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MOLECULAR DIAGNOSTICS OF *ENAPHALODES RUFULUS* (COLEOPTERA: CERAMBYCIDAE)

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Abstract

Oak-hickory forests in northwestern Arkansas, eastern Oklahoma and southern Missouri have recently experienced an oak decline event with widespread oak mortality. The oak mortality is associated with an outbreak of a native wood-boring cerambycid, *Enaphalodes rufulus* (Haldeman), the red oak borer. Taxonomic identification, below the family level, of larval Cerambycidae through traditional morphological methods is not usually possible. We employed molecular diagnostics, with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), to distinguish *E. rufulus* from other closely related species of cerambycids. A portion of the mitochondrial DNA 16S rRNA gene, isolated from legs or thoraxes of adult museum specimens, was amplified and digested with *Alu* I and *Hind* III restriction enzymes. Both restriction enzymes independently produced fragments for *E. rufulus* that were significantly different from any other cerambycid tested. *Alu* I had one restriction site for *E. rufulus* and two restriction sites for all other cerambycids tested, while *Hind* III did not cut for *E. rufulus* but did cut at one restriction site for all other cerambycids. Eggs, larvae, and pupae of *E. rufulus* along with an unknown cerambycid larva and pupa were successfully amplified and digested by this method to verify validity of this technique for multiple life stages.

Key Words: PCR-RFLP, genetics, red oak borer, longhorn beetle, native insect pest, taxonomic identification

Resumen

De manera continua en los bosques de roble de "nuez dura" de la region de Ozark National Forests en el norte de Arkansas, el este de Oklahoma y el sur de Missouri, se presentan eventos de disminución de las poblaciones de robles. La mortalidad de los robles está asociada con el aumento de las poblaciones de *Enapholodes rufulus*, el perforador rojo del roble, un cerambícido nativo perforador de Madera. Para distinguir *E. rufulus* de otras especies de Cerambycidae muy relacionadas, se utilizaron polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Una porción de DNA mitocondrial del gen 16rRNA fue aislada de las patas o tórax de adultos de especímenes de museos, amplificada y digerida con las enzimas de restricción *Alu I* y *Hind III*. Ambas enzimas produjeron fragmentos para *E. rufulus* que fueron significativamente diferentes a otros cerambícidos probados. *Alu I* tuvo un sitio de restricción para *E. rufulus* y dos sitios de restricción para todos los otros cerambícidos probados, mientras *Hind* III no presentó ninguna restricción para *E. rufulus* y no se encontró otro sitio de restricción para ninguno de los otros cerambícidos. Estos resultados indican que este método podría ser usado para determinar la presencia de *E. rufus* en otros eventos de disminución de las poblaciones robles, cuando se establece que éste es un factor que contribuye en la mortalidad de los árboles.

Translation provided by the authors.

The red oak borer, *Enaphalodes rufulus* (Haldeman) (Coleoptera: Cerambycidae), is an important wood-boring species native to eastern hardwood forests of the United States (Donley & Acciavatti 1980). A variety of oak species are attacked by *E. rufulus*, but trees in the red oak group *Erythrobalanus* are preferred, especially black oak, *Quercus velutina* Lam., scarlet oak, *Q. coccinea* Muenchh., and northern red oak, *Q. rubra* L. (Hay 1974). Since *E. rufulus* attacks and reproduces in living trees, significant degrade in lumber quality is an important issue in commercial stands (Hay 1964). Damage caused by borers often goes unnoticed until trees are felled and sawn for timber, and by this time as much as 40% of the 120-year value of the tree may be lost (Donley & Worley 1976). In comparison, damage caused by defoliators is much more noticeable, but defoliation typically causes only a 15-20% reduction in value (Donley & Worley 1976). Donley and Acciavatti (1980) estimated that 38% of oak wood used for lumber, cooperage and veneer in the Eastern United States is affected by *E. rufulus*.

Enaphalodes rufulus population densities historically have been documented at low levels. Hay (1969) found an average of 3.7, 2.8, and 2.5 attacks on the bottom 1.8 m of black oak, northern red oak, and scarlet oak, respectively in Ohio, and Donley and Rast (1984) found an average of 2.0 attack sites per red oak in Pennsylvania and 3.6

in Indiana. Recently, however, an unprecedented outbreak with significant economic and ecological impacts has occurred in the Ozark oak/hickory forests of northern Arkansas and southern Missouri (U.S.A.) (Stephen et al. 2001; Starkey et al. 2000). Analysis of recent data from the Ozark National Forest reveal an average of 599 active attacks (or current generation galleries) and 77 live larvae per tree in northern red oak (Fierke et al. 2005). USDA Forest Service estimates 450,000 ha of forest in the Ozark Mountains will be impacted by *E. rufulus* with an estimated 68,000 m^2 of timber loss or degradation (Guldin et al. 2005).

Removal of infested trees is a recommended control method for *E. rufulus* (Donley 1981, 1983), but diagnosis of infestation may be delayed as easily identifiable adults emerge only every two years and other identification methods are difficult and unreliable. Historically, attack sites were identified by observing frass (Hay 1969), but this method is limited by observer ability, weather conditions, and multiple insects with similar life histories. Larval keys for North American cerambycid species exist (Craighead 1923) but are difficult to follow and outdated. In addition, morphological differences among closely related cerambycid larvae are often minute. Larval identification is also important for detecting *E. rufulus* in tree hosts related to red oaks, which may harbor several cerambycid species, such as *Elaphidion* spp., *Goes* spp., or *Noeclytus* spp. (Yanega 1996). Molecular genetic techniques are an alternative to traditional methods for distinguishing *E. rufulus* larvae from other cerambycids.

The objective of this research was to develop a molecular diagnostic technique for all life stages of *E. rufulus* with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Genetic research has never been conducted on *E. rufulus* and molecular diagnostics have been reported for only one other cerambycid, *Anoplophora glabripennis* (Motschulsky) (Kethidi et al. 2003). The *A. glabripennis* diagnostic technique utilizes sequence characterized amplified regions (SCARs) derived from randomly amplified polymorphic DNA (RAPD). While the end result of this technique is a very simple polymerase chain reaction diagnostic, development of this procedure is time-consuming and expensive. On the other hand, PCR-RFLP is a simple, inexpensive, established and reliable technique (Taylor & Szalanski 1999) that has been used to identify many economically important insects, including termites, *Reticulitermes* spp. (Isoptera: Reticulitermatidae) (Szalanski et al. 2003), screwworm flies, *Cochliomyia* spp. (Diptera: Calliphoridae) (Litjens et al. 2001), and corn rootworm, *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Clark et al. 2001).

Materials and Methods

Specimen Collection

Enaphalodes rufulus adults were collected from two areas, Fly Gap and White Rock, in the Ozark Mountains of northwestern Arkansas with standard black-lighting techniques during the flight period between mid-June and late July of 2003 (UTM Zone 15–S NAD83: Fly Gap—0431660, 3954978, White Rock—412668, 3949429). Beetles were placed in 100% ethanol immediately upon capture.

Adult cerambycids, other than *E. rufulus*, that are common to the Ozark Mountain region and one closely related *Enaphalodes* species were collected during concurrent research at the University of Arkansas. Voucher specimens are stored in the Forest Insect Collection at the University of Arkansas Forest Entomology Lab (Table 1). Specimens were caught in clear plexiglass, passive flight intercept panel traps during the summer of

Table 1. List of adult specimens, collection location, collection date, and number used in this study.

Scientific name	Common name	Collection location (county, state)	Collection date	Number of specimens used
Enaphalodes rufulus	Red Oak Borer	Franklin Co., AR	19-VI-2001	29
Enaphalodes atomarius	N/A	Franklin Co., AR	21-VIII-2001	4
Distenia undata	N/A	Franklin Co., AR	10-VIII-2001	1
Orthosoma brunneum	Brown Prinoid	Franklin Co., AR	07-VIII-2001	2
Bellamira scalaris	N/A	Franklin Co., AR	03-VII-2001	1
Elaphidion mucronatum	Spine Bark Borer	Franklin Co., AR	06-VII-2001	3
Eburia quadrigeminata	Ivory Marked Beetle	Franklin Co., AR	07-VIII-2001	3
Neoclytus a. acuminatus	Red-headed Ash Borer	Franklin Co., AR	20-VII-2001	2
Goes tigrinus	White Oak Borer	Franklin Co., AR	10-VII-2001	1
Aegomorphus morrisii	N/A	Franklin Co., AR	10-VII-2001	1
Urographis fasciatus	N/A	Franklin Co., AR	27-VII-2001	3
Purpuricenus humeralis	N/A	Franklin Co., AR	10-VII-2001	3
Prionus imbricornis	Tile-horned Prionus	Franklin Co., AR	10-VIII-2001	2
Dorcaschema wildii	Mulberry Borer	Franklin Co., AR	06-VII-2001	1

TABLE 2. RESTRICTION FRAGMENT LENGTH POLYMORPHISMS FROM THE 16S RRNA GENE OF 14 CERAMBYICD SPECIES. PATTERNS A, B, AND C INDICATE DISTINCTIVE FRAGMENT LENGTHS.

Restriction enzyme	Species	Size of PCR Amplicon	Restriction site	Fragment(s)	Pattern
*Alu*I	*Enaphalodes rufulus*	414	291	291, 123	A
(AGCT)	*Enaphalodes atomarius*	415	168, 291	168, 124, 123	B
	Distenia undata	412	212, 288	212, 124, 76	C
	Orthosoma brunneum	417	217, 293	217, 124, 76	C
	Bellamira scalaris	413	216, 292	216, 121, 76	C
	Elaphidion mucronatum	413	215, 289	215, 124, 74	C
	Eburia quadrigeminata	414	215, 290	215, 124, 75	C
	Neoclytus a. acuminatus	418	218, 294	218, 124, 76	C
	Goes tigrinus	414	215, 290	215, 124, 75	C
	Aegomorphus morrisii	417	214, 289	214, 128, 75	C
	Urographis fasciatus	412	215, 290	215, 122, 75	C
	Purpuricenus humeralis	414	216, 290	216, 124, 74	C
	Prionus imbricornis	411	214, 287	214, 124, 73	C
	Dorcaschema wildii	412	213, 286	213, 126, 73	C
Hind III	*Enaphalodes rufulus*	414	—	414	A
(AAGCTT)	*Enaphalodes atomarius*	415	166	249, 166	B
	Distenia undata	412	210	210, 202	C
	Orthosoma brunneum	417	215	215, 202	C
	Bellamira scalaris	416	214	214, 202	C
	Elaphidion mucronatum	413	213	213, 200	C
	Eburia quadrigeminata	414	213	213, 201	C
	Neoclytus a. acuminatus	418	216	216, 202	C
	Goes tigrinus	414	213	213, 201	C
	Aegomorphus morrisii	417	212	212, 205	C
	Urographis fasciatus	412	213	213, 199	C
	Purpuricenus humeralis	414	214	214, 200	C
	Prionus imbricornis	411	212	212, 199	C
	Dorcaschema wildii	412	211	211, 201	C

2001. Specimens were collected in 50% ethylene glycol. Upon return to the lab, specimens were transferred to 95% alcohol until pinning. Morphological identification of adult specimens was made by Dr. J. K. Barnes, Arthropod Museum Curator at the University of Arkansas.

Eggs of *E. rufulus* were collected from a lab colony in March 2005. Larvae and pupae were collected from red oak trees harvested between October 2002 and March 2005. Eggs and early instars were frozen, and late instars and one pupa were stored in 95% alcohol until used. An unknown larva and pupa were collected from a white oak tree and stored in 95% alcohol until used.

PCR-RFLP Protocol

DNA was extracted from one leg or thorax of *E. rufulus* and the other 13 adult cerambycids used in this procedure. DNA extraction was accomplished by the protocol of the Qiagen DNeasy tissue kit (Valencia, CA). Re-suspended DNA was stored at -20°C until used. An approximately 420-bp portion of the 16S rRNA gene was amplified using the primers 16S-r (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al. 1994) and 16S-f (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati & Smith 1995). PCR reactions were conducted with 1 µl of extracted DNA as per Szalanski et al. (2000) with a thermocycler profile consisting of 40 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 45 s. PCR products were purified and concentrated with the Wizard SV Gel and PCR clean-up kit (Promega, Madison, WI). One sample from each adult was sent to the University of Arkansas for Medical Sciences DNA Sequencing Core Facility (Little Rock, AR), for direct sequencing in both directions. Consensus sequences for each species were acquired by manual alignment and editing of forward and reverse sequences in BioEdit (Hall 1999). GenBank accession numbers are DQ417758 to DQ2417771.

Webcutter 2.0 (Heiman 1997) was used to predict restriction sites from DNA sequence data. Amplified DNA was digested according to manufacturer's (Promega, Madison, WI) recommendations with the enzymes *Alu* I or *Hind* III. Fragments were separated by 2% agarose gel electrophoresis. Gels were stained with ethidium bro-

mide and photographed with the UVP BioDoc-it documentation system (Upland, CA).

RESULTS

The rRNA 16S amplicon ranged from 411 to 418 bp in all cerambycids studied (Table 1). Webcutter 2.0 analysis of the 16S sequences revealed that either *Alu* I or *Hind* III could effectively distinguish *E. rufulus* from all other species (Table 2). Either enzyme was also capable of distinguishing *E. atomarius* (Drury), a less common but closely related species. All other species produced similar restriction profiles.

To validate the diagnostic, this procedure was tested on 29 adult *E. rufulus* and as many replicates as possible for the additional adult species (Table 1). All species were replicated except *Distenia undata* (Fabricius), *Bellamira scalaris* (Say), *Dorcaschema wildii* Uhler, *Goes tigrinus* (DeGeer), and *Aegomorphus morrisii* (Uhler). There was only one individual available from the collection for these five species. Samples digested with *Alu* I revealed clearly defined results (Fig. 1) as did samples digested with *Hind* III (Fig. 2).

All life stages of *E. rufulus* also were tested with this procedure. Five *E. rufulus* eggs, four *E. rufulus* early larval head capsules, six *E. rufulus* late larval incised head capsules and one *E. rufulus* incised pupa head were used for confirmation of the use of this diagnostic procedure for multiple life stages. All samples produced positive results when either enzyme was used. One unknown cerambycid larva, and one unknown cerambycid pupa collected from a white oak tree also were tested. Results gave fragment lengths similar to those of all other cerambycids except *E. rufulus* and *E. atomarius.*

DISCUSSION

Results from this research show that *Enaphalodes rufulus* readily can be distinguished from all other cerambycids tested by PCR-RFLP and either of the two restriction enzymes, *Alu* I or *Hind* III. The ability to digest the PCR amplicon by either enzyme could be important for future studies where use of only one enzyme is possible.

PCR-RFLP is a well established means for species diagnostics of many organisms (Taylor & Szalanski 1999; Slade et al. 1993; Sperling et al. 1994; Roehrdanz 1997; Szalanski et al. 1997; Harrington and Wingfield 1995; Taylor et al. 1996). This method of identification requires less equipment and is less expensive than other practiced methods. It is also easy to repeat and can be used not only for diagnostics but also for phylogenetic analyses (Taylor & Szalanski 1999).

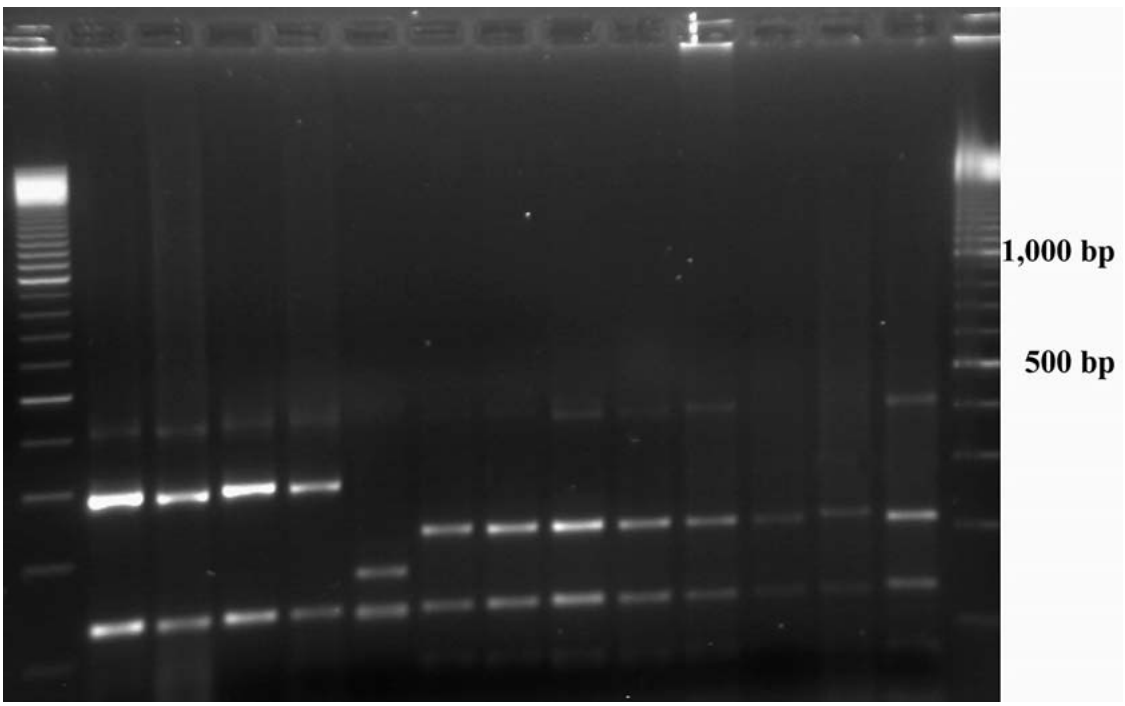

Fig. 1. *Alu* I digest of the 16S amplicon for 4 *Enaphalodes rufulus* (lanes 2-5), *Enaphalodes atomarius*, *Orthosoma brunneum*, *Purpuricenus humeralis*, *Prionus imbricornis*, *Elaphidion mucronatum*, *Eburia quadrigeminata*, *Urographis fasciatus*, *Neoclytus a. acuminatus*, and *Aegomorphus morrisii* on a 2% Agarose gel. The top band is undigested PCR product.

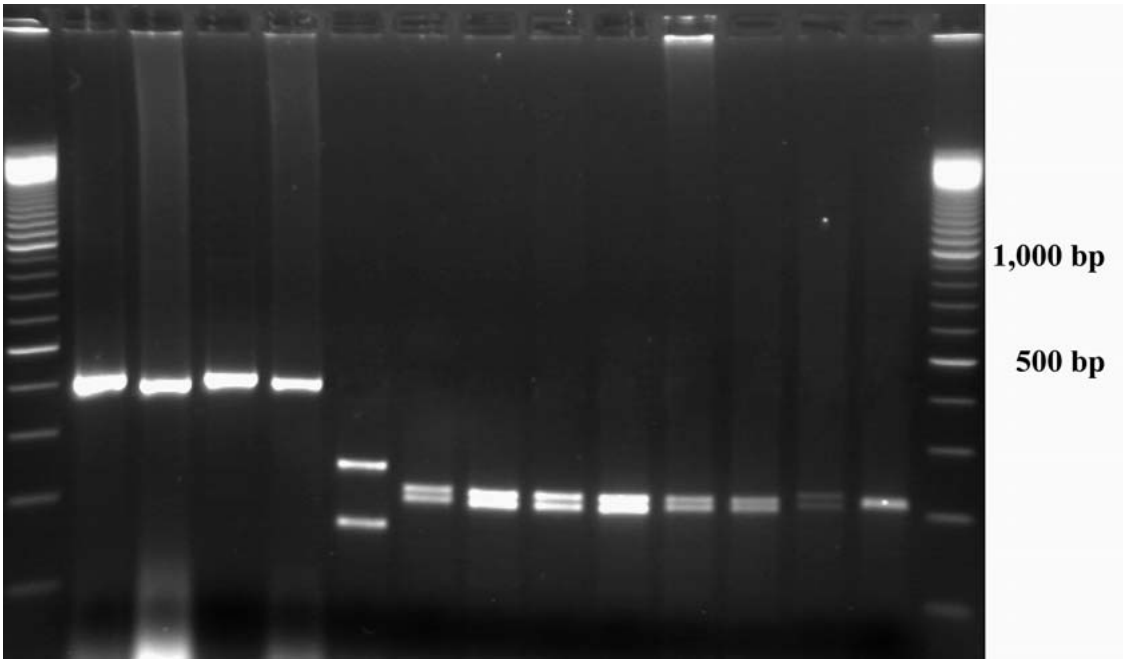

Fig. 2. *Hind* III digest of the 16S amplicon for 4 *Enaphalodes rufulus* (lanes 2-5), *Enaphalodes atomarius*, *Orthosoma brunneum*, *Purpuricenus humeralis*, *Prionus imbricornis*, *Elaphidion mucronatum*, *Eburia quadrigeminata*, *Urographis fasciatus*, *Neoclytus a. acuminatus*, and *Aegomorphus morrisii* on a 2% Agarose gel.

DNA extraction from dried adult legs or thoraxes worked well with Qiagen DNeasy extraction kit and PCR-RFLP. Extracting DNA from pinned, dried adult cerambycids could prove useful in future studies where genetic information may need to be extracted from stored museum specimens. Adults were used to create this procedure as they easily are identified by morphological characteristics, and positive identification was necessary for sequence comparisons.

Morphological identification of early instar cerambycid larvae is difficult if not impossible. PCR-RFLP is an established diagnostic tool for larval identification and easily could be used to distinguish morphologically similar, yet genetically distinct species of cerambycids that have similar life history strategies. This should prove useful in detecting *E. rufulus* as a contributing factor in other oak mortality events especially during the larval stage in which they spend about 90% of their two-year life cycle. Early detection of *E. rufulus* in other oak decline events should help foresters or landowners make informed management decisions in regard to harvest options and/or silvicultural remediation.

White oak mortality is prevalent throughout the Ozark Mountains in Arkansas. White oaks can harbor several species of cerambycids including *E. rufulus* and white oak borer, *G. tigrinus*. An unknown larva and pupa from a white oak tree, which may have been *G. tigrinus*, were tested to confirm the validity of this diagnostic technique and to show that this procedure works with larvae and pupae of species other than *E. rufulus*. The resulting larval and pupal fragments were similar to those of all other cerambycids tested, except *E. rufulus* and *E. atomarius*. A molecular diagnostic procedure possibly could be created for other cerambycids from the Ozarks. This would clarify to what extent *G. tigrinus* or other cerambycids are contributing to white oak mortality, and may offer insight into the prevalence of other immature cerambycids in economically important oak trees.

It was difficult to obtain samples from areas other than northern Arkansas as *E. rufulus* is normally at low population levels. However, this study provides a good foundation for *E. rufulus* identification with PCR-RFLP and our results can be expanded as specimens are collected from other regions of the eastern U.S.

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