzymes *Msp* I, *Dde* I and *Rsa* I all yielded one or more restriction sites, and the distribution of these restriction sites was consistently correlated with one genitalic morph or the other (Fig. 6). For weevils collected in Bamberg, South Carolina, *Dde* I yielded two restriction sites (three fragments) in individuals possessing *S. zeamais* genital morphology, while DNA from all *S. oryzae* morphs lacked these restriction sites and were not digested (Fig. 6). Similarly, *Msp* I cleaved the COI/COII sequence consistently at the same site for *S. zeamais* morphs, but did not digest the DNA from *S. oryzae* morphs. Restriction analysis with *Rsa* I (not shown) yielded two different cut sites, one fixed for *S. oryzae* morphs and the other fixed for *S. zeamais* morphs.

Our results indicate that two genetically distinct, hence probably reproductively isolated, species occur in sympatry, and that they can be assigned by genital morphology to either *S. oryzae* or *S. zeamais*. We have performed both RAPD-PCR and RFLP-PCR analyses on weevils from the five geographic locations listed above, each containing a mix of morphotypes, and genetic markers shown in Figure 5 have been consistently associated with the same genitalic morphs. If these sites harbored single breeding populations of weevils, and if crossing between morphotypes was occurring, we would expect to eventually see a random association of morphotypes with DNA markers. Since it is highly unlikely that both RAPD and mtDNA markers are linked with genes coding for sexually dimorphic genital characters, it is clear that these correlated characters signify the presence of two breeding populations. A single character, either morphological or genetic, would be inadequate for concluding the presence of two species because a plausible alternative hypothesis for polymorphism within one species could be made. Our mtDNA restriction site data represent just one character with two states (so far) because the mitochondrial genome is haploid and inherited intact with no recombination, acting as one locus (Avise et al. 1987). However the addition of at least one independent RAPD locus (band at 1400 kb, primer UBC431, Fig. 5) and the consistent association of these two molecular

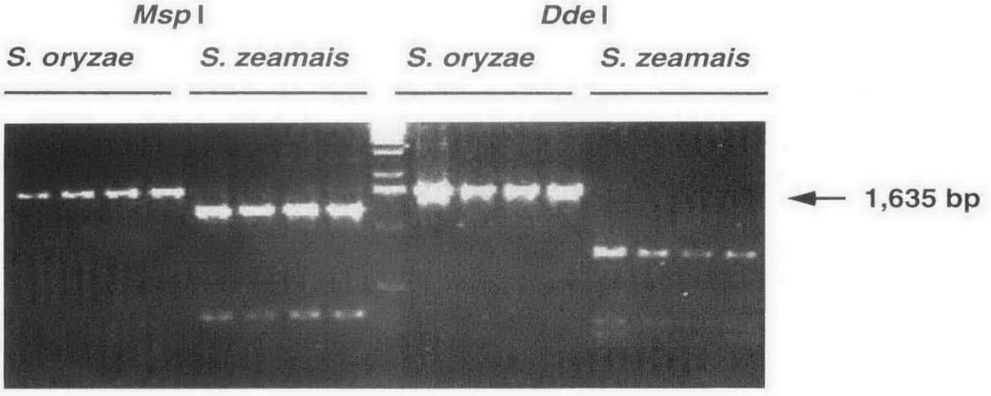

Fig. 6. Restriction digest of 1635 bp PCR product from mtDNA of *Sitophilus* weevils using enzymes *Msp*I and *Dde*I. Species designations refer to determinations from genital morphology. The undigested product is shown in all *S. oryzae* lanes.

markers with genital morphology make a strong case for two distinct lineages. We have confirmed Halstead's (1963) and Kuschel's (1978) conclusions of two species identified by genitalic characters, but show the lack of diagnostic utility for pronotal characters.

Acknowledgments

We greatly appreciate field collection of weevils by David Weaver, USDA ARS in Savannah, Georgia, and by John Sedlacek, Kentucky State University, Frankfort, Kentucky. We appreciate reviews of the manuscript by Nicola Anthony, University of Wisconsin, Madison, WI, and David Weaver, USDA ARS in Savannah, GA.

References

Avise, J.C., J. Arnold, R.M. Ball, E. Birmingham, T. Lamb, J. E. Neigel, C.A. Reeb and Saunders, N.C. 1987. Intraspecific phylogeography: the mitochondrial DNA Bridge between population genetics and systematics. Annual Review of Ecology and Systematics, 18, 489–522.

Beiras, M.J. and Petitpierre, E. 1981. Allozymic variability and genetic differentiation in three species of *Sitophilus* L. (Coleoptera: Curculionidae). Egyptian Journal of Genetics and Cytology, 10, 95–104.

Boudreaux, H.B. 1969. The identity of *Sitophilus oryzae*. Annals of the Entomological Society of America, 62, 169–172.

Burkholder, W. E. 1990. Practical use of pheromones and other attractants for stored-product insects. In: Ridgway, R.L., Silverstein, R.M. and Inscoe, M.N., ed. Behavior-modifying chemicals for insect management: applications of pheromones and other attractants. Marcel Dekker, Inc., N.Y., 497–516.

Floyd, E.H. and Newsom, L. D. 1959. Biological study of the rice weevil complex. Annals of the Entomological Society of America, 52, 687–695.

Halstead, D.G.H. 1963. The separation of *Sitophilus oryzae* (L.) and *S. zeamais* Motschulsky (Coleoptera: Curculionidae), with a summary of their distribution. Entomologist's Monthly Magasine, 99, 72–74.

Kiritani, K. 1965. Biological studies on the *Sitophilus* complex (Coleoptera: Curculionidae) in Japan. Journal of Stored Products Research, 1, 169–176.

Kuschel, G. 1961. On problems of synonymy in the *Sitophilus oryzae* complex (30th contribution, Col. Curculionidae). American Museum of Natural History. ser. 13, vol. iv, 441–444.

Kuschel, G. 1978. Notes on the identity of *Sitophilus zeamais* Motschulsky based on type material examination. Journal of Natural History, 12, 231.

Liu, H. and Beckenbach, A.T. 1992. Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. Molecular Phylogenetics and Evolution, 1, 41–52.

Pintureau, B., Grenier, A.M. and Nardon, P. 1991. Polymorphism of esterases in three species of *Sitophilus* (Coleoptera: Curculionidae). Journal of Stored Products Research, 27, 141–151.

Richards, O.W. 1944. The two strains of the rice weevil, *Calandra oryzae* (L.) (Coleoptera: Curculionidae). Transactionas of the Royal Entomological Society, London, 94, 187–200.

Smith, S.G. 1953. The cytology of *Sitophilus* (*Calandra*) *oryzae* (L), *S. granarius* (L), and some other Rhynchophora (Coleoptera). Cytologia, 17, 50–70.

Simon, C., McIntosh, C. and Deniega, J. 1993. Standard restriction fragment length analysis is not sensitive enough for phylogenetic analysis of identification of 17-year peridoical cicada broods (Hemiptera: Cicadidae): the potential for a new technique. Annals of the Entomological Society of America, 86, 228–238.

Throne, J.E. and Cline, L. D. 1989. Seasonal flight activity of the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curuclionidae) and the rice weevil, *S. oryzae* (L.), in South Carolina. Journal of Agricultural Entomology, 6, 183–192.

Throne, J.E. and Cline, L.D. 1991. Seasonal abundance of maize and rice weevils (Coleoptera: Curculionidae) in South Carolina. Journal of Agricultural Entomology, 8, 93–100.

Walgenbach, C.A., Phillips, J. K., Faustini, D.L. and Burkholder, W.E.. 1983. Male produced aggregation pheromone of the maize weevil, *Sitophilus zeamais*, and interspecific attraction between three *Sitophilus* species. Journal of Chemical Ecology, 9, 831–841.

Williams, J.G.K., Kubelik, A.R., Livak, K. J., Rafalski, J.A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research, 18, 6531–6535.

Yang, Z.-Y., Huang, P. and Wu, G.-X. 1989. Observations on the karyotypes of *Sitophilus oryzae* and *Sitophilus zeamais* and their hybrid offsprings. Acta Entomologica Sinica, 32, 4065–409.