Pitfalls in Applying Mitochondrial Markers Onto the Scolytid Species *Pityogenes chalcographus*

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Abstract—*Pityogenes chalcographus* is one of the major pests in Eurasian spruce stands. Crossing experiments performed in the mid-1970s suggested race differentiation, and mtDNA analysis of *P. chalcographus* gave evidence that today's populations are divided into several clades. The genetic distance between clades favours a model of allopatric origin with a separation about one million years ago while today haplotypes of the major clades exist sympatrically all over Europe. Within the last few years, the use of mtDNA as a sole genetic marker became a matter of critical discussion. It was shown that nuclear copies of mtDNA (*numts*) led to artefacts in some of the derived genealogies. A long PCR based approach for elimination of potential *numt* sequences was developed to validate the dataset of *P. chalcographus*. This method showed that the beetle's genome does not contain *numts*. Another factor that may influence mitogenomes is the presence of endosymbiotic *Wolbachia*, which causes alterations in insect reproduction and thus influences the population's mtDNA patterns. While *Wolbachia* was not found in *P. chalcographus* in past studies, the use of long and nested PCR, cloning and sequencing of PCR products, and *in situ* hybridization techniques gave evidence that at least a certain percentage of European populations harbour this intracellular endosymbiont. An influence on the mitochondrial dataset can not be excluded and further research is proposed to estimate the prevalence of *Wolbachia* in *P. chalcographus*.

In the mid-1970s, intraspecific variation and unidirectional incompatibility were detected in the Eurasian scolytid species *Pityogenes chalcographus* (Coleoptera, Scolytinae) when males from northern European regions were crossed with females from Central Europe (Führer 1976). This differentiation was further verified by morphological data and the existence of two races among the European populations of *P. chalcographus* was suggested (Führer 1978). Recently, mitochondrial markers were applied and a phylogenetic reconstruction assigned 58 haplotypes to six clades (Avtzis 2006). The two major clades exhibited a sympatric distribution in most of the European terrain, with clade I dominating in northern and clade IIIa in central Europe. The results supported the hypothesis of allopatric divergence of the mtDNA lineages, which postglacially came into sympatric existence in Europe. However, due to partial crossing incompatibility, the diverged lineages retained their genetic identity through the Ice Ages. During the re-colonization of Europe after the last Ice Age, diverged lineages of *P. chalcographus* perhaps also confronted differentiated geographic lineages of *P. abies*. Differential adaptation to diverse

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spruce biochemistry potentially played a role in shaping the current distribution of various *P. chalcographus* genotypes in Europe. The general pattern of geographic separation was in congruence with the findings of Führer (1976); however, it is not as strict as previously described.

High mutation rates established mtDNA as a popular genetic marker for inferring the demography of populations and speciation processes. The availability of conserved universal primers increased its extensive use and PCR amplification made mtDNA easily accessible for direct sequencing as well as for PCR-RFLP and SSCP techniques. After more than a decade of strong reliance on mtDNA, the last years brought emerging awareness that phylogenies derived solely from mtDNA may be biased by several influencing factors (Ballard and Whitlock 2004). Besides the fact that the comparatively small mtDNA molecule represents only one single locus and upcoming doubts if the evolution of the mitochondrial genome is strictly neutral, two main limitations for the reliability of mtDNA sequences must be considered:

First, transferred nuclear copies of mtDNA (*numts*) might be co-amplified using universal mitochondrial primers and grouped together into one distinct clade. Strategies to avoid *numt* based errors include *in silico* analysis of sequences (Bensasson and others 2001a) and PCR of long DNA regions following nested PCR (Thalman and others 2004).

Second, mtDNA transmission can be influenced by any selection for maternally selective traits. Several maternally transmitted endosymbionts are well known in invertebrates, with *Wolbachia* being the most common besides *Cardinium* and Rickettsia. *Wolbachia* infections are widespread among insects (Stouthamer and others 1999). While studies using standard PCR methods estimated a prevalence of the endosymbiont in about 20 percent of insect species (Werren and Windsor 2000), the application of long PCR (Jeyaprakash and Hoy 2000) indicated infection rates up to 70 percent. Due to manipulation of the hosts, reproduction by male killing, cytoplasmic incompatibility (CI), and parthenogenesis and feminization (Stouthamer and others 1999), *Wolbachia* influences mtDNA variation in infected populations (Shoemaker and others 2004). In a population newly infected with a reproductive parasite, the mtDNA associated with the initial infectious individuals will hitchhike along with the expanding reproductive parasite and replace the uninfected haplotypes (Hurst and Jiggins 2005). From a phylogenetic point of view, such a selective sweep may easily be mistaken for a population bottleneck.

The aim of our work was to analyze the mtDNA based phylogeny of *P. chalcographus* (Avtzis 2006) and to rule out any possible influence of *numt*- or *Wolbachia*-caused bias on its authenticity.

Numt Problem

As amplification of mtDNA of *P. chalcographus* was initially performed with universal primers, an erratic co-amplification of *numts* had to be excluded. *In silico* analysis of the *P. chalcographus* sequences was performed to identify non-synonymous base substitutions, additional stop codons, insertions and deletions, frameshifts, and the transition:transversion ratio. Observed patterns in the *P. chalcographus* dataset were all within a 5 percent confidence interval of the expected values. The fact that the GC is often methylated in nuclear DNA and that 5-methylcytosine mutates often to T (Bird 1980) was used as additional indicator to distinguish between mitochondrial and nuclear sequences. Only 12 percent of all observed C→T mutations were of

the GC→GT type indicating a non-methylated, and therefore, most probably non-nuclear molecular origin. Since in most cases *numts* are no longer than 1 kbp (Bensasson and others 2001b), we performed a long PCR amplifying a 3.5 kbp fragment that covered the whole ND2 and CO1, as well as parts of the CO2 gene. To achieve primers highly specific for coleopteran mitochondria, an alignment of currently known coleopteran mitogenomes was performed and conserved regions were selected as primer loci. Products of the long PCR were highly diluted to remove any amplifyable traces of original insect DNA and used as a template for nested PCR with CO1 universal primers. A comparison of the phylogenetic trees from 14 haplotypes of *P. chalcographus* derived from direct and nested PCR showed identical topologies. As *numts* co-amplified erroneously by universal primers tend to group together into a distinct clade (Bensasson and others 2001a), complete removal of ncDNA from the template and re-PCR with identical primers will lead to changes in tree topology. The absence of such changes allows exclusion of *numt* presence in the analyzed populations of *P. chalcographus*.

Wolbachia Problem

A sensitive detection system for *Wolbachia* is based on PCR amplification of the endosymbionts *wsp* gene using proofreading polymerases and high cycle numbers (Jeyaprakash and Hoy 2000, Zhou and others 1998). While Riegler (1999) did not detect *Wolbachia* infections in *P. chalcographus* screening only a limited number of Austrian individuals, a long PCR approach on 189 European individuals resulted in 14.3 percent positive reactions. In contrast to control experiments with *Wolbachia* in *Rhagoletis cerasi* (Riegler and Stauffer 2002), signals were often weaker. The PCR product was cloned and the sequence (GenBank DQ993183) revealed a high homology to a B-strain *Wolbachia pipientis* isolated from *Tipula aino* by Kittayapong and others (2003). Distribution of *Wolbachia* infection was compared with the distribution of mtDNA haplotypes and also haplotypes used in crossing experiments (Avtzis 2006) and no correlation of clade affiliation and *Wolbachia* infection was detected.

Possible sources of false positive results from PCR detection of *Wolbachia* are infected parasitoids harboured in *P. chalcographus*. This error source can be circumvented by *in situ* hybridization, which offers a possibility to detect *Wolbachia* directly in infected tissues (Chen and others 2005, Gómez-Valero and others 2005). Therefore, beetles were dissected and ovarial tissue recovered, split, and used for PCR and for *in situ* hybridization with *wsp* specific DIG-labelled probes (Chen and others 2005). Hybridization and PCR results were compared. As a control, ovarial tissues of *Drosophila simulans* with known infectious state were also objected to hybridization and showed accumulation of dark colour in ovarioles of infected samples, while uninfected tissue remained unstained. Hybridization of four specimens of *P. chalcographus* resulted in three cases of an accumulation of purplish brown color at different intensities. Although low sample numbers do not allow general conclusions, the binding of the *wsp* probe at ovarial tissue supports the hypothesis that positive PCR detections are not obtained due to amplification of contaminants.

It was shown that sympatric European lineages of *P. chalcographus* exhibit strong genetic divergence on mtDNA level. We have proven that these results are not biased by erratic co-amplification of *numts* but represent the authentic state of the beetle's mitochondrial genealogy. Furthermore, we have detected

the presence of the reproductive endosymbiont *Wolbachia* in *P. chalcographus* for the first time. Presently, little information is available on its abundance, geographic distribution, and phenotype. Further research is necessary to elucidate whether the current haplotype distribution of the beetle is solely an effect of differentiation driven by quaternary climate changes or if endosymbionts co-shaped its molecular history.

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