Identifying haplotypes of *Pityogenes chalcographus* (Col., Scolytidae) by Single Strand Conformation Polymorphism (SSCP)

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Zusammenfassung: Identifizierung von Haplotypen bei *Pityogenes chalcographus* (Col., Scolytidae) durch SSCP.

Der Kupferstecher *Pityogenes chalcographus* (Coleoptera, Scolytidae) gehört zu den am weitesten verbreiteten Borkenkäfern Europas. Käferpopulationen aus verschiedenen Gebieten Europas zeigten eine hohe Divergenz hinsichtlich der Nukleotidzusammensetzung des mitochondrialen Cytochrom Oxidase I Gens. Die Unterschiede betragen bis zu 2,26% zwischen einzelnen Herkünften. Die phylogenetische Auswertung der Sequenzen des kompletten Gens (1503bp) ergab einen 6-astigen Baum mit insgesamt 34 Haplotypen. Ausgehend von dieser Struktur wurden Primerpaare entworfen, um jeweils etwa 250 bp lange mutationsreiche Abschnitte des COI Gens zu amplifizieren. Die PCR Produkte wurden mittels Single Strand Conformation Polymorphism (SSCP) untersucht. Zwischen den einzelnen Haplotypen konnten zahlreiche Polymorphismen erkannt werden. Diese eignen sich als schnelles Werkzeug zum screening einzelner Individuen und verringern signifikant die Notwendigkeit klassischer Sequenzierreaktionen.

Key words: Scolytidae, *Pityogenes*, SSCP, phylogeography, mitochondrial DNA, PAGE

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Pityogenes chalcographus is a widely distributed spruce pest in Eurasia (KNIZEK et al. 2005). In the mid 70ies, E. Führer studied the intraspecific variation of this spruce bark beetle and detected race differentiation among European populations based on crossing experiments (FÜHRER 1977), morphological characters (FÜHRER 1978) and allozyme electrophoresis (RITZENGRUBER 1990). In order to verify the hypothesis of race differentiation, we analysed diverse European *P. chalcographus* populations using the Cytochrome Oxidase I gene (COI) of the mitochondrial DNA. The complete COI gene of 96 individuals was sequenced. In order to facilitate the screening of the European populations, we applied a PCR-SSCP method. This polyacrylamide electrophoresis technique offers a sensitive but inexpensive, rapid and convenient method for detecting DNA polymorphisms, reducing the amount of samples that require sequencing (SUNNUCKS et al. 2000).

Materials and Methods

Beetles were collected from several European locations given in table 1. They were directly put into absolute ethanol and stored there until use. Based on the sequences and mutational site distributions of 96 previously examined individuals of *P. chalcographus* several forward and reverse primers located in conserved DNA stretches along the COI gene were designed. Focus was laid to amplicon sizes between 200 and 260bp and annealing temperatures close to 60°C allowing the use of different primer combinations in the same cycling procedure. Finally two primer systems PC6 and PC4 (Table 2) were selected for subsequent investigations. PCR was performed in 10µl reactions containing 0.2µM forward and reverse primer, 50µM of each dNTP, 0.8µl template DNA, 0.4U Taq DNA polymerase (Sigma) and the PCR buffer provided by the manufacturer. Cycling conditions on a PTC-100 thermocycler (MJ Reseach) comprehend an initial denaturation at 94°C for 3min followed by 33 cycles of 94°C for 40sec, 60°C for 40sec and 72°C for 1min. Final elongation time was 15min at72°C.

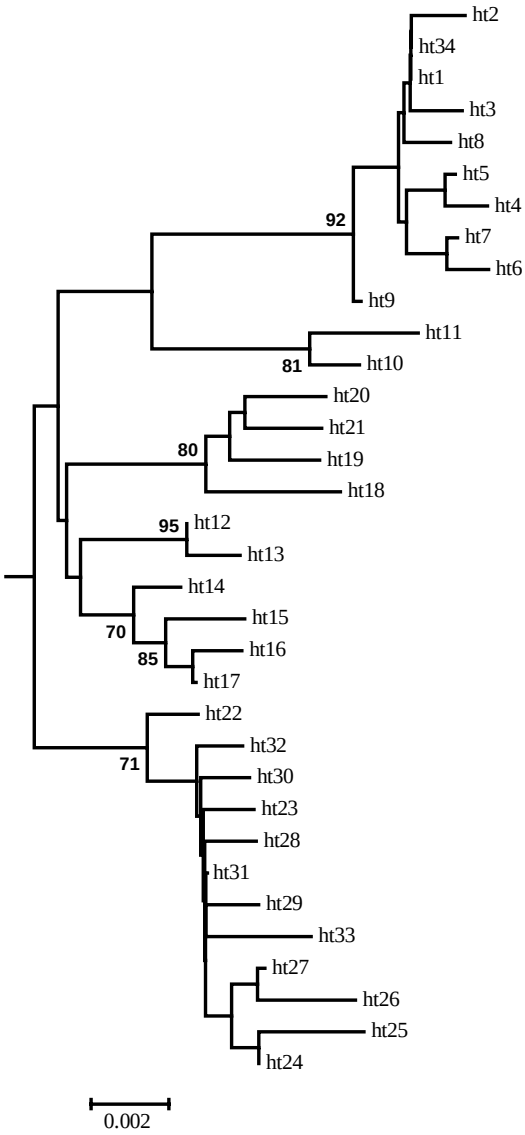

Fig. 1: Phylogenetic relationships of the 34 haplotypes of *P. chalcographus* calculated by NJ algorithm and based on the Tamura-Nei (TAMURA & NEI 1993) substitution model. *Pityogenes trepanatus* and *Pityogenes hopkinski* (AF:113395) were taken as outgroups.

PCR products were mixed with 1 vol of denaturing loading dye (96% formamide, 0.1% bromophenole blue, 0.1% xylene cyanol), incubated 5min at 95°C and immediately put on ice. 8µl of the mixture were loaded onto a gel containing 0.5x TBE, 5% glycerol, 10% acrylamide, 1% Bis and 0.2% of TEMED and APS. Electrophoresis was performed in a 200mm dual slab gel unit (C.B.S. Scientific) at 250V for 20 hrs. A constant gel temperature of 4°C for PC6 respectively 15°C for PC4 was provided by a circulating water flow. Gels were stained with 1x SYBR–Gold® (Molecular Probes) in 0.5x TBE for 15 min and visualized on a transilluminator.

A phylogenetic analysis was calculated using the Mega2 software (KUMAR et al. 2001) applying Neighbor Joining with the Tamura – Nei substitution model (TAMURA & NEI 1993).

Table 1: Information on the populations of *P. chalcographus.*

Collection site	Country	Host	Lon.	Lat.
Korgen	Norway	*Picea abies*	66°10 N	13°40 E
Eina	Norway	*Picea abies*	60°38 N	10°36 E
Kangashäkki	Finland	*Picea abies*	62°36 N	25°44 E
Rolfstorp	Sweden	*Picea abies*	57°06 N	12°17 E
Vilnius	Lithuania	*Picea abies*	54°41 N	25°19 E
Hajnowka	Poland	*Picea abies*	52°45 N	23°36 E
Bistra	Romania	*Picea abies*	46°30 N	23°10 E
Tharandt	Germany	*Picea abies*	50°59 N	13°35 E
Hofoldinger Forst	Germany	*Picea abies*	48°03 N	11°35 E
Zwardon	Slovakia	*Picea abies*	49°28 N	18°50 E
Banska Stiavnica	Slovakia	*Picea abies*	48°28 N	18°56 E
Uhlirske Janoviee	Czech Rep.	*Picea abies*	49°50 N	15°10 E
Bielersee	Swiss	*Chalcoprax*	47°10 N	7°20 E
Prinzensdorf	Austria	*Picea abies*	48°11 N	15°35 E
Kalkalpen	Austria	*Picea abies*	47°53 N	14°15E
Kärnten	Austria	*Picea abies*	46°37 N	14°37 E
Brixen	Italy	*Picea abies*	46°43 N	11°39 E
Tolmezzo	Italy	*Picea abies*	46°24 N	13°01 E
Asiago	Italy	*Picea abies*	45°52 N	11°30 E
Drama	Greece	*Chalcoprax*	41°09 N	24°08 E

Results and Discussion

COI revealed six significant clades with a maximum nucleotide divergence of 2,26% (Fig. 1). With the use of PC6 we determined three SSCP profiles that represent haplotypes of the clades I, IIa and IVa (Fig. 2a), while the application of PC4 enabled us to distinguish two more SSCP profiles (Fig. 2b) that link to haplotypes within clades IVb and IVc. A subsequent combination of the primer systems PC6 and PC4 (Table 2) achieved a sufficient resolution to assign any unknown sample directly to 5 out of 6 clades. Samples that fail to affiliate with any of these five clades, fall consequently into clade IIIb.

Table 2: Primers used in the SSCP analysis

Primer System	Sequence/F	Tm/ F	Sequence/R	Tm/ R	size of product (bp)
Pc 4	tcc aga tgc cta cct tct ctg	55,5	tcc aat gca cta atc tgc cat att a	53,8	252
Pc 6	gcc cca gat ata gca ttt cc	53,2	aat tcc tga tat atg aag gct g	49,9	200

Based on the SSCP profiles, we proceeded to the screening of populations from 20 different localities (Table 1). We analysed about 16 individuals per population. Thus out of about 322 individuals that were screened, 37% were determined after the application of PC6. These 117 samples are assigned to clades I (45,3%) and IVa (54,7%), while the gel phenotype of clade IIa was not found in any electrophoresis. The subsequent application of PC4 on the 63% of the samples that remain unidentified is required in order to determine their affiliation to the clades.

Even though, sequencing has become less expensive it remains a limiting factor in screening lots of individuals. In the analysis of *P. chalcographus*, the identification of 34 haplotypes encouraged us to turn to SSCP. Although this technique has been widely applied in biomedical research, only few studies have been published in entomological research (MARQUEZ & KRASFUR 2003). For the screening of European *P.*

chalcographus populations, this technique proved to be fast and highly sensitive to detect all the haplotypes within the single clades. Thus we will apply this technique now to detect the genetic basis of race differentiation within that species.

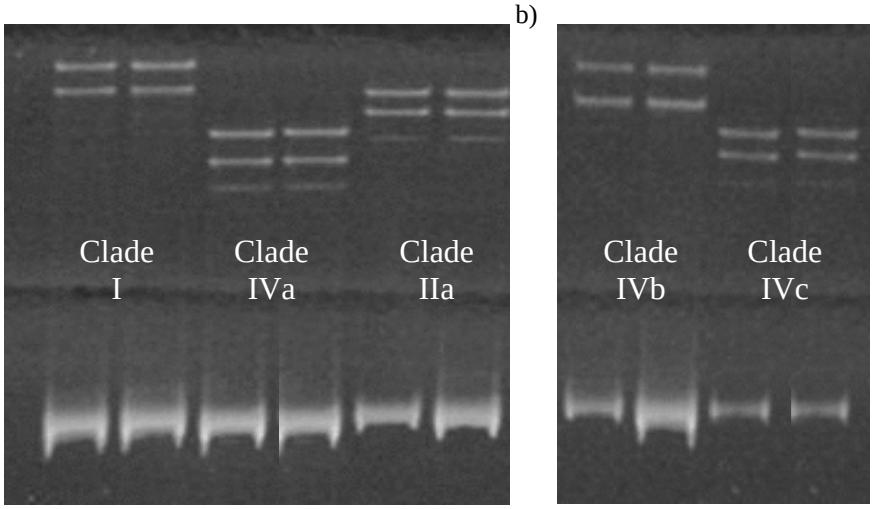

Fig. 2: a) Gel phenotypes of six individuals after PCR with primer system PC6. b) Gel phenotypes of four individuals after PCR with primer system PC4. Gels were stained with SYBR–Gold®.

Acknowledgements

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