Molecular Ecology 1995, 4, 777–780

SHORT COMMUNICATION

Molecular identification of six species of scale insects (*Quadraspidiotus* sp.) by RAPD-PCR: assessing the field-specificity of pheromone traps

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Abstract

The San-José Scale *Quadraspidiotus perniciosus* (SJS) is a quarantine pest in Switzerland. Its occurrence is monitored by trapping males on glue traps treated with artificial female sex pheromone that is supposed to be species-specific. However, large numbers of males were caught in locations where, for ecological reasons, this species was not expected to occur, suggesting that the pheromone is attractive for one or several other scale species. Because no morphological species determination key is described for males of this genus, it was not possible to test this hypothesis until now. We used randomly amplified polymorphic DNA (RAPD-PCR) to establish a molecular identification key for six European *Quadraspidiotus* species. Because the glue used on the traps proved to be an excellent preservative, this key enabled us to identify males caught on pheromone traps in the field and to assess the species-specificity of the SJS-pheromone.

Keywords: Diaspididae, pheromone specificity, polymerase chain reaction, RAPD markers, species identification, taxonomy

Received 9 January 1995; revision received 12 June 1995; accepted 21 June 1995

Introduction

In the genus *Quadraspidiotus* MacGillivray (Homoptera: Diaspididae), a species determination key based on morphological characters exists only for females (Kosztarab & Kozár 1988). Due to the low degree of differentiation, no general determination key has been published so far for males. The genus *Quadraspidiotus* is represented in Switzerland by eight species (Kosztarab & Kozár 1988). One of them, the San José Scale *Q. perniciosus* (Comstock) (SJS), is a quarantine pest in Switzerland and its occurrence is monitored by pheromone-treated sticky traps, on which only winged males are captured. Because the chemical compounds used for the pheromone-mimic were isolated from SJS females (Gieselmann *et al.* 1979; Anderson *et al.* 1981), it was assumed that these SJS-pheromone traps are species-specific (Smetnik *et al.* 1986). However, high trap catches indicated the presence of large populations of SJS in several locations in Switzerland where, for ecological reasons, this species was not expected to occur. Indeed, even the most intensive search for infested host plants was unsuccessful (E. Mani, personal communication). Based on these facts and on morphometric population data for *Quadraspidiotus* species (Kozàr *et al.*, in preparation), it was concluded that the traps at these locations had attracted another species, *Q. zonatus*, and that the pheromone used in these traps was not species-specific (Hippe *et al.*, in preparation). However, due to the lack of a determination key for males, this could not be verified until now.

We present a molecular identification key for six *Quadraspidiotus* species based on RAPD-PCR. We chose this technology mainly because these species are extremely small and because no information on the genome of *Quadraspidiotus* was available. Also, the specimens caught on pheromone traps were dead which made the use of biochemical methods impossible. All studied species are oligophagous or polyphagous herbivores of the genus *Quadraspidiotus*, living mostly on trees and shrubs (Kosztarab & Kozár 1988). *Q. pyri* mainly occurs on abundant host trees like *Aesculus*, *Betula*, *Carpinus*, *Crataegus*, *Malus* and *Populus*. SJS is polyphagous, attacking 239 host plant genera, most of which belong to the Rosaceae. It is an important fruit and ornamental tree pest in temperate regions, where it often attacks apple, pear, plum, peach,

red currant, gooseberry, rose and hawthorn. *Q. ostreaeformis* (Curtis) is reported from 48 plant genera and can reach high population densities. Its host range is widely overlapping with that of *Q. pyri*, and it is often found on fruit trees. *Q. marani* (Zahradnik) attacks *Crataegus, Fraxinus, Malus, Prunus, Pyrus* and *Sorbus*. It often appears as an orchard pest. *Q. gigas* only rarely appears as a pest. It mainly occurs on *Populus, Salix* and *Tilia*. *Q. zonatus* (Frauenfeld) is a common Palaearctic species that generally occurs on *Fagus* and *Quercus* but is reported from nine other host plant genera.

Our molecular identification key was successfully used to identify specimens of all six species caught on sticky traps in the field, and to answer the question on species-specificity of the SJS-pheromone.

Material and methods

Specimen and storage conditions

To study interpopulation variation of amplification patterns, specimens of SJS were collected from two different populations located *c.* 5 km apart near Zug, Switzerland, and from one population near Bern, Switzerland. Another population was sampled in Budapest, Hungary. The amplification patterns observed in these populations were compared to those produced with individuals from the laboratory culture at the Federal Research Station. *Q. gigas* was collected from *Populus* in Budapest, Hungary. The other species were collected in Switzerland: *Q. pyri* in Landquart from *Pyrus*, *Q. ostreaeformis* in Güttingen from *Malus*, *Q. marani* in Uttwil from *Malus*, and *Q. zonatus* in Fougères from *Quercus*.

We used specimens stored in alcohol, frozen, dry at room temperature, and on sticky traps. Males of the laboratory strain of SJS were used to screen for primers. The amplification patterns of good primer candidates were compared to those obtained from females and larvae of the same strain. For the other species, adult females collected in the field were identified morphologically and the amplification patterns compared to those of adult males caught on traps, and to those of larvae from the same populations.

DNA extraction procedure of single scales

DNA of single individuals was extracted using a DNA extraction protocol modified after Kawasaki (1990). Individual males or larvae were homogenised in 15 μL of lysis buffer (10-mM Tris-HCl, pH 8.0, 1-mM EDTA, 0.5% Tween 20, 100 μg/mL Proteinase K (Appligene) in a 500 μL microfuge tube using a sterile wooden toothpick. 15 μL of ddH_2O were added to make 30 μL final extraction solution

Primer	Sequence	PY	PE	OS	MA	GI	ZO
OPE-15	ACGCA	1900	1100	1910	1620	845	1050
	CAACC	820	870	1520	1100	680	970
		735	735	910	970	585	795
		660	480	795	660	330	700
		515	420	720	535		535
		490	320	610	420		460
		420	285	330			
OPF-02	GAGGA	950	715	1505	950	910	790
	TCCCT	745	585	1020	835	585	730
		520	365	635	745	485	635
		350	190	545	520	190	360
OPF-09	CCAAG	950	900	950	755	670	1590
	CTTCC	670	810	535	575	615	1170
		470	595	470	535	535	900
		340	470	340	470	455	500
		300	420	185	270	420	440
OPF-12	ACGGT	745	825	1320	1110	765	1015
	ACCAG	565	765	1110	1015	600	645
		475	520	665	600	520	490
		360	475	400	360	475	305
MIC-ECO	ATGAA	1110	885	1110	1805	425	760
	TTCGC	1045	695	1045	1045	340	615
		975	360	975	695		305
		510	260	510	490		
		425		425	425		

Table 1 DNA fragment sizes (base pairs ± *c.* 2%) for each species and primer, and sequence of each primer. Only the fragments involved in species identification are tabulated for each species. PY = *Quadraspidiotus pyri*; PE = *Q. perniciosus*; OS = *Q. ostreaeformis*; MA = *Q. marani*; GI = *Q. gigas*; ZO = *Q. zonatus*.

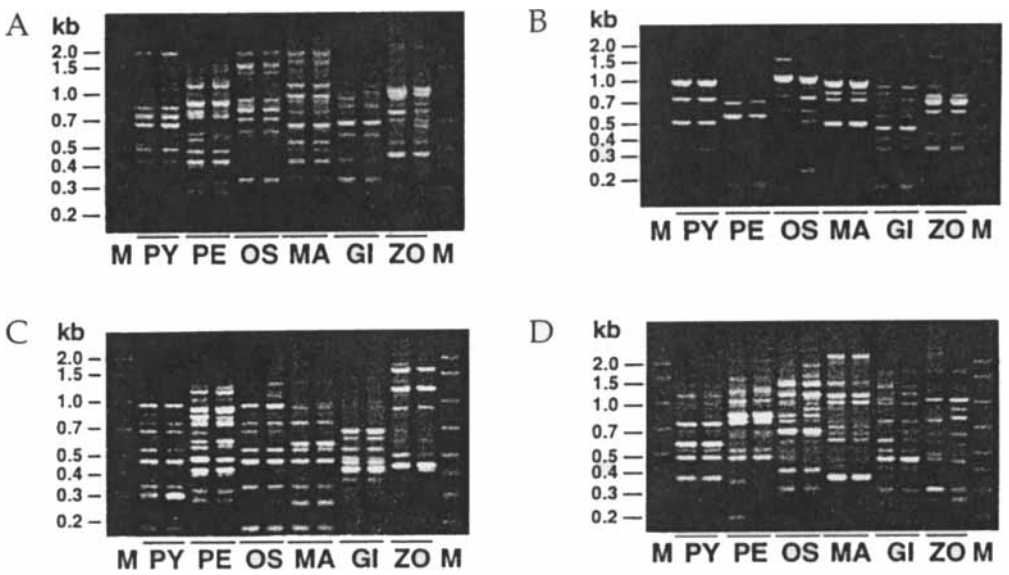

Fig. 1 Amplification patterns of two individuals of each species produced with (a) OPE-15, (b) OPF-02, (c) OPF-09, (d) OPF-12. M = DNA size marker, PY = *Quadraspidiotus pyri*, PE = *Q. perniciosus*, OS = *Q. ostreaeformis*, MA = *Q. marani*, GI = *Q. gigas*, ZO = *Q. zonatus*.

(100 μL for the larger females), and the tube was heated immediately for 10 min at 85 °C. The DNA was kept at –20 °C; it was stable for at least 12 month. DNA was quantified by extracting single individuals in 2 × TNE (20-mM Tris, pH 7.4, 20-mM EDTA, 2-M NaCl) using a TKO 100 fluorometer (Hoefer). On average, 80–160 ng DNA per individual was obtained with this DNA extraction method. Three μL of the extraction solution corresponding to *c.* 1–5 ng template DNA was used for amplification.

Polymerase chain reaction and analysis of fragment pattern

RAPD-PCR was performed as described by Williams *et al.* (1990) with the following specifications: The total reaction volume was 50 μL, containing 10-mM Tris-HCl, pH 8.3, 50-mM KCl, 1.5-mM $MgCl_2$, 0.01% gelatine, 200-μM dNTPs, 1.7 units of Taq polymerase, 1–5 ng template DNA and 0.5 μM of a single 10mer primer (Operon Technologies, Alameda CA, USA, with prefix OP; Microsynth, Windisch, Switzerland, MIC-ECO [Black *et al.* 1992]). The DNA was amplified on a Crocodile-II thermal cycler (Appligene; halogen lamp heating unit) using the following protocol: 3 min denaturation at 93 °C, then 45 cycles of 1 min at 93 °C, 1.5 min annealing at 38 °C, and 2 min extension at 72 °C. The amplification was completed by a final 7 min extension step at 72 °C. Twelve microlitres of the amplification product was electrophoresed on a 1.5% agarose gel at 9 V/cm for 60 min. The gels were photographed under UV light, the picture scanned and loaded into the program NCSA GelReader v.2.0.5 (National Centre for Supercomputing Applications, University of Illinois, Urbana-Champaign, IL, USA; public domain software) for calculation of fragment sizes.

Results

We screened a total of 44 primers for reproducible species-specific amplification patterns with low intraspecies variation. Many primers showed a 'fingerprint' pattern, i.e. extensive variation between conspecific individuals, while others did not produce any amplification product (data not shown). Table 1 lists the five primers we found to be most useful for species identification, together with the sizes of those amplified fragments that were used for species identification. Each of four of these primers individually distinguished all six species (Fig. 1a-d), and one other distinguished five of the six species (MIC-ECO;

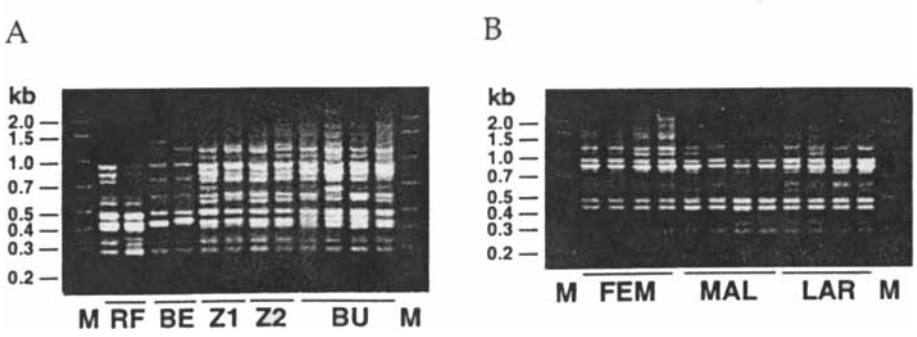

Fig. 2 Amplification patterns, produced with primer OPE-15, of (a) 12 *Q. perniciosus* males from different populations, RF = laboratory culture; BE = Bern, Z1 = Zug, population 1, Z2 = Zug, population 2 (all in Switzerland), BU = Budapest (Hungary); (b) 12 *Q. perniciosus* individuals of different sex and developmental stage; FEM = females, MAL = males, LAR = larvae.

Table 1). The major amplification products of these primers showed no intraspecific differentiation, although inter- and intrapopulation variation was observed in minor, mostly weak amplification products. The patterns of the major amplification products were identical within all tested populations, and no geographical variation was observed between populations as far as 1000 km apart (Fig. 2a). Furthermore, the same amplification patterns were observed for larvae, males and females within one species (Fig. 2b).

The same amplification patterns can be produced from dried specimens, from specimens stored in 70% ethanol, and from specimens trapped in insect glue. In general, any combination of at least two amplification products of a single primer can be used for accurate species identification (see Table 1, Fig. 1; primer MIC-ECO can not distinguish between *Q. pyri* and *Q. ostreaeformis*). In rare cases, not all fragments typical for a specific primer may appear, probably due to low DNA concentrations and/or low DNA quality. In such cases, using a second primer will still enable correct species identification, especially if electrophoresed alongside with control DNA of a known species. Problems were encountered only with dried specimens that were contaminated by high percentages of foreign DNA, such as decomposing fungi. We therefore excluded such specimens from our analysis. Negative controls (no DNA) with all five primers sometimes resulted in weak fragment patterns as described for other short primers (e.g. Hadrys *et al.* 1992). These fragments disappeared when DNA was included.

With our molecular species identification key, we were able to confirm that the SJS-pheromone trap is not entirely species-specific and that the second species attracted by this pheromone-mimic is indeed *Q. zonatus*, as was suggested earlier (Hippe *et al.*, in preparation). Only very low numbers of other species were found on few traps. We found that the glue used in the pheromone traps (Tanglefoot®: registered trademark of 'The Tanglefoot' company, Grand Rapids, MI 49504, USA) was conserving the trapped specimens for at least two years in an excellent condition for DNA analysis, resulting in amplification patterns identical to those of fresh individuals from the laboratory culture. We found that this sampling method is also suitable for other small insects, making it very useful for many ecological experiments where sampling can be done by traps.

Discussion

The use of RAPD-PCR allowed us to establish for the first time a species identification key for males of six *Quadraspidiotus* scale species. This was an essential prerequisite to test the specificity of the pheromone traps used to monitor occurrence and distribution of the important orchard pest species San-José Scale (SJS), *Q. perniciosus*, in the field.

Our results show that the pheromones currently used for SJS-monitoring are attractive for a second species of the genus *Quadraspidiotus*, namely *Q. zonatus*. Despite this fact, our results do not jeopardize the use of these pheromone traps for monitoring SJS in Switzerland. In this country, *Q. zonatus* is univoltine and males fly in late August and September, whereas SJS is bivoltine with the first period of male flights occurring in late April and May, and the second between July and September. Therefore, the period of potential interference of *Q. zonatus* males with SJS trap catches is restricted to the second phase of the second generation of this species (Hippe *et al.*, in preparation). Thus, if used only before mid-August, i.e. in the first to the first half of the second generation, the SJS-pheromone trap is a reliable monitoring tool to assess the occurrence and distribution of the San-José Scale.

Acknowledgements

We thank E. Mani, F. Kozár, F. Schwaller, C. Hippe, and C. Baroffio for many discussions and for providing the samples, and S. Mazza and C. Baroffio for laboratory assistance. E. Mani, C. Hippe and U. Hilber gave valuable comments on an earlier version of this paper. This work was supported in part by the Swiss Plant Protection Fund.

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