Validating mtDNA derived phylogenetic data of *Pityogenes chalcographus* in the light of nuclear pseudogenes and *Wolbachia* endosymbionts

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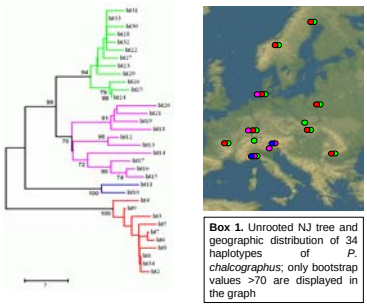
Introduction



Box 1. Unrooted NJ tree and geographic distribution of 34 haplotypes of *P. chalcographus*; only bootstrap values >70 are displayed in the graph

P. chalcographus is a widespread pest on spruce trees. Studies by E. Führer [1,2,3] indicated the possibility of race diffentiation among European populations.

Sequencing the whole mitochondrial COI gene of 96 individuals revealed 34 haplotypes forming a four-branched phylogenetic tree with a maximum divergence of 2.3%. Such high divergences indicate an allopatric origin of the observed clades with a separation about one million years before present. Anyway, the geographic distribution shows that different halplotypes coexist now sympatrically all over Europe (Box 1).

Reproductive incompatibility due to race diffentiation might be one explanation for this effect, but phylogenies derived solely from mitochondrial DNA (mtDNA) data should be validated carefully for other influencing factors. Mainly nuclear copies of mtDNA (numts) co-amplified by universal primers might group together into a distinct clade [4]; recently, a first coleopteran numt sequence has been described [5]. Furthermore, the presence of the endosymbiont *Wolbachia* may influence the distribution of mitochondrial haplotypes throughout populations [6].

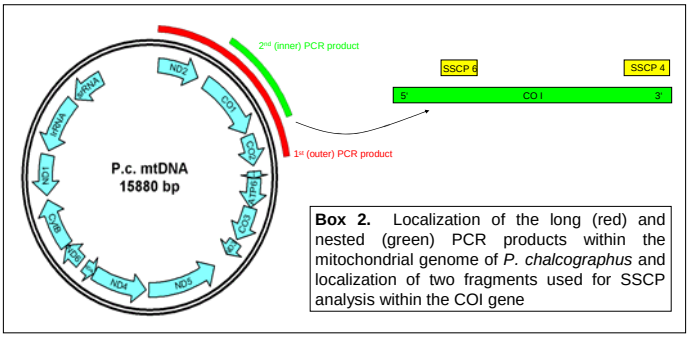
Material and Methods

***in silico* sequence analysis:** all mutations found in mtDNA sequences were characterized for codon position, nonsynonymus substitutions, frameshifts and transition-transversion ratio.

long PCR: using primers localized in the tRNA-Met and COII gene a 3463 bp product was amplified and utilized as template for nested PCR with COI universal primers (Box 2). 14 haplotypes were tested.

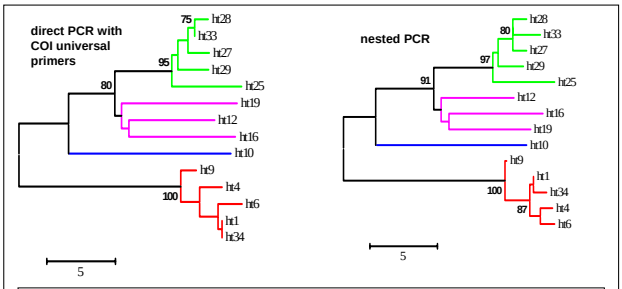
SSCP: two short fragments (SSCP 4 and SSCP 6) of the COI gene were amplified with primers highly specific for *P. chalcographus* mtDNA and used for subsequent SSCP analysis.

***Wolbachia* screening:** 189 individuals were tested by a PCR reaction amplifying a 586 bp fragment of the *wsp* gene of *Wolbachia*; PCR products were cloned into a pGEM vector, sequenced and characterized by a BLAST search



Box 2. Localization of the long (red) and nested (green) PCR products within the mitochondrial genome of *P. chalcographus* and localization of two fragments used for SSCP analysis within the COI gene

Results and Discussion



Box 3. Phylogenetic NJ trees of 14 individuals after dircet PCR using COI universal primers (left) and after nested PCR (right); only bootstrap values >80 are displayed

Possibility of *Wolbachia* infections in *P. chalcographus*

From 189 tested individuals 27 (= 14.3%) showed positive PCR reactions with *wsp* primers. After cloning, plasmids containing the fragment were sequenced, revealing identical clones with a high similarity to a *Wolbachia* seqeunce found in *Tipula aino* [8]. Further research will focus on *in situ* hybridization of dissected insects with specific DNA probes.

Screening for nuclear pseudogenes

The *in silico* analysis of mutations detected by direct sequencing of 96 individuals shows codon position distributions and a transition-transversion ratio as expected for mitochondrial coding sequences (Table 1).

Furthermore, phylogenetic analysis of sequences derived from direct and nested PCR reveals identical trees (Box 3). As the majority of numt insertions are quite small [7], a 3.5 kb amplicon should always originate from authentic mtDNA. Thus we do not expect one of the main clades of *P. chalcographus* originating from a nuclear pseudogene. SSCP analysis for a fast screening of popualtions has been established.

complete number of mutations	95
1st codon position	13
2nd codon position	2
3rd codon position	80
non synonymous substitutions	9
additional stop codons	0
frameshifts	0
transition – transversion ratio	8.5

Table 1. Properties of mutations found in the sequences of 96 individuals

References

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