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Identification of Seed Gall Nematodes of Agronomic and Regulatory Concern with PCR-RFLP of ITS1[1]

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Abstract: A molecular analysis of eight described species of seed gall nematode, along with six undescribed isolates from different hosts, has revealed a strong association between nucleotide sequence polymorphism and host status. Each anguinid nematode associated with a unique host produced a unique PCR-RFLP pattern for the ITS1 region. *Anguina* species that had been synonymized in the past, *Anguina agrostis, A. funesta,* and *A. wevelli* (*Afrina wevelli*), were readily discriminated. Two undescribed species from northern New South Wales and southeastern South Australia, reported to be vectors of *Rathyaibacter toxicus* in the disease called ''floodplain staggers,'' were differentiated by a single restriction enzyme, and both could be separated easily from *A. funesta,* the vector of *R. toxicus* in annual ryegrass toxicity. Other species differentiated in this study include *A. agropyronifloris, A. graminis, A. microlaenae, A. pacificae,* and undescribed species from host species *Dactylis glomerata, Agrostis avenacea, Polypogon monospeliensis, Stipa* sp., *Astrebla pectinata,* and *Holcus lanatus.* Phylogenetic analysis of the ITS1 region suggests that considerable anguinid genetic diversification has accompanied specialization on different host species.

Key words: Afrina wevelli, Anguina agrostis, Anguina tritici, diagnostics, nematode identification, PCR-RFLP, regulatory nematology, seed gall nematode.

Anguina species, the seed gall nematodes, have remarkably rich and convoluted taxonomic histories (Brzeski, 1981; Fortuner and Maggenti, 1987; Krall, 1991; Siddiqi, 2000). The genus includes the first recorded plant parasite, *Anguina tritici* (Steinbuch, 1799) Filipjev, 1936, and *A. funesta* Price, Fisher, & Kerr, 1979, the vector of the notoriously toxic bacterium, *Rathayibacter toxicus.* There are 11 valid species of *Anguina* according to the latest classification (Siddiqi, 2000). Species numbers, however, vary considerably among classifications, commensurate with the recognition of various genera or subgenera. For example, *Afrina wevelli* van den Berg, 1985, described originally from weeping lovegrass, *Agrostis curvula,* in South Africa, was synonymized with *Anguina agrostis* (Steinbuch, 1799) Filipjev, 1936, the bentgrass nematode, by Chizhov and Subbotin (1990). Ebsary (1991) did not recognize this synonymy and placed this species in the genus *Subanguina* Paramonov, 1967. Recently, Siddiqi (2000) transferred the species to *Anguina.* These taxonomic actions are not inconsequential when regulatory issues arise. The synonymy of *Afrina wevelli* and *Anguina agrostis* imposed quarantine status on seed gall nematodes found on weeping lovegrass because of earlier synonymies of *A. agrostis* and *A. funesta* (Siddiqi, 1985).

Underlying the taxonomic confusion is a conserved and nondescript morphology that complicates anguinid identification. Often, only juveniles are found in seed galls, which further complicates identification. Undoubtedly, host records of different anguinid species are compromised by misidentification. The large range of reported hosts for *Anguina agrostis* most likely includes records for several *Anguina* species (Krall, 1991). This study was initiated because of a need to rapidly identify anguinid species of regulatory concern. A polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) approach using the ribosomal DNA first internal transcribed spacer region (ITS1) has been found useful for DNA comparisons among individual nematodes (Powers et al., 1997). This study reports surprising host specificity among all isolates examined and provides a convenient guide for molecular recognition of anguinid species.

Materials and Methods

Nematode samples: The putative origin, host, and provider for the anguinid isolates are presented in Table 1. National and international seed trade creates ambiguity in determination of point-of-seed origin in some cases. For example, one isolate of *Anguina wevelli* arrived in our laboratory from USDA-APHIS offices in Oregon after seed from South Africa was imported into Texas and subsequently shipped to Oregon for re-export. Several nematology laboratories maintain vials of wheat seed galls for teaching purposes, although the last reported collection of *A. tritici* in the United States was in 1975 in Virginia (Carta, pers. comm.). Species determinations were made by morphological examination of nematodes extracted from plant galls, host association, and geographic location. Additional biochemical and life-cycle information of Australian isolates is available from T. Bertozzi. Photographic vouchers and dried galls of other anguinid species are deposited in the University of Nebraska Department of Plant Pathology nematology collection maintained by T. Powers.

DNA analysis: Single juveniles were processed for PCR

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TABLE 1. Seed gall nematode samples used for this study.

Species	Host	Location
Anguina wevelli[a]	*Agrostis curvula,* weeping lovegrass	South Africa
Anguina agrostis[b]	*Agrostis tenuis,* bentgrass	Oregon, U.S.A.
Anguina agropyronifloris[c]	*Agropyron smithii,* western wheatgrass	Kansas, U.S.A.
Anguina funesta[d]	*Lolium rigidum,* annual ryegrass	Australia
Anguina funesta[d]	*Lolium rigidum,* annual ryegrass	Geranium, Australia
Anguina funesta[d]	*Lolium rigidum,* annual ryegrass	Lochiel, Australia
Anguina graminis[b]	*Festuca ovina* var. *duriuscula,* hard fescue	Oregon, U.S.A.
Anguina microlaenae[d]	*Microlaena stipodes*	Australia
Anguina pacificae[e]	*Poa annua,* annual bluegrass	California, U.S.A.
Anguina tritici[f]	*Triticum aestivum,* wheat	Baja, Mexico
Anguina tritici[e]	*Triticum aestivum,* wheat	Baja, Mexico
Anguina tritici	*Triticum aestivum,* wheat	Virginia, U.S.A. 1975
Anguina sp.[b]	*Dactylis glomerata,* orchardgrass	Oregon, U.S.A.
Anguina sp.[d]	*Agrostis avenacea,* annual blowngrass	Australia
Anguina sp.[d]	*Polypogon monospeliensis,* annual beardgrass	Australia
Anguina sp.[d]	*Stipa* sp.	Australia
Anguina sp.[d]	*Astrebla pectinata*	Australia
Anguina sp.[d]	*Holcus lanatus,* velvetgrass	Australia

Collected by: [a] K. Ball, [b] J. Griesbach, [c] Nebraska Seed, [d] T. Bertozzi, [e] M. McClure, [f] T. Todd.

by placing them in a 15-µl drop of distilled water on a glass cover slip and manually disrupting them (Powers and Harris, 1993). Two PCR primers, rDNA2 (Vrain et al., 1992) and rDNA1.58S (Cherry et al., 1997) were used to amplify a 3′ portion of the 18S gene, the entire ITS1 region, and a 5′ section of the 5.8S gene. PCR reactions were conducted per Szalanski et al. (1997). Amplified DNA for DNA sequencing was purified using Geneclean II (Bio 101, Inc., Vista, CA). The Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) sequenced PCR products. Sequences were obtained for both DNA strands, and consensus sequences were derived from individual sequences in each direction using GAP (Genetics Computer Group, Madison, WI). The GenBank Accession Numbers for the DNA sequences are AF363093 to AF363109.

Restriction sites were predicted from the DNA sequence data using Web Cutter 2.0 (Heiman, 1997). Amplified DNA was digested according to manufacturer's (New England Biolabs, Beverly, MA) recommendations per Cherry et al. (1997) using the restriction enzymes Alu I, Bsr I, Eco RI, Hae III, Hha I, Hinf I, and Taq I. Fragments were separated by agarose (2.5% MetaPhor) gel electrophoresis following Cherry et al. (1997).

The distance matrix feature of PAUP* 4.0b2 (Swofford, 1999) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura, 1980) of sequence evolution. Seed gall nematode DNA sequences were aligned using rDNA sequences from *Ditylenchus phyllobius* (Thorne, 1934) Filipjev, 1936, *D. dipsici* (Kuhn, 1857) Filipjev, 1936, and *D. destructor* Thorne, 1945. GenBank accession numbers for the three *Ditylenchus* species are AF363110 to AF363112. Maximum likelihood and unweighted parsimony analysis of the alignments were conducted using PAUP* 4.0b2 (Swofford, 1999). Gaps were excluded for all analyses. Bootstrap analysis was used to compare the relative reliability of clades (Felsenstein, 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, either the single most-parsimonious tree generated in our parsimony search was used as the starting tree or a bootstrap analysis was conducted with 100 resamplings using the Heuristic algorithm of PAUP*. Rates were assumed to follow a gamma distribution, with shape parameter estimated via maximum likelihood based on the general-time-reversible model (GTR) (Yang, 1994). A total of 235 distinct data patterns were used for this model.

RESULTS

DNA sequencing of the ITS1 PCR-amplified product revealed a size range from 547 to 553 bp among each of the seed gall nematodes, with the exception of the anguinid species from *Astrebla,* which produced a 575-bp product. No nucleotide differences were observed among the intraspecific comparisons. Unique amplification products were obtained from every anguinid isolate examined in this study that was extracted from a different host species (Tables 2,3). The two most similar amplification products came from undescribed species collected from annual beardgrass and annual blowngrass in Australia. The single substitution that separated the ITS1 amplified product of these two nematodes also affected an HhaI site, allowing their discrimination by this enzyme (Table 2). Table 2 presents a summary of digestion patterns from enzymes useful for anguinid identification. Table 3 presents a composite genotype representing the seven restriction patterns that permit convenient identification. Digestion with the underlined enzymes in Table 3 provided unambiguous identification of the nematodes included in this study. Restriction digestion of amplified DNA from nematodes extracted from a wheat gall collected in 1974 resulted in restriction patterns identical to *A. tritici* patterns from recently collected galls.

The rDNA sequences of all of the seed gall nematodes were aligned using three *Ditylenchus* spp. as the outgroup taxa. The aligned data matrix including the outgroup taxa resulted in a total of 721 characters, including gaps, and is available at nematology.unl.edu/anguina.htm. Of these characters, 269 (37%) were vari-

TABLE 2. Seed gall nematode restriction fragment sizes and patterns.

Sample	Alu I		Bsr I		EcoRI		HaeIII		HhaI		HinfI		TaqI	
Anguina wevelli	550	A	467 83	A	301 249	A	550	A	550	A	375 129 46	A	357 135 58	A
A. agrostis	548	A	295 238 15	B	299 249	A	548	A	548	A	448 100	B	355 135 58	A
A. agropyronifloris	547	A	547	C	547	B	317 230	B	547	A	447 100	B	489 58	B
A. funesta	548	A	295 238 15	B	299 249	A	548	A	548	A	448 100	B	490 58	B
A. graminis	548	A	310 238	B	548	B	548	A	548	A	249 199 100	C	490 58	B
A. microlaenae	550	A	550	C	301 249	A	550	A	550	A	449 52 49	D	357 135 58	A
A. pacificae	549	A	239 225 85	D	549	B	319 230	B	549	A	448 101	B	491 58	B
A. tritici	277 274	B	550	C	550	B	550	A	462 88	B	550	E	492 58	B
A. sp. *Dactylis*	550	A	297 253	B	550	B	320 230	B	550	A	252 198 100	C	357 135 58	A
A. sp. *Agrostis*	553	A	301 237 15	B	553	B	333 220	B	303 250	C	454 99	B	360 135 58	A
A. sp. *Polypogon*	553	A	301 237 15	B	553	B	333 220	B	553	A	454 99	B	360 135 58	A
A. sp. *Stipa*	548	A	467 81	A	299 249	A	548	A	548	A	375 98 46 29	F	355 135 58	A
A. sp. *Astrebla*	359 216	C	575	C	575	B	575	A	575	A	401 128 46	A	517 58	B
A. sp. *Holcus*	550	A	310 240	B	301 249	A	550	A	550	A	450 100	B	303 135 58 54	C

able and 124 (17%) were informative for parsimony. Bootstrap analysis of the aligned seed gall and *Ditylenchus* spp. rDNA ITS1 sequences resulted in a consensus tree (Length = 529, CI = 0.781), which delineated four clades (Fig. 1). Regardless of whether the starting tree was the most parsimonious one or was obtained via step-wise addition, the maximum likelihood search found only one tree (Fig. 2). This maximum likelihood tree (–Ln likelihood = 3441.96403, total number of rearrangements tried = 2,920) was identical in topology to the parsimony tree, and to the maximum likelihood tree derived by bootstrap analysis.

Discussion

Amplification and digestion of the ITS1 region could distinguish each of the anguinid species examined during this study. As a diagnostic tool, this process could eliminate much of the ambiguity involved in morphological identification of juvenile specimens. Anguinid specimens are usually encountered as juveniles in galled plant tissue or residue following seed testing for germination (Griesbach et al., 1999). Importantly, this method could provide standardization for identifications in regulatory settings where misidentification can result in a significant economic impact. Several of the samples provided for this study were actually nematodes intercepted on shipments of seed. *Anguina wevelli* has been intercepted in the United States on imported shipments of weeping lovegrass seed, *Agrostis curvula,* on several occasions. The possibility that this nematode may be identical to *A. funesta,* as indicated by Krall (1991), restricts the movement of this seed. The ITS1

TABLE 3. Seed gall nematode patterns for the restriction enzymes Alu I, Bsr I, EcoR I, Hae III, Hha I, Hinf I, and Taq I.

Sample	Restriction enzyme pattern
Anguina wevelli	AAAAAAA
A. agrostis	ABAAAABA
A. agropyronifloris	ACBBBBBB
A. funesta	ABAAAABB
A. graminis	ABBAACB
A. microlaenae	ACAAADA
A. pacificae	ADBBABB
A. tritici	BCBABEB
A. sp. *Dactylis*	ABBBACA
A. sp. *Agrostis*	ABBBCBA
A. sp. *Polypogon*	ABBBABA
A. sp. *Stipa*	AAAAAFA
A. sp. *Astrebla*	CCBAAAB
A. sp. *Holcus*	ABAAABC

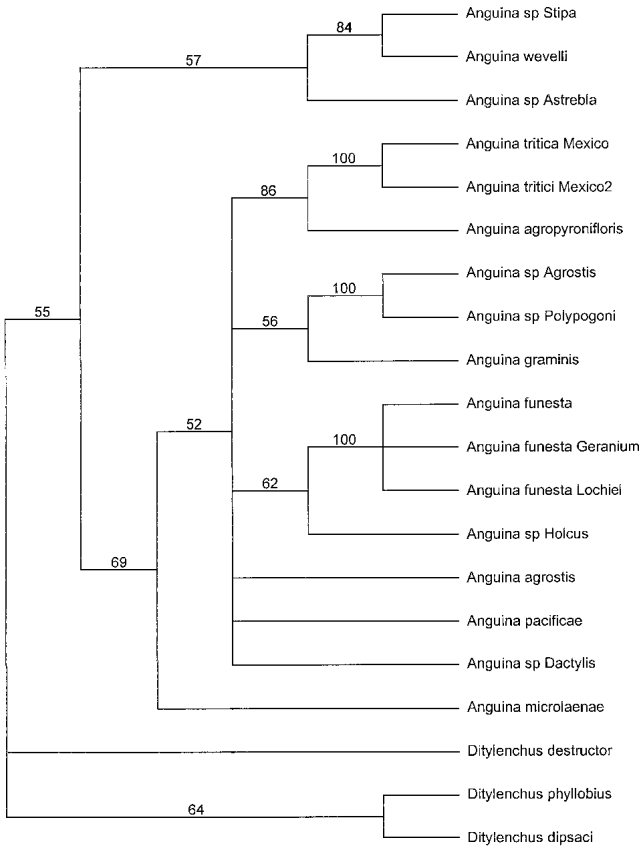

FIG. 1. Single most-parsimonious tree found using a branch and bound search using PAUP* of rDNA ITS1 sequences with gapped characters excluded. Bootstrap values for 1,000 branch and bound replicates are listed above branches supported at ≥50%.

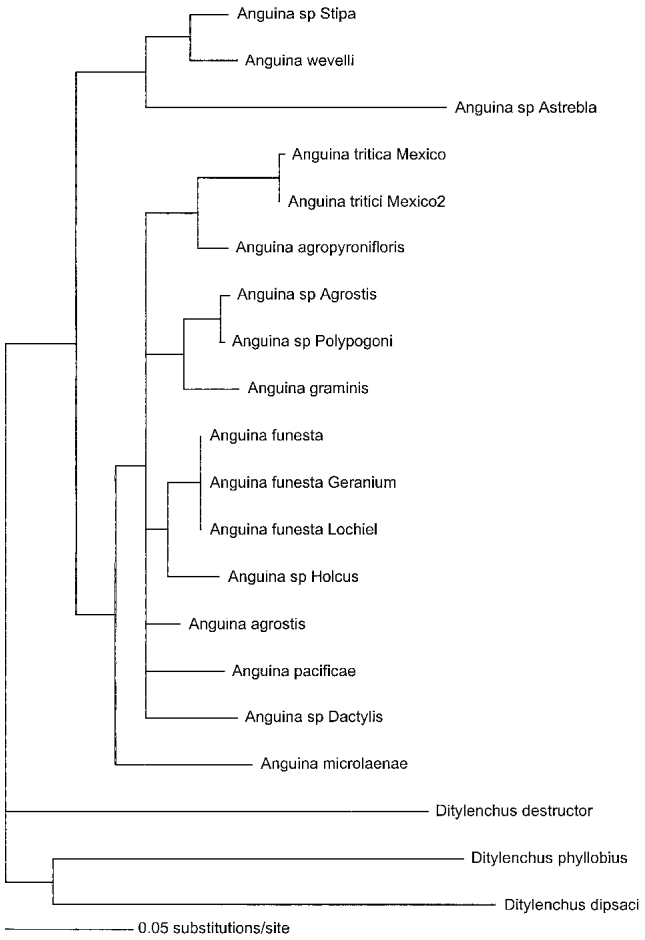

FIG. 2. Best maximum likelihood tree based on rDNA ITS1 sequences. Branch lengths are proportional to the number of inferred changes.

nucleotide sequence of *A. wevelli*, *A. funesta,* and *A. agrostis* clearly demonstrates that these are all separate species. The phylogenetic analysis presented in this paper suggests that *A. wevelli* is a member of a grouping separate from those species and possibly separate from other nominal species in Anguina.

Shipments of seed of orchardgrass, *Dactylis glomerata,* have been rejected based on the presence of a nematode believed to be *A. agrostis*. Digestion of the ITS1 amplification product with EcoR I, Hae III, or Hinf I differentiates between the anguinid isolate from orchardgrass and *A. agrostis*. The sensitivity of the PCR-RFLP approach is illustrated by the discrimination of the two undescribed anguinid species associated with ''floodplain staggers'' (Bertozzi and McKay, 1995). A single G→A substitution in ITS1 is the only nucleotide difference observed between these nematodes, yet the creation of an Hha I site in the isolate found on annual blowngrass, *Agrostis avenacea,* provided a means to differentiate it from the isolate found associated with annual beardgrass, *Polypogon monospeliensis*.

It is tempting, based on the phylogenetic analysis of these nematodes, to imagine that specialization on different host species has served as an isolating mechanism in anguinid evolution. Greater resolution of species relations and more conspecific populations are necessary before theories of speciation can be tested.

Many of the nematodes included in this study are seldom encountered in the field. Other than *A. funesta* and *A. tritici,* all of the anguinid species examined in this study came from a restricted geographic locality. Currently, we are attempting to collect anguinid-induced galls that permit a more extensive comparison of geographic and temporal variation.

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