Polymerase Chain Reaction Techniques for Distinguishing Three Species and Two Strains of *Aphelinus* (Hymenoptera: Aphelinidae) from *Diuraphis noxia* and *Schizaphis graminum* (Homoptera: Aphididae)

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ABSTRACT Ribosomal DNA sequences for the internal transcribed spacer 2 (ITS2) were cloned and sequenced from *Aphelinus albipodus* Hayat & Fatima, *A. varipes* (Foerster), and 2 strains of *A. asychis* Walker, all endoparasitoids that have been released against the Russian wheat aphid, *Diuraphis noxia* (Mordvilko). The polymerase chain reaction (PCR) using primers designed on the basis of these rDNA sequences, followed by agarose gel electrophoresis, successfully distinguishes all 4 *Aphelinus* populations from each other and from the Russian wheat aphid and another economically important cereal aphid, the greenbug, *Schizaphis graminum* (Rondani). Two additional strains of *A. asychis* and 4 postrelease populations of *A. albipodus* are also correctly identified to species with these primers. An additional strain of *A. varipes* was not separable from *A. albipodus*, but on microscopic analysis it proved to be morphologically anomalous. Using this technique, *A. asychis* DNA is detectable in a parasitized Russian wheat aphid 24 h after parasitization. The sensitivity is 10^{-3} adult wasp DNA equivalent.

KEY WORDS Aphelinidae, *Aphelinus*, aphid, Aphididae, biological control, parasitoid

ACCURATE IDENTIFICATION OF natural enemies is critical to the success of classical biological control programs, not only in the initial phases when candidate natural enemies are chosen and released, but also during subsequent manipulation and evaluation phases (Delluchi et al. 1976). The identification problems can be particularly daunting when a complex of congeners, which may include sibling exotic species, or disjunct populations of the same exotic species, is released into an area containing resident populations of native species and previously released exotic species.

A case in point is the biological control program launched against the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), after its appearance in North America in the late 1980s (Stoetzel 1987, Lajeunesse and Johnson 1992, Yu 1992). A large effort was mounted to collect and release natural enemies of this pest (Hopper et al. 1998, Prokrym et al. 1998a). Among numerous exotic parasitoids released were 3 species of *Aphelinus: A. albipodus* Hayat & Fatima, *A. asychis* Walker, and *A. varipes* (Foerster) (Elliott et al. 1995). The *A. asychis* individuals released in this program compose a group of geographical isolates that may constitute a sibling species complex (Kazmer et al. 1996); furthermore, *A. asychis* and *A. varipes* were already present in North America from previous releases against other aphid species (van den Bosch et al. 1959, Jackson et al. 1970, Jackson and Eikenbary 1971), and may also have been accidentally introduced, undetected, along with their exotic hosts (Wharton 1983). Another North American aphelinid, *A. semiflavus* Howard, may or may not be synonymous with *A. asychis* (Mackauer and Finlayson 1967). Also, there have been reports of an undescribed species near *A. varipes* (Lajeunesse and Johnson 1991, Yu 1992).

This taxonomic uncertainty has made it difficult to evaluate the success of the classical biological control program mounted against the Russian wheat aphid (Prokrym et al. 1998a). Our inability to distinguish these species reliably leaves us unable to determine their effectiveness in suppressing populations of the Russian wheat aphid and other cereal aphids, and to incorporate them into integrated pest management (IPM) programs in the dynamically changing cropping systems landscape in which small grains production is practiced in North America.

Because these and other aphelinid taxa are in many cases very difficult or impossible to distinguish morphologically, several biochemical and molecular methods have been tried to differentiate them, including allozyme electrophoresis (Walton et al. 1990, Strong 1993), random amplified polymorphic DNA variability (Kazmer et al. 1995), and microsatellite

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DNA variability (Vanlerberghe-Masutti and Chavigny 1997). Other nucleic acid markers used to characterize parasitoid taxa have included genomic fragments of undetermined identity (Greenstone and Edwards 1998) and the ribosomal internal transcribed spacers (ITS) 1 and 2 (Campbell et al. 1993, Orrego and Aguidelo-Silva 1993, Sappal et al. 1995).

Fragments of the ITS2, located between the 5.8 S and 28 S ribosomal DNAs, are good candidates for markers. They have highly repetitive and relatively divergent sequences among closely related populations, and have proven useful for comparison of closely related insect species, subspecies, or populations (Black et al. 1989, Collins et al. 1990, Porter and Collins 1991). We describe a sensitive PCR-based technique employing variability in the ITS2 region for distinguishing *Aphelinus albipodus*, *A. varipes*, and Chinese and French (Antibes) strains of *A. asychis*, from one another and from 2 aphid hosts, *D. noxia* and the greenbug, *Schizaphis graminum* (Rondani). We show further that the technique correctly distinguishes other populations of the same species.

Materials and Methods

Insects. Protocols for rearing aphids and parasitoids have been described elsewhere (Reed et al. 1991). They were maintained in cages in a Conviron model 123 incubator (Controlled Environments, Pembina, ND) at 20°C and a photoperiod of 16:8 (L:D) h. Founding stocks for colonies of *A. asychis* from Antibes, France, and Ningxia, People's Republic of China, and of *A. varipes* from Montpellier, France, were provided by K. R. Hopper of the USDA–ARS Beneficial Insects Introduction Research Unit in Newark, DE; *A. albipodus* from Tacheng, People's Republic of China, were provided by D. Gonzáles of the University of California, Riverside. To reduce the risk of contamination, only 1 *Aphelinus* colony was maintained at a time.

Isolation of Genomic DNA. DNA was extracted from insects by the method of Sambrook et al. (1989). Adult wasps or aphids were placed individually in 0.5-ml microcentrifuge tubes and homogenized using a battery-powered homogenizer (Midwest Scientific, St. Louis, MO) in 50 μl of isolation buffer containing 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl (pH 9.1), 0.05 M EDTA, 1% SDS, and 200 μg/ml RNase A. The homogenate was spun briefly and incubated for 30 min at 65°C. Ten microliters of 8 M potassium acetate was added to the tube and mixed by tapping. The tube containing homogenate was incubated on ice for 30 min and centrifuged for 15 min at 1,000 $\times$ *g*. Supernatant was transferred to a new tube and was extracted once with 1 volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated overnight at –20°C, pelleted by centrifugation, and resuspended in 200 μl distilled water.

PCR Amplification, Cloning, and Sequencing of ITS2. The DNA fragment was amplified using the forward primer 58SF and reverse primer 28SR of Porter and Collins (1991):

58SF5′-GTGAATTCTGTGAACTGCAGGACACATGAAC-3′

28SR 5′-ATGCTTAAATTTAGGGGGTA-3′.

PCR products were separated on a 0.8% low melting point agarose gel. DNA fragments were sliced from the gel and extracted using a Wizard PCR Preps DNA purification system (Promega, Madison, WI). The isolated DNA fragments were ligated into a pGEM-T vector (Promega) overnight at 14°C. Transformed *Escherichia coli* cells with vectors were plated on LB/ampicillin/IPTG/X-Gal medium. White colonies were subjected to PCR amplification to confirm the presence of inserts and the expected sizes of fragments. PCR-confirmed colonies were inoculated into 75 ml LB/ampicillin cultures and plasmid DNA was extracted using a Qiagen Plasmid Midi kit (Qiagen, Santa Clarita, CA). DNA inserts were sequenced using an automated sequencer located at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater, OK.

Primer Design and PCR Amplification of Individual Wasp and Aphid DNA. After ITS2 DNA sequences were obtained from the 4 wasp populations, GCG UNIX version 9 (Genetics Computer Group, Madison, WI) was used to align and analyze the sequences. Primers were designed to separate all wasp populations from one another and from the greenbug and Russian wheat aphid. The single base detection technique (Kwok et al. 1990) was used to design primers for separating *A. albipodus* from *A. varipes* and for separating the Chinese and Antibes *A. asychis* strains. Five individuals of each wasp population and each aphid species were subjected to individual DNA extraction and PCR amplification. PCR reactions (25 μl) contained 10 mM Tris-HCl pH 9, 1.5 mM $MgCl_2$, 1 μM of each primer, 50 mM KCl, 0.1 mM of each dNTP, 0.05 U/μl of *Taq* DNA polymerase, and 2 μl of DNA template, and were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA). DNA was initially denatured for 3 min at 94°C, and the PCR amplification was conducted for 50 cycles, with 30 s denaturing at 94°C, 30 s annealing at 50–55°C depending on the primers, and 1 min extension at 72°C. PCR products (10 μl) were separated on 1% agarose gel, stained with 0.5 μg/ml ethidium bromide, and photographed under UV light.

Validation of Specificity. To determine whether the primers we developed from laboratory colonies would be useful for species-level identification of other populations, we tested additional *A. asychis* strains from Kazakhstan and Montpellier, France, an *A. varipes* population from Lattes-Juvignac, France (all from K. R. Hopper, USDA–ARS–BIIRU), and populations of *A. albipodus* recovered from the field in California (from D. Gonzáles, University of California, Riverside), Colorado, Montana, and Utah (from D. R. Prokrym, USDA–APHIS, Niles, MI). DNA extraction and PCR amplification were performed as for the principal strains.

Detection Sensitivity. Two pairs of primers were selected for the detection sensitivity test. DNA of Antibes strain *A. asychis* was 10-fold serially diluted

Table 1. Primer sequences and fragment size produced in PCR amplification

Name	Sequence	Optimal temp	Target species	Fragment size
58S-F	TGTGAACTGCAGGACACATGAAC	50	*A. albipodus*	531 bp
28S-R	ATGCTTAAATTTAGGGGGTA		*A. asychis*-C[a]	528 bp
			A. asychis-A[b]	527 bp
			A varipes	533 bp
58S-F	TGTGAACTGCAGGACACATGAAC	55	*A. albipodus*	480 bp
Aalv-R	GTAATTTATTTCGTACACAC		*A. varipes*	482 bp
Ava-FA	GAAATGAAATTTCGAACGAA	50	*A. varipes*	300 bp
Aalv-R	GTAATTTATTTCGTACACAC			
Aas-F	GCGCGCGCACCAGCGTGCCG	53	*A. asychis*-C	202 bp
28S-R	ATGCTTAAATTTAGGGGGTA		*A. asychis*-A	202 bp
58S-F	TGTGAACTGCAGGACACATGAAC	55	*A. asychis*-C	495 bp
Aas-CRG	TGAGGTCGTCGCAATAATAG			

[a] Chinese strain.
[b] Antibes strain.

from 10^{-2} to 10^{-9} equivalent of an adult wasp in a constant concentration of 10^{-2} equivalent of *D. noxia* DNA in water. Wasp DNA without aphid DNA and aphid DNA without wasp DNA were included as controls. PCR amplification was performed for 60 cycles and 20 μl PCR product was subjected to gel electrophoresis.

Parasitization Detection. Groups of 60 *D. noxia* were exposed to 25 pairs of adult Antibes *A. asychis*. Aphids were collected at 24 and 48 h after initial exposure to wasps. Ten aphids were individually extracted, and DNA was PCR-amplified and analyzed on agarose gel for parasitoid DNA.

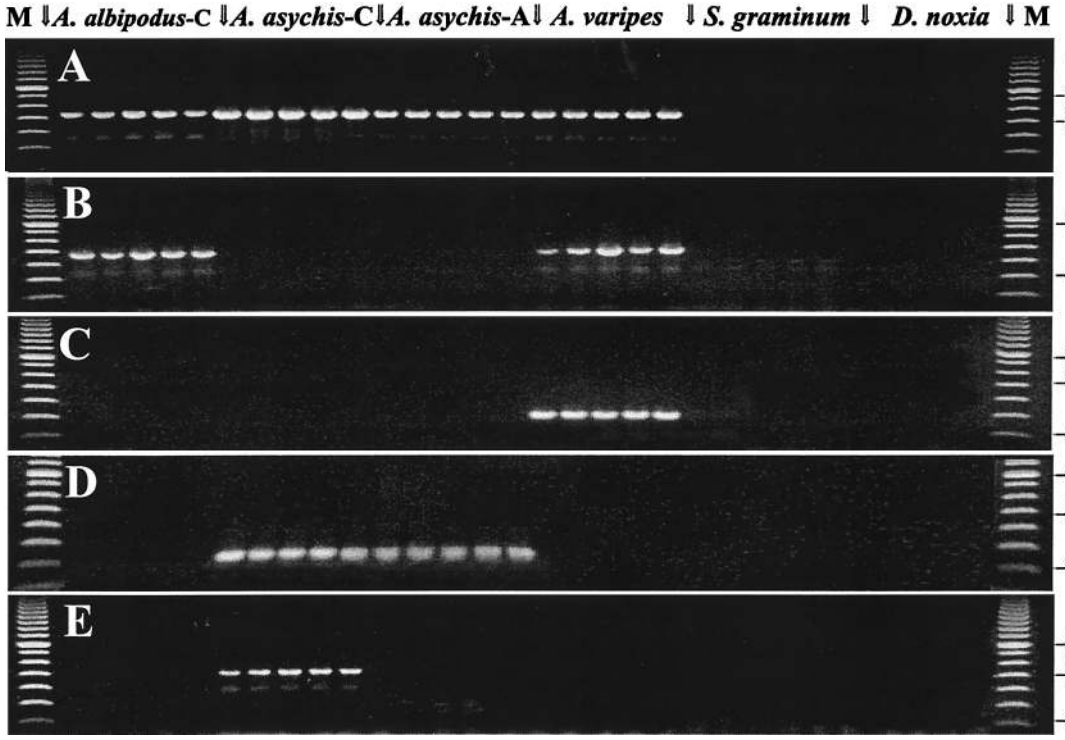

Fig. 1. PCR amplifications for distinguishing 4 *Aphelinus* populations from each other and from the greenbug and the Russian wheat aphid. (A) PCR amplification of ≈530 bp fragments in *Aphelinus* species to separate the wasps from *S. graminum* and *D. noxia* using primer 58S-F and 28S-R. (B) Approximately 480 bp fragments were amplified from *A. albipodus* and *A. varipes* using primer 58S-F and Aalv-R for separating *A. albipodus* and *A. varipes* from *A. asychis* and aphids. (C) 300 bp fragments were amplified from *A. varipes* only using primer Ava-F and Aalv-R. (D) 202 bp fragments were amplified from both Chinese and Antibes strains of *A. asychis* using primer Aas-F and 28S-R for separating *A. asychis* from other *Aphelinus* species and aphids. (E) 495 bp fragments were amplified from Chinese strain *A. asychis* using primer 58S-F and Aas-CRG. M, 100 bp DNA marker from Pharmacia; -C, Chinese strains; -A, Antibes strain.

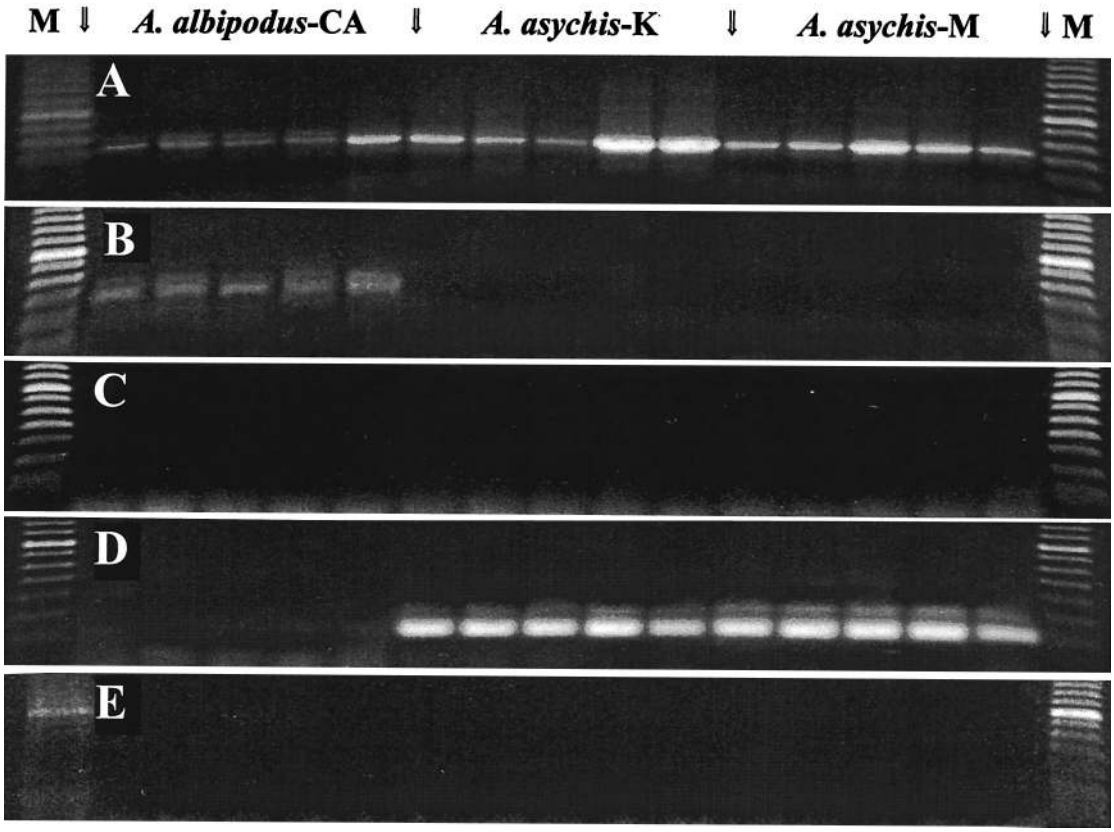

Fig. 2. Primer tests on 1 additional *A. albipodus* population and 2 *A. asychis* strains. (A) 28S-F and 58S-R. (B) 58S-F and Aalv-R. (C) Ava-FA and Aalv-R. (D) Aas-F and 28S-R. (E) 58S-F and Aas-CRG. -M, 100 bp DNA marker from Pharmacia; -CA, California population; -K, Kazakhstan strain; -M, Montpellier strain.

Voucher Specimens. Voucher materials under numbers T92/023 (*A. albipodus*), T91/004 (*A. varipes*), and T89/119 and T92/051 (*A. asychis* from Antibes and the PRC, respectively) have been previously deposited in the Texas A&M University Insect Collection.

Results

ITS2 fragments of ≈530 bp were easily amplified using primers 58S-F and 28S-R from all wasp populations, but not from either aphid species. Sequences were confirmed as ITS2 by similarity searching the GenBank Blastn database (National Institutes of Health). From the known ribosomal DNA ITS2 sequences, several sets of primers were designed to separate wasps from *S. graminum* and *D. noxia*, and to separate the 4 original wasp strains from one another. Primer sequences, target species, and expected PCR fragment size are listed in Table 1.

The primer 58S-F binding site is located on the 3′ downstream end of the 5.8 S ribosomal DNA, and the primer 28S-R binding site is located on the 5′ upstream end of the 28 S ribosomal DNA. These sequences are highly conserved, and the corresponding primers have worked for many mosquito species (Wesson et al. 1992) and hymenopteran species (van Kan et al. 1996). In our experiment, 5 individuals of each wasp and aphid were subjected to PCR amplification using these 2 primers. Results (Fig. 1A) showed that ≈530 bp fragments were consistently amplified from all wasps, and no difference was observed among individuals within species or populations. However, there was no corresponding fragment amplification from *S. graminum* or *D. noxia* under the same conditions. Unsuccessful PCR amplification represents mismatches between the primers and aphid DNA which, therefore, can be used for separating selected *Aphelinus* species or strains from both the greenbug and Russian wheat aphid.

Reverse primer Aalv-R was designed from the ITS2 DNA sequences of *A. albipodus* and *A. varipes*. PCR amplification using this primer with the common primer 58S-F produced a ≈480 bp fragment only from *A. albipodus* and *A. varipes*, and no corresponding fragment from *A. asychis*, *S. graminum*, or *D. noxia* (Fig. 1B). To separate *A. albipodus* from *A. varipes*, forward primer Ava-FA was designed based on the ITS2 DNA sequence of *A. varipes*. Adenine is the only

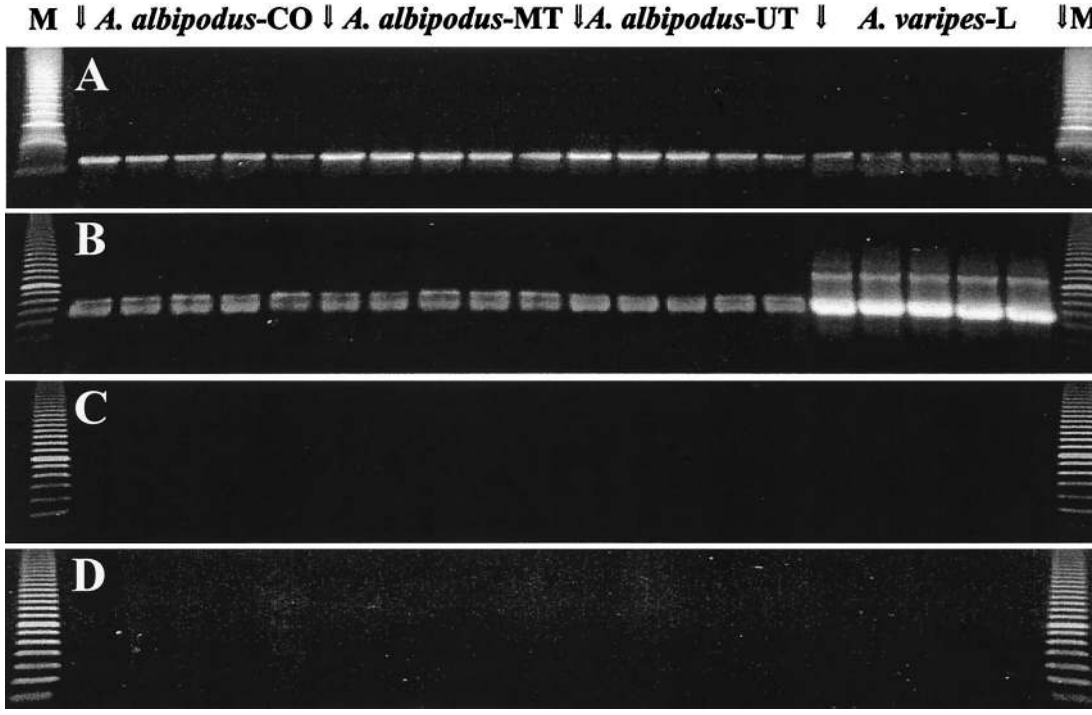

Fig. 3. Primer tests on additional 3 *A. albipodus* and 1 *A. varipes* populations. (A) 28S-F and 58S-R. (B) 58S-F and Aalv-R. (C) Ava-FA and Aalv-R. (D) Aas-F and 28S-R. M, DNA size marker: 200 bp DNA marker from Promega in A, and 100 bp DNA marker from Pharmacia in B–D; -CO, Colorado population; -MT, Montana population; -UT, Utah population; -L, Lattes-Juvignac strain.

potential nucleotide that can be placed at the 3′ end of the primer to produce a perfect A/T match between the primer and the DNA of *A. varipes*, while producing an A/G mismatch between the primer and the DNA of *A. albipodus.* PCR amplification using primer Ava-FA and the common primer Aalv-R yielded a 300 bp fragment only from *A. varipes:* no corresponding fragment was produced from any other wasp or aphid examined (Fig. 1C).

Forward primer Aas-F was designed from the ITS2 DNA sequences of both Chinese and Antibes strains of *A. asychis.* PCR amplification using this primer with the common primer 28S-R produced a 202 bp fragment only from the 2 strains of *A. asychis*, and no corresponding fragment was produced from *A. albipodus*, *A. varipes*, *S. graminum*, or *D. noxia* (Fig. 1D). To separate the Antibes and Chinese strains of *A. asychis*, the reverse primer Aas-CRG was designed based on the ITS2 DNA sequence of the Chinese strain. Guanine is the only potential nucleotide that could be placed at the 3′ end of the primer to produce a G/A mismatch between the primer and the DNA of the Antibes strain of *A. asychis*, and this resulted in a dramatic reduction of PCR product yield. Primer Aas-CRG was not perfectly matched with DNA of the Chinese *A. asychis:* this particular primer/DNA match resulted in no significant reduction of PCR product. PCR amplification using primer Aas-CRG and the common primer 58S-F yielded a 495 bp fragment only from Chinese *A. asychis*, and no corresponding fragment was produced from any other wasp or aphid examined (Fig. 1E).

PCR amplification using primers 58S-F and 28S-R yielded expected ≈530 bp fragments from all individual wasps of all the Kazakhstan and Montpellier *A. asychis* strains and the *A. albipodus* population recovered from California (Fig. 2A). Approximately 480 bp fragments were amplified using primers 58S-F and Aalv-R only from individuals of California *A. albipodus*, and no corresponding fragment was produced from wasps of Kazakhstan and Montpellier *A. asychis* (Fig. 2B). No fragment was amplified from any of these wasps using primers Ava-FA and Aalv-R (Fig. 2C). These results indicate that those primers are specific for *A. varipes*, and that the California population of *A. albipodus* behaves in this assay like the Tacheng, China, strain from which it was derived (Prokrym et al. 1998a). By using primers Aas-F and 28S-R, a 202 bp fragment was amplified from each wasp of both the Kazakhstan and Montpellier strains of *A. asychis*, and no corresponding fragment was produced from the California *A. albipodus* (Fig. 2D). No DNA fragment was produced in PCR amplification using primers 58S-F and Aas-CRG (Fig. 2E), indicating that the Kazakhstan and Montpellier strains of *A.*

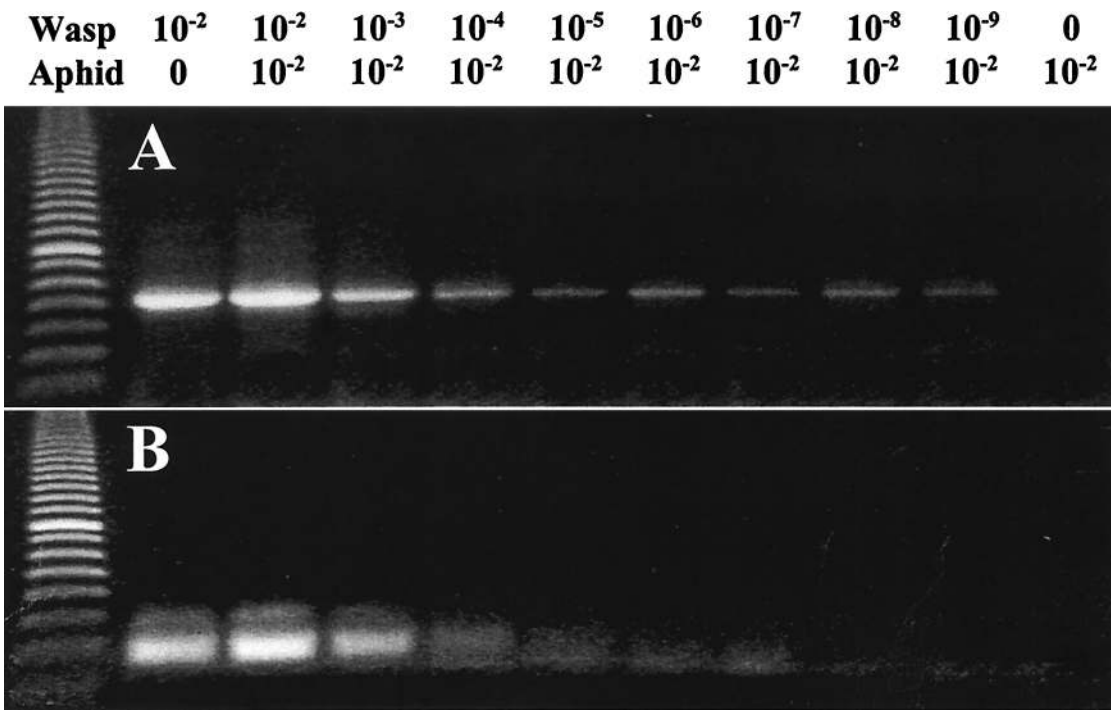

Fig. 4. PCR amplification of aphid DNA spiked with different amount of wasp DNA. (A) PCR amplification using primer 58S-F and 28S-R. (B) PCR amplification using primer Aas-F and 28S-R. Wasp = amount of DNA in adult wasp DNA equivalent of Antibes strain *A. asychis;* Aphid = amount of DNA in adult aphid DNA equivalent of Russian wheat aphid. Left-most column is 100 bp DNA marker from Pharmacia.

asychis have somewhat different ITS2 sequences than the Chinese strain.

The 3 additional populations of *A. albipodus* and the Lattes-Juvignac strain of *A. varipes* were subjected to PCR amplification using the ITS2 primers. An expected fragment of ≈530 bp was produced from 58S-F and 28S-R priming PCR amplification of individual wasp DNA of all 4 groups (Fig. 3A). Approximately 480 bp fragments were also amplified from all 4 groups using primer 58S-F and Aalv-R (Fig. 3B). PCR amplification using primers Ava-FA and Aalv-R (Fig. 2C) yielded no detectable product from any individual (Fig. 3C). By using primers Aas-F and 28S-R, no 202 bp fragment was amplified from any wasp of 3 *A. albipodus* and 1 *A. varipes* populations of *Aphelinus* (Fig. 3D).

Failure to amplify a ≈300 bp fragment from *A. varipes* using primers Ava-FA and Aalv-R indicates that the Lattes-Juvignac and Montpellier strains of *A. varipes* differ in their ITS2 sequences. These anomalous results prompted us to reexamine the morphology of all our *A. varipes* and *A. albipodus* collections in light of the current taxonomic consensus (Prokrym et al. 1998b). We found that whereas the other collections all conformed to the consensus, the Lattes-Juvignac *A. varipes* collection combined mostly *A. varipes* characters (ovipositor difficult to see, hind tibiae with color, abdomen uniform in color) with the *A. albipodus* character, male 3rd antennal funciular segment 1.5–2 times longer than broad.

To test primer sensitivity, a variable amount of wasp DNA was added to a constant concentration of *D. noxia* DNA to simulate a homogenate of a parasitized host. A fragment with the expected size was produced only from samples spiked with wasp DNA and a positive wasp DNA control. No corresponding fragment was amplified from the aphid DNA control. PCR amplification using primers 58S-F and 28S-R yielded expected fragments that could be visually differentiated from negative controls at and above a concentration of 10^{-3} adult wasp DNA equivalent (Fig. 4A). Trace DNA amplifications were observed when wasp DNA was reduced to below 10^{-4} adult wasp DNA equivalent. Similar results were obtained in the PCR amplifications using primers Aas-F and 28S-R (Fig. 4B). Both PCR amplifications were repeated with different batches of DNAs, and the results were repeatable. From these data we are confident that our current PCR system can detect as little as 10^{-3} adult wasp DNA equivalent, and it may be possible to detect even lower wasp DNA amounts in aphid DNA mixtures by adjusting PCR conditions or adding secondary PCR amplification.

Detection of parasitism was tested on *D. noxia* collected at 24 and 48 h after initial exposure to Antibes *A. asychis.* DNA was extracted from 10 aphids parasitized for 24 h and from 10 aphids parasitized for 48 h. PCR amplification was performed using primers Aas-F and 28S-R. Results (Fig. 5) indicate that wasp DNA is detectable as early as 24 h. No corresponding fragment

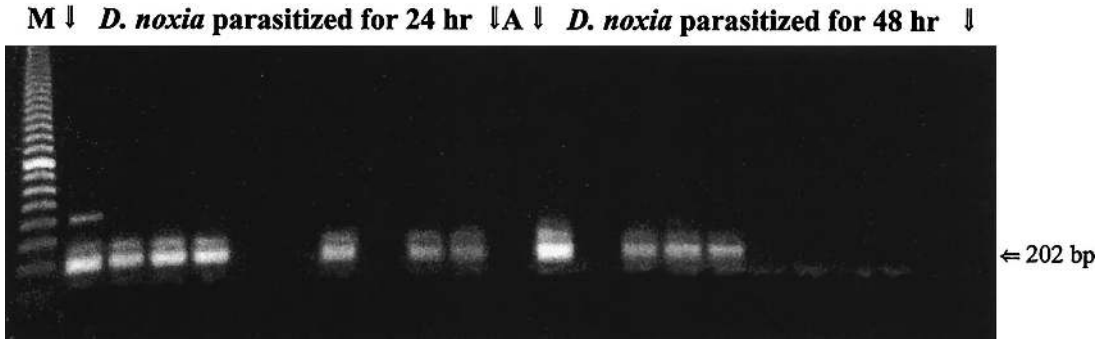

Fig. 5. PCR amplification for detecting parasitoids within Russian wheat aphids collected 24 and 48 h after initial exposure to Antibes *A. asychis* female wasps. M, 100 bp DNA marker from Pharmacia; A, aphid DNA control.

was amplified from the nonparasitized aphid DNA control. Seven of 10 aphids collected 24 h after initial exposure to wasps were determined as parasitized, and 4 of 10 aphids collected 48 h after initial exposure were determined as parasitized.

Discussion

Detection of aphelinid parasitism is made challenging by the small size of both host and parasitoid. Early detection by allozyme electrophoresis of aphelinid wasps within aphids was attempted by Walton et al. (1990). First-instar parasitoids were not detectable until 84 h after removal of female wasps, and electrophoretic detection of wasp larvae was 24 h behind detectability by dissection.

PCR amplification using specific primers allows rapid and accurate early detection of aphelinids. In this pilot experiment, we used traditional overnight DNA precipitation. Many commercial kits allow finishing DNA purification within 2 h. The whole PCR detection processes could be completed in <8 h, including 3–4 h for the PCR reaction and 1–2 h for agarose gel electrophoresis. The only other available technology, holding aphid mummies in the laboratory to await parasitoid emergence, does not enable parasitoid identification when large numbers of mummies fail to produce parasitoids (Wraight et al. 1993).

The ability to process large numbers of aphids in this fashion will facilitate research on the impact of aphelinids on cereal aphid populations, while contributing to the continuing evaluation of the classical biological control program against the Russian wheat aphid. More work is needed simply to determine the importance of aphelinids in suppressing cereal aphid populations. In France, where the Russian wheat aphid is attacked by *A. asychis*, *A. varipes*, and 2 aphidiid species, total parasitism seldom exceeds 20% and is usually <10% (Hopper et al. 1995, Chen and Hopper 1997). In Idaho, <1% of the populations of 4 species of cereal aphids and only17% of mummies collected from a variety of irrigated grains yielded aphelinid adults, and aphidiids appeared to be more important in suppressing aphid populations (Feng et al. 1992).

Aphelinids could become more important now that *A. albipodus* has been successfully established by the Russian wheat aphid classical biological control program (Prokrym et al. 1998a) and as mixed cropping systems, which provide more opportunities for retaining parasitoids on alternative hosts, gain favor (Dhuyvetter et al. 1996, Holtzer et al. 1996, Peterson et al. 1996). The techniques described here will be useful in documenting the extent of and any changes in aphelinid parasitism of cereal aphids. We look forward to testing our primers on additional distinct populations of these aphelinids, as they become available.

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