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Quick detection of *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) in chestnut dormant buds by nested PCR

Chiara Sartor, Daniela Torello Marinoni, Roberto Botta

University of Turin Department of Arboriculture and Pomology, Via Leonardo da Vinci 44, Grugliasco 10095 (TO), Italy

KEY WORDS

Gall wasp, Castanea, DNA, 28S, diagnosis

ABSTRACT

Dryocosmus kuriphilus Yasumatsu (Hymenoptera: Cynipidae) lives in chestnut asymptomatic buds from oviposition (June-July) until budburst. It is thus easily spread by plant material used in propagation. It is thus of interest to identify infested plant batches before their release. Unfortunately a non-destructive method for checking buds has not yet been developed and the only technique available is the screening of a buds sample. The visual investigation is long and requires highly skilled and trained staff. The purpose of this work was to set up an effective and fast method able to identify the presence of the larva at first instar in a large number of buds by PCR. Four primer pairs were designed on nuclear and mitochondrial sequences of a set of seven gall wasp taxa and tested on the insect DNA. The 28S sequence was chosen for nested PCR carried out on DNA extracted from samples of 2 g buds simulating four levels of infestation (larvae were added to uninfested buds). The marker was able to detect 1 larva out of 2 g buds and the method showed a potential efficiency of 5000 to 15000 buds per week, depending on bud size.

INTRODUCTION

In spring 2002 the presence of the insect *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) was reported in chestnut groves and forest of Cuneo Province (Piedmont Region, Italy). After 9 years, the cynipid is spread all over Italy and was found in France and Slovenia. This gall-wasp is indigenous to China and was previously introduced into Japan (1941), Korea (1963) and USA (1974) where it caused serious yield losses to chestnut. *D. kuriphilus* is a univoltine and thelytokous species; it lays eggs in chestnut buds in summer. At the time of budburst the larva induces the formation of greenish-red, 8–15 mm large galls, which develop in mid-April on new shoots. Gall development suppresses shoot elongation, reduces fruiting, and causes twig dieback. Severe infestation can result in mortality of young trees (Payne *et al.,* 1975).

Since buds remain asymptomatic until the following spring, it is impossible to note the presence of the cynipid in the period between oviposition and budburst, before the gall formation. Yet, a rapid and unequivocal identification of *D. kuriphilus* presence in dormant buds is very useful to stop infected material before cynipid leak and to reduce the spread of the infestation. Larvae detection can be done by cutting buds and searching for their presence at the stereomicroscope; the procedure is time consuming and often inaccurate.

The development of an efficient and reliable technique able to detect the insect in dormant buds would be highly valuable and useful for the application of protective measures against the spread in the European Community of this harmful organism, in compliance to European Communities Decision 2006/464/EC stating that “Member States shall conduct official annual surveys for the presence of the organism or evidence of infestation by the organism in their territory”.

Polymerase chain reaction (PCR) analysis of species-specific mitochondrial DNA and ribosomal nuclear sequences is currently the most commonly used method for species identification. Eukaryotic nuclear rDNA is tandemly organized with high copy numbers up to ca. 5,000. Each repeat unit consists of genes coding for the nuclear small subunit (18S), large subunit (25-28S), and 5.8S rDNAs. (Hwang and Kim 1999).

The mtDNA of multicellular animals consists of a closed circular DNA molecule. It ordinarily contain 36 or 37 genes: 2 for ribosomal RNAs (16S rRNA and 12S rRNA), 22 for tRNAs and 12 or 13 for subunits of multimeric proteins of the inner mitochondrial membrane (cytochrome oxidase, ATP synthase, NADH dehydrogenase and cytochrome b apoenzyme. (Wolstenholme, 1992). Mitochondrial DNA is known to evolve much faster than the nuclear genome; 12S rDNA, however, is highly conserved like the nuclear small subunit (18S) rDNA, and it has been employed to illustrate phylogeny of higher categorical levels such as in phyla or subphyla (Ballard et al., 1992). Compared to the nuclear rDNA, it is more difficult to design universal primers for amplifying specific regions in mtDNA due to a high variability. This is why only a few mitochondrial genes such as 12S rDNA, 16S rDNA, Cytb, ND1 and COI have been employed in phylogenetic studies.

All information considered, we tested four primers pairs designed on nuclear and mitochondrial sequences of a set of seven gall wasp taxa, in order to develop a simple method based on PCR reaction. The goal was to identify the presence of *D. kuriphilus* larva at first instar in dormant buds.

MATERIAL AND METHODS

Multiple alignment and primer design

All available full-length nuclear and mitochondrial sequences of 18S, 28S, COI and Cytb of gall wasp taxa (Table 1) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and aligned by ClustalW (Thompson *et al*., 1994).

Four primers pairs were designed on conserved regions; sequences and expected amplified fragment length are reported in table 2.

Sample preparation

D. kuriphilus DNA was obtained from a single larva, fresh or stored in 70% ethanol, by crushing it in 40 µl of TE buffer inside a well of an ELISA plate. The suspension was transferred into a 1.5 ml Eppendorf tube, briefly sonicated (2 min) in an ultrasonic bath, and then boiled for 5 min; a final centrifugation (1 min at 8000 rpm) was done to precipitate insect debris.

To test the sensitivity of the technique and to exclude the possibility that false positives may occur, the following levels of infestation were simulated by adding the larvae to uninfested buds before crushing tissues for DNA extraction:

1) 2 g. of buds without larvae

2) 2 g. buds with one larva

3) 2 g. buds with two larvae

4) 2 g. buds with five larvae

The buds were collected from chestnut plants grown in pots under screen house with insect-proof barriers, and then added with larvae at the first instar which is the same developmental stage they are found in dormant buds.

DNA was extracted with EZDNA Plant Maxi Kit (Omega Bio-Tek, Norcross, Georgia).

Direct and nested PCR

The 4 primer pairs (18S, 28S, COI, Cyt b), designed as described above were initially tested for amplification using the pure *D. kuriphilus* DNA. Insect universal primers for the 12S DNA (Simon *et al.*, 1991) were used as control.

Five µl of the extracted DNA (about 50 ng/ µl) were amplified in a 20µ1 direct PCR using 0.5 units of Taq polymerase (Bioline, London UK) per reaction. Each 20 µ1 reaction consisted of 2 µl of buffer 10X, 0.9 µl of $MgCl_2$ 50 mM (both solutions supplied with the polymerase), 1 µl of forward and 1 µl of reverse primer (20 pM/µl), 0.2 µl nucleotide mix (20 mM), 0.5 Unit of BIOTAQ polymerase (Bioline, London, UK) and 9.8 µl sterile distilled water. After 3 minutes at 95°C, DNA fragments were amplified through 37 cycles at the following steps: 30" at 95°C, 45" at 55°C and 1 min. at 72°C; a final extension step was carried out at 72°C for 10 min. In the case of DNA extracted from buds, 2 µl of the amplification product was then used as the template for the nested PCR, carried out for 28 cycles at the same conditions as the direct PCR.

Amplification products were run on 1% agarose gel and visualized with a UV transilluminator, after ethidium bromide staining.

RESULTS AND DISCUSSION

All 4 primers pairs and the control ones produced amplicons with *D.kuriphilus* DNA, but only those designed on 28S gave an excellent result with a single fragment of the expected length. This may be due to better conservation of nuclear than mitochondrial DNA.

Consequently, this primer pair was chosen to amplify the bud DNA with the four different levels of infestation. Results of nested PCR were visualised with ethydium bromide on agarose gel (fig.1) and showed no amplification in buds without larvae (first lane) and increasing signal intensity in the three samples with increasing level of infestation (one, two five larvae in lane 2 to 4). The fragment length corresponded to the size of the amplicon from the pure insect DNA (fifth lane).

The proposed technique can quickly detect the presence of genetic material belonging to the cynipid in the total DNA extracted from buds. The use of the 28S primer pair can thus reliably detect *D. kuriphilus* since it is the only insect found in chestnut buds in winter. In a basic laboratory, equipped with a normal centrifuge (x 8 vials) and a thermal cycler, one unskilled operator can safely process up to 16 g per day of buds. This quantity corresponds to a number of buds which can vary from 480 to 1280 approx., depending on the cultivar. Optimising the extraction step by performing more sets of extractions/day, it is possible to process up to 192 g of buds in 5 days. The current method is the bud check at the stereomicroscope; it is able to process approx. 200 to 500 buds/day depending on the required precision level.

This technique could help phytosanitary services to contain *D. kuriphilus* diffusion, speeding up the analysis and identifying the insect at its first larval instar.

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TABLES

ACCESSION	GENE	SPECIES	REFERENCE
AF395137.1	Cytb	*Synergus gallaepomiformis* (Fonscolombe)	Rokas *et al*. 2002
AF395139.1	Cytb	*Barbotinia oraniensis* (Barbotin)	Rokas *et al*. 2002
AF395140.1	Cytb	*Panteliella bicolor* (Ionescu & Roman)	Rokas *et al*. 2002
AF395141.1	Cytb	*Periclistus brandti* (Ratzeburg)	Rokas *et al*. 2002
AF395142.1	18S	*Diplolepis rosae* (Linnaeus)	Rokas *et al*. 2002
AF395143.1	18S	*Synergus gallaepomiformis*	Rokas *et al*. 2002
AF395145.1	18S	*Andricus curvator* (Hartig)	Rokas *et al*. 2002
AF395147.1	18S	*Barbotinia oraniensis*	Rokas *et al*. 2002
AF395148.1	18S	*Panteliella bicolor*	Rokas *et al*. 2002
AF395149.1	18S	*Periclistus brandti*	Rokas *et al*. 2002
AF395150.1	28S	*Barbotinia oraniensis*	Rokas *et al*. 2002
AF395151.1	28S	*Synergus gallaepomiformis*	Rokas *et al*. 2002
AF395152.1	28S	*Periclistus brandti*	Rokas *et al*. 2002
AF395153.1	28S	*Panteliella bicolor*	Rokas *et al*. 2002

AF395154.1	28S	*Plagiotrochus quercusilicis* (Fabricius)	Rokas *et al*. 2002
AF395155.1	28S	*Andricus curvator*	Rokas *et al*. 2002
AF395157.1	28S	*Diplolepis rosae*	Rokas *et al*. 2002
AF395174.1	COI	*Diplolepis rosae*	Rokas *et al*. 2002
AF395177.1	COI	*Andricus curvator*	Rokas *et al*. 2002
AF395179.1	COI	*Barbotinia oraniensis*	Rokas *et al*. 2002
AF395180.1	COI	*Panteliella bicolor*	Rokas *et al*. 2002
AF395181.1	COI	*Periclistus brandti*	Rokas *et al*. 2002
AJ228453.1	Cytb	*Andricus curvator*	Stone and Cook 1998
DQ012610.1	28S	*Synergus gallaepomiformis*	Unpublished
DQ012621.1	COI	*Andricus curvator*	Unpublished
DQ012652.1	COI	*Synergus gallaepomiformis*	Unpublished
DQ217993.1	Cytb	*Andricus curvator*	Unpublished
DQ218030.1	Cytb	*Plagiotrochus quercusilicis*	Unpublished
DQ218031.1	Cytb	*Plagiotrochus quercusilicis*	Unpublished
DQ218032.1	Cytb	*Plagiotrochus quercusilicis*	Unpublished
DQ218033.1	Cytb	*Plagiotrochus quercusilicis*	Unpublished

Tab.1: Accessions retrieved from GenBank

CODE	**SEQUENCE**	**EXPECTED AMPLIFIED FRAGMENT LENGTH**
18S F	GTACAAAGGGCAGGGACGTA	399 bp

18S R	ATGGCCGTTCTTAGTTGGTG	
COI F	ACCCCCTCCTATTGGATCA	410 bp
COI R	CCTGATATAGCTTTCCCTCGAT	
28S F	CGCACCTCCAGGATAACACT	320 bp
28S R	CAAAAGATCGAATGGGGAGA	
Cyt b F	TCAGGTTGGATATGAATTGGTG	337 bp
Cyt b R	GAAATTGTAATTTGATTATGAGGAGGA	

Tab. 2: Primer sequences and expected amplified fragment length

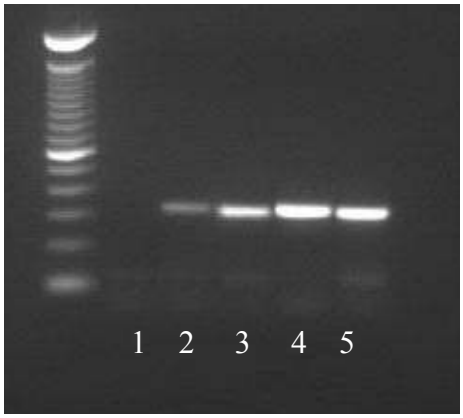

Fig. 1: 28S electrophoretic profile (1: no larva, 2: 1 larva, 3: 2 larvae, 4: 5 larvae, 5: *D. kuriphilus*)