Mitochondrial and Nuclear Markers for Analyzing the Phylogeography of *Pityogenes chalcographus* (Coleoptera, Scolytidae): Development, Applications and Pitfalls

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I would rather have a mind opened by wonder
than one closed by belief

Gerry Spence

Abstract

Pityogenes chalcographus is one of the major pests in Eurasian spruce stands and, besides others, the beetle's ability to aggregate facilitates periodical mass outbreaks. Crossing experiments performed in the mid 1970ies showed differences in reproductive compatibility when insects from various geographic origins were mated. Furthermore, morphological differences between European populations were observed. A race differentiation due to past separation events during Quarternary glaciations was hypothesized.

Today genetic markers offer a possibility to reconstruct the history of taxa in time and space by analyzing changes in the insects DNA sequence. In this thesis molecular tools for phylogenetic investigations on *P. chalcographus* are presented and compared.

Mitochondrial DNA was among the first markers to be introduced in phylogenetic studies. The availability of cross-species amplifying universal primers and its comparable high mutation rate made mtDNA an ideal molecule to investigate population genetic events ranging back several myr. MtDNA analysis of *P. chalcographus* gave evidence that today's populations are divided into several clades. The genetic distance between clades suggests an allopatric origin with a separation about one myr ago while today haplotypes of the major clades coexist sympatrically all over Europe. Based on the detected mutational sites specific single strand conformation polymorphisms (SSCP) were developed in frame of this thesis. The suitability of the SSCP system for fast genotyping was tested and proved positive for distinguishing the main clades.

Within the last years the use of mtDNA as a sole genetic marker became matter of critical discussion. It was shown that nuclear copies of mtDNA (numts) led to artefacts in some of the derived genealogies. To validate the dataset of *P. chalcographus* a long PCR based approach for elimination of potential numt sequences was developed. Comparison of direct and long PCR derived phylogenies

showed that the beetle's genome does not contain amplifiable nuclear copies of the mitochondrial target gene.

Another factor that may influence mitogenomes is the presence of endosymbiotic *Wolbachia*, causing alterations in insect reproduction and thus influencing the populations 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 the endosymbiont. An influence on the mitochondrial dataset can not be excluded and further research is suggested to estimate the prevalence of *Wolbachia* in *P. chalcographus*.

Nuclear markers are a possibility to overcome the limitations of mtDNA. Investigations on the applicability of these marker systems, with a focus on microsatellite sequences, were performed. Microsatellites are short tandem repeats made up of 1-6 nucleotide motifs. Proprietary mutational mechanisms cause high substitution rate and polymorphism at such loci. Locus specific primers must be developed *de novo* for each species to be analyzed for the first time. Different methods for microsatellite isolation were compared and seven polymorphic loci identified by use of a library enrichment strategy. Compared to many other insect species, microsatellites in *P. chalcographus* seem to be less abundant, shorter and less polymorphic.

As an alternative approach towards a nuclear marker system the isolation of transposable genetic elements by degenerate primed PCR was investigated. Three sequences with homology to known transposons were identified and may serve as a starting point for subsequent marker development.

Kurzfassung

Der Kupferstecher *Pityogenes chalcographus* gehört zu den wichtigsten Schädlingen in eurasischen Fichtenstandorten. Seine Fähigkeit zur Aggregation führt neben anderen Schadfaktoren zu regelmäßig wiederkehrenden Massenausbrüchen. Versuche in der 1970er Jahren zeigten Unterschiede in der Kreuzungskompatibilität von Käfern verschiedener Herkünfte. Weiters wurden morphologische Unterschiede zwischen diversen europäischen Populationen festgestellt. Diese Befunde wurden als Hinweis auf eine Rassendifferenzierung infolge eiszeitlicher Trennungsereignisse interpretiert.

Heute ermöglichen genetische Marker, die räumliche und zeitliche Bewegung von Arten durch Analyse von DNA-Sequenzen zu rekonstruieren. In dieser Arbeit werden molekulare Systeme zur Untersuchung der Phylogenetik von *P. chalcographus* vorgestellt und verglichen.

Mitochondriale DNA wurde als einer der ersten Marker in der Phylogenetik eingeführt. Die Verfügbarkeit von über Artgrenzen hinaus anwendbaren Universalprimern und die vergleichsweise hohe Mutationsrate machten mtDNA zu einem idealen Molekül um vergangene populationsgenetische Ereignisse über einige Millionen Jahre hinweg zu untersuchen. Eine Analyse der mtDNA von *P. chalcographus* zeigte eine Unterteilung heute lebender Populationen in mehrere Clades. Der genetische Abstand zwischen diesen Gruppen deutet auf eine allopatrische Entstehung vor etwa einer Million Jahre hin, obgleich die meisten von ihnen heute sympatrisch in ganz Europa vorkommen. Auf Basis der detektierten Mutationen wurden im Rahmen dieser Dissertation spezifische SSCP Primer entwickelt. Die Tauglichkeit der SSCP für die rasche Typisierung von DNA wurde untersucht; die Clades konnten dabei erfolgreich unterschieden werden.

In den letzten Jahren wurde die alleinige Verwendung von mtDNA als genetischer Marker Gegenstand kritischer Diskussion. Mehrere Fälle wurden beschrieben, in denen nukleare Kopien mitochondrialer Gene (numts) zu Artefakten in den

abgeleiteten Stammbäumen führten. Um den vorliegenden mtDNA-Datensatz zu überprüfen wurde eine Strategie verwendet, die durch Amplifikation langer DNA-Abschnitte mögliche numts eliminiert. Der Vergleich dieser Daten mit direkt sequenzierter DNA zeigte, dass im Genom des Kupferstechers keine nuklearen Kopien des untersuchten Zielgens vorkommen.

Ein weiterer Faktor der auf die Zuverlässigkeit mitochondrialer Daten Einfluss nehmen kann ist die Infektion mit *Wolbachia*. Dieser Endosymbiont greift in die Fortpflanzung der Insekten ein und verändert damit auch die Verteilungsmuster der mtDNA. Während in früheren Studien *Wolbachia* in *P. chalcographus* nicht nachgewiesen wurde, zeigten die hier angewandten Methoden (long und nested PCR, Klonierung, *in situ* Hybridisierung), dass zumindest ein gewisser Prozentsatz der europäischen Populationen infiziert ist. Ein Einfluss auf die Verteilung der mtDNA kann nicht gänzlich ausgeschlossen werden und weitere Untersuchungen sind erforderlich, um die Infektionsraten verschiedener Populationen zu ermitteln.

Nukleare Marker bieten eine Möglichkeit, die Einschränkungen der mtDNA zu umgehen. Die Anwendbarkeit solcher Marker, mit einem Schwerpunkt auf Mikrosatelliten, wurde untersucht. Mikrosatelliten sind kurze Sequenzen, in denen ein 1-6 Nukleotide umfassendes Motiv vielfach wiederholt wird. Besondere Mutationsmechanismen führen zu hohen Substitutionsraten und Polymorphismen in solchen Sequenzen. Lokus-spezifische Primer müssen für jede Spezies, die erstmalig untersucht wird, von neuem entwickelt werden. Verschiedene Methoden zur Isolation von Mikrosatelliten wurden verglichen und sieben polymorphe Loci identifiziert. Verglichen mit vielen anderen Insektenarten sind die Mikrosatelliten des Kupferstechers eher selten, kurz und weniger polymorph.

As alternativer Ansatz zur Entwicklung nuklearer Marker wurde die Isolierbarkeit von mobilen genetischen Elementen mittels degenerierter Primer untersucht. Drei Sequenzen mit großer Homologie zu bekannten Transposons konnten identifiziert werden und bieten einen Ansatzpunkt für weitere Markerentwicklung.

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1. Introduction

1.1 Evolution, speciation and phylogeography

It is widely accepted that any particular organism is the product of a great number of interplaying historical contingencies commonly referred to as evolution. Increases of size and complexity seem an overall long term trend, leading to the expansion of organisms into new ecological niches and an increase in species diversity. The proposed initial conditions of life and the fact that simple, unicellular life forms have not been replaced gives evidence for a passive trend where the direction of change is imposed by boundaries of initial minimum values while the total variance within a clade increases (Carroll, 2001). Alternative hypothesis proposing actions of an internal finality on the direction of evolutionary processes (Schönborn, 2005) recently became subject to extensive public discussion, generally characterized by a substantial lack of scientific evidence.

Speciation is a complex process. In the beginning differentiation of separated populations takes place at a few loci and intermediate forms would exist if extrinsic barriers were absent. While divergence increases, hybrids are still possible but biased towards infertility or reduced fitness. At this time the great majority of genomic regions is still shared and undifferentiated. After passing a point of no return the groups are so divergent in some aspects of their reproductive biology and behaviour that a fusion, even under conditions faciliating massive hybridization, is impossible. In sympatry they will either coexist or experience competitive exclusion. The biological species concept (Wu, 2001) defines a species as formed as soon as a group of interbreeding individuals shows complete genome-wide reproductive isolation from other groups.

Inferences about evolutionary processes are fossil records and the phylogenetic tree of life (Carroll, 2001). Since 1981 the number of annually published reports about phylogenies based on gene-sequence information has increased exponentially (Pagel, 1999).

The terminus 'phylogeography' was introduced by Avise et al. (1987). Based upon the fact that the discipline of population genetics had remained separate from fields associated with phylogeny and macroevolution and realizing the potential of the fast evolving mitochondrial DNA as a molecular system highly suitable for phylogenetic analysis, Avise et al. anticipated it as a 'bridge between population genetics and systematics'. Phylogeography describes the geographical distributions of genealogical lineages, taking inputs from molecular and population genetics, demography, ethology, historical geography and phylogenetics (Avise, 1998), approaching a view of genes in space and time (Hewitt, 2001).

The European land mass provides an interesting field for phylogeographic studies. 2.5 myr ago the arctic ice shields formed and quarternary climatic oscillations went along with enormous temperature shifts of 7 – 14° C within only a few decades. The climatic stability of the last 8000 years seems to be the exception rather than the rule (Hewitt, 2000; 2004; Taberlet et al., 1998). Europe, a large peninsula of Asia, is structured by Alpine mountains and the Mediterrenian sea forming strong east-west oriented barriers. Thus the northward expansion of cold tolerant species during warm periods as well as the occupation of warmer southern biota during glaciations were restricted. With each oscillation species went extinct over large parts of their range, dispersed to new locations or separated into refugia. Repeated expansions and contractions of species range should imply successive bottlenecks, a loss of genetic diversity in northern populations and speciation events due to survival in different refugia and adaptation to new environments and neighbours (Hewitt, 2000) .

Refugial areas in the Carpathes, the present-day Moscow area, the Balkans, Italy and the Iberian peninsula were the main sources of the postglacial recolonization of Europe. Today the distribution of many species throughout the continent is subdivided by suture zones which were created when diverged populations from separate glacial refugia met during their range expansion (Fig. 1.1).

Most important suture zones are located (Taberlet et al., 1998)

- along the Alpine barrier, separating Italian lineages from others in western and northern Europe
- around the French-German border, where populations from the Iberic peninsula meet others from more eastern refugia; due to the flat landscape without substantial barriers the suture zone is relatively broad
- in the Pyrenees; it may be considered as a special case of the zone described before
- along a west to east line across Scandinavia, that was colonized from north and south by populations originating from different refugia.

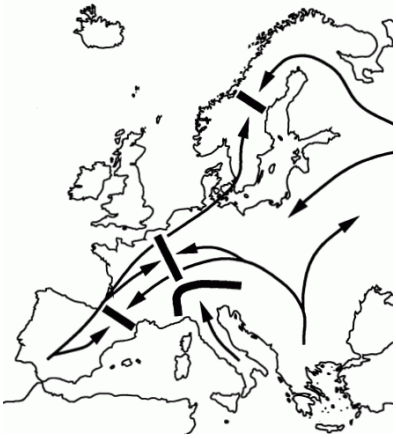

Fig. 1.1. Postglacial colonization routes and suture zones in Europe (Taberlet et al., 1998): Quarternary ice ages led to the extinction of northern populations. Recolonization from southern refugia during the recent warm period followed typical pathways and created suture zones, indicated by black bars, where populations from different refugial areas meet.

1.2 *Pityogenes chalcographus*

1.2.1 Biology

The six toothed bark beetle *Pityogenes chalcographus* (L.) (Coleoptera, Scolytidae) is a 1.5 to 3 mm sized insect with a black head and thorax and red-brownish elytra (Fig. 1.2). *P. chalcographus* shows a sex dimorphism with males having six clearly distinguishable spines on the elytral declivity. In females declivities show only rudimentary spines (Pfeffer, 1995).

Fig. 1.2. *Pityogenes chalcographus*. Body length ~ 2 mm; the spines on the declivity are characteristic for a male individual.
Source: Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany

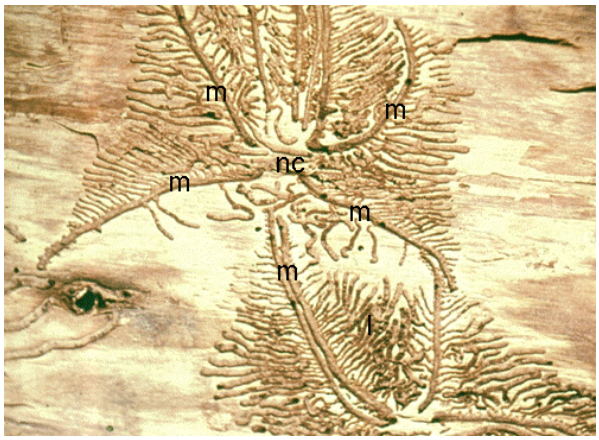

Fig. 1.3. Galleries of *P. chalcographus*. Several mother galleries (m) derive from a nuptial chamber (nc). Larval galleries (l) with much smaller diameter are horizontally oriented to the mother galleries.
Source: IFFF, BOKU

P. chalcographus belongs to the main pests in *Picea abies* stands. The beetle is not strictly monophagous and other members of the genus *Picea* as well as *Larix sp.*, *Pinus sp.* and *Pseudotsuga sp.* may serve as hosts (Pfeffer, 1995). The swarming season of the parent generation starts between mid April to mid June, depending on altitude and geographic site. Adult animals try to locate a suitable host tree either by plant volatiles or aggregation pheromones (Byers, 2004). Males bore into the phloem and excavate a nuptial chamber that usually does not touch the wood (Postner, 1974). In the following days they attract 3-6 females using sexual pheromones. After mating females bore mother galleries, carving the wood, and lay 40 to 80 eggs in nieches (Fig 1.3). If females are disturbed or if the tree is too heavily infested by other *P. chalcographus*, females can emerge and create another mother gallery without further mating. Such sister broods are characterized by the lack of a nuptial chamber (Sittichaya, 2005).

Larval galleries originate in egg nieches and are oriented horizontally to the mother galleries (Fig. 1.3). After three larval stages young imagines emerge from the tree (Postner, 1974).

Depending on altitude and temperature two to three generations exist per year. All ontogenetic stages are able to hibernate, either in the bark or the soil (Postner, 1974). *P. chalcographus* is common on fresh windfall and timber, while its ability to kill healthy trees seems low (Hedgren, 2004). Under stimulating environmental conditions the beetle's high reproductive rate may faciliate a rapid increase of attacks to identical host trees. Such circumstances exhaust host defence and explain why during mass outbreaks even trees that were not previously weakened are object to severe damage (Lieutier, 2004).

The beetle is often associated with blue stain fungi from the genera *Ophiostoma* and *Ceratocystis* (Kirisits, 2004). Both organisms gain benefit with the fungi transported to new host trees by the beetles and helping them to overcome the host trees defence response (Paine et al., 1997).

1.2.2 Race differentiation in *P. chalcographus*

Crossing experiments among Northeast and Central European populations of *P. chalcographus* revealed differences in fertility and reproductive compatibility (Führer, 1976; 1977). Incompatibilities were also detected between low-land populations from southern Germany and Alpine populations. Allopatric females were partly rejected by males in certain strain combinations, in which sympatric and allopatric females were simultaneously offered as pairing partners (Sturies and Führer, 1979). Crossings of Scandinavian, Polish and Alpine strains resulted in different degrees of inter-population heterosis (Führer and Klipstein, 1980).

Also morphological parameters – the proportion of the antennae and the spine formation on the elytra – showed significant differences between northeast and Central European populations (Führer, 1978). Allozyme electrophoresis revealed two groups. However, races were not clustered according to previous findings (Ritzengruber, 1990).

It has been suggested that race differentiation in *P. chalcographus* occured due to glacial separation and postglacial recolonization of the European continent (Führer 1976; 1977; 1978)

1.2.3 Geographic distribution and postglacial recolonization

Climatic oscillations in the Quarternary caused repeated local extinction and recolonization effects (Hewitt, 2004). After the last temperature amelioration at the end of the Wuerm glacial about 10 000 ybp, vegetation and fauna began to reinvade the previously frozen northern zones. *Picea abies* did not survive in the Iberian peninsula and had its three refugial areas in the Dinaric Alps, the Carpathes and the area north of Moscow - Kostroma (Schmidt-Vogt, 1977; Lagercrantz and Ryman, 1990). Further the Apennines were important for the recolonization of the South Alps (Giannini et al., 1991).

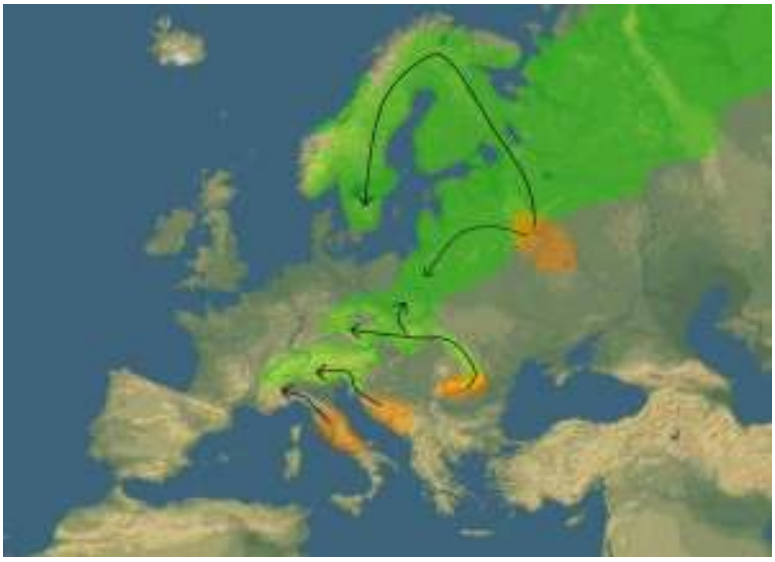

Fig. 1.4. Occurrence of *Picea abies* in Europe. Green areas show the recent distribution while orange areas represent the refugial zones (southwest to northeast: Apennine, Dinaric Alps, Carpathes, Kostroma) during the last glacial (Schmidt-Vogt, 1977; Giannini et al., 1991). Arrows indicate the expected recolonization routes.

It is suggested that *P. abies* remigrated from the Dinaric back to the Central Alps, from the Apennine into the southern Alps, from the Carpathic back to the northern Alps and from the Kostroma area back to Eastern Europe, with an introgression into Poland, and to Scandinavia (Fig. 1.4).

As *P. abies* represents the main host of *P. chalcographus* (Pfeffer, 1995) it may be hypothesized that both species shared at least some refugial areas and remigration routes.

1.2.4 Influence of *Wolbachia*

The α-proteobacterium *Wolbachia* is endosymbiontic in numerous arthropod species (Stouthamer et al., 1999). Conservative estimations based on standard PCR detection suggest that 16 to 22% of insect species are infected with *Wolbachia* (Werren and Windsor, 2000), while other assays using the more sensitive long PCR revealed up 76% infection rate (Jeyaprakash and Hoy, 2000).

In the host species *Wolbachia* is usually maternally transmitted and the endosymbiont is capable of manipulating the hosts reproductive system (Stouthamer et al., 1999). Most important effects are

- cytoplasmic incompatibility (CI), leading to death of fertilized eggs when infected males are mated with infected femals (Hoffmann and Turelli, 1997)
- thelytokous parthenogenesis, causing the production of parthenogenetically females in species that usually have parthenogenetically male offspring (Stouthamer et al., 1999)
- male killing, where male offspring of infected femals will die during embryogenesis; infected populations will therefore show a female biased sex ratio. Male killing *Wolbachia* were identified in numerous insect species (Majerus et al., 2000; Hurst et al., 2000; Jiggings et al., 1998).

Maternal inheritence and reproductive manipulations male cause alterations in the distribution patterns of infected populations' mtDNA (Turelli, 1992).

Riegler (1999) found no evidence for *Wolbachia* infections in *P. chalcographus*, while the endosymbiont was isolated from three other members of the scolytid family, *Ips typographus* (Stauffer et al., 1997), *Hypothenemus hamperi* (Vega et al., 2002), and *Xylosandrus germani* (Koivisto et al., 2004).

1.3 Phylogenetics: molecular approaches

1.3.1 Genetic markers

Genetic markers can be defined as distinguishable segments of DNA, other biomolecules derived from DNA sequences like RNA or proteins or, more general, any traits of an organism which can be linked to its genome. Before the emergence of electrophoretic and molecular genetic techniques only few markers for Mendelian traits like body pigmentation (True, 2003) were available. In the last decades a fast development resulted in several classes of markers to be used in population biology (Sunnucks, 2000), differing in resolution, precision, reproducibility and also technical difficulty and analytical effort. Thus, the choice of an appropriate genetic marker not only depends on the initial genetic questions but also on the availability of marker systems for a given species (Nève and Meglécz, 2000), the quantity and quality of the provided specimen (Dean and Ballard, 2001) and the time and equipment which may be utilized for a study. Ideal markers – as far as they exist – should combine appropriate substitution rates, reproducible sequences and easy alignment with well described universal primers (Cruickshank, 2002).

The following section will provide an overview of commonly used marker systems, their typical applications, benefits and limitations. Besides allozymes, all markers presented here are based on DNA techniques. These offer the big advantage to rely on a chemical very stable molecule; DNA is found in almost all cells and tissues of an organism. Fast and easy to use extraction kits are available and will often work well also with old and dried samples. Trace amounts may be amplified by PCR and the amount of DNA contained in a single insect may be sufficient for many tests and reactions. Among molecules that might serve as markers, DNA remains to be least cumbersome in long term storage, even under field conditions (Dillon et al., 1996).

i) Allozymes

The term 'allozyme' refers to different allelic forms of nuclear encoded enzymes (Parker et al., 1998) which can be identified by protein electrophoresis. Homozygotic organisms show typically one band per locus in the electropherogram, while

heterozygotes yield two or more bands, depending on the proteins secondary structure. Allozymes represent codominant Mendelian loci and were first introduced to population studies in the 1960s (Harris, 1966; Hubby and Lewontin, 1966).

While the basic procedures for allozyme analysis are inexpensive and straightforward, it is crucial to extract active and nondenatured protein. Although samples were successfully conserved for long periods by freeze-drying, field sampling remains cumbersome and especially older or dried tissues often fail in analysis (Thorpe and Solé-Cava, 1994).

For minimal statistical confidence 10 to 20 independent polymorphic loci with at least two alleles and a minimum allele frequency of 0.05 should be considered (Berg and Hamrick, 1997). As many species are monomorphic for most commonly used allozymes and loci with more than three alleles are generally rare, quite often species are encountered where this genetic marker is not applicable.

Most models consider the phenotypic differences among allozymes as minimal and there is strong evidence that most observed variations are in fact selectively neutral. Anyway, differences in metabolic function were described (Patarnello and Battaglia, 1992), so in some cases selection might act on allozyme distribution.

ii) Restriction fragment length polymorphism (RFLP)

RFLP was the first DNA based marker to be introduced in population genetics by Avise (1994). It is based on the fact that restriction endonucleases cleave DNA at specific nucleotide sequence recognition sites. Over 300 restriction enzymes are commercially available with 4-, 5-, 6-, or 8-base recognition sites. Mutations may create or destroy recognition sites within a genome, so that the electrophoretic patterns of digested DNA will differ between mutated and native genotype. Allele frequencies are quantified by the presence or absence of specific restriction sites among individuals (Parker et al., 1998).

While the isolated short molecules of animal mtDNA will often yield a manageable amount of restriction fragments which can be resolved by agarose gel electrophoresis, nuclear DNA will contain thousands of restriction sites and appear as smear after digestion (Chapman and Brown, 1987). Therefore transfer of the DNA to a membrane and hybridization to a specific probe (Southern, 1975) must be performed to visualize polymorphic banding patterns; the selection of a suitable

probe is often challenging and the procedures require large amounts of high quality DNA, the handling of radioactive substances and are time-consuming and expensive.
An efficient application of the RFLP technique is the restriction digest of genome fragments first amplified by a PCR reaction. These fragments may be randomly selected from a genomic library or, more common, known parts of the genome amplified by specific primers. PCR targets may be located in nuclear as well as in mitochondrial DNA. PCR-RFLPs are usually visualized by agarose gel electrophoresis and ethidium bromide staining.

iii) Random amplified polymorphic DNA (RAPD)
For the production of RAPD markers genomic DNA is amplified with short (~10 bp) oligonucleotide primers with random sequence (Welsh and McClelland, 1990). Only one primer is used per reaction, resulting in the amplification of sequences with inward-oriented flanking regions. The amplification products are separated on agarose gels and allelic variation consists on the presence or absence of distinct bands. Suitable primers will reveal several polymorphic bands within a population.
While the method itself is rather fast and cheap, results might be affected by the purity, quantity and quality of the template DNA, by the primer-template ratio and even by the brands of enzymes and thermocyclers used (Dassanayake and Samaranayake, 2003). Therefore often reproducibility is poor. Furthermore, most RAPD markers are not inherited codominantly, complicating the identification of heterozygosis.

iv) Amplified fragment length polymorphism (AFLP)
The technique of AFLP was extensively reviewed by Blears et al. (1998). Genomic DNA is digested with a rare and a frequent cutting restriction enzyme; simultaneously oligonucleotide adaptors are ligated to the fragments. These adaptors provide primer binding sites for subsequent PCR amplification. The primers are supplemented with additional 'selective' nucleotides at the 3' end, reducing the complexity of generated amplicons, and the primer corresponding to the rare-cutting enzyme is labelled. Amplicons are separated on denaturing polyacrylamide gels and visualized by autoradiography. Like RAPD the technique does not depend on prior sequence knowledge and produces a multi-locus fingerprint. The specifity of the

enzymes and the stringent amplification conditions provide reliable and reproducible results between experiments and laboratories. The technique is quite insensitive to the amount of template DNA used, while a high quality and purity of the template is crucial (Reineke et al., 1998). In the meantime several kits providing all reagents for AFLP analysis are commercially available and fluorescent labels are replacing the use of radioactivity.

v) Single strand conformation polymorphism (SSCP)

Short (< 500 bp), single stranded DNA molecules fold into a sequence-dependent three dimensional shape. This shape has strong influence on the migration rate of the molecule in non-denaturing polyacrylamide gels and hence may be used for the detection of even single base differences between DNA samples (Nataraj et al., 1999). Suitable fragments of a single locus are first amplified by specific PCR primers. While PCR-RFLP will only detect mutations within the recognition sites of the applied endonuclease, SSCP has the potential to detect the great majority of all sequence variation within the fragment (Sunnucks et al., 2000).

PCR products are mixed with a denaturing agent and separated on a polyacrylamide gel. After visualization by autoradiography, silver staining or SyBr dyes a slower migrating, fine, multibanded and a faster, coarser, single banded pattern will be seen on the gel. The bands are formed by different stable conformations of the two renatured DNA strands and re-annealed double stranded DNA.

While SSCP analysis is extensively used in biomedical research, phylogenetic applications are still rare. The main limitations of the technique are the rather short size of applicable PCR fragments, the lack of information on the kind of sequence variation and the sometimes difficult interpretation of the gels. Furthermore there does not exist a precise theory which can predict whether a particular mutation will be detected by the method or not (Nataraj et al., 1999). Anyway SSCP provides a simple, fast and cheap method for the comparison of DNA fragments to be considered where the expenses for sequencing might be a limiting factor.

vi) Transposable Elements

Transposable elements (TEs) are stretches of DNA with the ability to move from one site of the genome to another. They are the most abundant class of DNA in higher eucaryots, contributing to more than 50% to the total genome size in some taxa (Lewin, 2004).

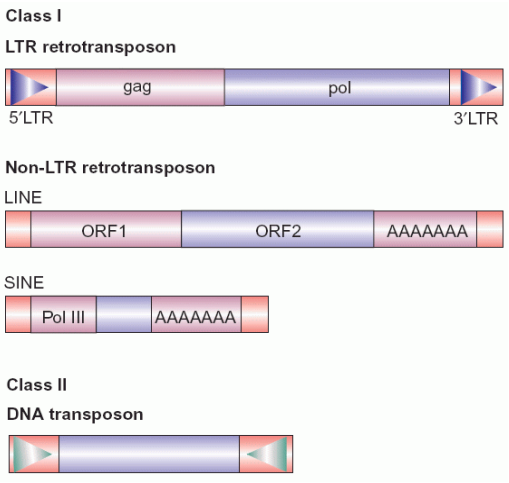

Fig. 1.5. Structure of different TEs (Wong and Choo, 2004): LTR = long terminal repeat; gag = capsid protein encoding gene; pol = polyprotein encoding gene; ORF = open reading frame; AA... = polyA region

Depending on the mode of transposition they are divided into two classes (Fig. 1.5): DNA transposons (Class II) like *Tc* or *mariner* encode transposase genes flanked by terminal-inverted repeats. They are able to move through the genome by a cut-and-paste mechanism. Retrotransposons (Class I) generate an RNA intermediate that is transcribed to DNA by a reverse transcriptase before insertion into new locations. Unlike DNA-transposons they enter the new loci as a copy while the mother element remains fixed in the genome.

Class I transposons can further be subdivided into long terminal repeat (LTR)-retrotransposons like *Ty*, *copia* or *gypsy* and long (LINE) and short (SINE) interspersed nuclear elements. LTR-TEs with a length of several kb contain the capsid-protein encoding *gag* and a polyprotein *pol* gene with protease, reverse transcriptase, RNase H and integrase activities. LINEs have a size of ~ 6 kb and harbour two open reading frames with RNA-binding, endonuclease and reverse transcriptase activity and a poly-A tail. SINEs are shorter (~ 0.4 kb), do not encode proteins and their mobility depends on the activity of LINEs (Wong and Choo, 2004). While LINEs are strongly biased to gene-poor regions, SINEs are most frequent in GC- and gene-rich parts of the genome.

Retrotransposons share many similarities in their organisation, gene products and life cycles with retroviruses, suggesting that they derived from a common ancestor (Xiong and Eickbush, 1990). Opposite to retroviruses, retrotransposons lack an envelope domain and thus the ability for forming an infectious particle necessary for the passage through the cell membrane.

If novel TE insertions take place in the germline, the trait will become inheritable. This stable integration of large DNA stretches into dispersed chromosomal loci with reproducible ancestral and derived states gives TEs some potential as a genetic marker (Schulman et al., 2004).

'Sequence-specific amplified polymorphism' (SSAP), the first marker system using LTRs was introduced by Waugh et al. (1997). It is a modification of the AFLP system, where genomic DNA is digested with two restriction enzymes and, after adaptor ligation, fragments containing LTRs are amplified by an adaptor- and a retrotransposon-specific primer.

The 'retrotransposon based insertional polymorphism' (RBIP; Flavell et al., 1998) detects the presence or absence of an LTR at a specific site by a PCR reaction with two site-specific flanking primers and one LTR specific primer. While at unoccupied sites the two flanking primers will produce an amplicon, occupied sites are amplified by one flanking and the LTR specific primer. In this case, due to the long insertion of several kb, the combination of the flanking primers will not yield any product. RBIP is in difference to SSAP a single-locus, co-dominant technique.

The technical challenge of RBIP is the identification of suitable insert sites and primers. It contains steps to identify LTR sequences within a given genome, southern blotting to determine the copy numbers of the LTR and library screening to obtain LTR flanking regions.

TEs were established as phylogenetic markers in plant biology (Kumar and Hirochika, 2001) as well as in humans (Rowold and Herrara, 2000) or whales (Shimamura et al., 1997).

vii) Minisatellites

Minisatellite DNA, also known as 'variable number tandem repeats' (VNTRs), is a noncoding DNA that occurs at scattered sites throughout the genome (Jeffreys et al., 1985). It is composed of short (8 – 100 bp), tandemly repeated motifs and the minisatellite regions are hypervariable due to variation in the repeat number of units at each locus (Parker et al., 1998).

For a multilocus approach, genomic DNA is digested with a frequent cutting restriction endonuclease, separated by agarose gel electrophoresis, blotted to a nylon membrane and hybridized with standard repeat sequence probes. As even

closely related individuals do not share identical banding patterns, minisatellites are the marker of choice for paternity exclusion and the identification of individuals.
A single-locus approach by constructing locus-specific probes is principally possible. As this technique is more labour intensive and probably less informative than the use of microsatellite markers it is only rarely used (Burke, 1991).

viii) Microsatellites

Microsatellites, also known as 'simple sequence repeats' (SSRs) or 'short tandem repeats' (STRs) are short DNA stretches in which a 1-6 nucleotide motif is tandemly repeated. They were found in all pro- and eucaryotic genomes analyzed so far. Di- and trinucleotide motifs seem to be the most abundant category and they are vastly overrepresented in the genome assuming a chance association of nucleotides (Ellegren, 2004). Most SSRs are embedded in non-coding DNA, but the frequency distribution varies between species and also within the chromosomes of the same organism with some tendency to form clusters (Schlötterer, 2000). While SSRs contribute approximately 3% to the human genome, some species seem to be poor in microsatellites (Day and Ready, 1999; Fagerberg et al., 2001; Navajas et al., 1998; Ross et al., 2003).
Microsatellites show a high degree of length polymorphism and mutation rates range from 10^{-6} to 10^{-2} per generation, being thus significant higher than base substitution rates in other parts of the genome and two to three orders of magnitude higher than values known for allozymes (Jarne and Lagoda, 1996). These high mutation rates are caused by slippage events during DNA replication, where nascent and template strand realign out of register (Fig. 1.6). With the rise of PCR and the development of high throughput fragment analysis methods SSRs became one of the most powerful codominant markers ever found. In principle, several thousand potentially polymorphic loci should exist in any species (Schlötterer, 2000).

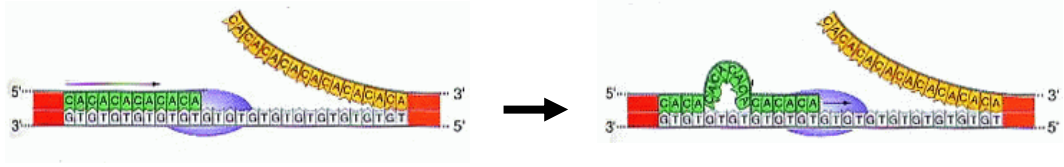

Fig. 1.6. DNA replication slippage. Misalignment due to a loop-forming DNA strand at the replication fork will lead to the gain (if the newly synthesized strand loops out) or loss (if the loop occurs in the template strand) of repeat units. Source: www.virtuallaboratory.net

Due to the fact that most of the SSRs are located in noncoding regions with high base substitution rates, the development of universal flanking primers applicable for larger groups of taxa is not possible. Thus, microsatellite markers need to be isolated *de novo* from most species to be examined for the first time (Zane et al., 2002). The traditional isolation procedure consists of the construction of partial genomic libraries and the screening of several thousand clones by colony hybridization with SSR specific probes. Although this approach is technically simple, it is extremely laborious and time consuming, especially for genomes with a low microsatellite density. Refined or alternative approaches for SSR isolation include the construction of enriched libraries (Ostrander et al., 1992; Chenuil, 2003), vectorette PCR (Lench et al., 1996), the use of expressed sequence tags (Rungis et al., 2004) and RAPD based techniques (Lunt et al., 1999; Ender et al., 1996). From 127 articles on microsatellite isolation published in the first two issues of the 2005 volume of Molecular Ecology Notes, a vast majority of 101 used enriched genomic libraries for this task. 15 authors used conventional libraries without enrichment, the remaining 11 publications describe other techniques.

1.3.2 Animal genomes

The animal cell contains two independent genomes: the nuclear (ncDNA) and the mitochondrial (mtDNA). The nuclear genome is big (e.g. 1.4 x 10^8 bases in *Drosophila melanogaster*; Hoskins et al., 2002), encodes for several thousand proteins and exists in two copies per diploid cell. Heterozygosity is frequent in the nuclear genome of outbreeding diploid species. Protein-coding genes, scattered over the chromosomes, form only the minor part of the genome while the vast majority is made up of non-coding sequences like introns, transposons or untranslated intergenic regions (Lewin, 2004).

The mutation rate and the distribution of polymorphic sites is not random along the nuclear genome. While 18S-rDNA and coding single-copy ncDNA is quite conserved, other loci like introns, the ribosomal internal transcribed spacer (ITS) and mini- or microsatellites evolve at much faster rates. Differences in recombination rate, gene density and selection strength contribute to this unevenness. Reduced effective

population size may contribute to the fact that sex chromosomes vary less than others in humans and *Drosophila* (Zhang and Hewitt, 2003).

Nuclear primers often fail to amplify between taxonomic groups and some properties of the molecular evolution of ncDNA like gene duplication, formation of pseudogenes or intron sliding counteracts the search for 'versatile' primers like those available for mitochondrial genes (Zhang and Hewitt, 2003). Thus, *de novo* development of applicable primers will be a major constraint in nuclear genome analysis for foreseeable times (Wan et al., 2004).

The mitochondrial genome of metazoans is a much smaller (about 1.6 x 10^4 bases) haploid, circular DNA molecule (Lewin, 2004) (Fig. 1.7). In all animals analyzed so far it contains the genes for two ribosomal RNAs, 22 transfer RNAs and 13 proteins belonging all to subunits of the respiratory electron transport chain. The genes are tightly packed with only few noncoding parts; introns or repetitive elements are missing. A cell may contain many mitochondria occupying up to 25% of the volume of its cytoplasm and each mitochondrion contains many copies of the mtDNA (Ballard and Whitlock, 2004).

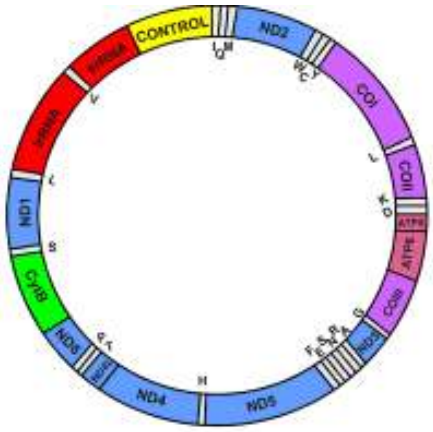

Fig. 1.7. Organisation of insect mtDNA: a circular strand of DNA containing 37 genes with only few noncoding regions

It has been shown that the mitochondrion and its genome originated in the endosymbiosis of α-proteobacteria in ancestral eucaryot cells (Gray et al., 2001). Yet, mitochondrial genomes contain one to three orders of magnitude fewer genes than those of free-living α-proteobacteria and up to 99% of mitochondrial protein content is a product of nuclear genes. This gene loss of mitochondria may be explained by the functional replacement of still needed genes with pre-existing nuclear sequences ('gene substitution'), the loss of no longer necessary genes and, finally, the functional transfer of mitochondrial genes to the nucleus. The latter case is demanding as the transferred gene must gain regulatory elements for proper

expression, targeting signals for downstream transport into the mitochondrion and point mutations to overcome the differences between the mitochondrial and the standard code. Due to this reasons functional gene transfer remains a rare event described almost exclusively in plants, while mtDNA transferred to the nucleus of animals is probably always dead on arrival (Adams and Palmer, 2003).

Anyway the transfer of mtDNA derived sequences to the nucleus remains an ongoing process in eucaryots and mitochondrial pseudogenes have been identified in the nuclear genome of many species (Zhang and Hewitt, 1996; Thalmann et al., 2005). Such nuclear mitochondrial (numt) sequences can derive from any part of the mtDNA and occur typically as single copies at dispersed genomic locations. They are usually short (less than 1 kb), but large fragments as well as tandemly repeated pseudogenes have been reported in mammals (Bensasson et al., 2001; Thalmann et al., 2004).

While the nuclear genome of metazoans has undergone dramatic changes in size and complexity during evolution the organisation of mtDNA has remained almost unchanged (Saccone et al., 2002). It is not fully understood why mitochondria still posses individual genomes. The genetic system requires numerous enzymes for DNA maintainance, RNA processing and gene regulation that could be eliminated if all genes were transferred to the nucleus. Hypothesis for the retaining of an own genome include that the highly hygrophobic mitochondrial *CoB* and *CoxI* proteins are difficult to import through the organellar membrane and toxic if present in the cytosole. A major factor preventing further functional gene transfer in animals is probably the non-standard genetic code of mtDNA (Adams and Palmer, 2003).

While ncDNA is inherited biparentally and undergoes frequent recombinations, mtDNA shows uniparental, usually maternal, inheritance without or at least almost without recombination (Rand et al., 2004). This reduces the effective population size of the mitochondrial genome to a fourth of that of nuclear autosomal DNA, guarantees an identical genealogical history of the whole molecule and weakens the impact of natural selection as it acts as one selective unit. It was found that the mutation rate of mtDNA is in average higher than those of ncDNA (Brown et al.,

1979). A detailed analysis in *Drosophila* by Moriyama and Powell (1997) showed that the synonymous substitution rate in mitochondrial genes is 1.7 to 3.4 times higher than that of the fastest and 4.5 to 9.0 times higher than that of average nuclear genes.

High mutation rates established mtDNA as a popular genetic marker for inferring the demography of populations and species evolution and the availability of conserved 'universal' primers (Simon et al., 1994) increased its extensive use once more. 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. 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 (Gerber et al., 2001), two main limitations for the reliability of mtDNA sequences must be considered:

- First, nuclear pseudogenes might be co-amplified using universal mitochondrial primers and group together into one distinct clade. Strategies to avoid numt based errors include *in silico* analysis of sequences for increased numbers of non-synonymous base substitutions, frameshifts, additional stop codons and reduced transition:transversion ratios (Bensasson et al., 2001). Any of these findings should gain doubt on the mitochondrial origin of the retrieved sequences. With currently available methods authenticity of mtDNA can only be guaranteed by long PCR making use of the circular nature of the animal mitochondrial genome (Thalman et al., 2004).

- Second, mtDNA due to its strictly maternal inheritance is always a marker for processes in females. In case of asymmetrical male and female history within a species the marker only reflects the female portion of the genealogy. Further, mtDNA transmission will be influenced by any selection for maternally transmitted genes or other maternally selective traits. Unfortunately several maternally transmitted endosymbionts are well known in invertebrates, with

Wolbachia as the most important infecting at least 16% of insect species (Werren et al., 1995). The effects of *Wolbachia* infection range from cytoplasmatic incompatibility over male-killing to the induction of thelytokous parthenogenesis. In a population newly infected with the endosymbiont, the mtDNA associated with the initial infectious individuals will hitchhike trough the population and replace the haplotypes that were present before (Hurst and Jiggins, 2005). From a phylogenetic point of view this selective sweep may easily be mistaken for a population bottleneck of a founder effect. The situation is further complicated by the fact that different *Wolbachia* strain might co-infect the same individual (Dobson et al., 2002; Riegler and Stauffer, 2002; Sinkins et al., 1995).

Considering the effects mentioned above it seems advisable to validate any mtDNA derived data by a suitable set of nuclear markers and by additional tests for possible bias caused by known influencing factors.

2. Research objectives

2.1 Mitochondrial DNA

2.1.1 Development of SSCP markers

Although sequencing remains the most reliable method for mtDNA genotyping it is time consuming and expensive (Nataraj et al., 1999). To overcome these limitations the potential of SSCP analysis (Sunnucks et al., 2000) as a high-throughput genotyping system has been evaluated.

Direct sequencing of the CO1 gene of 96 *P. chalcographus* individuals revealed 34 haplotypes grouping into four main clades (Avtzis, 2005). Seven fragments of the CO1 gene covering relevant point mutations were selected as source DNA for SSCP. Primers based on the direct sequences were designed and the amplicons were denatured and run on native polyacrylamide gels at different temperatures. After optimization of electrophoresis conditions the banding patterns showed several polymorphisms and combined analysis of two fragments proved to be sufficient to distinguish between the main clades. Some hundred individuals so far have been genotyped using this method.

2.1.2 Validation of mitochondrial origin

The possibility of race differentiation in *P. chalcographus* was indicated by crossing experiments (Führer, 1976, 1977; Sturies and Führer, 1979; Führer and Klipstein, 1980), morphological parameters (Führer, 1978) and allozyme analysis (Ritzengruber, 1990). An investigation on a molecular basis was performed by Avtzis (2005) using the mitochondrial CO1 gene, revealing a four-branched phylogenetic tree for beetles of different European origins.

As amplification of mtDNA was initially performed with universal primers (Simon et al., 1994; Lunt et al., 1996) an erratic co-amplification of nuclear pseudogenes (Bensasson et al., 2001; Thalman et al., 2004) had to be excluded. An *in silico*

analysis of the obtained sequences was performed to identify non-synonymous base substitutions, additional stop codons, insertions and deletions, frameshifts and the transition:transversion ratio. Furthermore, primers for a long PCR resembling a 3.5 kb fragment that covers the whole ND2 and CO1 and parts of the CO3 gene were designed. To achieve primers highly specific for coleopteran mitochondria an alignment of the mitogenomes of *Pyrocoelia rufa* (Bae et al., 2004), *Tribolium castanaeum* (Friedrich and Muqim, 2003) and *Crioceris duodecimpunctata* (Stewart and Beckenbach, 2003) was performed using Clustal X (Thompson et al., 1997) and conserved regions were selected as primer loci. Long PCR products were diluted and used as a template for nested PCR with CO1 universal primers. Products from direct and nested PCR were cloned and compared for sequence polymorphisms.

2.1.3 Detection of *Wolbachia* infections

Wolbachia, a maternally transmitted endosymbiont which infects at least 16% of insect species (Werren, 1997) causes hitchhiking of the endosymbiont related mtDNA through a newly infected population (Turelli et al., 1992). In mtDNA derived phylogenies such an infection-induced sweep might be mistaken for a past bottleneck event, thus making an analysis of the *Wolbachia* infectious state of the population inevitable to identify possible artefacts.

The most sensitive detection system for *Wolbachia* known so far is based on PCR amplification of the endosymbionts *wsp* gene using proofreading polymerases (Zhou et al., 1998; Jeyaprakash and Hoy, 2000). *Taq*, *Pfu* and *Pwo* DNA polymerase brands (Fermentas, Lithuania; GeneCraft, Germany; peqlab, Germany; Sigma, USA) were used to amplify *wsp* sequences of selected individuals with different mitochondrial haplotypes. Authenticity of *Wolbachia* origin of obtained amplicons was verified by cloning and sequencing. A *Taq-Pwo* mixture which yielded highest sensitivity was used for the screening of individuals from different European origins. Distribution of *Wolbachia* infection was compared with the distribution of mtDNA haplotypes.

False positive results from PCR detection of *Wolbachia* could occur due to the amplification of *wsp* sequences derived from infected parasitoids harboured in *P.*

chalcographus. Therefore beetles were dissected, ovarial tissue recovered, split and used for PCR and for *in situ* hybridization with *wsp* specific DIG-labelled probes (Chen et al., 2005). Hybridization and PCR results were compared.

2.2 Isolation and characterization of microsatellites

To obtain a full picture of the evolutionary history of populations the use of nuclear DNA markers is essential (Zhang and Hewitt, 2003). Microsatellites have proven to belong to the most powerful traits nowadays available (Jarne and Lagoda, 1996). Thus, the establishment of SSR markers seemed a method to verify the already obtained mitochondrial datasets and phylogenies and to gain deeper insight into the population structure of *P. chalcographus*.

The majority of microsatellite loci is isolated using enriched genomic libraries. Modified protocols based on the FIASCO method published by Zane et al. (2002) were used to isolate SSR markers from *P. chalcographus*. Primer pairs flanking microsatellite loci were developed.

As enrichment strategies remained cumbersome in some coleopterans, revealing only few SSR sequences (Keller and Largiadèr, 2003), short and interrupted repeat motifs (Alvarez et al., 2003; Gaublomme et al., 2003; Lagisz and Wolff, 2004), loci with low allele numbers (Gauthier and Rasplus, 2004; Patt et al., 2004a) or low levels of genetic diversity among populations (Kim and Sappington, 2005), vectorette PCR was tested as an alternative strategy for microsatellite isolation (Lench et al., 1996).

Preliminary genotyping was performed by amplifying microsatellite loci of beetles from different mitochondrial clades using fluorescent labelled forward primers, capillary electrophoresis on an ABI 310 genetic analyzer (Applied Biosystems, USA) and determination of allele sizes by the Genotyper software (Applied Biosystems, USA).

2.3 Transposons

Transposons have shown some potential as a nuclear genetic marker (Berenyi et al., 2002; Flavell et al., 1998). Due to the described difficulties connected with the use of SSR markers in coleopterans, TEs have been considered as a nuclear marker system for *P. chalcographus*.

As the prevalence of TEs in the eucaryot genome is high (Lewin, 2004) there is a good chance to pick TE sequences in any random stretch of nuclear DNA. Therefore, library clones for SSR isolation that did not harbour repeat sequences were further analyzed for TEs by submission to the CENSOR data base (Jurka et al., 2005).

A targeted search for class I and class II elements was performed using degenerate primed PCR (Arkhipova and Meselson, 2000). Multi-banded PCR products were obtained and several bands excised, cloned and sequenced, revealing three potential TE isolates.

3. Material and methods

3.1 General laboratory procedures

3.1.1 Frequently used buffers and media

Standard buffers (Tris.HCl, 0.5 M ETDA, 1 M DTT, 10% SDS, 20 x SSC, TE, 50 x TAE, 10 x TBE) were prepared and stored following the guidelines in Sambrook et al. (1989).
LB Agar and LB Broth (Bertani, 1951) was purchased as a dry medium from Fluka (USA) and prepared following the instructions of the manufacturer.
Church Buffer containing 0.25 M sodium phosphate, 1 mM EDTA, 1% BSA, 7% SDS was prepared according to Church and Gilbert (1984).
SOC Broth containing 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 20 mM magnesium chloride hexahydrate, 20 mM glucose, pH=7 was prepared according to Hanahan (1983).

3.1.2 Insect collection

Specimens of *Pityogenes chalcographus* were collected by Stauffer and Avtzis during summer 2004 at different locations throughout Europe and stored in ethanol at -20° C. Further specimens were provided from different European institutions and researchers. A complete overwiew is given in appendix 1.

A laboratory stock of populations obtained from Germany, Italy and Poland was maintained by Avtzis and provided a continuous source of fresh insects.

Specimens of *Messor structor* and *Lasius austriacus* were provided by Schlick-Steiner and Steiner. Samples of *Ips typographus*, *Ips duplicatus*, *Tomicus piniperda* and *Rhagoletis cerasi* used for some experiments were obtained from the collection of the Institute of Forest Entomology, Department of Forest- and Soil Sciences, University of Natural Ressources and Applied Life Sciences.

3.1.3 Extraction and storage of insect DNA

For most purposes insect DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, USA). Insects were put into 1.5 ml Eppendorf tubes, overlayed with 180 µl lysis solution T (all buffers and solutions provided with the Kit) and homogenized with a pestle. 20 µl Proteinase K-solution were added and the mixture incubated at 55° C for 1–2 h. After an RNase digest for 2 min at room temperature 200 µl lysis solution C were added, the mixture incubated for further 10 min at 70° C, supplemented with 200 µl ethanol and loaded onto a binding column. The columns were washed twice with 500 µl washing solution and DNA was eluted in 50 µl elution buffer.

Up to 3 weeks eluted DNA was stored at 4° C. Long term storage was performed at -20° C. When necessary, DNA content was determined using a SmartSpec spectrophotometer (Biorad, USA).

Only for use in southern blot applications DNA of single beetles was extracted by a salt precipitation method following the protocol Junakovic (2004). Insect tissue was ground in Eppendorf tubes with 100 µl ice cold homogenisation buffer (0.1 M Tris.HCl, 0.1 M EDTA, 1% SDS, pH=9) using an electric driven pestle and incubated for 60 min at 68° C. After cooling down to room temperature 14 µl 8 M potassium acetate solution were added, mixed thoroughly and incubated 30 min on ice. The mixture was centrifuged in a microfuge at maximum speed for 10 min and the supernatant transferred to a fresh Eppendorf tube. DNA was precipitated for 1–12 h with one volume of i-propanol and recovered by centrifugation. The pellet was washed with 70% ethanol, air-dried and dissolved in 50 µl TE buffer.

3.1.4 PCR amplifications

PCR reactions were carried out in 10 µl reactions containing 1.5 mM magnesium chloride, 50 µM dNTPs (Fermentas, Lithuania), 0.2 µM of each primer, 0.4 U Biotherm *Taq* DNA polymerase (Genecraft, Germany) and the reaction buffer provided by the manufacturer of the polymerase. Thermocycling was performed in a

Primus 25 advanced thermocycler (peqlab, Germany) in 200 µl thin walled tubes (Biozym, Germany).

3.1.5 Primer design

For design of new PCR primers target sequences were analyzed using the Primer3 software (Rozen and Skaletsky, 2000). Proposed oligos were further modified manually. For the calculation of the molecular weight, annealing temperatures and secondary structures like dimers or hairpins the Fast-PCR software (Kalendar, 2005) was used. Primers were synthesized by Invitrogen Corporation (USA) and by Ingenetix GmbH. (Austria).

Table 3.1. Universal degenerate code

Index	Base(s)
M	A / C
R	A / G
W	A / T
S	G / C
Y	C / T
K	G / T
V	A / G / C
H	A / C / T
D	A / G / T
B	C / G / T
N	A / C / G / T

Universal degenerate code (Table 3.1) was used for the description of primer sequences.

3.1.6 Preparation of competent cells

E. coli JM 109 cells (Promega, USA) were maintained as frozen stock (-80° C) in the laboratory. An aliquot of this stock was thawed, plated onto LB agar and grown over night at 37° C. A single colony was inoculated into a tube with 2.5 ml LB broth, incubated at 37° C for 12 h and transferred to a 500 ml bottle containing 250 ml LB broth for further growth until OD_{600} values between 0.3 and 0.4 were reached. The culture was split in 50 ml aliquots into Falcon tubes, cooled on ice and centrifuged for 10 min at 3000 rpm and 4° C. The supernatant was discarded and each pellet dissolved in 5 ml ice cold transformation and storage solution (TSS; Chung et al., 1989), an LB broth containing 10% (w/v) polyethylene glycol 3350, 5% (v/v) dimethyl sulfoxide and 25 mM magnesium chloride. Aliquots of 300 µl were filled into pre-chilled Eppendorf tubes, shock frozen in liquid nitrogen and stored at -80° C.

3.1.7 Cloning procedures

PCR products were purified with the QiaQuick PCR purification kit (Qiagen, USA) and ligated into plasmids using the pGEM-T vector system (Promega). For a single ligation 1 to 5 ng PCR product were mixed with 0.2 µl pGEM vector, 0.2 U T4 ligase and 2 µl rapid ligation buffer (all reagents provided with the kit). A final volume of 4 µl was set with sterile water and the reaction incubated over night at 4° C.

Older PCR products and products derived from reactions containing polymerases with proofreading activity were additionally objected to a tailing procedure resembling 3'-adenosin overhangs: 10 to 20 ng of PCR product were mixed with 0.4 U Biotherm *Taq* DNA polymerase (Genecraft), 1 µl Biotherm Buffer and 0.2 µM dATP (Fermentas), the reaction volume was adjusted to 10 µl with sterile water and the mixture incubated at 70° C for 30 min. 2 µl of the reaction product were subsequently used as DNA source in the above described ligation procedure.

Ligated DNA was split into two aliquots of 2 µl, transferred in 1.5 ml Eppendorf tubes and placed on ice. Meanwhile an aliquot of competent *E. coli* cells was thawed on ice, mixed gently and 50 µl cell suspension were placed into each tube using pre-chilled pipette tips. The plasmid-cell mixture was incubated on ice for 20 min, heat-shocked at 42° C for 50 sec in a water bath and put back to ice for 2 more min. 950 µl SOC broth were added to each tube and the cells grown for 1 h at 37° C. Afterwards the cells were pelleted by centrifugation at 2500 rpm for 5 min in a chilled centrifuge and resuspended in 100 µl LB broth. The suspension was plated onto one or two petri dishes with LB Agar containing 100 µg/ml ampicillin, 50 µg/ml IPTG and 40 µg/ml X-Gal. The plates were grown at 37° C over night and isolated white colonies were picked for further characterization.

For fast determination of a colonies insert length standard PCR reactions containing T7 and SP6 primers but no template DNA were prepared on ice. A transformed bacterial colony was probed with a sterile wooden toothpick that was afterwards shortly submerged in the PCR mastermix; the toothpick was subsequently used to inoculate LB broth for plasmid purification. PCR amplification was performed with 5

minutes cell lysis at 96° C followed by 32 cycles of 94° C (30 sec), 54° C (45 sec) and 72° C (60 sec). The length of the amplicon was determined by gel electrophoresis and resembled the insert length plus 164 bp.

3.1.8 Plasmid purification

Plasmid purification was achieved by an alkaline lysis procedure (Sambrook et al., 1989). All centrifugation steps were performed at 13000 rpm in a cooled microfuge (Sigma, USA). 1.5 ml of an overnight culture of transgenic *E. coli* were transferred to an Eppendorf tube and centrifuged for one min. The supernatant was discarded and the pellet resuspended in 100 µl suspension buffer (50 mM Glucose, 10 mM EDTA, 25 mM Tris.HCl, pH=7.5). 5 U RNase A were added and the mixture incubated at room temperature for 5 min. Afterwards 200 µl lysis solution (0.2 N sodium hydroxide, 1% SDS) and 150 µl ice cold neutralisation solution (3 M K^+, 5 M Ac^-) were added, with vigorous mixing after each step. The tubes were incubated on ice, centrifuged for 5 min and the supernatant transferred to a new tube. Plasmid DNA was precipitated for 2 min with two volumes ethanol, recovered by centrifugation, washed with 70% ethanol, air dried and dissolved in 100 µl sterile water or 10 mM Tris.HCl, pH=8.

3.1.9 Agarose gel electrophoresis

Agarose slab gel electrophoresis for the determination of length and concentration of DNA fragments was performed in a submarine horizontal gel system (C.B.S. Scientific, USA) using 1x TAE as running buffer. Depending on the fragment size gels with 1 to 2% agarose concentration and supplemented with 0.5 µg/ml ethidium bromide were used. DNA was visualized on a UV transilluminator (UVP Inc., USA) and documented with a digital camera (Sony, Japan).

3.1.10 Southern hybridization techniques

Digoxigenin (DIG) labelled specific probes were constructed by PCR labelling. Standard PCR reactions at a 25 µl scale where dNTPs were replaced by a mixture

containing 2 mM of each dATP, dCTP and dGTP, 1.9 mM dTTP and 0.1 mM DIG-11-dUTP (Roche, Germany) were performed using appropriate template, primers and cycling conditions. Parallel a standard reaction with unlabelled dNTPs was performed at a 10 µl scale.

5 µl of the labelled and unlabelled product were loaded to an agarose minigel to control PCR efficiency and DIG-dUTP incorporation. Bands of similar brightness were expected with a reduced migration rate for the labelled DNA. Remaining labelled product was mixed with 15 µl Church buffer and 0.15 mg denatured salmon sperm (Roche). The probe solution was stored in Falcon tubes at -20° C and thawed and denatured for 10 min in a boiling water bath immediately before use.

Transfer of DNA from agarose gels to nylon membranes was performed as described by Sambrook et al. (1989). The DNA was crosslinked by 2 min of UV exposition on a transilluminator. Membranes were placed in hybridization tubes and pre-hybridized for 1 h at 60° C in 15 ml Church buffer containing 1.5 mg denatured salmon sperm (Roche) under constant agitation. The prehybridization solution was discarded and the membranes hybridized over night with specific probe solution. Probe solutions were recovered, stored at -20° C and re-used several times.

The membranes were washed two times 5 min in 2x SSC/0.1% SDS at room temperature, 15 min in 0.5x SSC/0.1% SDS at 60° C and 15 min in 0.1x SSC/0.1% SDS at 60° C. Subsequent steps were performed at room temperature. Membranes were equilibrated 2 min in wash buffer (0.1 M maleic acid, 0.15 M sodium chloride, 0.3% Tween 20, pH=7.5), incubated 30 min in 20 ml DIG blocking agent (Roche) and 30 min in 20 ml DIG blocking agent supplemented with 2 µl Anti-DIG antibody conjugated to alkaline phosphatase (Roche). Antibody solution was recovered, stored at 4° C and re-used several times. Membranes were washed two times 15 min in wash buffer and equilibrated 5 min in detection buffer (0.1 M Tris.HCl, 0.1 M sodium chloride, pH=9.5). Excess liquid was removed with blotting paper and membranes were placed in a plastic envelope. Approximately 1 ml CSPD solution (Roche) per 100 cm^2 was spread over the membranes, the envelopes sealed and incubated at

37° for 10 min. Membranes were exposed to Biomax x-ray films (Kodak, USA) for 2 to 12 h.

For re-probing membranes were briefly soaked in distilled water, incubated two times 15 min in stripping solution (0.2 M sodium hydroxide, 0.1% SDS) at 37° C under constant agitation and equilibrated in 2x SSC.

3.1.11 DNA sequencing

Sequencing of plasmids or PCR products with automated sequencing machines was performed externally by Genterprise Genomics GmbH. (Mainz, Germany) and by the Cancer Research Center DNA Sequencing Facility (University of Chicago, USA), following the guidelines of these institutions. Sequence chromatograms and listings were provided electronically.

3.2 Mitochondrial DNA

3.2.1 Design and application of SSCP primers

Fragments of 96 individuals covering parts of the CO1 gene were amplified and sequenced by Avtzis (2005) using the primers K698 (Caterino and Sperling, 1999; 5′-TACAATTTATCGCCTAAACTTCAGCC-3′) and UEA10 (Lunt et al., 1996; 5′-TCCAATGCACTAATCTGCCATATTA-3′). Based on these sequences seven forward- and six reverse primers were designed as described in 3.1.5. Primer sequences and amplicon sizes are given in table 3.2.

Amplifications were carried out under standard PCR conditions with 3 min initial denaturation at 94° C followed by 32 cycles of 94° C (30 sec), 60° C (45 sec) and 72° C (45 sec) and a final extension step at 68° C (10 min).

Table 3.2. SSCP primers. Sequences (5'→3') of 7 primer pairs amplifying 200-250 bp regions within the CO1 gene. PCR products were used for SSCP genotyping.

Fragment	Forward primer		Reverse primer		size (bp)
	Start[a]	Sequence	Start[a]	Sequence	
SSCP 1	2200	AAAAGAAGCTTTCGGAG	2424	AGGGCTGAGGGACTAAAAG	224
SSCP 2	2553	CAATAGGGGCTGTATTTGCC	2764	AATGTTTCAGAGAAGGTAGGC	211
SSCP 3	2341	CATTGCTGTTCCTACAGGAAT	2590	CACAATGCCTGCAATAATGG	249
SSCP 4	2737	TCCAGATGCCTACCTTCTCTG	2989	TCCAATGCACTAATCTGCCATATTA[b]	252
SSCP 5	1481	TTTTGGAGCATGATCA	1695	CGTGGAAATGCTATATCTG	214
SSCP 6	1673	GCCCCAGATATAGCATTTCC	1873	AATTCCTGATATATGAAGGCTG	200
SSCP 7	1889	GCCATCAATTTCATTTCTACAA	2140	ATAAACTTCTGGGTGGCC	251

[a] relative position of 5′ end to the *Drosophila yakuba* genome (Clary and Wolstenholme, 1985)
[b] SSCP 4 reverse primer is identical with UEA10 (Lunt et al., 1996)

PCR products were mixed with one volume SSCP loading dye (50% formamide, 0.2% bromophenole blue, 0.2% xylene cyanole), denatured for 5 min at 95° C and cooled immediately on ice. A 10 µl aliquot was loaded to a native polyacrylamide gel (10.5% acrylamide, 5% glycerol, 0.55% bis-acrylamide, 0.2% TEMED, 0.05% ammonium persulphate in 0.5x TBE) and separated on a dual vertical slab gel system (C.B.S. Scientific) at 200–250 V for 10–20 h. The temperature of the apparatus was held constant by a cooled circulating water bath (MGW Lauda, Germany). DNA was visualized by silver staining (Caetano-Anollés and Gresshof, 1994) or by submerging

the gel in 1x SyBr Gold I (Molecular Probes, USA) for 10 min under constant agitation followed by UV transillumination.

Table 3.3. Individuals for SSCP evaluation. DNA of 36 individuals with known CO1 sequence and thus assigned to a specific clade was used for implementation of SSCP.

Clade	Haplotype	Individual
I	1	59SW9
	1	38RO10
	1	30F9
	1	53PL5
	2	65DE3
	4	52SLOVA10
	7	66DE5
	9	59SW2
II	10	1678IT11
	11	12IT8
IIIb	12	12IT10
	12	1891IT10
	12	1896IT12
	12	12IT3
	13	1680IT11
IIIa	14	66DE6
	15	66DE2
	16	65DE8
IIIc	18	12IT2
	19	1894IT12
	20	1679IT11
	21	12IT7
IIId	22	52SLOVA8
	23	62NO10
	23	53PL7
	23	59SW6
	23	65DE5
	25	65DE10
	26	1882PL4
	27	62NO1
	29	1895IT12
	30	66DE8
	33	38RO6
	33	1888CH
	33	65DE6
	33	1885FR5

For implementation optimal electrophoresis temperature was determined. DNA of 6 sequenced individuals identified as haplotypes 1, 2, 3, 5 and 6, amplified with the primers SSCP1, SSCP2, SSCP3 and SSCP4 and of further 6 individuals of haplotype 6, 11, 13, 15, 20 and 33 amplified with primers SSCP5, SSCP6 and SSCP7. Products were separated at 4°, 15° and 25° C. Optimal running temperature and separation time were determined empirically.

To test the ability of the primers to produce unique banding patterns for clade discrimination, 36 individuals representing 24 haplotypes in 7 clades were selected for further analysis (Table 3.3). The haplotypes of all individuals had been determined by Avtzis (2005) based on sequencing the region flanked by the primers UEA5 and UEA10 (Lunt et al., 1996). To examine stability of the observed patterns haplotypes 1, 12, 23 and 33 were represented with four individuals each.

Mass screening was carried out with primers SSCP6 and SSCP3 using an identical setup at optimized temperature and runtime conditions.

3.2.2 *In silico* analysis of mutations detected in CO1 sequences

Following molecular characteristics were selected to discriminate numt and mtDNA:

- overall AT bias of mtDNA
- codon substitution bias

- abundance of nonsynonymous substitutions
- CT substitution bias
- presence or absence of insertions/deletions and additional stop codons
- transition-transversion ratio

CO1 consensus sequence and observed mutational sites were listed in an Excel file; comparisons were computed using Excel and GeneRunner Version 3.05.

3.2.3 Development of long PCR primers

Mitogenomic sequences of the coleopteran species *Pyrocoelia rufa* (Lampyridae; Acc. Nr. NC_003970), *Tribolium castanaeum* (Tenebrionidae; Acc. Nr. NC_003081) and *Crioceris duodecimpunctata* (Chrysomelidae; Acc. Nr. NC_003072) were obtained from GeneBank and aligned using Clustal X (Thompson et al., 1997). To faciliate identification of conserved regions sequences of *Apis mellifera* (Hymenoptera, Apidae; Acc. Nr. NC_001566), *Bombyx mori* (Neoptera, Bombycidae; Acc. Nr. NC_002355) and *Drospohila simulans* (Diptera, Drosophilidae; Acc. Nr. NC_005781) were further included to the alignment. Conserved regions were selected for primer design. In case of SNPs within conserved regions bases from the coleopteran species were included preferably. Developed primers are shown in table 3.4.

Table 3.4. long PCR primers. Sequences (5' → 3') of 3 forward and 3 reverse primers located in different regions of mtDNA.

Primer	Region	Start[a]	Orientation	Sequence
Met/F	methionin tRNA	148	forward	gct wht ggg ttc ata ccc
CO2/R	CO2 gene	3611	reverse	caa att tct gaa cat tg
CO3/R	CO3 gene	5270	reverse	cca tga aat cct gtt gc
ND4/F	ND4 gene	8437	forward	acc atg agh aat tat ta
CyB/R	cytochrome B gene	11093	reverse	aat atc att ctg gtt kaa tat g
sRN/F	s-rRNA	14286	forward	agg gta tct aat cct ag

[a] relative position of 5' end to the *Drosophila yakuba* genome (Clary and Wolstenholme, 1985)

Fresh *P. chalcographus* DNA was extracted with the GenElute kit (Sigma), stored at 4° C for up to 6 weeks and used as template for PCR optimization. Reactions were set up at a 10 µl scale containing 0.4 µM of each primer, 200 µM dNTPs, 0.4 U *Taq* DNA polymerase (Sigma), 0.01 units Sawady *Pwo* polymerase (peqlab) and 1 µl

template DNA in the buffer provided with the *Pwo* polymerase. Magnesium was provided at concentrations from 2 to 6 mM at 1 mM steps. PCR conditions were 3 min initial denaturation at 94° C followed by 32 cycles of 94° C (30 sec), 55° C (1 min) and 68° C (t_e min) and a final extension step at 68° C (10 min). Extension times were set to t_e=1.5 and t_e =2.5 min.

To determine the influence of template concentration reactions identical to those described above were set up. Magnesium was supplied at 2, 4 and 6 mM and DNA at 0.25 and 2.5 µl. Extension time was 2.5 min.

For estimation of suitable template dilution factors, a long PCR under optimized conditions was performed at a 10 µl scale with 1 µl template DNA. The product was diluted 1:100, 1:1000 and 1:10000 with sterile water. 1 µl of the dilutions was used as template in a nested PCR reaction using the CO1 specific primers UEA10 and UEA5 (Lunt et al., 1996). As a control, genomic DNA was diluted 1:1000, 1:10000 and 1:100000 corresponding to the genomic DNA contents in the diluted long PCR products, and amplified the same way.

To evaluate the influence of DNA degradation after prolonged storage, DNA of 16 individuals from different origins and with different storage history was amplified with Met/F and CO2/R primers under optimized conditions. Freshly extracted DNA was used as positive control. After amplification, 6 µl PCR product were visualized on a 1% agarose gel. The remaining product was diluted 1:10000 and used as template in a nested PCR as described before. Products were again visualized on a 1% agarose gel.

3.2.4 Nested PCR

For exclusion of potential numt contamination outer PCR was performed in 10 µl reactions using 0.4 µM of each Met/F and CO2/R primer, 6 mM magnesium sulphate, 200 µM dNTPs, 0.4 U *Taq* DNA polymerase (Sigma), 0.01 U Sawady *Pwo* polymerase (peqlab) and 1 µl DNA template in the buffer provided with the *Pwo* polymerase. Cycling conditions were 3 min initial denaturation at 94° C followed by 32 cycles of

94° C (30 sec), 55° C (1 min) and 68° C (2.5 min) and a final extension step at 68° C (10 min).

Products were diluted 1:10000 with sterile distilled water and 1 µl diluted amplicon was used as template for any inner PCR with CO1 specific primers.

3.2.5 Identification of *Wolbachia* infections by long and nested PCR

Table 3.5. Samples for *Wolbachia* detection. The DNA had been analyzed before by Avtzis (2005); corresponding results are shown in the left column. Results indicated with ± represent weak PCR amplicons.

Id. #	Ind.	Clade	*Wolbachia*
1	53.4	I	+
2	59.1		+
3	62.10		+
4	65.8		+
5	52.6		±
6	59.10		±
7	62.5		±
8	62.6		±
9	5.1		-
10	52.10		-
11	62.7		-
12	65.2		-
13	59.4	IIIb	±
14	65.3		±
15	69.5		±
16	69.8		±
17	12.4		-
18	65.6		-
19	69.4		-
20	75.1		-

A set of 20 DNA extracts from single *P. chalcographus* beetles already examined by Avtzis (2005) was chosen for optimization of *Wolbachia* detection methods (Table 3.5)

For PCR detection of *Wolbachia* the primers wsp-f (5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3') and wsp-r (5'-AAAAATTAAACGCTACTCCAGCTTCTGCAC-3') amplifying a 600 kb fragment of the *wsp* gene (Jeyaprakash and Hoy, 2000) were used. PCR was performed at a 10 µl scale with 1 µl genomic DNA as template. Three different reaction chemistries were used: Mixture A contained the standard PCR ingredients (see chapter 3.1.4). Mixture B contained 0.5 µM of each primer, 1.5 respectively 3 mM magnesium sulphate, 100 µM dNTPs, 0.4 U *Taq* polymerase (Sigma) and 0.01 U Sawady *Pwo* polymerase (peqlab) in the magnesium free buffer provided with the *Pwo* polymerase. Mixture C contained 0.5 µM of each primer, 100 µM dNTPs, 0.08 µl of the long PCR enzyme mix (Fermentas) and the complete reaction buffer provided with the enzyme mix.

Cycling conditions for mixture A were 3 min initial denaturation at 94° C followed by 35 cycles of 94° C (30 sec), 65° C (1 min) and 72° C (1 min) and a final extension step at 68° C (10 min). Cycling conditions for mixtures B and C were 3 min initial denaturation at 94° C followed by 10 cycles of 94° C (10 sec), 65° C (30 sec) and

68° C (1 min), 25 cycles of 94° C (10 sec), 65° C (30 sec) and 68° C (1 min plus additional 20 sec for every consecutive cycle) and a final extension step at 68° C (10 min).

For nested PCR reaction products were diluted 1:100 with sterile water. 1 µl of this dilution was used as template in a standard PCR reaction with primers wsp81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and wsp691R (5'-AAAAATTAAACGCTACTCCA-3') (Zhou et al., 1998). Cycling conditions were 2 min initial denaturation at 94° C followed by 35 cycles of 94° C (1 min), 55° C (1 min) and 72° C (1 min) and a final extension step at 72° C (10 min).

One uninfected (Manaos) and two infected (California, Rio de Janeiro) strains of *Drosophila simulans* were provided by Wolfgang Miller (Center for Anatomy and Cell Biology, Medical University of Vienna) and used as negative and positive controls.

3.2.6 Identification of *Wolbachia* infections by *in situ* hybridization

Insects were vivisected under a stereo microscope using sterile forceps and scalpel blades. Ovarial tissue was recovered, objected to microscope slides, pre-fixed with a drop of methanol and air-dried over night. Final fixation was carried out in a drop of 0.4% formaldehyde at 4° C for 5 min. Slides were washed twice by pipetting 2 ml buffer 1 (100 mM Tris.HCl, 150 mM sodium chloride, pH=7.4) atop the tissue, letting stand for 30 sec and decanting the liquid (Chen et al., 2005).

After 10 min air-drying 10 µl of a hybridization solution containing 1 ng/µl of a DIG-labelled *wsp* specific probe, 5% (w/v) dextrane sulphate, 2% (v/v) denatured salmon sperm, 1x SSC, 1x Denhart's reagent (Sambrook et al., 1989) and 50% (v/v) formamide were placed on the slide under a cover slip. Tissue was denatured for 5 min at 96° C, cooled on ice and hybridized over night at 42° C in a humid chamber.

The cover slip was removed and the slide washed two times 5 min with 2x SSC at room temperature and once 5 min with 0.1x SSC at 42° C. All subsequent steps were carried out at room temperature. The slide was exposed to buffer 2 (100 mM

Tris.HCl, 150 mM sodium chloride, 0.5% (w/v) blocking reagent (Roche), pH=7.4) for 15 min, briefly washed with buffer 1 and air-dried for 10 min. 10 µl Anti-DIG antibody conjugated to alkaline phosphatase (Roche; 1:500 in buffer 2) were placed atop each tissue specimen and incubation was performed for one h in a humid chamber. Slides were washed two times 5 min in buffer 1 and equilibrated 5 min in buffer 3 (100 mM Tris.HCl, 150 mM sodium chloride, 1% (w/v) BSA, 0.3% (v/v) Triton X-100, pH=7.4).

Staining was performed with 20 µl NBT/BCIP solution (Amresco, USA) in the dark under a cover slide. As soon as a purple color became visible (30 min up to several h) the cover slip was removed, the sample washed briefly with distilled water, mounted and microscopy was performed to detect cells infected with *Wolbachia*.

For positive and negative control *D. simulans* strains as described in 3.2.5 were used.

3.3 Microsatellites

3.3.1 Production of DIG labelled oligoprobes

DIG labelled dinucleotide oligoprobes were synthesized by a PCR reaction following the protocol of Kruckenhauser (2000). Each 0.4 mM of asymmetric priming oligos (5′-GTGTGTGTGTGTGTG-3′ and 5′-CACACACAC-3′ for $(AC)_x$ probes, 5′-GAGAGAGAGAGAGAG-3′ and 5′-CTCTCTCTC-3′ for $(GA)_x$ probes) were mixed with 10 µl PCR DIG labelling mix (Roche), 5 U Biotherm *Taq* DNA polymerase (Genecraft) and the buffer provided with the enzyme in a total volume of 100 µl. The mixture was objected to 35 cycles of 39° (1 min), 72° (1 min) and 94° (1 min). Repeated terminal extensions result in a population of repeat containing DNA molecules ranging from 200 to about 2500 bp, seen as an intensive smear after agarose gel electrophoresis.

The labelled product was loaded to a preparative 1.5% agarose gel with the DNA in the range 300 to 700 bp excised and recovered. $(AC)_x$ and $(GA)_x$ probes were mixed together with 1.5 mg denatured salmon sperm (Roche) and 15 ml Church buffer. Storage of the probes was as described in chapter 3.1.10.

3.3.2 Construction of enriched libraries by agarose bound avidine

Based on the 'Microsatellite Manual' (Glenn, 2000) and the FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) protocol (Zane et al., 2002) a protocol was developed for the construction of genomic libraries enriched for microsatellite sequences. The construction of such a library was performed using the following steps:

i) digestion and adaptor ligation

100 to 250 ng of insect genomic DNA were digested with *MseI* and ligated to AFLP adaptors in a one-step reaction. The reaction mix contained the DNA, 1x REact 1 buffer (Invitrogen), 5 mM DTT, 50 µg/ml BSA, 200 µM ATP, 1 µM of each *MseI* AFLP adaptor (5'-TACTCAGGACTCAT-3', 5'-GACGATGAGTCCTGAG-3'), 2.5 U of *MseI* (Invitrogen) and 1 U of T4 DNA ligase (Promega) in a total volume of 25 µl and was incubated for 3 h at 37° C. After incubation the enzymes were inactivated by heating to 70° C for 5 min and 225 µl of sterile water were added to the mixture. The efficiency of the ligation was tested with a standard PCR reaction containing 2.5 µl of the inactivated, diluted DNA and 0.2 µM of each *MseI*-AFLP adaptor primer (5'-GATGAGTCCTGAGTAAn-3'; 4 different primers with the possible bases A/C/T/G at the 3' position were used simultaneously). To determine the onset of the logarithmic amplification phase reactions were exposed to 14, 17, 20, 23, 26 and 29 cycles of 94° C (30 sec), 53° C (60 sec) and 72° C (60 sec). 5 µl product were loaded onto a 2% agarose gel and a cycle number where a faint smear became visible on the gel was selected for a production PCR at a 100–200 µl scale to yield approximately 1 µg amplified DNA.
The AFLP procedure offers a smart possibility to exclude a biased overrepresentation of multicopy elements in the library. Such elements appear as distinct bands embedded in the smear of fragments. By exploring the reaction products of combinations of only 3 or even 2 of the primers conditions might be found where the multicopy bands disappear.

ii) avidin capture of microsatellite sequences

PCR products of step (i) were pooled, purified using the QiaQuick PCR purification kit (Quiagen) and the DNA content was determined photometrically. 500 ng DNA were

mixed with 20 pmoles of biotinylated SSR oligos in 100 µl reaction volume containing 4.2x SSC and 0.7% SDS. Multiple combinations of compatible oligoprobes were tested during development of the method. The mixture was denatured for 5 min in a boiling water bath and allowed to cool down to room temperature for 15 min. In the meantime 75 µl of Vectrex agarose bound avidin D beads (Vector Labs, USA) were pelleted by centrifugation (all centrifugations with Vectrex beads were carried out at 4° C and 15000 rpm for 2 min). The supernatant was removed and the beads washed twice with 400 µl TBST (100 mM Tris.HCl, 150 mM sodium chloride, 0.1% Tween 20, pH=7.5) and resuspended in 350 µl TBT (100 mM Tris.HCl, 150 mM sodium chloride, pH=7.5). The DNA-probe mix was added to the beads and hybridized 1 h at 50° C under constant agitation.

Afterwards the beads were pelletet and the supernatant containing unbound fragments was removed. The beads were washed three times with 400 µl TBST and three times with 400 µl 0.2x SSC, 0.1% SDS. The last washing solution was recovered for further use. 100 µl TLE (10 mM Tris.HCl, 0.1 mM EDTA, pH=8.0) were added to the beads and the DNA eluted by 5 min incubation at 96° C, cooling on ice, centrifugation and recovery of the supernatant. A second elution was performed by adding 12 µl 0.15 M sodium hydroxide to the remaining beads, incubating 5 min at room temperature, neutralizing with 38 µl 0.1 M Tris.HCl pH=7.2 and recovery of the supernatant.

Unbound fragments, the last washing solution and the two elutions were precipitated with two volumes 96% ethanol at -20° C over night. DNA was recovered by centrifugation, washed with 70% ethanol, air dried and dissolved in 50 µl sterile water. 1 µl of each fraction was used as template in PCR reactions at similar conditions as described in (i) and with 22 cycles to estimate the specific DNA content at the different enrichment steps.

iii) cloning of enriched DNA

One or both eluates were used for a recovery PCR at similar conditions as described in (i) over 32 cycles. The products were purified using the QiaQuick PCR purification kit and up to five parallel ligation and transformation reactions were set up to maximize the number of transformed colonies. White colonies were transferred to masterplates with 82 specimens each.

iv) screening of the library

After 16 h growth the master plates were probed with sterile nylon membranes (Roche), sealed with parafilm and stored at 4° C. The membranes were incubated on the surface of blotting paper soaked with 10% SDS for 3 min, denaturing solution (0.5 N sodium hydroxide, 1.5 M sodium chloride) for 15 min, neutralisation solution (1.5 M sodium chloride, 0.5 M Tris.HCl, pH=7.4) for 5 min and 2x SSC for 5 min. Afterwards the membranes were air dried for 30 min and DNA was crosslinked by UV exposition an a transilluminator for 2 min. 1 ml of 2x SSC containing 60 U of proteinase K (Sigma) was spread on each membrane. The membranes were placed on plastic trays, covered with saran wrap and incubated for 1 h at 37° C. Cell debris was removed by pressing wet blotting paper against the membranes. Separated by nylon nets the membranes were placed in hybridization tubes and prehybridized at 60° C for 1 h in 15 ml Church buffer containing 1.5 mg denatured salmon sperm (Roche).

The prehybridization solution was discarded and either 5'-biotinylated oligoprobes in Church buffer or a digoxigenin labelled $(AC)_x$+$(GA)_x$ oligoprobe solution was hybridized to the membranes at 60° C over night.

After hybridization membranes were placed in individual plastic petri dishes and washed two times in 2x SSC, 0.1% SDS at room temperature (5 min), once in 0.5x SSC, 0.1% SDS at 60° C (15 min) and once in 0.1x SSC, 0.1% SDS at 60° C (15 min). The presence of probe-target hybrids was detected using the Biotin respectively DIG luminescent detection kit (Roche) following the instructions of the manufacturer. Membranes were exposed to Biomax x-ray film (Kodak) for 4 to 24 h.

v) retrieval of candidate clones

After development of x-ray films colonies with strongly positive signals were located on the masterplates, evaluated for insert length as described in (3.1.7) and transferred to LB broth. Plasmids carrying inserts longer than 300 bp were extracted for further investigations and sequencing.

3.3.3 Construction of high stringency enriched libraries using PMPs

Streptavidine MagneSphere Paramagnetic Particles (PMPs, Promega, USA) were evaluated as an alternative system for capturing microsatellite sequences with biotinylated oligos.
Sections i, iii, iv and v of the protocol presented in the last section remained unchanged.

ii) streptavidin capture of microsatellite sequences
1 µg purified PCR product was diluted with sterile water to a total volume of 250 µl, denatured 5 min in a boiling water bath and cooled on ice. Each 20 pmoles of $(AC)_8$ and $(GA)_8$ biotinylated oligoprobe and 13 µl 20x SSC were added and the final volume set to 500 µl. The mixture was hybridized one h at 50° C under constant agitation.
In the meantime 300 µl of PMPs were separated on a two-position magnetic separation stand (Promega). The liquid fraction was removed and discarded, the PMPs washed three times with 300 µl 0.5x SSC and resuspended in 100 µl 0.5x SSC. The DNA-probe mixture was added to the PMPs and incubated 20 min at room temperature under constant agitation.
PMPs were separated and the unbound DNA fraction recovered for further use. Afterwards PMPs were washed four times with 300 µl 0.1x SSC recovering the last washing solution. For DNA elution 100 µl of pre-warmed sterile water were added to the PMPs, incubated at 50° C for 3 min and the liquid phase was removed fast after magnetic separation.
The unbound fraction, last wash solution and eluted DNA were precipitated with two volumes 96% ethanol at -20° C over night, DNA was recovered by centrifugation, washed with 70% ethanol, air dried and dissolved in 50 µl sterile water. 1 µl of each fraction was used as template in PCR reactions at similar conditions as described in (i) and with 22 cycles.

3.3.4 Construction of low stringency enriched libraries using PMPs

Reaction conditions were the same as described before, but after coincubation of PMPs and DNA-probe hybrids the PMPs were washed twice with 300 µl 0.5x SSC and twice with 0.2x SSC.

3.3.5 PCR pretesting of plasmid DNA for the presence of SSRs

0.2 µl plasmid DNA retrieved after enrichment was used as template in a standard PCR reaction containing the primers $(AC)_8$, $(GA)_8$ and SP6. Amplification was performed with an initial denaturation at 94° C (3 min) followed by 32 cycles of 94° C (30 sec), 59° C (45 sec) and 72° C (45 sec). Microsatellites in the plasmid insert formed primer binding sites for the SSR oligos resulting in a visible band shorter than the determined insert length. Plasmids that gave negative results in this step were objected to a further PCR with $(AC)_8$, $(GA)_8$ and T7 as primers. Plasmids with positive reaction in any of the two PCRs were classified as potential SSR isolates and selected for sequencing.

3.3.6 Search for microsatellite sequences by vectorette PCR

500 ng of insect DNA were mixed with 5 U of *SauIII* (Invitrogen) in 50 µl total volume containing 1x the reaction buffer provided with the restriction enzyme. The mixture was incubated at 37° C for 3 hours and the enzyme inactivated by heating to 70° C for 10 minutes.

In the meantime a mixture containing 2.5 µM of each vectorette adaptor V1 (5'-GATCTCCGGTACATGATCGAGGGGACTGACAACGAACGAACGGTTGAGAAGGGAGAG-3') and V2 (5'-CGCTCTCCCTTCTCCTAGCGGTAAAACGACGGCCAGTCCTCGATCATGTACCGGA-3'), 100 mM Tris.HCl pH7.2 and 10 mM magnesium chloride was denatured at 100° C for 5 min and ramped to 4° C over 90 min.

10 µl of digested genomic DNA and 5 µl of the adaptors were mixed with 0.25 U of T4 DNA ligase (Promega) in a total volume of 20 µl containing 1x ligase buffer. The mixture was incubated over night at 4° C, the ligase inactivated by heating to 70° C for 10 minutes and the final volume was set to 100 µl with sterile water.

First vectorette PCR was performed at a 25 µl scale containing 1x GeneAmp PCR buffer (Applied Biosystems, USA), 2 mM magnesium chloride, 100 µM dNTPs, 0.5 µM vectorette primer (5'-TAGCGGTAAAACGACGGCCAG-3'), 0.5 µM of SSR specific primer, 3 U AmpliTaq Gold (Applied Biosystems) and 1 µl adaptor-DNA-ligate. PCR conditions were 3 min initial denaturation at 94° C followed by 34 cycles of 94° C (30 sec), 63° C (60 sec) and 72° C (60 sec), and 10 min final extension at 72° C. Repeat-oligos $(AC)_8$, $(GA)_8$ and $(CTT)_6$ were used as SSR specific primers.
Products from the first vectorette PCR were loaded onto a 1.5% preparative agarose gel. Smeared products with a size between 300 and 1000 bp were excised and DNA recovered using the GenElute gel extraction kit (Sigma), cloned into pGEM vector (Promega) and used for subsequent *E. coli* transformation. White colonies were picked, plasmid extracted and sequenced using vector primers.
Based on sequences containing microsatellite repeats specific primers with annealing temperatures close to 60° C, located in the SSR flanking region and facing towards the SSR were developed. Reaction conditions for the second vectorette PCR were similar as for the first PCR with one specific primer per reaction replacing the SSR primer. Products were loaded to a preparative gel and distinct bands, usually embedded in light smears, were excised, cloned and sequenced as described above.

3.3.7 Microsatellite genotyping

After identification of microsatellite carrying sequences flanking primers were designed as described in 3.1.5 and tested in standard PCR reactions on plasmid and genomic DNA. Primer pairs giving strong bands of correct size were selected and labelled forward primers with either 5'-FAM, 5'-HEX or 5'-TET fluorophores were obtained from Ingenetix GmbH.

DNA of beetles representing 12 haplotypes from the four major mitochondrial clades was selected for preliminary genotyping. PCR reactions followed the standard protocol. Cycling conditions were 3 min initial denaturation at 94° C followed by 32 cycles of 94° C (30 sec), 60° C (60 sec) and 72° C (45 sec), and a final extension time of 20 min at 68° C to ensure complete 3'-adenylisation of all products. Products with compatible allele sizes and fluorescent dyes were mixed, supplemented with

GeneScan 500-TAMRA size standard (Applied Biosystems) and separated on an ABI 310 genetic analyzer (Applied Biosystems). Allele sizes were determined using the Genotyper software (Applied Biosystems).

3.4 Transposable genetic elements

Highly degenerate primer sets developed by Arkhipova and Meselson (2000) were used to isolate transposable element (TE) sequences in two subsequent PCR reactions.

1 µl genomic DNA extract of single individuals from two Austrian (A, B) and one Norway (C) populations was used as template in a first PCR containing 1x Sawady *Pwo* polymerase buffer (peqlab), 3.75 mM magnesium sulphate, 125 µM dNTPs, 25 µM of each primer, 0.075 U Sawady *Pwo* polymerase (peqlab) and 3.375 U *Taq* DNA polymerase (Sigma) in 10 µl total reaction volume. To isolate reverse transcriptase (RT) sequences of *line*- and *gypsy*-like TEs the primer pair RTase A (5′-GAYITIINNNVNGSNTWY-3′) and RTase E (5′-ANININAINCCNARRWM-3′) was used. *Mariner*- and *Tc1*-like sequences were first amplified with marI (5′-RWARTYNVWNGGNGC-3′) and marDE (5′-TNNTNWBNDBNGAYGARA3′). Cycling conditions were 5 min initial denaturation at 95° C followed by 10 cycles of 94° C (1 min), 47° C (1 min) and 72° C (1 min), 50 cycles of 94° C (20 sec), 52° C (45 sec) and 72° C (45 sec), and 10 min final extension at 72° C.
4 µl of the reaction products were loaded to a 2% agarose gel. Visible smears or bands above the primer dimers were interpreted as a sign of successful first step amplification.

In the second PCR step the primer concentration was reduced to 12.5 µM while the concentrations of other components remained unchanged. Reaction volume was increased to 20 µl, 1 µl of the first PCR product was used as template.

Gypsy-like elements were amplified from the RTase products with the primers B-gyp (5′-HIIDBNNTNCCNTTYGG-3′) and C-gyp (5′-ANNANRTCRTCNANRTA-3′) and 2 min initial denaturation at 95° C followed by 10 cycles of 94° C (1 min), 48° C (45 sec) and

72° C (45 sec), 55 cycles of 94° C (20 sec), 53° C (30 sec) and 72° C (20 sec), and 10 min final extension at 72° C.

For further amplification of *line*-like elements the RTase products and the primers B-line (5′-INGGNIBNCSNCARGG-3′) and C-line (5′-RNNRNRTCRTCNGCRWA-3′) were used at 2 min initial denaturation at 95° C followed by 10 cycles of 94° C (1 min), 50° C (45 sec) and 72° C (45 sec), 35 cycles of 94° C (20 sec), 55° C (30 sec) and 72° C (20 sec), and 10 min final extension at 72° C.

Isolation of *mariner* elements was performed with the primers marII (5′-GCNARRTCNGGNSWRTA-3′) and marD (5′-TNNTNYWNGAYAAYGM-3′), for *Tc1*-like elements the primers TcI (5′-WNYTCDATNGKRTT-3′) and TcII (5′-RTTNARRTCNGGNSWYTG-3′) were used. In both reactions the temperature profile was the same as for *line*-like elements.
Reaction products were separated on a 2% preparative agarose gel and distinct bands in the range 200 – 500 bp were excised, cloned into pGEM vector and used for *E. coli* transformation. White colonies were picked, plasmid DNA extracted and sequenced using vector primers.

Obtained sequences were submitted to the CENSOR database (Jurka et al., 2005) to identify TE homologues. DIG labelled hybridization probes of TE derived inserts were synthesized as described in 3.1.10.

4. Results and Discussion

4.1 SSCP genotyping of mtDNA

4.1.1 European haplotypes of the *P. chalcographus* CO1 gene

Avtzis (2005) amplified and sequenced a 1543 bp stretch covering almost the whole CO1 gene of 262 individuals from different European origins. PCR error (Kobayashi et al., 1999) and sequence ambiguities had been excluded by sequencing PCR products from each 5' and 3' direction and repetitive amplification of products showing SNPs compared to the mitochondrial consensus sequence. 58 haplotypes were identified and NJ algorithms revealed a four branched phylogenetic tree that is further subdivided into six distinct clades (Fig. 4.1).

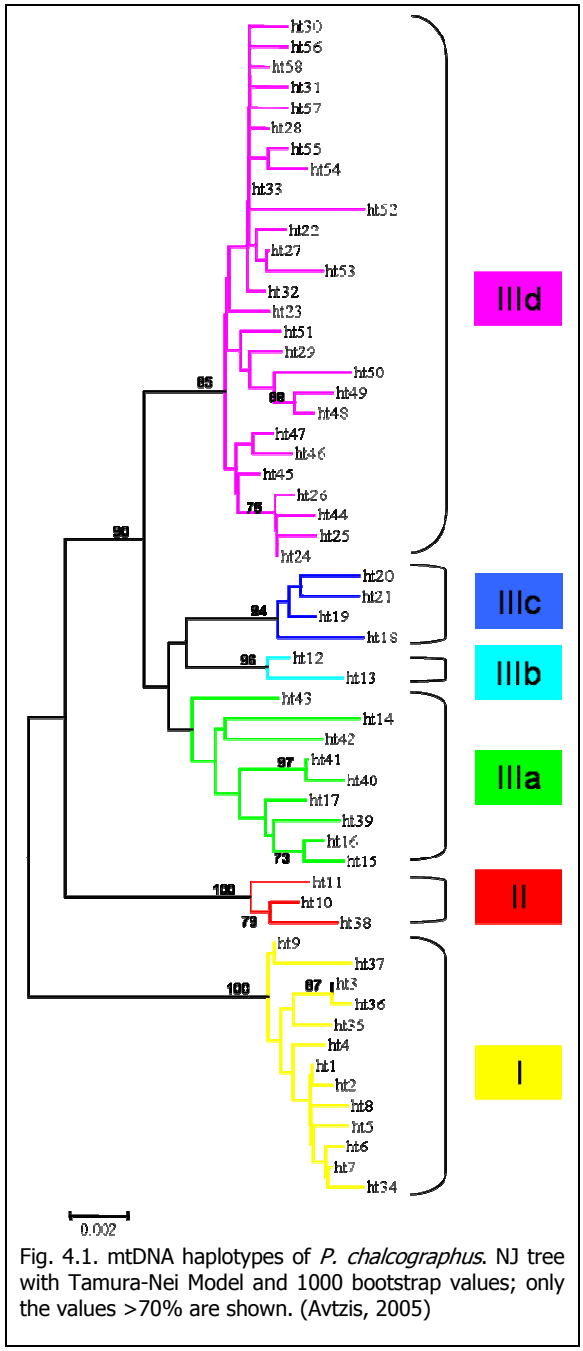

Fig. 4.1. mtDNA haplotypes of *P. chalcographus*. NJ tree with Tamura-Nei Model and 1000 bootstrap values; only the values >70% are shown. (Avtzis, 2005)

Genetic divergence between clades ranges up to 2.3% suggesting an allopatric origin of the observed pattern. The insect molecular clock (Gaunt and Miles, 2002) dates back the separation about one myr. Today most of the clades coexist sympatrically all over Europe. An in depth view on haplotype frequencies reveals some distributional bias: While clade I dominates the north-eastern parts of Europe, clade IIId is mainly found in the Alpine area. Clade IIIa is overrepresented in the Balkans and finally clade II is restricted to the Apennine peninsula.

The observed distribution of mitochondrial haplotypes may originate in glacial isolation and speciation events followed by recolonization of the continent in the recent warmer period. For further testing of this hypothesis a larger number of individuals had to be genotyped to determine clade affiliation. As direct sequencing is still a limiting factor due to its high costs (Sunnucks et al., 2000) the usability of SSCP for fast genotyping was evaluated.

4.1.2 Potential of clade discrimination using SSCP primers

Primers with suitable product sizes were developed from CO1 sequences; details are summarized in table 3.2. As a first step in evaluation of SSCP primers a suitable electrophoresis temperature for DNA fragments had been determined (Nataraj et al., 1999). Highest degrees of band separation and polymorphism were observed at 4° C for primers SSCP1, SSCP5, SSCP6 and SSCP7, at 15° C for primers SSCP3 and SSCP4 and at 25°C for primer SSCP2 (Fig. 4.2 and 4.3).

In a larger run with 36 individuals at optimized temperature primers showed remarkably differences in their ability to produce distinguishable banding patterns for different clades (Fig. 4.4 and Table 4.1).

Table 4.1. Separation potential of different SSCP primers. Products that showed unique banding patterns with at least one electrophoresis regime are marked with a colored background. Different primers were able to distinguish between 0 and 3 different clades.

Primer	Unique patterns for single clades				Unique patterns for clade combinations	Unseparated clades
SSCP1	I	II				IIIa, b, c, d
SSCP2	II	IIIa			I + IIIb	IIIc, d
SSCP3	IIIb				I + II + IIIa	IIIc, d
SSCP4	I	II	IIIc	IIId	IIIa + IIIb	
SSCP5	no clade specific separation					
SSCP6	I	II	IIIa			IIIb, c, d
SSCP7	I	II				IIIa, b, c, d

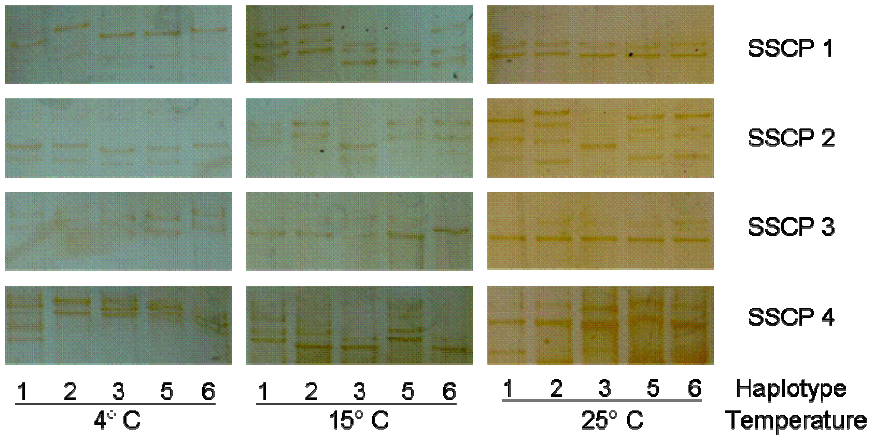

Fig. 4.2. Temperature optimization for primers SSCP1, SSCP2, SSCP3 and SSCP4. PCR products of 5 haplotypes were denatured and exposed to PAGE electrophoresis at different running temperatures. Banding patterns are strongly temperature dependant.

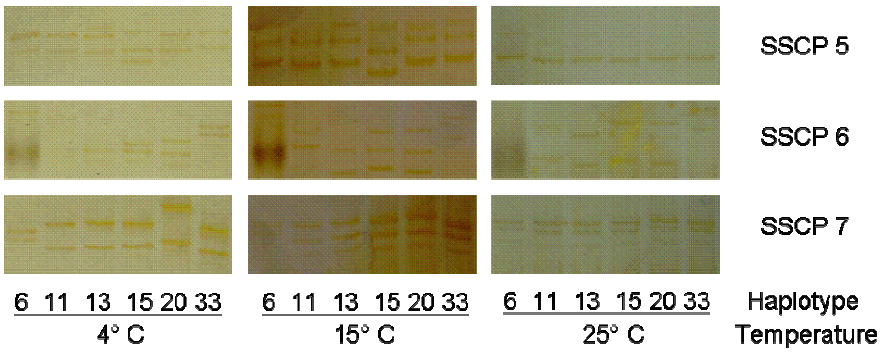

Fig. 4.3. Temperature optimization for primers SSCP5, SSCP6 and SSCP7. For this analysis 6 haplotypes were used. Pattern polymorphisms vary with electrophoresis temperature.

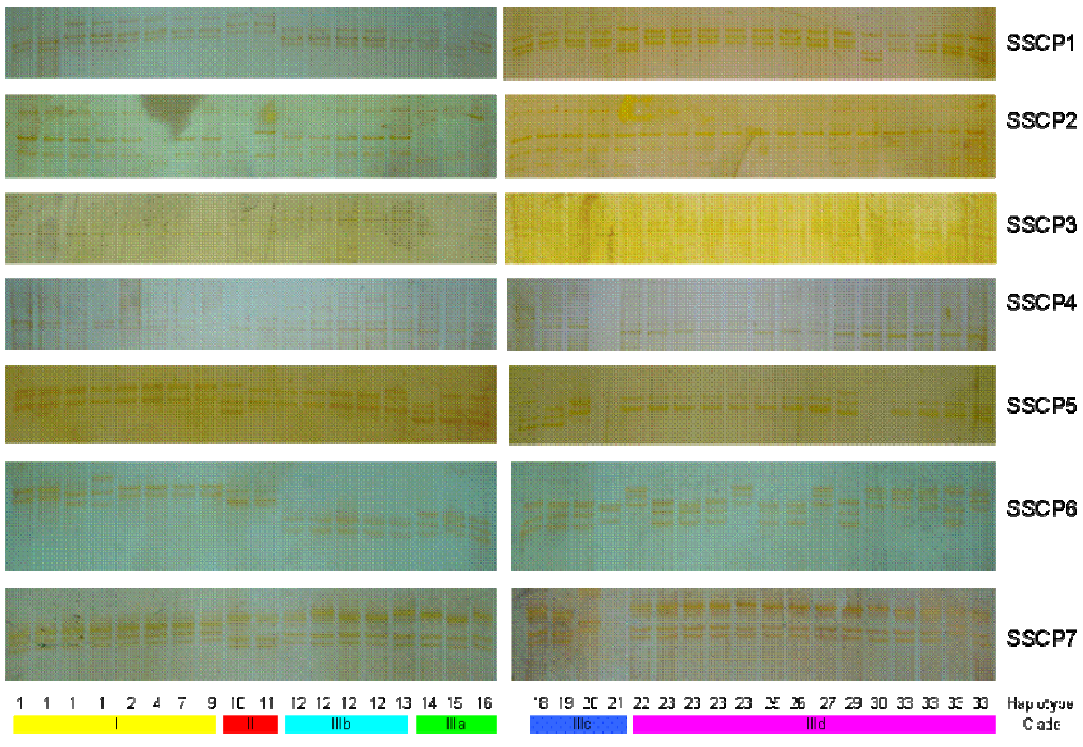

Fig. 4.4. Separation potential of SSCP primers for clade discrimination. 36 individuals representing 24 haplotypes in 6 clades were amplified with primer SSCP1-SSCP7. PAGE separation was carried out at optimized temperatures.

As primers SSCP5, SSCP6 and SSCP7 amplify targets outside the region used for initial haplotyping of the specimens, polymorphisms within repeats of one haplotype were expected and finally observed for SSCP6 among haplotypes 1, 23 and 33.

SSCP5 was the only primer pair that did not yield any clade specific separation. The distant clades I and IIa showed unique patterns with all other primers tested except SSCP3. The separation of the closer related subtypes of clade III was more problematic.

For maximum resolution screening the following procedure was suggested:

- First separation with primer pair SSCP6 will identify clades I, II, IIIa
- All samples that were not identified in the first reaction are separated with primer pair SSCP4. By this clades IIIc and IIId are identified by unique patterns. Any sample with the group pattern of IIIa + IIIb can be assigned to clade IIIb as IIIa was already identified in the first step.
- Still unidentified samples may represent new haplotypes and must be objected to sequencing.

In case a separation between IIIc and IIId is not required, the PCR reaction using SSCP4 may be replaced by the use of SSCP3, directly identifying clade IVb. Compared to the use of SSCP4 more clearly distinguishable patterns may be achieved. This approach was used by Avtzis (2005) to perform a screening of 427 individuals from different European origins. In total 353 individuals (82.7%) could be assigned to one of the clades, 250 of them (58.5%) in the first PCR. 74 (17.4%) were not clearly identified and had to be sequenced.

4.1.3 Comparison of SSCP and direct sequencing of PCR products

Major arguments for the use of SSCP are fast and on-site availability and lower cost compared to direct sequencing performed in external centers. A calculation for the cost of consumables of the described screening procedure is given in table 4.2.

A single PCR reaction was calculated with €0.20 for primers, *Taq* and plastic consumables. The cost of one purification with the QiaQuick PCR purification kit is €1.90. For sequencing, the price for a home-run reaction at Genterprise (Mainz, Germany), actually €10.80, was calculated. The prize of one polyacrylamide-gel including running buffer and costs for staining was estimated with €2.20; 16 samples fit onto one gel.

Table 4.2. Comparison of costs for SSCP analysis and direct sequencing. SSCP costs in the presented dataset containing 427 samples. Other samples with different clade distributions may result in altered costs due to different amounts of PCR and sequencing reactions required for complete genotyping. As the analyzed 1.5kb region of the mtDNA can not be sequenced in one single run, two sided sequencing of PCR products was calculated.

Direct sequencing		**SSCP genotyping**	
427 PCR reactions (CO1 amplification)	85.40 €	427 PCR reactions (SSCP6 amplification)	85.40 €
427 PCR product purifications	811.13 €	427 PAGE separations	58.71 €
854 sequencing reactions (two sides seq.)	9223.20 €	103 PCR reactions (SSCP3 amplification)	20.60 €
		103 PAGE separations	14.16 €
		74 PCR reactions (CO1 amplification)	14.80 €
		74 PCR product purifications	140.60 €
		148 sequencing reactions (two sides seq.)	1598.40 €
Total cost	**10119.73 €**	**Total cost**	**1932.67 €**
Average total cost per sample	**23.70 €**	**Average total cost per sample**	**4.53 €**

Under the given characteristics (~60% of the samples identified with one PCR-SSCP, ~20% identified in direct sequencing) the average cost of genotyping a single individual was €4.53 when SSCP technique was applied, compared to €23.70 for sequencing of all samples. Thus, the use of SSCP allowed a cost reduction of about 80%.

Another factor to be considered in cost calculation is working time. From the experiences in our lab the hands-on time per sample, under the assumption that 24 samples are processed in parallel, will be about 2.5 minutes for complete SSCP genotyping compared to 2 minutes for direct sequencing. On basis of the current FWF salary for laboratory technicians (€0.15 minute^{-1}) the influence of hands-on time will always be below 10% of the total cost.

4.2 Evaluation of possible numt influence on the mtDNA dataset

4.2.1 *In silico* analysis of mutations detected in CO1 sequences

Results of *in silico* analysis of the sequences of 262 individuals representing 58 different haplotypes and expected values for authentic mtDNA are summarized in table 4.3.

Table 4.3. *In silico* analysis of CO1 mutations. Total number and relative amount of mutational patterns observed in a 1557 bp stretch of n=262 individuals is compared with expected values for authentic mtDNA.

Pattern	Total	Relative (%)	Expected value for mtDNA [a]
Single base substitutions	125	100.0	-
1st codon position substitutions	15	12.0	14.9 ± 9.4% [b]
2nd codon position substitutions	2	1.6	4.5 ± 3.5% [b]
3rd codon position substitutions	108	86.4	80.6 ± 21% [b]
Nonsynonymous substitutions	13	10.4	7.47 ± 5.4% [c]
C→T substitutions	25	20.0	-
GC→GT substitutions	3	12.0 [d]	25 ± 14.0% [e]
Insertions	0	0	none [f]
Deletions	0	0	none [f]
Additional stop-codons	0	0	none [f]
Transitions (3rd codon position)	95	88.0 [g]	84.9% ± 18.1% [h]
Transversions (3rd codon position)	13	12.0 [g]	15.1 ± 7.6% [h]
Trasition-transversion ratio	7.31	-	-
GC content	-	34.6	28.66 ± 10.5% [i]

[a] expected relative values as given in reference ± χ^2 confidence interval at α=0.05 (Sachs, 1999)
[b] Blouin et al., 1998
[c] Shoemaker et al., 2004; data of *D. subaquinaria*
[d] percentage GC→GT substitutions of total C→T substitutions
[e] Bulmer, 1986; Bensasson et al., 2001
[f] Zhang and Hewitt, 1996
[g] percentage of total transitions/transversions on 3rd codon position
[h] Tamura, 1992
[i] Lin and Danforth, 2004; data for CO1 genes

Mitochondrial DNA shows some characteristics in base composition and mutational patterns that are different from the nuclear genome. Such differences may thus be helpful to discriminate between authentic mtDNA and numts (Bensasson et al, 2001). Numts originate in the ongoing transfer process of mtDNA into the nucleus (Adams and Palmer, 2003). Numt DNA was observed frequently in vertebrate (Arctander, 1995; Thalman et al., 2004; Thalmann et al., 2005) as well as in nonvertebrate (Sunnucks and Hales, 1996; Bensasson et al., 2001; Pons and Vogler, 2005) species

and may be coamplified by conserved universal primers. A compilation of reported numts is avilable online (http://www.pseudogene.net).

Most obvious, mtDNA is strongly AT biased (Lewis et al., 1995) and possesses a 5 to 10 times faster evolutionary rate than single copy nuclear DNA (Brown et al, 1979). Most probably this fast evolution is explained by inefficient repair mechanisms at the mitochondrial replication complex (Clayton et al., 1974) and by the higher turnover rate in tissues compared to nuclear DNA (Rabinowitz and Swift, 1970). As soon as a sequence is transferred into the nucleus it will further evolve with the typical patterns of a pseudogene. Compared to the authentic sequence which is under some selective constraint there will be less codon position bias and a higher proportion of nonsynonymous base replacements (Sunnucks and Hales, 1996). Observed patterns in the *P. chalcographus* dataset are all within a 5% confidence interval of the expected values.

Transition-transversion ratio is significantly higher in mtDNA than in corresponding pseudogenes (Arctander, 1995). The ratio of 7.31 in the actual dataset is typical for authentic mtDNA.

The GC dinucleotide is often methylated in nuclear DNA and 5-methylcytosine mutates abnormally often to T (Bird, 1980). Therefore the rate of GC→GT mutations among the four possible nC→nT combinations is highly overrepresented in the nucleus but not in mtDNA where methylation does not occur (Bulmer, 1986). Only 12% of all observed C→T mutations in *P. chalcographus* were of the GC→GT type indicating a non-methylated and therefore unlikely to be of non-nuclear molecular origin.

4.2.2 Development of long PCR primers

The goal of developing long PCR for *P. chalcographus* was to provide a technical tool to overcome possible influences of nuclear pseudogene co-amplification in mtDNA analysis. Several strategies to avoid such co-amplifications are known so far. The purification of mtDNA by caesium chloride gradient centrifugation (Nishiguchi et al., 2002) still remains kind of a gold standard but is inapplicable when the amounts of source DNA are limited. Besides, the procedure is slow and laborsome and therefore not suitable for the screening of large populations. Other enrichment techniques (Burgener and Hübner, 1998) provide a DNA that may still be contaminated with some nuclear sequences. In cases where the sequences of authentic mtDNA and the corresponding pseudogenes are known the development of target specific primers may be recommended (Zhang and Hewitt, 1996).

Most nonvertebrate numt insertions described so far are rather small with less than 1000 bp transferred to the nucleus in one piece (Thalmann et al., 2004). Thus, a long PCR utilizing primers based on coleopteran sequences and amplifying several thousand base pairs should exclude any amplicons derived from nuclear DNA.

Mitochondrial genomes of only three coleopteran species are completely sequenced until now: the spotted asparagus beetle *Crioceris duodecimpunctata* (Stewart and Beckenbach, 2003), the firefly *Pyrocoelia rufa* (Bae et al., 2004) and the flour beetle *Tribolium castaneum* (Friedrich and Muqim, 2003). Alignments performed by Clustal X (Thompson et al, 1997), including the mitochondrial sequences of three non-coleopteran insect species honeybee *Apis mellifera ligustica* (Crozier and Crozier, 1993), silkworm *Bombyx mori* (Lee et al., unpubl., direct submission to GenBank NC_002355) and fruit fly *Drosophila simulans* (Ballard, unpubl., direct submission to GenBank NC_005781), resulted in 6 candidate primers shown in table 4.4.

Table 4.4. Primer sequences, alignments and GenBank accession numbers for long PCR

Met/F (18-mer)		5′ GCTWHTGGGTTCATACCC 3′
C. duodecimpunctata	NC_003372	...AT.....
P. rufa	NC_003970	...TT.....
T. castaneum	NC_003081	...AT.A.....
A. mellifera ligustica	NC_001566	...AACA.....
B. mori	NC_002355	...AT....C.....
D. simulans	NC_005781	...AC.....
CO2/R (17-mer)		5′ CAAATTTCTGAACATTG 3′
C. duodecimpunctata	NC_003372	.....
P. rufa	NC_003970	.G.....
T. castaneum	NC_003081	.....
A. mellifera ligustica	NC_001566	.....
B. mori	NC_002355	.....
D. simulans	NC_005781	.....
CO3/R (17-mer)		5′ CCATGAAATCCTGTTGC 3′
C. duodecimpunctata	NC_003372	.....A..
P. rufa	NC_003970	.....A..A.....
T. castaneum	NC_003081	..G..G..C..A.....
A. mellifera ligustica	NC_001566	.....
B. mori	NC_002355	.....
D. simulans	NC_005781	.....
ND4/F (17-mer)		5′ ACCATGAGHAATTATTA 3′
C. duodecimpunctata	NC_003372	.....C.....
P. rufa	NC_003970	.....A.....
T. castaneum	NC_003081	C.....C.C..C.....
A. mellifera ligustica	NC_001566	.....A...C....
B. mori	NC_002355	.....CT.....
D. simulans	NC_005781	.....C.....
CyB/R (22-mer)		5′ AATATCATTCTGGTTKAATATG 3′
C. duodecimpunctata	NC_003372	.....T.....
P. rufa	NC_003970	.....G....G.....
T. castaneum	NC_003081	.....G...G..
A. mellifera ligustica	NC_001566	.....A....T.....
B. mori	NC_002355	.....G....G.....
D. simulans	NC_005781	.....G.....
sRN/F (17-mer)		5′ AGGGTATCTAATCCTAG 3′
C. duodecimpunctata	NC_003372	.....
P. rufa	NC_003970	.....
T. castaneum	NC_003081	.....
A. mellifera ligustica	NC_001566	.....
B. mori	NC_002355	.....
D. simulans	NC_005781	.....

4.2.3 Optimization of long PCR conditions

Table 4.5. Primer combinations. Due to similar T_m different combinations of the primers are possible.

Forward	Reverse	Amplicon (bp)
Met/F	CO2/R	3463
Met/F	CO3/R	5122
Met/F	CyB/R	10945
sRN/F	CO2/R	5205
sRN/F	CO3/R	6864
sRN/F	CyB/R	12687
ND/4	CO2/R	11054
ND/4	CO3/R	12713

The described primer set allows several combinations covering the entire CO1 gene (Table 4.5). Preliminary tests (data not shown) suggested that the binding of primer CO3/R on *P. chalcographus* DNA is rather poor while the other primers gave good results. Amplicon sizes over 5 kb require long extension periods and highest quality DNA. The latter factor must be considered when analysis is performed with samples stored for extended time (Dean and Ballard, 2001). Some degradation of DNA must be expected, specially if frozen individuals or extracts were exposed to repeated freeze-thaw cycles. While short products might still be amplifiable from such templates, the yield will dramatically decrease with the amplicon size. Thus, the primer pair Met/F and CO2/R was selected for further optimization.

i) influence of Mg^{++} concentration and extension time

The concentration of magnesium ions in the PCR buffer is crucial for successful amplification (Gelfand and White, 1990) and optimal concentrations have to be determined empirically. A lack of magnesium will cause poor product yields while an oversupply may result in smearing or unspecific by-products. As long PCR requires higher dNTP concentrations than standard reactions and magnesium is bound by dNTPs, its concentration usually also has to be increased.

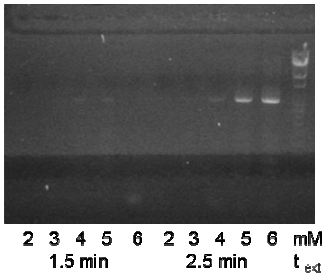

Fig. 4.5. Influence of Mg^{++} concentration and extension time; Marker IV on the very right lane

Extension time is amplicon size dependant. Estimations for the incorporation rate for *Taq* DNA polymerase range from 30 to 60 bases per second (Innis et al., 1988). Thus, a 3.5 kb amplicon should require 1 to 3 minutes for complete strand synthesis. Incorporation rate depends on the brand of the enzyme, buffer composition, possible inhibitors in the template DNA and will usually decrease towards the end of a PCR reaction due to a depleted pool of available dNTPs and heat inactivation of the

enzyme. Over-optimal extension times will not increase the product yield or quality and prolong the total reaction time and thus the time the polymerase is exposed to high temperature.

The use of 2.5 minutes extension time combined with high magnesium concentrations resulted in best product yields (Fig. 4.5). The total duration of such a PCR reaction was about 3.5 hours which allows the setup of up to three amplification batches on the same thermocycler within a single working day.

ii) influence of template DNA concentration

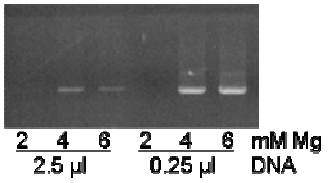

Fig. 4.6. Influence of template concentration on PCR product yield

While bands observed with 0.25 µl template DNA were more or less of the same brightness as in the former experiment where 1 µl template had been used, 2.5 µl DNA caused substantial reduction of product yield at all magnesium concentrations (Fig. 4.6).

Theoretically, the amount of PCR product doubles in each cycle of the reaction. Thus, a higher concentration of template DNA should increase product yield. Anyway, above a critical threshold concentration PCR yields are not necessarily correlated this way and enzyme inhibiting substances like polysaccharides or proteins, often co-extracted with the DNA, may increasingly disturb the reaction. The single insect extraction using the GenElute kit (Sigma) provides DNA at a concentration where 0.5 to 1 µl extract contain an optimal amount of template DNA for the 10 µl reaction scale.

iii) evaluation of dilution factors for nested PCR

During long PCR a large amount of fragments originating from authentic mitochondrial DNA is produced that can be used as template for nested PCR with any CO1 specific primers. As genomic template DNA – and so potential numt elements – is still present in the reaction mix after first PCR, a dilution factor must be found where

- original template DNA is so highly diluted that nested primers will not yield any product from it
- long PCR derived fragments are still present in a concentration to amplify sufficient product for subsequent applications.

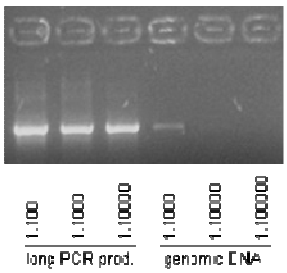

Fig. 4.7. Influence of template dilution on nested PCR. At dilution factors of 1:10000 and above no product from original template DNA is formed.

While genomic DNA diluted 1:1000 still yielded a faint band, higher dilutions did not provide any visible product. On the other hand a dilution factor of 1:10000 of the long PCR product still gave a strong band in the nested PCR (Fig. 4.7). Trace amounts of genomic DNA from the first reaction mixture will not contribute to this product which will therefore represent only fragments with authentic mitochondrial origin.

iv) influence of storage time on PCR results

Specimens of *P. chalcographus* were stored as whole individuals submerged in absolute ethanol at -20° C. Long term storage of extracted DNA is also performed at -20° C. Results of nested PCR from 16 individuals with different storage history are compiled in table 4.6. Figure 4.8 shows the corresponding electrophoresis gels.

Table 4.6. Effects of storage time on long and nested PCR results

Nr.	Sample	Origin	Storage time in EtOH	Time since DNA extraction	long PCR			nested PCR
					expected band	additional bands	smears	expected band
1	38R – 1	Romania	> 2 years	1 month				+
2	65D – 3	Germany	1 year	1 month	+	+		+
3	75GR – 5	Greece	1 year	1 month	+			+
4	62NO – 9	Norway	1 year	1 month	+			+
5	48D – 12	Germany	2 months	6 months	+		+	+
6	52SL – 1	Slovakia	2 months	1 year	+	+		+
7	69AT – 6	Austria	some days	6 months	+	+		+
8	72CZ – 7	Czechia	some days	6 months				
9	IT10 – 12	Italy	1 year	1 year	+	+		+
10	4PL3	Poland	some months	2 years	+	+		+
11	F4	Finland	1 week	1 month	+	+		+
12	1IT3	Italy	1 year	2 years	+		+	+
13	1IT18	Italy	2 years	6 months	+		+	+
14	24K2	Croatia	2 months	2 years	+	+		+
15	25R1	Russia	2 months	2 years				+
16	28AT – 1	Austria	some months	6 months	+	+		+

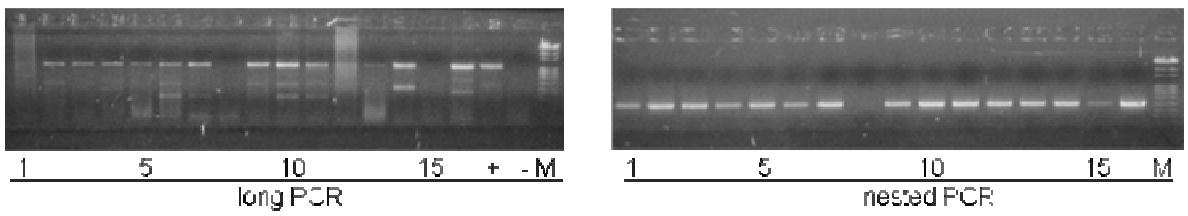

Fig. 4.8. Effects of storage time: long and nested PCR products; M = molecular weight marker IV. After long storage additional bands may be observed in long PCR products while nested PCR yields sharp single bands of expected size.

The experiment has shown that the long PCR system using Met/F and CO2/R primers is robust enough to amplify products of correct size from insects which were stored up to two years in ethanol as well as from extracted DNA of the same age. The most obvious effect of using old and thus sheared or degenerated DNA is the formation of additional unspecific bands and smears in long PCR. After dilution and nested PCR strong products of the correct size could be obtained from 15 out of 16 DNAs examined. Even templates where no band was visible in the long PCR (samples nr. 1 and 15) had formed enough product to be amplified in the subsequent nested reaction. The only sample in this test that did not yield any product (nr. 8) was not among the oldest DNA extracts tested. Therefore problems during extraction seem more plausible than degenerative effects during storage.

4.2.4 long PCR for validation of mtDNA derived phylogenetic data

As described in chapter 4.1.1 direct sequencing of a 1543 bp PCR product of the CO1 gene revealed a four-branched phylogenetic tree. A partial tree from 14 haplotypes from all major clades and the reconstruction of the tree using sequenced nested PCR products of the same individuals showed identical topologies (Fig. 4.9).

Numts co-amplified erratically by universal primers tend to group together into a distinct clade (Arctander, 1995; Bensasson et al, 2001; Zhang and Hewitt, 1996). In such cases complete removal of ncDNA from the template and re-PCR with identical primers will lead to changes in tree topology.

It was proven that at high dilutions of the long PCR product original DNA will not serve as a template for nested PCR with universal primers. Thus, all sequences derived from nested PCR originate in long PCR products which themselves represent authentic mtDNA.

As no changes in tree topology were observed the presence of numts in the analyzed populations of *P. chalcographus* can be excluded.

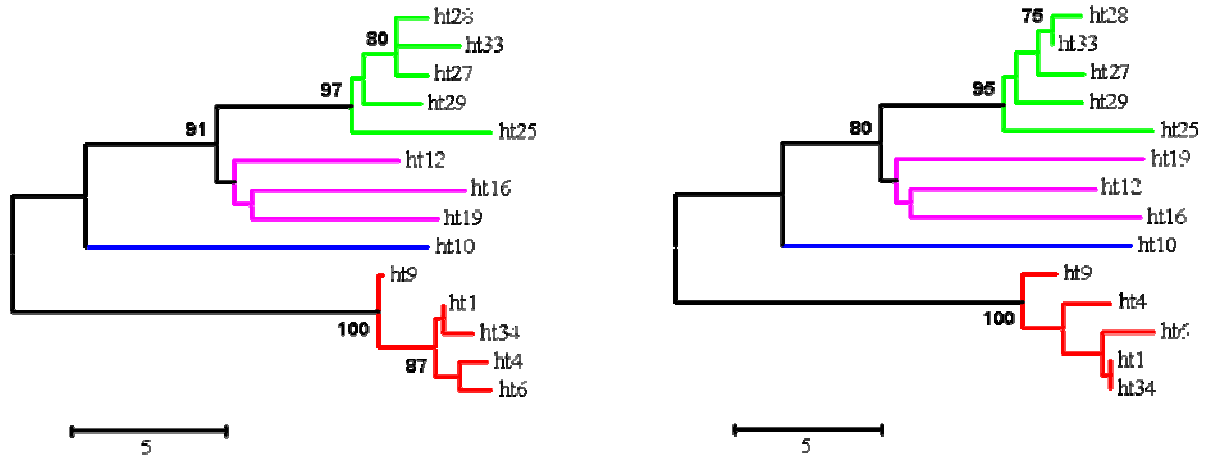

Fig. 4.9. Phylogenetic NJ trees derived from long and nested PCR (left) and from direct PCR (right); only bootstrap values over 70 are displayed. Bar length indicates 5 bp separation.

4.3 Detection of *Wolbachia* in *P. chalcographus*

4.3.1 Detection of *Wolbachia* by PCR

The use of a long PCR technique introduced by Jeyaprakash and Hoy (2000) is expected to improve *Wolbachia* amplification compared to conventional PCR reactions (Zhou et al., 1998). While Riegler (1999) did not detect *Wolbachia* infections in *P. chalcographus* by conventional PCR, a long PCR approach by Avtzis (2005) on 189 individuals resulted in 14.3% positive reactions. Different to experiences from conventional PCR, signals were often weak. One PCR product derived from an individual from Rora, Norway, was successfully cloned and showed high sequence homology to a B-strain *Wolbachia pipientis* isolated from *Tipula aino* by Kittayapong et al. (2003) (GenBank Acc. Nr. AF481165.1).

The repetition of this approach on 20 individuals already tested by Avtzis (2005) using different PCR chemistries revealed a poor reproducibility of the results, summarized in table 4.7. Most positive bands were extremely weak and few individuals showed bands with unexpected size. Modifications of the annealing temperature did not increase signal strength (data not shown). The use of a mixture of *Pwo* and *Taq* polymerases at high Mg^{++} concentrations yielded the highest number of positive reactions and strongest bands, although 6 individuals negative

under this conditions gave positive signals in other PCR reactions. Still even the strongest signals were about one order of magnitude weaker than the *D. simulans* DNA used as positive control. Besides it is remarkable that also the *Wolbachia*-free *D. simulans* strain used as negative control showed a faint band (Fig. 4.10).

Table 4.7. Results of long PCR for *Wolbachia* detection. 20 individuals were analyzed with different PCR chemistries. d = double band; ↑ = larger product than expected; ↓ = shorter product than expected; empty fields represent negative reactions; in the last column the number of positive reactions with expected product size is summarized.

Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	$\Sigma_{pos.}$
Expected[a]	+	+	+	+	±	±	±	±					±	±	±	±					12
PCR 1[b]	+	+	+										+						+		5
PCR 2[c]	d	+	↓	+				+	+	+	+	+	+	+	+	+				+	13
PCR 3[d]	+	d	+	+	+		+		+	↑	+		+		+	+		+		+	12
PCR 4[e]			+			+						+	+			+	+			+	7
ntd. PCR[f]	+																				

[a] results by Avitzis (2005)
[b] conventional PCR
[c] long PCR using *Pwo* (peqlab) and *Taq* (Sigma) polymerase and 3 mM Mg^{++}
[d] long PCR using *Pwo* (peqlab) and *Taq* (Sigma) polymerase and 1.5mM Mg^{++}
[e] long PCR using Fermentas enzyme mix
[f] nested PCR using diluted template of PCR 2 and wsp81F + wsp691R primers

As attempts for cloning and sequencing the faint long PCR products failed, a nested PCR technique was chosen for further verification of the obtained results. A 1:100 dilution of the products of PCR 2 (Table 4.7) used as template in nested PCR with wsp81F and wsp691R primers (Zhou et al., 1998) resulted in only one positive reaction and a clear positive control derived from DNA of infected *D. simulans* (Fig. 4.10).

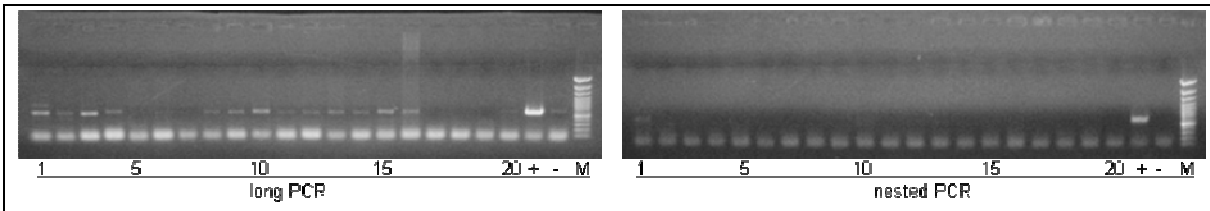

Fig. 4.10. long and nested PCR of 20 individuals of *P. chalcographus*. Weak bands are observed in long PCR of 13 individuals. Nested PCR shows only one positive band. + = infected *D. simulans* Rio de Janeiro; - = uninfected *D. simulans* Manaos; M = 100bp ladder

As a clear signal of the *Wolbachia* infected positive control was detected in nested PCR the functionality of the method itself seems doubtless. Observed results could thus be generated by

a. very low titers of *Wolbachia* combined with high levels of PCR inhibiting substances in the template which inhibit any amplification by *Taq* polymerase used as a single enzyme
b. false positive results from the long PCR primers
c. mutations at the primer binding site of wsp81F and/or wsp691R of the possible *Wolbachia* strain found in *P. chalcographus*, leading to failure of nested PCR.

ad a. Failure of *Wolbachia* detection due to low titers is identified as a frequent phenomenon in conventional PCR by Jeyaprakash and Hoy (2000) and long PCR is suggested as potential method to overcome the constraint. While this would be an explanation for the positive detection in long PCR, the presence of inhibitors of *Taq* polymerase seems implausible, as the same DNA extracts have been used successfully for amplification of other DNA loci. Furthermore, it is widely experienced that PCR products showing just faintest bands on agarose gel will still be suitable templates at much higher dilutions than used in this experiment.

ad b. Extensive literature search did not give evidence of widespread false positive results using the described long PCR primes. One PCR product of *P. chalcographus* was cloned and sequenced successfully and identified as authentic *wsp* sequence. Without further investigations the assumption of false positive results seems therefore not acceptable.

ad c. Riegler (pers. comm.) mentions that *wsp* primers often fail to detect B-strain *Wolbachia*. Nevertheless, an alignment of the only *wsp* sequence derived from *P. chalcographus* primers and derived sequences show perfect matches, making false negative reactions in nested PCR due to primer binding site mutations rather unlikely. For further testing other *Wolbachia* loci like *ftsZ* should also be considered.

4.3.2 Detection of *Wolbachia* by *in situ* hybridization

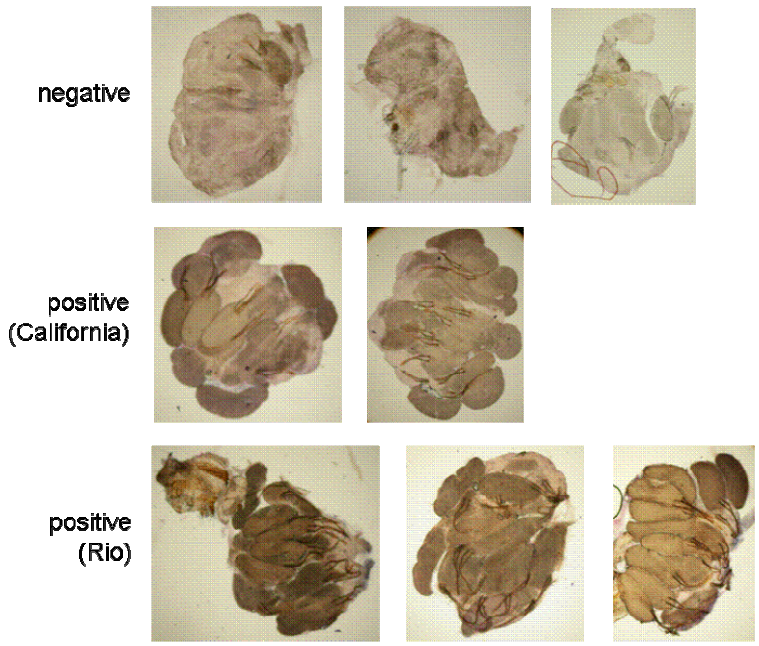

Fig. 4.11. Ovarial tissue of *D. simulans*, 40-fold magnification. After *in situ* hybridization with *wsp* specific probe and staining with NBT/BCIP solution an accumulation of purple/dark color is observed only in ovarioles of *Wolbachia* infected insects.

In situ hybridization offers a possibility to detect *Wolbachia* directly in infected tissues (Chen et al., 2005; Gómez-Valero et al., 2005), reducing the risk of false positive results due to contamination with infected parasites. A DIG-labelled *wsp* specific probe applied to ovarial tissue of one uninfected and two infected strains of *Drosophila simulans* showed accumulation of dark color in ovarioles of infected samples, while uninfected tissue remained unstained (Fig. 4.11).

Living insects are an absolute requirement for preparation of ovarial tissue. As the described experiments were carried out in late autumn, only a small number of suitable *P. chalcographus* samples was available and it must be considered that *Wolbachia* titers may be low short before insects diapause (Riegler, 2002). Ovarial tissue of four specimens was hybridized and in three cases an accumulation of purplish brown color was observed at different intensities (Fig. 4.12).

Although low sample number and missing PCR data in this experiment 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. Preparation of a larger number of specimens in different ontogenetic stages and from different geographic origins, combined with PCR testing of the same

insects, is needed for further interpretation of the current prevalence of *Wolbachia* in *P. chalcographus*.

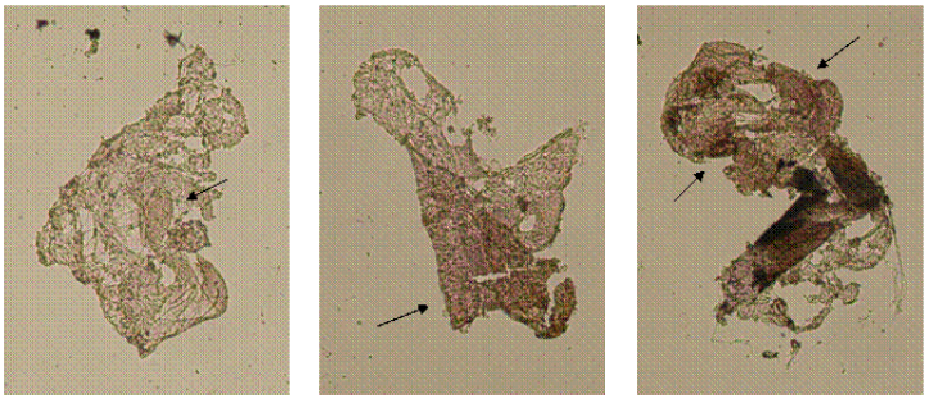

Fig. 4.12. Results of *in situ* hybridization of ovarial tissue excised from *P. chalcographus* individuals. Three specimens with accumulation of purple color (arrows) are shown. 40-fold magnification.

4.4 Interpretation of the integrity of the mtDNA derived dataset

Extensive testing has been performed to exclude the presence of numts within the dataset. All parameters assessed by *in silico* analysis fitted into the ranges expected for mtDNA. Long and nested PCR resulted in identical tree topologies. Thus, no method applied here gave any hint that some of the sequences might be of non-mitochondrial origin. It may therefore be concluded that only authentic mtDNA was included in phylogenetic analysis.

The possibility of *Wolbachia* influence remains more conundrous. A remarkable percentage of *P. chalcographus* DNA extracts yielded products in long PCR and one product was sequenced and identified as part of a *wsp* gene. An infection with the endosymbiont or, due to double bands observed in long PCR, superinfections with more than one *Wolbachia* strain may thus be not excluded. Hurst and Jiggins (2005) argue that mtDNA should not be used as a sole marker for phylogenetic studies, even if PCR analysis indicates current absence of *Wolbachia*, as this does not proof past absence or contamination below detection limit. The mtDNA derived phylogenetic tree of *P. chalcographus* should therefore be treated with some caution until in depth studies suggested in chapter 5 of this thesis are carried out.

4.5 Microsatellites

4.5.1 Isolation of microsatellites using agarose bound avidine

The principle of microsatellite isolation using library enrichment by agarose bound avidine is described in chapter 3.3.2. After restriction enzyme digestion, adaptor ligation and recovery PCR the amplicons are hybridized to SSR containing, biotinylated oligonucleotides. Hybrids are captured by avidin coated agarose particles; as the avidin- and streptavidin-biotin bonds exhibit the highest known protein-ligand affinity in nature (Livnah et al., 1993), the complex will withstand harsh washing conditions and the captured sequence will be released rather by denaturation of the probe-target DNA hybrid than by breaking of the protein-ligand structure. Denaturation may be obtained by high temperature as well as by alkaline substances.

In total nine enriched genomic libraries were constructed using agarose bound avidine. An overview is given in table 4.8.

Table 4.8. Agarose bound avidin derived genomic libraries

Library	**Ms1**	**Ms2**	**Ms3**	**Ms4**	**La1**	**Pc1**	**Pc2**	**Pc3**	**Pc4**
DNA source	*Messor structor*				*Lasius austriacus*	*Pityogenes chalcographus*			
Reference date	16 Feb 2004	15 Apr 2004	27 Apr 2004	21 Jun 2004	04 Feb 2005	15 Apr 2004	27 Apr 2004	21 Jun 2004	26 Aug 2004
Capture probes	$(GT)_8$, $(CT)_8$	$(GT)_8$, $(CT)_8$	$(GT)_8$, $(CT)_8$, $(CTT)_6$	$(AC)_8$, $(GA)_8$	$(AC)_8$, $(GA)_8$	$(GT)_8$, $(CT)_8$	$(GT)_8$, $(CT)_8$, $(CTT)_6$	$(AC)_8$, $(GA)_8$	$(AC)_8$, $(GA)_8$
Total colonies screened	200	164	164	231	246	69	237	324	232
Detection probe	biotin	biotin	biotin	biotin	DIG	biotin	biotin	biotin	DIG
Positive hybrids	19	36	20	21	41	14	18	34	32
Seq. clones	19	29	20	13	11	14	18	32	14
SSR cont. clones	13	8	2	3	2	0	0	1	0
Unique seq's	12	7	2	3	2	0	0	1	0

Hymenopteran genomes are considered to be rich in microsatellites (Estoup et al., 1993; Thoren et al., 1995). Thus isolation attempts from a hymenopteran species were chosen as a method to calibrate the efficiency of library enrichment protocols.

After preliminary results, several optimization strategies were applied, including

i) combinations of capture oligoprobes
ii) release of captured sequences
iii) probe designs for colony hybridization

i) combinations of capture oligoprobes

Principally parallel capture of different microsatellite motifs in the same enrichment reaction is possible, as long as probes are used that (a) do not form dimeric probe-probe-complexes and (b) do not highly differ in annealing temperature. Several libraries derived from parallel enrichment are described (Berg et al., 2003; Chenuil et al., 2003; Keller and Largiadèr, 2003).

After successful isolation of SSRs from libraries Ms1 (68.4% of sequenced plasmids contained SSRs) and Ms2 (27.6%), where the two compatible dinucleotide oligoprobes $(GT)_8$ and $(CT)_8$ had been used, the trinucleotide probe $(CTT)_6$ was additionally introduced in library Ms3. $(GT)_8$ and $(CT)_8$ have a melting temperature of 45.9° C, $(CTT)_6$ of 44.6° C. FastPCR (Kalendar, 2005) did not detect any expected probe-probe complexes.

Anyway, the simultaneous enrichment resulted in a reduced yield of only 10% positive plasmids. As a possible interaction between capture probes cannot be excluded, for further library construction only combinations of two compatible dinucleotide probes were used.

ii) release of captured sequences

To release the captured DNA strand from the biotinylated oligoprobe the hydrogen bonds between probe and hybrid must be broken either by heat or by denaturing substances. Zane et al. (2002) recommends exposition of probe-hybrid complexes to 95° C in a buffer containing 10 mM Tris and 1 mM EDTA, pH=9 for 5 min followed by an elution with small amounts of 0.15 M sodium hydroxide, which will be subsequently neutralized by titration with Tris.HCl. While other enrichments were only thermally eluted, in Ms3 and Pc3 both methods were compared for library construction (Fig 4.13 and Table 4.9).

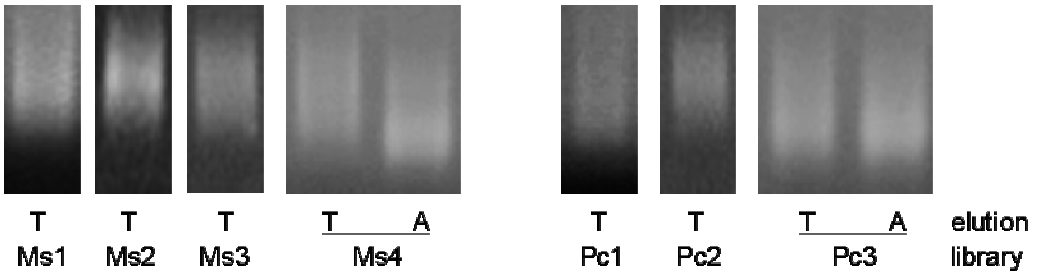

Fig. 4.13. Products of 32 cycle control PCR of the enriched library eluate; T = thermal elution, A = alkaline elution. The clips are arranged that the upper side of the frame represents 800 and the lower 100 bp. Comparable results were obtained in all experiments, but note that in library Ms4 alkaline elution forms a brighter smear made up of shorter fragments.

The intensity and size of smears after 32 cycle control PCR of the enrichment eluate was comparable for all libraries besides Ms4, where alkaline elution resulted in more intense but shorter sized smear.

Table 4.9. Comparison of thermal and alkaline elution. While DNA content is similar with both methods, alkaline eluates show highly decreased transformation rates.

Library	Elution	DNA content (ng/µl)	Transformed colonies	Positive hybrids
Ms4	thermal	73.5	216	21
	alkaline	66.5	15	0
Pc3	thermal	56.0	246	34
	alkaline	70.0	78	0

Photometric determination of DNA content after recovery PCR approved the visual estimation (Table 4.9). Identical amounts of DNA were used for subsequent ligation and transformation. Cloning results revealed significant difference between elution methods and highly reduced colony counts for the alkaline extract. Although complete neutralization of the eluate was verified using pH indicator tape and recovery PCR itself seemed not influenced, sodium hydroxide highly affected the subsequent ligation efficiency. The molecular mechanistics of this effect remain unclear. Alkaline eluates, although promising from DNA content, were not used for further library construction.

iii) probe designs for colony hybridization

Colony hybridization is an essential step in screening genomic libraries (Grunstein and Hogness, 1975). Besides La1 and Pc4 all libraries were screened using the same biotinylated oligoprobes that were used for avidin capture within the enrichment

step. While this technique reduces the amount of different reagents used in the isolation process, biotinylated probes exhibit two major limitations in colony hybridization:

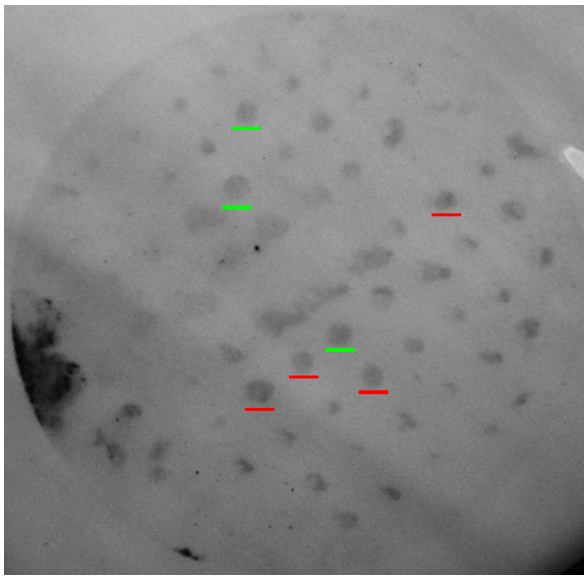

Fig. 4.14. Colony hybridization blot of 82 colonies derived from library Ms1. Colonies selected for sequencing are underlined; green lines represent colonies positive for SSR insert, red lines represent negatives.

- each probe is labelled only with one biotin molecule at its 5' terminal, so the total amount of biotin is low
- *E. coli* cellular biotin, even after prolonged proteinase-K digests, may contribute to high background levels and poor signal-noise ratio.

Indeed screening results showed a demand for optimization. All colonies gave at least some signal that can be interpreted as cellular biotin and sequencing revealed that even darkest colonies often did not contain SSRs (Fig. 4.14). More stringent washing conditions only influenced the overall density of the film but did not increase signal-noise ratios.

Therefore the digoxigenin (DIG) system was chosen for hybrid detection (Schmitz et al., 1991). DIG is a hapten derived from the plant *Digitalis purpurea* and detectable by alkaline phosphatase conjugated antibodies. The detection procedure is identical to that used for biotin labels, but as about 5% of the probe's nucleotides will carry a DIG molecule much higher signals compared to 5'-labelled biotin may be expected. Furthermore *E. coli* cells do not contain DIG resulting in lower background noise. DIG labelled probes were applied for screening of the libraries La1 and Pc4 and for all PMP derived libraries. As La1 and Pc4 were extremely poor in SSR content, results from the use of the DIG system are presented in chapter 4.2.3.

In total 24 unique microsatellite loci of *Messor structor*, 2 loci of *Lasius austriacus* and one locus of *Pityogenes chalcographus* were isolated using agarose bound avidin enriched libraries. For all experiments presented here the same batch of avidin

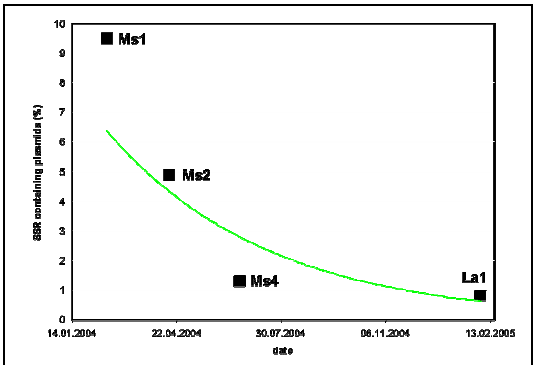

Fig. 4.15. Percentage of SSR containing plasmids among total number of transformed colonies; only libraries derived from hymenopteran species are displayed and library Ms3 is skipped due to possible probe-probe interactions.

coated beads had been used. While the low yield from coleopteran species may be explained by general difficulties isolating SSRs for some taxa (Day and Ready, 1999; Fagerberg et al., 2001; Navajas et al., 1998; Primmer et al., 1997; Zhang, 2004), the discouraging result in library La1 gained doubts on the stability of the avidin beads itself. Indeed, time analysis of the rate of SSR containing inserts among screened colonies suggests an exponential decay in hybridization efficiency (Fig. 4.15). The manufacturer of the avidin-agarose beads recommends storage at 4° C but does not provide any half-life or expiry dates for the product. Due to failure of isolating microsatellites from *P. chalcographus* and decreasing efficiency on hymenopterans the use of agarose bound avidin for library construction had been discontinued.

Table 4.10 summarizes all unique SSRs isolated using agarose bound avidine.

Table 4.10. Unique microsatellite motifs isolated using agarose bound avidin

Species	Library	Isolate	Motif
Messor structor	Ms1	1 A	$(AG)_{10}$ --- $(AG)_8$
		1 B	$(TC)_{17}$
		1 C	$(TG)_5$
		1 E	$(CG)_4$ --- $(TG)_{13}$ – $(TA)_6$
		1 F	$(CA)_{17}$ – TG – $(CG)_5$
		2 A	$(AG)_{21}$
		2 C	$(AG)_{11}$
		2 D	$(TC)_4$ – TT – $(TC)_{25}$ --- $(TC)_4$
		2 E	$(AG)_8$ --- $(AG)_4$
		3 A	$(TA)_4$ --- $(TC)_7$
		3 B	$(AG)_5$ – GG – $(GA)_{21}$
		3 E	$(TG)_{12}$ --- $(TG)_4$
	Ms2	12 C	$(AG)_6$ – GG – $(AG)_3$ – GG – $(AG)_3$ – GG – $(AG)_7$ – GG – $(AG)_3$ – GG – $(AG)_9$
		12 E	$(GA)_{39}$
		12 I	$(AC)_{10}$ --- $(TC)_{15}$
		12 O	$(AC)_{25}$
		13 D	$(AC)_6$
		13 J	$(CT)_{11}$
		13 K	$(GA)_4$ – AA – $(GA)_4$
	Ms3	6 A	$(AG)_8$ – AA – $(AG)_6$
		8 J	$(GA)_{11}$ – $(GT)_{10}$
	Ms4	21 C	$(AT)_5$
		24 B	$(CT)_{12}$ – G – $(TC)_3$
		24 E	$(AG)_8$
Lasius austriacus	La1	29 F	$(AG)_9$
		30 P	$(AGGGTG)_x$, numerous interrupts
P. chalcographus	Pc3	11 L	$(TG)_3$ – A – $(TG)_3$

4.5.2 Development of a PCR based pre-test for SSR content

Plasmid DNA containing an SSR insert should yield PCR products if amplified using a vector sequence and an oligo representing a compatible tandem repeat as primers. Pre-testing of plasmids by this method was suggested by Gaublomme et al. (2003). After some library construction, when SSR carrying plasmids became available, a PCR test for $(AC)_x$ and $(AG)_x$ microsatellites was established.

For first evaluation, 5 positive and 2 negative plasmids (Table 4.11) were objected to a PCR reaction containing SP6 vector primer and $(AC)_8$, $(CA)_8$ or a combination of both oligos as second primer. Fig. 4.16. shows PCR products of all reactions.

Table 4.11. PCR pretests (1). 5 plasmids containing and 2 lacking SSR sequences were tested with $(AC)_8$, $(GA)_8$ and a multiplex of both oligos. (+) = weak bands

Plasmid	SSR motif	$(AC)_8$		$(GA)_8$		$(AC)_8 + (GA)_8$	
		exp.	der.	exp.	der.	exp.	der.
1 C	$(TG)_5$	(+)	-	-	-	(+)	-
2 A	$(AG)_{21}$	-	(+)	+	+	+	+
2 C	$(AG)_{11}$	-	(+)	+	+	+	+
3 E	$(TG)_{12}$ --- $(TG)_{11}$	+	+	-	-	+	+
12 K	$(AC)_{10}$ --- $(TC)_{14}$	+	+	+	-	+	+
11 D	none	-	-	-	-	-	-
24 F	none	-	-	-	-	-	(+)

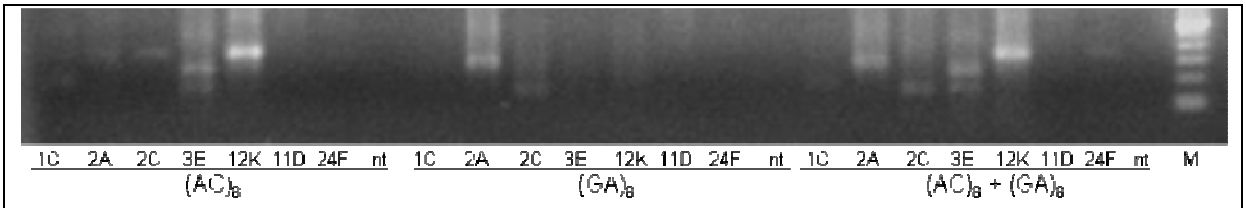

Fig. 4.16. PCR pretests with mono and multiplex SSR oligos and SP6 vector primer. nt = no template; M = 100 bp marker

The short tandem repeat $(TG)_5$ formed no suitable primer binding site for the $(AC)_8$ oligo, all other SSRs were detected correctly in the mono- as well as in the multiplex reactions. Therefore a larger reaction using the multiplexed primers $(AC)_8$, $(GA)_8$ and SP6 (Table 4.12) was set up to further investigate the accuracy of the method.

Table 4.12. PCR pretests (2). 20 plasmids were tested with an $(AC)_8$ + $(GA)_8$ multiplex reaction.

Plasmid	SSR motif	Result expected	Result derived
11 F	$(TC)_{19}$	+	-
11 I	$(GA)_4$ – AGA – $(AG)_{19}$	+	+
11 L	$(TG)_3$ – A – $(TG)_3$	-	-
12 E	$(GA)_{39}$	+	+
13 J	$(CT)_{11}$	+	+
21 C	$(AT)_5$	-	-
24 E	$(AG)_8$	+	+
11 A	none	-	-
11 B	none	-	-
11 C	none	-	+
18 A	none	-	-
18 B	none	-	-
18 C	none	-	+
19 A	none	-	-
19 B	none	-	-
19 D	none	-	+
24 G	none	-	-
24 H	none	-	-
24 J	none	-	-
24 K	none	-	+
Total plasmids tested		**20**	**100 %**
Correct results		**15**	**75 %**
False positives		**4**	**20 %**
False negatives		**1**	**5 %**

A fair rate of correct detections using the PCR pretest was observed. While false positive results will only lead to higher sequencing cost, false negatives imply the risk of losing SSRs already cloned. Thus, the low rate of false negatives combined with the overall performance gave reason to include a PCR pretest in all subsequent enrichment experiments. In order to detect SSRs located close to the SP6 promoter region of the plasmid and therefore giving extremely short amplicons maybe overseen in gel electrophoresis, negative reactions were further tested using the combination of $(AC)_8$, $(GA)_8$ and the T7 vector primer.

4.5.3 Isolation of microsatellites by PMPs

Streptavidine coated paramagnetic particles (PMPs) use the same principle as agarose bound avidin: biotinylated oligonucleotides and oligo-bound sequences are co-captured from a solution by the strong strepavidin-biotin and the much weaker DNA hydrogen bonds. Opposite to the particles used in 4.2.1 PMPs have much smaller diameters (0.5-1 µm compared to 20-40 µm for agarose beads) resulting in an increase of active surface and are separated from the hybridization buffer by a

strong magnetic field rather than by centrifugation. The latter case allows a faster and more complete phase separation without application of strong gravitational or shearing forces (Safarik and Safarikova, 2004).

Again an enrichment attempt with hymenopteran DNA was chosen to calibrate the efficiency of the system. In total one library for *Lasius austriacus* and five libraries for *Pityogenes chalcographus* were constructed using PMPs. An overview is given in table 4.13.

Table 4.13. PMP derived genomic libraries

Library	**La2**	**Pc5**	**Pc6**	**Pc7**	**Pc8**	**Pc9**
DNA source	*Lasius austriacus*	*Pityogenes chalcographus*				
Reference date	11 Apr 2005	18 May 2005	27 Jun 2005	10 Aug 2005	02 Sep 2005	02 Sep 2005
Capture probes	$(AC)_8$, $(GA)_8$	$(AC)_8$, $(GA)_8$	$(AC)_8$, $(GA)_8$	$(AC)_8$, $(GA)_8$	$(AC)_8$, $(GA)_8$	$(CTT)_6$
Stringency	high	high	low	low	low	low
Total colonies screened	410	302	410	10	410	48
Positive hybrids	44	61	47	-	58	-
Positive pre-PCR	28	24	27	10	25	1
Seq. clones	28	24	27	10	25	1
SSR cont. clones	26	15	24	6	20	0
Unique seq's	25	1	11	1	7	0

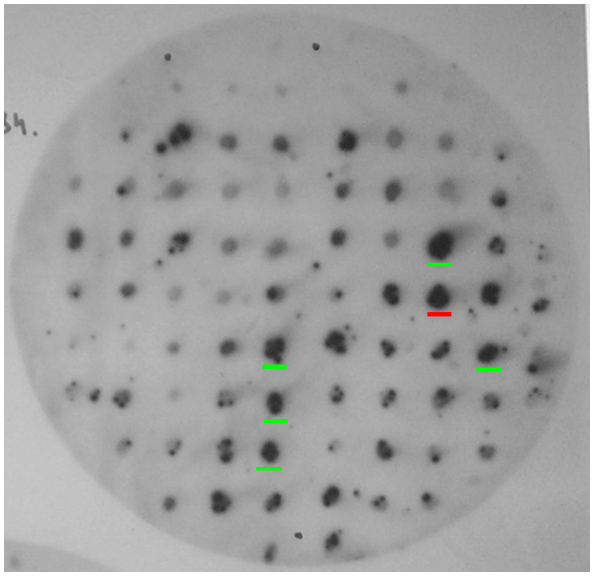

Fig. 4.17. Colony hybridization blot of 82 colonies derived from library La2. Colonies selected for further PCR screening are underlined; green lines represent colonies positive for SSR insert after sequencing; the red lined colony gave negative pre-PCR result and has not been sequenced.

All libraries presented in this section except Pc9 have been hybridized using a gel-purified DIG labelled $(AC)_x$+$(GA)_x$ probe. Compared to the biotinylated probes a highly increased signal-noise ratio was observed (Fig. 4.17). Colonies presenting the darkest signals on film were picked and objected to a PCR pretest for the presence of SSRs. Only colonies with a positive pre-test were further submitted for sequencing.

The PCR pretest significantly increased the rate of positive among sequenced clones from 18.6% to 65.7%, making the isolation procedure faster and more cost efficient. Details are shown in fig. 4.18.

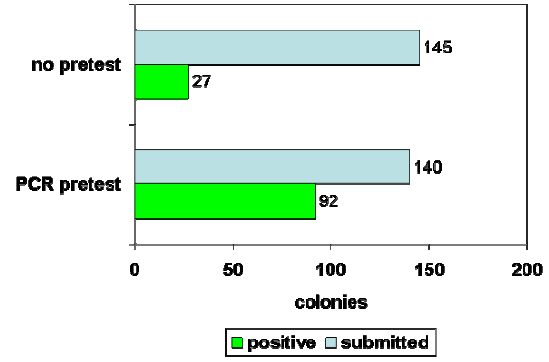

Fig. 4.18. Rate of SSR positive clones among those submitted for sequencing; libraries without pre-test: Ms1-4, Pc1-3; libraries with pre-test: La1-2, Pc4-9

The successful isolation of 25 unique microsatellite motifs from one *L. austriacus* library (La2) showed the high potential of PMPs for selective enrichment of target sequences. In direct comparison, 29 out of 1867 picked colonies (1.6%) from agarose bound avidin libraries were sequenced positive for SSR inserts. For PMP generated libraries the ratio was 91 out of 1590 picked colonies (5.7%).

Isolation of microsatellites from *P. chalcographus* remained problematic. Most obvious was the isolation of a SSR sequence referred to as '37C-type'. The clone was first observed in Pc5. From this library 15 colonies containing SSRs were sequenced, but all of them showed identical flanking regions ligated into the vector in sense and antisense orientation. The motif itself was present and in 5 different allelic forms, thus multiple ligation of the same PCR product can be excluded.

To avoid biased enrichment of a single sequence further libraries were objected to less stringent washing conditions in the bead capture step, and for library Pc8 the biotinylated oligoprobe $(GA)_8$ was removed. By this it was possible to isolate other SSR motifs, but still the 37C-type dominated retrieved sequences (Table 4.14).

Table 4.14. Presence of 37C-type sequence in libraries			
Library	**Colonies sequenced**	**37C – type**	**%**
Pc5	15	15	100.0
Pc6	27	11	40.8
Pc7	10	3	30.0
Pc8	25	11	44.0

Primers for the 37C-type locus were developed (see chapter 4.2.4) but could not contribute to solve the nature of this sequence' predominance. It is known that some microsatellites occur in large gene families with similar flanking regions (Meglecz et al., 2004), but amplicons of the locus always showed one or two alleles in genotyping. Therefore it must be assumed that the locus is single-copy in the *Pityogenes* genome. One should be aware that the restriction enzyme used for source DNA fragmentation and the twofold recovery PCR may result in non-representative pools of microsatellite clones in the library (Chambers and MacAvoy, 2000). Thus, a single perfect-fit locus within a genome low in microsatellites may outrule most other SSR loci in enrichment procedure.

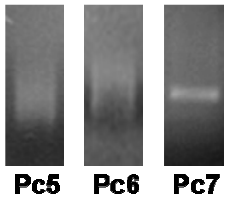

Fig. 4.19. Products of recovery PCR in libraries Pc5-Pc7

Among enrichment experiments, libraries Pc7 and Pc9 exhibit some exceptions. In Pc7 gel electrophoresis of recovery PCR did not show the expected smear but instead one distinct band (Fig. 4.19). As Pc5 and Pc6 already showed multiple isolations of 37C-type inserts and the size of the band fitted to the expected size for this clone, a large ligation-transformation reaction was cancelled for Pc7. After small scale ligation only 10 colonies were picked randomly without further pre-screening and sequencing revealed 3 clones of the 37C-type. From the other clones two were unreadable and one carried a unique SSR sequence, others did not contain microsatellites.

Library Pc9 was enriched using $(CTT)_6$ as capture probe. As no DIG labelled hybridization probe was available for this SSR motif, screening of 48 colonies was directly carried out using the PCR pre-test with $(CTT)_6$, SP6 and T7 primers respectively. Only one out of 48 colonies gave positive PCR signal but did not contain any microsatellite.

A complete overview of unique microsatellites isolated using the PMP system is given in table 4.15.

Table 4.15. Unique microsatellite motifs isolated using PMPs

Species	Library	Isolate	Motif
Lasius austriacus	La2	32 A	$(TG)_{11} – TA – (TG)_4$
		32 B	$(GAAA)_7$ --- $(AG)_4 – AA – (AG)_{11} – G_9$
		32 E	$(TG)_4 – CGTGG – (GT)_7$
		32 F	$(GA)_8$ --- $(TG)_2 – TA – (TG)_{36}$
		32 G	$(AG)_{26}$
		32 I	$(CT)_{12}$
		33 A	$(TG)_7 – TATGTA – (TG)_4$
		33 B	$(CT)_{23}$
		33 C	$(TC)_{23} – (AC)_{16}$
		33 E	$(AG)_{30}$
		34 A	$(AG)_{16}$
		34 C	$(AG)_{22}$
		34 D	$(GT)_7$
		34 E	$(GT)_{15}$
		34 F	$(TG)_8$
		35 E	$(TC)_{23}$
		35 G	A_{18} --- $(AG)_{26}$
		35 H	$(TG)_2 – TC – (TG)_2 – TC – (TG)_{11}$
		35 K	$(AC)_5 – AG – (CA)_{20}$
		35 L	$(GT)_{17}$
		36 A	$(AC)_{20} – (AG)_8 – AA – (AG)_8$
		36 C	$(GA)_4 – A – (AG)_{34}$
		36 D	$(CA)_{17} – (TA)_6$
		36 G	$(GA)_{34} – GT – (GA)_2$
		36 H	$(TC)_{29}$
Pityogenes chalcographus	Pc5	37 C	$(TC)_{16} – TTCT – (TC)_4$
	Pc6	41 E	$(GA)_{11}$
		41 F	$(TG)_4 – C – (GT)_4$
		41 H	$(GA)_{21}$
		41 M	$(AC)_7$
		43 A	$(TG)_9$
		44 A	$(TG)_{15}$
		44 D	$(TG)_4 – (AG)_{10}$
		44 I	$(GA)_9$
		44 K	$(GT)_6$
		44 N	$(AC)_6 – C – (CA)_4$
		45 B	$(GA)_{18} – GG – (GA)_9$
	Pc7	46 J	$(TG)_{12} – (TG)_{11}$
	Pc8	46 B	$(GT)_{15} – T – (TG)_3$
		46 C	$(AC)_6 – G – (CA)_3 – CG – (CA)_2$
		46 F	$(CA)_{13}$
		47 H	$(CA)_2 – CC – (CA)_2 – CG – (CA)_6$
		48 B	$(AC)_7$
		48 E	$(CA)_7$
		48 L	$(AC)_{10} – ATACA – (CT)_{17}$

4.5.4 Primer development and genotyping of *P. chalcographus* microsatellite loci

21 unique microsatellite loci were isolated throughout all experiments described. After excluding isolates with extremely short repeat motifs and unsuitable flanking regions, a subset of 17 loci was selected for primer development (Table 4.16).

Table 4.16. *P. chalcographus* microsatellite loci and primers. Primer sequence, size of the cloned allele and computed $T_{anneal.}$ are shown for all tested loci. Only primers that yielded amplicon with *P. chalcographus* genomic DNA were labelled with fluorescent dye for subsequent fragment analysis.

Locus	Primer F (5′ – 3′)	Primer R (5′ – 3′)	size (bp) [a]	$T_{anneal.}$ [b]	Label
41 E	GACGTTCTCCGAAAGCAG	AGCGACATCATTTTTGGC	198	55.7 – 60.7	TET
41 F	CTGCTTTACGGCGAGGTTACG	GAACTCGCGTATTTTGTCGAA	178	56.2 – 61.2	FAM
41 I	ACACGTAGGACGAACGTTGA	CGTCATTTGTTTCGTTGAGG	230	55.9 – 60.9	HEX
43 A	TTGTTGGAAGCGCAACAGTA	AGTGGCTGAGTGGCTAGTGC	178	56.7 – 61.7	TET
44 A	ACTTCTACTCTTTGATTAGAGAACG	CACGAACACGCGAACTACTCG	237	55.7 – 60.7	
44 D	GAATGAATTGATGCGAGAGTA	GTCATCCTGCCAAGCTTTCAC	251	55.3 – 60.3	FAM
44 K	AATCCACAATGGTGTACC	TCGTTTTCCACCACCTTTTGTG	244	55.2 – 60.2	
44 N	TCACACTGGTCTTCGAGCTG	GTCACTGCAAGGGGAGATTGC	228	57.2 – 62.2	FAM
45 B	GCAACACAGACGAGGACGT	ATATTCCCGAAAGAATCCAAG	240	55.3 – 60.3	HEX
46 B	AGCGCGTCTCTCTGTAAAGG	ATATTGGCACGTCGCATAG	200	56.0 – 61.0	
46 C	ACGCCAAAACGAAGTGTGATG	CCGAGAGTTTTTCTCGTTCAAT	249	56.1 – 61.1	TET
46 F	TGGCACGTCGCATAGAGAA	AGCGCGTCTCTCTGTAAAGG	196	57.0 – 62.0	
46 J	GGTTACTAACTAAGACTGTGTG	ATTCCCGATGTCAACCGTAG	225	55.3 – 60.3	
47 H	TGCTTCAAAACTCACCACA	GGGAAAGTGTTTCCTTATTGG	222	55.8 – 60.8	
48 B	GTCAAACCCCTCAGCACTG	GATTCTCCCCCACAACAAG	212	56.3 – 61.3	HEX
48 E	CATGGCACTGCTTCACCCAG	CGCTGGTGCTGTATTCTTGA	246	56.8 – 61.8	
48 L	CAACACGCTTTGCCTTTGTAA	CGATGACTGGACAGCTTGTG	245	56.6 – 61.6	

[a] size of amplicon in the cloned allele
[b] calculated by FastPCR (Kalendar, 2005)

The '37C-type' locus was represented by the primer pair 41 I. Eight loci did not yield any PCR product from *P. chalcographus* genomic DNA. Forward primers labelled with a fluorescent dye (either 5'-FAM, -TET or -HEX) and used in subsequent fragment analysis revealed allele sizes and distributions of nine remaining loci. Results are shown in table 4.17.

Few publications are available on coleopteran microsatellite isolation and most of them cover carabid species. Rather short SSR motifs containing less than 10 repeat units (Alvarez et al., 2003; Dhuyvetter and Desender, 2003; Gaublomme et al., 2003) and low allele numbers (Brouat et al., 2002; Gaublomme et al., 2003; Keller and Largiadèr, 2002; Keller and Largiadèr, 2003; Lagisz and Wolff, 2004; Patt et al., 2004a) are commonly reported, with few remarkable exceptions (Margraf et al.,

2005; Patt et al., 2004b). Observations for scolytid species (Berg et al., 2004; Kerdelhué et al., 2003; Sallé et al., 2003; Gauthier and Rasplus, 2004) support these trends.

Table 4.17. Preliminary *P. chalcographus* genotyping. Homozygotic individuals are represented with one allele size, heterzygotic with two. n.i. = not interpreted due to unclear chromatogram. The expected size is the size of the cloned allele derived from the library.

Locus	**41 E**	**41 F**	**41 I**	**43 A**	**44 D**	**44 N**	**45 B**	**46 C**	**48 B**
expected size (bp)	198	178	230	178	251	228	240	249	212
Haplotype	detected allele sizes								
1	196	177	230	173/178	237/249	226/228	200	246	211/213
7	216	177	216/228	173/178	237	220	200	246	208/213
8	204	177	224/228	173/178	249	226	200	246	211/213
14	190	177	230	173	237	226	200	246	208/213
16	196	177	230	173	237	n.i.	200	246	211/213
17	208	177	n.i.	173/178	237	n.i.	200	246	211/213
18	206	177	228/230	173/178	237	216	200	238	211/213
19	198	177	230	173/178	237	228	200	n.i.	211/213
21	226	177	230	173/178	251	214/220	200	246	211/213
23	216/230	177	230	173/178	237/244	226	200	246	211/213
29	198	177	230	173/178	239/246	n.i.	200	248	211/213
33	190/200	177	228	173	237/239	214/228	200	246	211/213
Alleles	**10**	**1**	**4**	**2**	**5**	**5**	**1**	**3**	**3**

The results of enriched libraries and preliminary genotyping of *P. chalcographus* fit to the traits found for all scolytid and many other coleopteran microsatellites. Seven microsatellite loci were polymorphic with at least two different alleles. The isolated microsatellites provide the first nuclear marker system established for *P. chalcographus*.

4.5.5 Vectorette PCR

Vectorette PCR was tested as an alternative approach to isolate microsatellites from genomes where SSR content is considered to be low. Isolation procedure consists of a first (target specific) vectorette PCR, cloning and sequencing of the products, construction of a flanking primer and a second, sequence specific vectorette PCR (Hui et al., 1998).

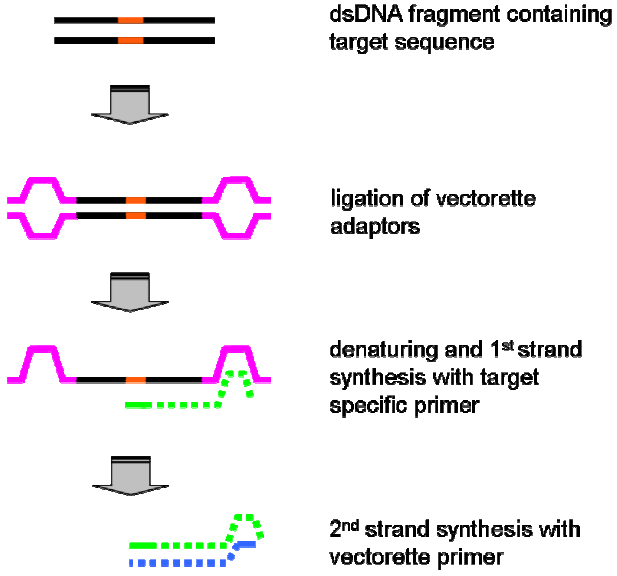

Fig. 4.20. Principle of 1st vectorette PCR

The principle of first vectorette PCR is outlined in figure 4.20. An adaptor with homologous DNA termini enclosing a nonhomologous, unpaired sequence ('bubble') is ligated to DNA fragments. The vectorette primer is identical to a sequence in the unpaired region of the adaptor and therefore not yet priming any PCR. Only fragments carrying a specific sequence are amplified in first cycle with target specific primers, thus restoring a perfect binding site for the vectorette primer. Second strand is synthesized when the vectorette primer anneals to the product of the first PCR cycle. Products of first vectorette PCR are sequenced and a primer inside the flanking region and facing towards the target is developed.

Adaptor ligated fragments are then reprobed in a second vectorette PCR using flanking and vectorette primer (Fig. 4.21).


Fig. 4.21. Principle of 2nd vectorette PCR; orange: 1st PCR product; blue: 2nd PCR product

In case microsatellite sequences are considered as targets, tandem oligorepeat primers will be used in first vectorette PCR.

While the use of a primer specific for a single copy gene should result in one sharp band after the first vectorette reaction, oligonucleotide primers will bind to many microsatellite loci present in the genome thus resembling a smear. Gel electrophoresis revealed the expected smear for SSR primers and a distinct band for the primer UEA5 (Lunt et al., 1996) targeting a single mitochondrial gene and used as positive control for adaptor ligation. (Fig. 4.22).

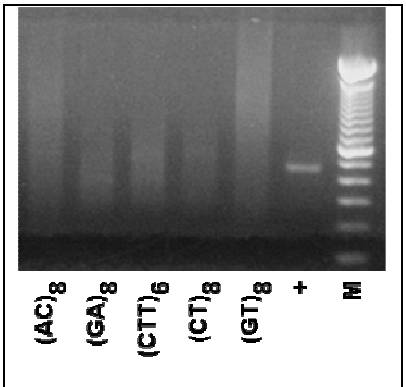

Fig. 4.22. Results of 1st vectorette PCR; + = UEA5; M = 100 bp marker

Sequencing of 27 plasmids containing gel excised products of the first vectorette PCR resulted in 8 inserts flanked by unique microsatellite motifs (Table 4.18). One of the inserts carried two separated SSRs.

Table 4.18. Results of 1st vectorette PCR

Plasmid	Motif	Derived primer(s)
101 A	$(GT)_8$	5' – TCCAGGAATCCACCATTG – 3'
101 B	$(TG)_8$	5' – CGAGTAAACAGGCAATGG – 3'
101 C	$(GT)_{12}$	5' – GCGGAAGAAACAGTCCG – 3'
101 D	$(AC)_8$ $(AC)_9$	5' – GACGTACAGTGGGTTCAG – 3' 5' – GGACAGCACTCAAAACG – 3'
101 E	$(AC)_8$	5' – TCCGCCCCGAAGTGACC – 3'
101 I	$(GT)_{57}$	5' – TTGGTACTGCGCTACAG – 3'
102 G	$(AG)_4$	none
103 C	$(CAG)_7$	5' – GCGATTTATGGACGACG – 3'

In case that primers were derived from sequences which are not located in multigene families, sharp bands are expected in second vectorette reaction. Anyway, even after several attempts to optimize PCR conditions, second reaction only resulted in more or less described bands embedded in remarkable background smears (Fig. 4.23). Narrow excision of the bands, cloning and sequencing did not reveal any applicable microsatellite loci.

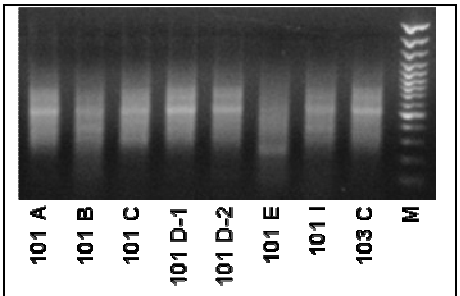

Fig. 4.23. Results of 2nd vectorette PCR; M = 100 bp marker

While at least some groups succeeded in isolation of SSRs by vectorette PCR (Lench et al., 1996), an unforeseeable amount of optimization steps would be needed to achieve comparable results in *P. chalcographus*. Eastlund et al. (2002) recommend touchdown PCR protocols, variations in the ratio of specific and vectorette primers and shifting of GC clamps in primer design to maximize vectorette specifity and yield. It should be considered that both vectorette reactions require independent optimization. Given timeframe in the recent project did not allow to further touch the implied ambiguities (Phettberg, 2005) of this technique.

4.6 Transposable genetic elements

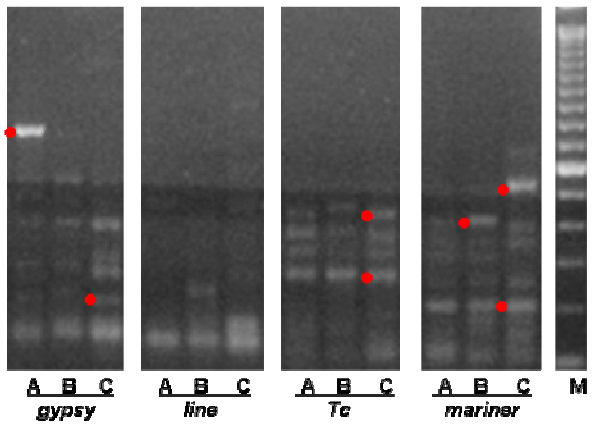

Fig. 4.24. Degenerate primed PCR, 2nd step; A, B: Austrian populations 15/6 and 15/9; C: Norway population 61/10; M: 100 bp ladder. Excised bands are marked with a red dot on the left side.

Degenerate primed PCR following the protocol of Arkhipova and Meselson (2000) yielded multi-banded products for all primer combinations tested except for *line* specific primers. 7 bands were selected for excision and cloning and are further referred to (Fig. 4.24: from left to right and from top to bottom in each electropherogram): gyp3, gyp5, tc2, tc3, mar1, mar2, mar3. As eluted and cloned DNA in mar2 showed two distinct product lengths, plasmids were named as mar2A and mar2B.

Submission of the insert sequences to the CENSOR database (Jurka et al., 2005) and protein homology search in BLASTX (http://www.ncbi.nlm.nih.gov/blast/) resulted in three positive identifications:

- mar2A shows homology to a retrotransposon originally isolated in *Aedes aegypti* (Tu and Hill, 1999), GenBank AAF20019
- mar2B shows homology to the reverse transcriptase sequence of a retrotransposon first described in *Monascus pilosus* and widespread among filamentous fungi (Chen et al., unpubl., direct submission to GenBank

AAX14028) as well as to the reverse transcriptase of a *Drosophila simulans* retrotransposon (Petrov and Hartl, unpubl., direct submission to GenBank AAC24969)

- mar3 contains a complete open reading frame for a polyprotein with homology to a *Schistosoma mansoni* retrotransposon (Demarco et al., 2005), GenBank CAJ00251.

It is remarkable that the isolated TEs, although fished with class II specific primers, show homology to class I elements. Following Miller (pers. comm.), longer sequences than those available now (205-524 bp) would be recommendable for a definite characterization of the TE.

Retrotransposon sequences were not described in *P. chalcographus* before. Due to the difficulties developing nuclear markers based on microsatellites (chapter 4.5) and the necessity of a non-mitochondrial system for phylogenetic analysis (chapter 4.4) TEs can be suggested as a considerable alternative markers.

5. Perspectives for future research

5.1 *Wolbachia* infection status of *P. chalcographus*

Wolbachia infections are widespread among insects (Werren et al., 1995; Stouthamer et al., 1999). While studies using standard PCR methods estimated a prevalence of the endosymbiont in ~20% of insect species (Werren and Windsor, 2000), long PCR (Jeyaprakash and Hoy, 2000) and determination of sex ratio in large samples (Jiggins et al., 2001) indicated infection rates up to 70%. Due to manipulation of the hosts reproduction by male killing, CI, parthenogenesis and feminization (Werren, 1997) *Wolbachia* influences mtDNA variation in infected populations (Turelli et al., 1992; Jiggins, 2003; Shoemaker et al., 2004) making the inference of phylogenetic data based on mitochondrial sequences unsafe and unsatisfactory (Hurst and Jiggins, 2005).

Evidence for a possible infection of *P. chalcographus* was presented in this thesis. Furthermore, reproductive incompatibility effects described by Führer (1976; 1977) and interpreted as manifestation of speciation processes could as well originate from *Wolbachia*. As current phylogenies of *P. chalcographus* are mainly based on mitochondrial sequences (Avtzis, 2005), an in depth analysis of prevalence and distribution of the endosymiont in the beetles European populations seems of high interest.

The long PCR and *in situ* hybridization techniques used in this thesis should be completed by

- long and nested PCR of other *Wolbachia* specific genes like *ftsZ* (Jeyaprakash and Hoy, 2000; West et al., 1998) or 16S-DNA (Gómez-Valero et al., 2004; Werren, 1997)
- cloning and sequencing of PCR products to identify endosymbiont strains and possible superinfections

- the use of hybridization probes specific for other *Wolbachia* genes (Gómez-Valero et al., 2004; Chen et al., 2005) or by *in situ* immunostaining procedures (McGraw et al., 2002).

The establishment of an infected laboratory stock population would not only provide a source of DNA and tissues which may be used as positive controls but would also allow to investigate infection dynamics over several generations (Johanowicz and Hoy, 1999) and in different ontogenic stages of the insect. An infected laboratory stock would also faciliate the development of a reliable and robust detection system for subsequent broad screening of wildlife populations.

Unidirectional reproductive incompatibility was described in *P. chalographus* (Führer, 1977). Breeding experiments with *Wolbachia* positive and negative insects are necessary to determine whether these effects were caused by the endosymbiont and to classify the CI phenotype (Werren, 1997).

A comparison of geographic distribution of *Wolbachia* infections and mitochondrial haplotypes of *P. chalographus* should gain insight if the endosymbiont influenced the hosts mitogenome (Shoemaker et al., 2004; Jiggins, 2003; Turelli et al., 1992).

Artificial transfer of the endosymbiont into new hosts (McGraw et al., 2002; Riegler et al., 2004) may further complete the understanding of *Wolbachia* effects on the beetle.

5.2 Nuclear markers

Essential limitations of the authenticity of mtDNA derived phylogenies (Hurst and Jiggins, 2005; Thalman et al., 2004) request further development of nuclear markers for *P. chalcographus*. Although isolation proved to be laborsome along scolytid species (Kerdelhué et al., 2003; Sallé et al., 2003), the high potential of microsatellites regarding theoretical availability and expected mutation rates (Schlötterer, 2000) still makes them a marker of choice.

Enriched libraries generated with paramagntic particles led to the isolation of seven polymorphic microsatellite loci for *P. chalcographus* and further isolation attempts may still increase this number, especially if capture and detection probes for other di-, tri- and tetranucleotide repeat motifs are included. Some trinucleotide motifs are expected to escape cellular mismatch repair systems better than dinuclotides (Li et al., 2002), enhancing mutation rate and polymorphism in such SSRs.

Single nucleotide polymorphism (SNP) and single copy nuclear polymorphic sequences (scnp) may become an alternative to microsatellites (Zhang and Hewitt, 2003). Like for SSRs there is no shortcut for finding these markers in an organism, neither are universal primers with broad cross-species amplification available, but protocols for isolation are established (Karl and Avise, 1993; Bagley and Gall, 1997) and may serve as a starting point for further analysis of nuclear DNA.

Transposable elements (Flavell et al., 1998; Berenyi et al., 2002) also offer some potential to serve as a nuclear marker. The possibility to isolate TEs by PCR techniques from the *P. chalcographus* genome has been demonstrated in this thesis. Identification of a higher number of TE sequences and subsequent development of multi- (Waugh et al., 1997) or single-locus (Flavell et al., 1998) approaches seems possible.

6. References

Adams K.L., and Palmer J.D. (2003) Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Molecular Phylogenetics and Evolution 29, 380-395.

Alvarez N., Aebi A., Risterucci A.M., Hosseart-McKey M., and Benrey B. (2003) Isolation and characterization of polymorphic microsatellite loci in *Acanthoscelides obvelatus* Bridwell (Coleoptera: Bruchidae). Molecular Ecology Notes 2003, 12-14.

Arctander P. (1995) Comparison of a mitochondrial gene and a corresponding nuclear pseudogene. Proceedings of the Royal Society of London, B 262, 13-19.

Arkhipova I., and Meselson M. (2000) Transposable elements in sexual and ancient asexual taxa. Proceedings of the National Academy of Sciences 97, 14473-14477.

Avise J.C., Arnold J., Ball R.M., Bermingham E., Lamb T., Neigel J.E., Reeb C.A., and Saunders N.C. (1987) Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. Annual Review of Ecology and Systematics 18, 489-522.

Avise J.C. (1998) The history and purview of phylogeography: a personal reflection. Molecular Ecology 7, 371-379.

Avise J.C. (1994) Molecular Markers, Natural History, and Evolution. Chapman and Hall, New York.

Avtzis D.N. (2005) Phylogeography of *Pityogenes chalcographus* (Col., Scoyltidae) based on mtDNA. Ph. D. thesis, Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU, Vienna.

Bae J.S., Kim I., Sohn H.D., and Jin B.R. (2004) The mitochondrial genome of the firefly, *Pyrocoelia rufa*: complete DNA sequence, genome organization, and phylogenetic analysis with other insects. Molecular Phylogenetics and Evolution 32, 978-985.

Bagley M.J., and Gall G.A.E. (1998) Mitochondrial and nuclear DNA sequence variability among populations of rainbow trout (*Oncorhynchus mykiss*). Molecular Ecology 7, 945-961.

Ballard J.W.O., and Whitlock M.C. (2004) The incomplete natural history of mitochondria. Molecular Ecology 13, 729-744.

Bensasson D., Zhang D.X., Hartl D.L., and Hewitt G.M. (2001) Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends in Ecology and Evolution 16, 314-321.

Berenyi M., Gichuki S.T., Schmidt J., and Burg K. (2002) *Ty1-copia* retrotransposon-based S-SAP (sequence-specific amplified polymorphism) for genetic analysis of sweetpotato. Theoretical and Applied Genetics 105, 862-869.

Berg E.E., and Hamrick J.L. (1997) Quantification of genetic diversity at allozyme loci. Canadian Journal of Forest Research 27, 415-424.

Berg P.R., Dawson D.A., Pendhal J., Kirkendall L.R., and Burke T. (2003) Isolation and characterization of microsatellite loci from two inbreeding bark beetle species. Molecular Ecology Notes 2003, 423.

Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. Journal of Bacteriology 62, 293-300.

Bird A.P. (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Research 8, 1499-1504.

Blears M.J., De Grandis S.A., Lee H., and Trevors J.T. (1998) Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. Journal of Industrial Microbiology & Biotechnology 21, 99-114.

Blouin M.S., Yowell C.A., Courtney C.H., and Dame J.B. (1998) Substitution Bias, Rapid Saturation, and the Use of mtDNA for Nematode Systematics. Molecular Biology and Evolution 15, 1719-1727.

Brouat C., Mondor-Genson G., Audiot P., Sennedot F., Lesorbe L., and Rasplus J.Y. (2002) Isolation and characterization of microsatellite loci in the ground beetle *Carabus nemoralis* (Coleoptera, Carabidae). Molecular Ecology Notes 2, 119-120.

Brown W.M., George M.G., and Wilson A.C. (1979) Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences 76, 1967-1971.

Bulmer M. (1986) Neighboring Base Effects on Substitution Rates in Pseudogenes. Molecular Biology and Evolution 3, 322-329.

Burgener M., and Hübner P. (1998) Mitochondrial DNA enrichment for species identification and evolutionary analysis. Zeitschrift für Lebensmitteluntersuchung und -forschung Serie A 207, 261-263.

Burke T., Hanotte O., Bruford M.W., Cairns E. (1991) Multilocus and single locus minisatellite analysis in population biological studies. in: Burke T., Dolf G., Jeffreys A.J., Wolff R. (eds.) DNA Fingerprinting: Approaches and Applications, 154-168. Birkhäuser Verlag, Basel.

Byers J.A. (2004) Chemical ecology of bark beetles in a complex olfactory landscape. In: Lieutier F., Day K.R., Battisti A., Grégoire J.C., Evans H.F. (eds.), *Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis*. pp. 89-134. Kluwer Academic Publishers, Dordrecht.

Caetano-Anollés G., and Gresshoff P.M. (1994) Staining Nucleic Acids with Silver: An Alternative to Radioisotopic and Fluorescent Labeling. Promega Notes Magazine 45, 13-21.

Carrol S.B. (2001) Chance and necessity: the evolution of morphological complexity and diversity. Nature 409, 1102-1109.

Caterino M.S., and Sperling F.A.H. (1999) *Papilio* phylogeny based on mitochondrial cytochrome oxidase I and II genes. Molecular Phylogenetics and Evolution 11, 122-137.

Chambers G.K., and MacAvoy E.S. (2000) Microsatellites: consensus and controversy. Comparative Biochemistry and Physiology Part B 126, 455-476.

Chapman R.W., and Brown B.L. (1989) Two methods to detect DNA fragments produced by restriction enzymes. Analytical Biochemistry 177, 199-202.

Chen W.J., Tsai K.H., Cheng S.L., Huang C.G., and Wu W.J. (2005) Using In Situ Hybridization to Detect Endosymbiont *Wolbachia* in Dissected Tissues of Mosquito Host. Journal of Medical Entomology 42, 120-124.

Chenuil A., le Gac M., and Thierry M. (2003) Fast isolation of microsatellite loci of very diverse repeat motifs by library enrichment in echinoderm species, *Amphipholis squamata* and *Echinocardium cordatum*. Molecular Ecology Notes 2003, 324-327.

Chung C.T., Niemela S.L., and Miller R.H. (1989) One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. Proceedings of the National Academy of Sciences 86, 2172-2175.

Church G.M., and Gilbert W. (1984) Genomic sequencing. Proceedings of the National Academy of Sciences 81, 1991-1995.

Clayton D.A., Doda J.N., and Friedberg E.C. (1974) The Absence of a Pyrimidine Dimer Repair Mechanism in Mammalian Mitochondria. Proceedings of the National Academy of Sciences 71, 2777-2781.

Crozier R.H., and Crozier Y.C.(1993) The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics 133, 97-117.

Cruickshank R.H. (2002) Molecular markers for the phylogenetics of mites and ticks. Systematic and Applied Acarology 7, 3-14.

Dassanayake R.S., and Samaranayake L.P. (2003) Amplification-Based Nucleic Acid Scanning Techniques to Assess Genetic Polymorphism in *Candida*. Critical Reviews in Microbiology 29, 1-24.

Day J.C., and Ready P.D. (1999) Relative abundance, isolation and structure of phlebotomine microsatellites. Insect Molecular Biology 8, 575-580.

Dean M.D., and Ballard J.W.O. (2001) Factors affecting mitochondrial DNA quality from museum preserved *Drosophila simulans*. Entomologia Experimentalis et Applicata 98, 279-283.

Demarco R., Machado A.A., Bisson-Filho A.W., and Verjovski-Almeida S. (2005) Identification of 18 new transcribed retrotransposons in *Schistosoma mansoni*. Biochemical and Biophysical Research Communications (2005) in press.

Dhuyvetter H., and Desender K. (2003) Isolation and characterization of microsatellite loci in the saltmarsh beetle *Pogonus chalceus* (Coleoptera, Carabidae). Molecular Ecology Notes 3, 460-461.

Dillon N., Austin A.D., and Bartowsky E. (1996) Comparison of preservation techniques for DNA extraction from hymenopterous insects. Insect Molecular Biology 5, 21-24.

Dobson S.L., Marsland E.J., and Rattanadechakul W. (2002) Mutualistic *Wolbachia* Infection in *Aedes albopictus*: Accelerating Cytoplasmic Drive. Genetics 160, 1087-1094.

Eastlund E., Walker C., Song K., and Mueller E. (2002) Improved Universal Vectorette System and Pre-Made Vectorette Genomic DNA Libraries. Life Science Quarterly 3, 5-10.

Ellegren H. (2004) Microsatellites: Simple Sequences with Complex Evolution. Nature Reviews: Genetics 5, 435-445.

Ender A., Schwenk K., Städler T., Streit B., and Schierwater B. (1996) RAPD identification of microsatellites in *Daphnia*. Molecular Ecology 5, 437-441.

Estoup A., Solignac M., Harry M., and Cornuet J.M. (1993) Characterization of $(GT)_n$ and $(CT)_n$ microsatellites in two insect species: *Apis mellifera* and *Bombus terrestis*. Nucleic Acids Research 21, 1427-1431.

Fagerberg A.J., Fulton R.E., and Black W.C. (2001) Microsatellite loci are not abundant in all arthropod genomes: analyses in the hard tick, *Ixodes scapularis* and the yellow fever mosquito, *Aedes aegypti*. Insect Molecular Biology 10, 225-236.

Flavell A.J., Knox M.R., Pearce S.R., and Noel Ellis T.H. (1998) Retrotransposon-based insetion polymorphisms (RBIP) for high throughput marker analysis. The Plant Journal 16, 643-650.

Friedrich M., and Muqim N. (2003) Sequence and phylogenetic analysis of the complete mitochondrial genome of the flour beetle *Tribolium castanaeum*. Molecular Phylogenetics and Evolution 26 (3), 502-512.

Führer E. (1976) Fortpflanzungsbiologische Unverträglichkeit beim Kupferstecher (*Pityogenes chalcographus* L.) - ein neuer Ansatz zur Borkenkäferbekämpfung? Forstarchiv 47, 114-117.

Führer E. (1977) Studien über intraspezifische Inkompatibilität bei *Pityogenes chalcographus* L. (Col., Solytidae). Zeitschrift für angewandte Entomologie 83, 286-297.

Führer E. (1978) Rassendifferenzierung bei *Pityogenes chalcographus* L. (Col., Scolytidae). Zeitschrift für angewandte Entomologie 86, 392-402.

Führer E., and Klipstein E.L. (1980) Rassendifferenzierung bei *Pityogenes chalcographus* L. (Col., Scolytidae). III. Fertilität intraspezifischer F1-Bastarde. Forstwissenschaftliches Centralblatt 99, 85-90.

Gaublomme E., Dhuyvetter H., Verdyck P., Mondor-Genson G., Rasplus J Y, and Desender K. (2003) Isolation and characterization of microsatellite loci in the ground beetle *Carabus problematicus* (Coleoptera, Carabidae. Molecular Ecology Notes 3, 341-343.

Gaunt M.W., and Miles M.A. (2002) An Insect Molecular Clock dates the Origin of the Insects and Accords with Palaeontological and Biogeographic Landmarks. Molecular Biology and Evolution 19, 748-761.

Gauthier N. and Rasplus J.Y. (2004) Polymorphic microsatellite loci in the Coffee Berry Borer, *Hypothenemus hampei* (Coleoptera, Scolytidae). Molecular Ecology Notes 4, 294-296.

Gelfand D.H. and White T.J. (1990) Thermostable DNA polymerases. in: PCR protocols: a guide to methods and applications. Academic Press, San Diego.

Gerber A.S., Loggins R., Kumar S., and Dowling T.E. (2001) Does nonneutral evolution shape observed patterns of DNA variation in animal mitochondrial genomes? Annual Review of Genetics 35, 539-566.

Giannini R., Morgante M., and Vendramin G.G. (1991) Allozyme variation in in Italian populations of *Picea abies* (L.) Karst. Silvae Genetica 40, 160-166.

Gómez-Valero L., Soriano-Navarro M., Pérez-Brocal V., Heddi A., Moya A., García-Verdugo J.M., and Latorre A. (2004) Coexistence of *Wolbachia* with *Buchnera aphidicola* and a Secondary Symbiont in the Aphid *Cinara cedri*. Journal of Bacteriology 186, 6626-6633.

Glenn T.C., Cary T., Dust M., Hauswaldt S., Prince K., Ramsdell C., and Shute I. (2000) Microsatellite isolation 2000. http://www.uga.edu/srel/DNA_Laboratory/protocols.htm

Gray M.W., Burger G., and Lang B.F. (2001) The origin and early evolution of mitochondria. Genome Biology 2, 1018.1-1018.5.

Grunstein M., and Hogness D.S. (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proceedings of the National Academy of Sciences 72, 3961-3965.

Hanahan D. (1983) Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology 166, 557-580.

Harris H. (1966) Enzyme polymorphism in men. Proceedings of the Royal Society of London, B, 164, 298-310.

Hedgren P.O. (2004) The bark beetle *Pityogenes chalcographus* (L.) (Scolytidae) in living trees: reproductive success, tree mortality and interaction with *Ips typograhus*. Journal of Applied Entomology 128, 161-166.

Hewitt G.M. (2000) The genetic legacy of the Quaternary ice ages. Nature 405, 907-913.

Hewitt G.M. (2001) Speciation, hybrid zones and phylogeography - or seeing genes in space and time. Molecular Ecology 2001, 537-549.

Hewitt G.M. (2004) Genetic consequences of climatic oscillations in the Quarternary. Philosophical Transactions of the Royal Society of London B 359, 183-195.

Hoffmann A.A., and Turelli M. (1997) Cytoplasmatic incompatibility in insects. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds.) *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*, 42-80. Oxford University Press, Oxford.

Hoskins R.A., Smith C.D., Carlson J.W., Carvalho A.B., Halpern A., et al. (2002) Heterochromatic sequences in a *Drosophila* whole-genome shotgun assembly. Genome Biology 3, 1-16.

Hubby J.L., and Lewontin R.C. (1966) A molecular approach to the study of genetic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura*. Genetics 52, 203-215.

Hui E.K.-W., Wang P.-C., and Lo S.J. (1998) Strategies for cloning unknown cellular flanking DNA sequences from foreign integrants. Cellular and Molecular Life Sciences 54, 1403-1411.

Hurst G.D.D., and Jiggins F.M. (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effect of inherited symbionts. Proceedings of the Royal Society of London, B 272, 1525-1534.

Hurst G.D.D., Johnson A.P., G. van der Schulenberg J.H., Fuyama Y. (2000) Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. Genetics 156, 699-709.

Innis M.A., Myambo K.B., Gelfand D.H., and Brow M.A.D. (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proceedings of the National Academy of Sciences 85, 9436-9440.

Jarne P., and Lagoda P.J.L. (1996) Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution 11, 424-429.

Jeffreys A.J., Wilson V., and Thein S.L. (1985) Individual-specific 'fingerprints' of human DNA. Nature 316, 76-79.

Jeyaprakash A., and Hoy M.A. (2000) Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Molecular Biology 9, 393-405.

Jiggins F.M., Bentley J.K., Majerus M.E.N., and Hurst G.D.D. (2001) How many species are infected with *Wolbachia*? Cryptic sex ratio distorters revealed to be common by intensive sampling. Proceedings of the Royal Society of London, B 268, 1123-1126.

Jiggins F.M., Hurst G.D.D., and Majerus M.E.N. (1998) Sex ratio distortion in *Acraea encedon* is caused by a male-killing bacterium. Heredity 81, 87-91.

Jiggins F.M. (2003) Male-Killing *Wolbachia* and Mitochondrial DNA: Selective Sweeps, Hybrid Introgression and Parasite Population Dynamics. Genetics 164, 5-12.

Johanowicz D.L., and Hoy M.A. (1999) *Wolbachia* infection dynamics in experimental laboratory populations of *Metaseiulus occidentalis*. Entomologia Experimentalis et Applicata 93, 259-268.

Junakovic N. (2004) Southern blot analysis of individual *Drosophila* flies. Methods in Molecular Biology 260, 41-57.

Jurka J., Kapitonov V.V., Pavlicek A., Klonowski P., Kohany O., Walichiewicz J. (2005) Repbase Update, a database of eukaryotic repetitive elements. Cytogentic and Genome Research 110, 462-467.

Kalendar R. (2005) FastPCR, PCR primer design, DNA and protein tools, repeats and own database searches program. (http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm)

Karl S.A., and Avise J.C. (1993) PCR-based Assays of Mendelian Polymorphisms from Anonymous Single-Copy Nuclear DNA: Techniques and Applications for Population Genetics. Molecluar Biology and Evolution 10, 342-361.

Keller I., and Largiadèr C.R. (2002) Identification of one X-linked and five autosomal microsatellite loci in *Carabus violaceus* (Coleoptera, Carabidae) in their applicability to related taxe. Molecular Ecology Notes 2, 290-292.

Keller I., and Largiadèr C.R. (2003) Five microsatellite DNA markers for the ground beetle *Abax parallelepipedus* (Coleoptera, Carabidae). Molecular Ecology Notes 2003, 113-114.

Kerdelhué C., Mondor-Genson G., Rasplus J.Y., Robert A., and Lieutier F. (2003) Characterization of five microsatellite loci in the pine shoot beetle *Tomicus piniperda* (Coleoptera: Scolytidae). Molecular Ecology Notes 3, 100-101.

Kim K.S., and Sappington T.W. (2005) Polymorphic microsatellite loci from the western corn rootworm (Insecta: Coleoptera: Chrysomelidae) and cross-amplification with other *Diabrotica spp*. Molecular Ecology Notes 5, 115-117.

Kirisits T. (2004) Fungal associates of European bark beetles with special emphasis on the Ophiostomatoid fungi. In: Lieutier F., Day K.R., Battisti A., Grégoire J.C., Evans H.F. (eds.), *Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis*. pp. 181-235. Kluwer Academic Publishers, Dordrecht.

Kittayapong P., Jamnongluk W., Thipaksorn A., Milne J.R., and Sindhusake C. (2003) *Wolbachia* infection complexity among insects in the tropical rice-field community. Molecular Ecology 12, 1049-1060.

Kobayashi N., Tamura K., and Aotsuka T. (1999) PCR error and Molecular Population Genetics. Biochemical Genetics 37, 317-321.

Koivisto R.K.K., Peer K., Braig H.R. (2004) Wolbachia lineages associated with outbreeding depression in ambrosian beetles. 3rd International Wolbachia Conference, Heron Island, QL, Australia.

Kruckenhauser L. (2000) Molekulare Analyse der intra- und interspezifischen Variation in der Gattung Marmota. Ph.D. thesis, University of Vienna.

Kumar A. and Hirochika H. (2001) Applications of retrotransposons as genetic tools in plant biology. Trends in Ecology and Evolution 6, 127-134.

Lagercrantz U., and Ryman N. (1990) Genetic structure of Norway spruce (*Picea abies*): concordance of morphological and allozyme variation. Evolution, 44, 38-53.

Lagisz M., and Wolff K. (2004) Microsatellite DNA markers for the ground beetle *Pterostichus oblongopunctatus* F. Molecular Ecology Notes 4, 113-115.

Lench N.J., Norris A., Bailey A., Booth A., and Markham A.F. (1996) Vectorette PCR isolation of microsatellite repeat sequences unsing anchored dinucleotide repeat primers. Nucleic Acids Research 24, 2190-2191.

Lewin B. (2004) *Genes VIII*. Pearson Education Intl., London.

Lewis D.L., Farr C.L., and Kaguni L.S. (1995) *Drosophila melanogaster* mitochondrial DNA: completion of the nucleotide sequence and evolutionary comparisons. Insect Molecular Biology 4, 263-278.

Li Y.C., Korol A.B., Fahima T., Beiles A., and Nevo E. (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Molecular Ecology 11, 2453-2465.

Lieutier F. (2004) Host resistance to bark beetles and its variations. in: Lieutier F., Day K.R., Battisti A., Grégoire J.C., Evans H.F. (eds.), *Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis*. pp. 135-180. Kluwer Academic Publishers, Dordrecht.

Lin C.-P., and Danforth B.N. (2004) How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined datasets. Molecular Phylogenetics and Evolution 30, 686-702.

Livnah O., Bayer E.A., Wilchek M., and Sussmann J.L. (1993) Three-dimensional structures of avidin and the avidin-biotin complex. Proceedings of the National Academy of Sciences 90, 5076-5080.

Lunt D.H., Zhang D.X., Szymura J.M., and Hewitt G.M. (1996) The insect cytochrome oxidase I gene: evolutionary pattern and conserved primers for phyolgenetic studies. Insect Molecular Biology 5, 153-165.

Lunt D.H., HutchinsonW.F., and Carvalho G.R. (1999) An efficient method for PCR-based isolation of microsatellite arrays (PIMA). Molecular Ecology 1999, 891-894.

Majerus M.E.N., G. van der Schulenburg J.H., and Zakharov I.A. (2000) Multiple causes of male-killing in a single sample of the two-spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae) from Moscow. Heredity 84, 605-609.

Margraf N., Gautschi B., and Rahier M. (2005) Isolation and characterization of microsatellite loci in the Alpine leaf beetle, *Oreina elongata*. Molecular Ecology Notes 5, 419-420.

McGraw E.A., Merritt D.J., Droller J.N., and O'Neill S.L. (2002) *Wolbachia* density and virulence attenuation after transfer into a novel host. Proceedings of the National Academy of Sciences 99, 2918-2923.

Meglecz E., Petenian F., Danchin E., Coeur d'Acier A., Rasplus J.Y., and Faure E. (2004) High similarity between flanking regions of different microsatellites detected within each of two species of Lepidoptera: *Parnassius apollo* and *Euphydryas aurinia*. Molecular Ecology 13, 1693-1700.

Moriyama E.N., and Powell J.R. (1997) Synonymous Subtitution Rates in *Drosophila*: Mitochondrial Versus Nuclear Genes. Journal of Molecular Evolution 45, 378-391.

Nataraj A.J., Olivos-Glander I., Kusukawa N., and Highsmith W.E.jr. (1999) Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. Electrophoresis 20, 1177-1185.

Navajas M.J., Thistlewood H.M.A., Lagnel J., and Hughes C. (1998) Microsatellite sequences are under-represented in two mite genomes. Insect Molecular Biology 7, 249-256.

Nève G., and Meglecz E. (2000) Microsatellite frequencies in different taxa. Tree 15, 376-377.

Nishiguchi M.K., Doukakis P., Egan M., Kizirian D., Phillips A., Prendini L., Rosenbaum H.C., Torres E., Wyner Y., DeSalle R., and Giribet G. (2002) DNA isolation procedures. Methods and Tools in Biosciences and Medicine 249-287.

Ostrander E.A., Jong P.M., Rine J., and Duyk G. (1992) Constrction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. Proceedings of the National Academy of Sciences 89, 3419-3423.

Pagel M. (1999) Inferring the historical patterns of biological evolution. Nature 401, 877-884.

Paine T.D., Raffa K.F., and Harrington T.C. (1997) Interactions among scolytid bark beetles, their associated fungi, an live host trees. Annual Reviews in Entomology 42, 179-206.

Parker P.G., Snow A.A., Schug M.D., Booton G.C., and Fuerst P.A. (1998) What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79, 361-382.

Patarnello T., and Battaglia B. (1992) Glucosephosphate isomerase and fitness: Effects of temperature on genotype dependent mortality and enzyme activity in two species of the genus *Gammarus* (Crustacea: Amphipoda). Evolution 46, 1568-1573.

Patt A., Naumann C.M., Misof B., and Wagner Th. (2004) Isolation and characterization of microsatellite loci in *Monolepta vincta* Gerstaecker, 1871 (Coleoptera, Chysomelidae, Galerucinae). Molecular Ecology Notes 4, 572-574.

Patt A., Misof B., Wagner T.H., and Naumann C.M. (2004) Characterization of microsatellite loci in *Amphitmetus transversus* (Kolbe, 1897) (Coleoptera, Curculionidae). Molecular Ecology Notes 4, 188-190.

Pfeffer A. (1995) Zentral- und westpaläarktische Borken- und Kernkäfer (Coleoptera: Scolytidae, Platypodidae). Pro Entomologia, Naturhistorisches Museum Basel.

Phettberg H. (2005) Schönborn zwischen Darwin und dem Herrn. Der Standard 20050716, 34.

Pons J., and Vogler A.P. (2005) Complex Pattern of Coalescence and Fast Evolution of a Mitochondrial rRNA Pseudogene in a Recent Radiation of Tiger Beetles. Molecular Biology and Evolution 22, 991-1000.

Postner M. (1974) Scolytidae (= Ipidae), Borkenkäfer. In: Schwenke W. (ed.) *Die Forstschädlinge Europas*. Band 2, pp. 334-482. Parey, Berlin.

Primmer C.R., Raudsepp T., Chowdhary B.P., Moller A.P., and Ellegren H. (1997) Low Frequency of Microsatellites in the Avian Genome. Genome Research 7, 471-482.

Rabinowitz M., and Swift H. (1970) Mitochondrial nucleic acids and their relation to the biogenesis of mitochondria. Physiological Reviews 50, 376-427.

Rand D.M., Haney R.A., and Fry A.J. (2004) Cytonuclear coevolution: the genomics of cooperation. Trends in Ecology and Evolution 19, 645-653.

Reineke A., Karlovsky P., and Zebitz C.P.W. (1998) Preparation and purification of DNA from insects for AFLP analysis. Insect Molecular Biology 7, 95-99.

Riegler M. (1999) Untersuchungen zu *Wolbachia* in *Ips typographus* und anderen Arten der Rhynchophora. Diploma thesis, Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU, Vienna.

Riegler M. (2002) The Endosymbiont *Wolbachia* (α-Proteobacteria) in the European Cherry Fruit Fly *Rhagoletis cerasi* (Diptera, Tephritidae): Population Dynamics & Influence on Pupulation Genetics. Ph. D. thesis, Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU, Vienna.

Riegler M., Charlat S., Stauffer C., and Mercot H. (2004) *Wolbachia* Transfer from *Rhagoletis cerasi* to *Drosophila simulans*: Investigating the Outcomes of Host-Symbiont Coevolution. Applied and Environmental Microbiology 70, 273-279.

Riegler M., and Stauffer C. (2002) *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). Molecular Ecology 11, 2425-2434.

Ritzengruber O. (1990) Isoenzymanalyse verschiedener *Pityogenes chalcographus* L. (Col., Scolytidae) I. Methodenanpassung, Enzympolymorphismus. Journal of Applied Entomology 55, 55-63.

Ross C.L., Dyer K.A., Erez T., Miller S.J., Jaenike J., and Markow T.A. (2003) Rapid Divergence of Microsatellite Abundance Among Species of *Drosophila*. Molecular Biology and Evolution 20, 1143-1157.

Rowold D.J., and Herrera R.J. (2000) *Alu* elements in the human genome. Genetica 108, 57-72.

Rozen S., and Skaletsky H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. in: Krawetz S, Misener S (eds.) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386.

Rungis D., Bérubé Y., Zhang J., Ralph S., Ritland C.E., Ellis B.E., Douglas C., Bohlmann J., and Ritland K. (2004) Robust simple sequence repeat markers for spruce (*Picea spp.*) from expressed sequence tags. Theoretical and Applied Genetics 109, 1283-1294.

Saccone C., Barome P.O., D'Erchia A.M., D'Errico I., Pesole G., Sbisà E., and Tullo A. (2002) Molecular strategies in Metazoan genomic evolution. Gene 300, 195-201.

Sachs L. (1999) *Angewandte Statistik*. Springer Verlag, Berlin.

Safarik I., and Safarikova M. (2004) Magnetic techniques for the isolation and purification of proteins and peptides. BioMagnetic Research and Technology 2, doi 10.1186/1477-044X-2-7.

Sallé A., Kerdelhué C., Breton M., and Lieutier F. (2003) Characterization of microsatellite loci in the spruce bark beetle *Ips typograhus* (Coleoptera: Scolytinae). Molecular Ecology Notes 3, 336-337.

Sambrook J., Fritsch E.F., and Maniatis T. (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbour Laboratory Press, New York.

Sauvard D. (2004) General biology of bark beetles. In: Lieutier F., Day K.R., Battisti A., Grégoire J.C., Evans H.F. (eds.), *Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis*. pp. 63-88. Kluwer Academic Publishers, Dordrecht.

Schlötterer C. (2000) Evolutionary dynamics of microsatellite DNA. Chromosoma 109, 365-371.

Schmidt-Vogt H. (1977) *Die Fichte*. Band 1. Paul Parey, Hamburg

Schmitz G.G., Walter T., Seibl R., and Kessler C. (1991) Nonradioactive labeling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. Analytical Biochemistry 192, 222-231.

Schönborn C. (2005) Finding Design in Nature. The New York Times, Late Edition July 7th, Section A, 23.

Schulman A.H., Flavell A.J. and Noel Ellis T.H. (2004) The Application of LTR Retrotransposons as Molecular Markers in Plants. in: Miller W.J., Capy P. (eds.) Mobile Genetic Elements. Protocols and Genomic Applications, 145-175. Humana Press, Totowa.

Shoemaker D.D.W., Dyer K.A., Ahrens M., McAbee K., and Jaenike J. (2004) Decreased Diversity but Increased Substitution Rate in Host mtDNA as a Consequence of *Wolbachia* Endosymbiont Infection. Genetics 168, 2049-2058.

Shimamura M., Yasue H., Ohshima K., Abe H., Kato H., Kishiro T., Goto M., Munechika I., and Okada N. (1997) Molecular evidence from retrotransposons that whales form a clade within even-toed ungulates. Nature 388, 666-670.

Simon C., Frati F., Beckenbach A.T., Crespi B., Liu H., and Flook P. (1994) Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. Annals of the Entomological Society of America 87, 651-701.

Sinkins S.P., Braig H.R., and O'Neill S.L. (1995) *Wolbachia* superinfections and the expression of cytoplasmatic incompatibility. Proceedings in Biological Science 261, 325-330.

Sittichaya W. (2005) Untersuchungen über das Geschwisterbrutverhalten des Kupferstechers, *Pityogenes chalcographus* L. (Col., Scolytidae). Diploma thesis, Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU, Vienna.

Southern E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98, 503-517.

Stauffer C., Lakatos F., and Hewitt G.M. (1999) Phylogeography and postglacial colonization routes of *Ips typographus* L. (Coleoptera, Scolytidae). Molecular Ecology 1999, 763-773.

Stauffer C., van Meer M.M.M., Riegler M. (1997) The presence of the proteobacteria *Wolbachia* in European *Ips typograhus* (Col., Scolytidae) populations and the consequences for genetic data. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 11, 709-711.

Stewart J.B., and Beckenbach A.T. (2003) Phylogenetic and genomic analysis of the complete mitochondrial DNA sequence of the spotted asparagus beetle *Crioceris duodecimpunctata*. Molecular Phylogenetics and Evolution 26, 513-526.

Stouthamer R., Breeuwer J.A.J., and Hurst G.D.D. (1999) *Wolbachia* pipientis: Microbial Manipulator of Arthropod Reproduction. Annual Reviews in Microbiology 53, 71-102.

Sturies H.J., and Führer E. (1979) Rassendifferenzierung bei *Pityogenes chalcographus* L. (Col., Scolytidae). II. Partnerdiskriminierung bei der Paarung. Allgemeine Forst- und Jagdzeitung, 150, 99-101.

Sunnucks P., and Hales D.F. (1996) Numerous Transposed Sequences of Mitochondrial Cytochrome Oxidase I-II in Aphids of the Genus *Sitobion* (Hemiptera: Aphididae). Molecular Biology and Evolution 13, 510-524.

Sunnucks P., Wilson A.C.C., Beheregaray L.B., Zenger K., French J., and Taylor A.C. (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Molecular Ecology 9, 1699-1710.

Sunnucks P. (2000) Efficient genetic markers for population biology. Trends in Ecology and Evolution 15, 199-203.

Taberlet P., Fumagalli L., Wust-Saucy A.-G., and Cosson J.-F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. Molecular Ecology 7, 453-464.

Tamura K. (1992) The Rate and Pattern of Nucleotide Substitution in *Drosophila* Mitochondrial DNA. Molecular Biology and Evolution 9, 814-825.

Thalmann O., Hebler J., Poinar H.N., Pääbo S., and Vigilant L. (2004) Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. Molecular Ecology 13, 321-335.

Thalmann O., Serre D., Hofreiter M., Lukas D., Eriksson J., and Vigilant L. (2005) Nuclear insertions help and hinder inference of the evolutionary history of gorilla mtDNA. Molecular Ecology 14, 179-188.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., and Higgins D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24, 4876-4882.

Thoren P.A., Paxton R.J., and Estoup A. (1995) Unusually high frequency of $(CT)_n$ and $(GT)_n$ microsatellite loci in a yellowjacket wasp, *Vespula rufa* (L.) (Hymenoptera: Vespidae). Insect Molecular Biology 4, 141-148.

Thorpe J.P. and Solé-Cava A.M. (1994) The use of allzyme electrophoresis in invertebrate systematics. Zoologica Scripta 23, 3-18.

True J.R. (2003) Insect melanism: the molecules matter. Trends in Ecology and Evolution 18, 640-647.

Tu Z., and Hill J.J (1999) *MosquI*, a novel family of mosquito retrotransposons distantly related to the *Drosophila* I factors, may consist of elements of more than one origin. Molecular Biology and Evolution 16, 1675-1686.

Turelli M., Hoffmann A.A., and McKechnie S.W. (1992) Dynamics of Cytoplasmatic Incompatibility and mtDNA Variation in Natural *Drosophila simulans* Populations. Genetics 132, 713-723.

Vega F.E., Benavides P., Stuart J.A., and O'Neill S. (2002) *Wolbachia* Infection in the Coffee Berry Borer (Coleoptera: Scolytidae). Annals of the Entomological Society of America 95, 374-378.

Wan Q.-H., Wu H., Fujihara T., and Fang S.-G. (2004) Which genetic marker for which conservation gentics issue? Electrophoresis 25, 2165-2176.

Waugh R., McLean K., Flavell A.J., Pearce S.R., Kumar A., Thomas B.B.T., and Powell W. (1997) Genetic distribution of *Bare-1*-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms. Molecular and General Genetics 253, 687-694.

Welsh J., and McClelland M. (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18, 7213-7218.

Werren J.H., Windsor D.M., and Guo L. (1995) Distribution of *Wolbachia* among neotropical arthropods. Proceedings of the Royal Society of London, B 262, 197-204.

Werren J.H. (1997) Biology of *Wolbachia*. Annual Reviews in Entomology 42, 587-609.

Werren J.H. and Windsor D.M. (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proceedings of the Royal Society of London, B 267, 1277-1285.

West S.A., Cook J.M., Werren J.H., and Godfray H.C.J. (1998) *Wolbachia* in two insect host-parasitoid communities. Molecular Ecology 7, 1457-1465.

Wolstenholme D.R., and Clary D.O. (1985) The mitochondrial DNA molecular of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. Journal of Molecular Evolution 22, 252-271.

Wong L.H. and Choo K.H.A. (2004) Evolutionary dynamics of transposable elements at the centromere. Trends in Genetics 20, 611-616.

Wu C.I. (2001) The genetic view of the process of speciation. Journal of Evolutionary Biology 14, 851-865.

Xiong Y. and Eickbush T.H. (1990) Origin and evolution of retroelements based upon their reverse transcriptase sequences. The EMBO Journal 9, 3353-3362.

Zane L., Bargelloni L., and Patarnello T. (2001) Strategies for microsatellite isolation: a review. Molecular Ecology 11, 1-16.

Zhang D.X. and Hewitt G.M. (1996) Nuclear integrations: challenges for mitochondrial DNA markers. Trends in Ecology and Evolution 11, 247-251.

Zhang D.X. and Hewitt G.M. (2003) Nuclear DNA analysis in genetic studies of populations: practice, problems and prospects. Molecular Ecology 12, 563-584.

Zhang D.X. (2004) Lepidopteran microsatellite DNA: redundant but promising. Trends in Ecology and Evolution 19, 507-509.

Zhou W., Rousset F., and O'Neill S. (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of the Royal Society of London, B 265, 509-515.

7. Appendix

7.1 *Pityogenes chalcographus* sample collection

Id. Nr.	Country	Location	Host tree / Trap	Longitude	Latitude
1IT	Italy	Brixen	*Pinus strobus*	46°43	11°39
2SL	Slovenia	Kranj (Hastje)	*Picea abies*	46°15	14°21
3AT	Austria	Hainfeld	Chalcoprax	48°02	15°46
4PL	Poland	Beskid Slaski	unknown	50°06	18°32
5FR	France	unknown	*Picea abies*	45°64	02°84
6Li	Lithuania	Vilnius	*Picea abies*	54°41	25°19
7CH	Switzerland	Birmersdorf	Pheroprax	47°22	08°27
8FR	France	Mouterhouse	*Pinus sylvestris*	48°59	07°27
9PL	Poland	Bialystok	*Picea abies*	53°13	23°32
10IT	Italy	Asiago	*Picea abies*	45°52	11°30
11IT	Italy	Tolmezzo	*Picea abies*	46°24	13°01
12IT	Italy	Pavullo	*Picea abies*	44°20	10°50
13AT	Austria	Murau-Neumarkt	*Picea abies*	47°07	14°01
14AT	Austria	Muhrursprung	*Picea abies*	47°14	13°26
15AT	Austria	Kärnten	*Picea abies*	46°37	14°37
16IT	Italy	Brixen	*Picea abies*	46°43	11°39
17SL	Slovenia	Kranj (Cegelnica)	*Picea abies*	46°15	14°21
18SL	Slovenia	Kranj (Hastje)	*Picea abies*	46°15	14°21
19SL	Slovenia	Medvode (Mavcice)	*Picea abies*	46°15	14°21
20SL	Slovenia	Kocevsko	*Picea abies*	45°38	14°52
21SL	Slovenia	Kranj (Brdo)	Chalcoprax	46°15	14°21
22SL	Slovenia	Kranj (Brdo)	Pheroprax	46°15	14°21
23SL	Slovenia	Kranj (Brdo)	Linoprax	46°15	14°21
24K	Croatia	Trakoscan	*Picea abies*	46°15	15°52
25R	Russia	Ladoga Lake Shore (nearby Kuznechnoye - Priazensk district)	*Picea abies*	61°09	29°52
26R	Russia	Ban'Koora - Luga district (Leningrad)	*Picea abies*	58°44	29°52
27AT	Austria	Günsels	*Picea abies*	48°23	15°21
28AT	Austria	Prinzensdorf	*Picea abies*	48°11	15°35
29FR	France	Raon sur plaine	*Picea abies*	48°31	7°06
30FR	France	Mazoires - Massif central	*Picea abies*		
31FR	France	Vagney	*Picea abies*	48°01	6°43
32AT	Austria	Braunau	*Picea abies*	48°15	13°04
33CH	Switzerland	Bielersee	Chalcoprax	47°10	7°20
34K	Croatia	Jastrebarsko	*Picea abies*	45°40	15°39
35AT	Austria	Donatikpl.	*Picea abies*	47°19	16°25
36AT	Austria	Waldviertel	*Pinus sylvestris*	48°30	15°00
37AT	Austria	Raxe	*Picea abies*	47°20	15°30
38RO	Romania	Sacele	*Picea abies*	45°37	25°42
39RO	Romania	Bistra	*Picea abies*	46°30	23°10
40SLOVA	Slovakia	Banska Stiavnica	*Picea abies*	48°28	18°56
41AT	Austria	Rotwald	*Pinus cembrae*	47°40	15°10
42SLOVA	Slovakia	Zvolen	*Picea abies*	48°35	19°08
43NO	Norway	Ås	Chalcoprax	59°40	10°48

Id. Nr.	Country	Location	Host tree / Trap	Longitude	Latitude
44FI	Finland	Oulunsalo	*Picea abies*	65°01	25°28
45	Poland	Chojnice	*Pinus strobus*	53°42	17°34
46DE	Germany	Essenhof	*Picea abies*	52°16	10°31
47SLOVA	Slovakia	Kysucké Nové Mesto	*Picea abies*	49°20	18°50
48DE	Germany	Hofuldinger Forst	*Picea abies*	48°03	11°35
49DE	Germany	Ebersberger Forst	*Picea abies*	48°05	11°58
50DE	Germany	Tharandt	*Picea abies*	50°59	13°35
51DE	Germany	unknown	Pheromone trap		
52SLOVA	Slovakia	Zwardon	*Picea abies*	49°28	18°50
53PL	Poland	Hajnowka	*Picea abies*	52°45	23°36
54EST	Estonia	Varbola	*Picea abies*	59°02	24°30
55FI	Finland	Jarvenpää	*Picea abies*	60°28	25°06
56FI	Finland	Jarvenpää	*Pinus sylvestris*	60°28	25°06
57FI	Finland	Kangashäkki	*Picea abies*	62°36	25°44
58SW	Sweden	Overkalix	*Pinus sylvestris*	66°21	22°56
59SW	Sweden	north of Overkalix	*Picea abies*	66°25	22°50
60NO	Norway	40 km out of MoiRana	*Picea abies*	66°25	14°30
61NO	Norway	Korgen	*Picea abies*	66°10	13°40
62NO	Norway	Rora	*Picea abies*	63°50	11°22
63NO	Norway	Eina	*Picea abies*	60°38	10°36
64SW	Sweden	Rolfstorp	*Picea abies*	57°06	12°17
65DE	Germany	Kropp	*Picea abies*	54°24	9°31
66DE	Germany	Heubronn	*Picea abies*	47°45	7°45
67DE	Germany	Harz - Hasselfelde	*Picea abies*	51°45	11°00
68DE	Germany	Nedlitz	*Pinus strobus*	52°04	12°14
69AT	Austria	Kalkalpen	*Picea abies*		
70DE	Germany	Wohhalbe	*Picea abies*	47°59	7°51
71DE	Germany	Zipfelweg - Kirchzarten	*Picea abies*	48°	7°53
72CZ	Czech Republic	Uhlirske Janoviee env. Nechyba	*Picea abies*	49°50	15°10
72CZ	Czech Republic	Pribram env. Obecnice	Pheromone trap	49°42	14°01
74NL	Netherlands	Benekom	*Picea abies*	52°0	5°40
75GR	Greece	Drama	Pheromone trap	41°09	24°08
76SLOVA	Slovakia	Tanap - Javorina	*Picea abies*	49°14	20°08
77RO	Romania	Codlea	*Picea abies*	45°42	25°27
78HU	Hungary	Velf (Velem)	*Picea abies*		
79KR	Croatia	Pozega	*Picea abies*	43°50	20°02
80PL	Poland	Tatra	*Picea abies*	49°18	20°07
81PL	Poland	Bialowieza	*Picea abies*	52°43	23°55
82SB	Serbia	Inga Mountain	*Picea abies*	43°52	18°25
83Li	Lithuania	Girionys - Kaunas	*Picea abies*	54°54	23°54
84Li	Lithuania	Rietavas	*Picea abies*	55°44	21°56
85SW	Sweden	Umnäs	*Picea abies*	65°24	16°10
86NO	Norway	Koppera	*Picea abies*	63°24	11°51
87NO	Norway	Selbu	*Picea abies*	63°13	11°02
88NO	Norway	Trondheim	*Picea abies*	63°25	11°25
89DÄ	Denmark	Jyderup skov	*Picea abies*	55°40	11°26
90HU	Hungary	Zalf (Zalaegesreg)	*Picea abies*	46°51	16°51
91HU	Hungary	Örlf (Szentgotthard)	*Picea abies*	46°57	16°17
92HU	Hungary	Budafa (Lenti)	Pheromone trap	46°37	16°33
93BE	Belgium	Wellin	Pheromone trap	50°05	5°07
94BE	Belgium	Bruxelles	Pheromone trap	50°50	4°20

7.2 Microsatellite sequences

SSR targets are printed **bold**.

Primer binding sites are printed underlined.

7.2.1 *Messor structor*

1A

TAAGGAAAAAAAGAGGATGGAAGATAATANAGGAAGGATGGGAACGTATTATCTCGTCTCGCGCGCGGGAATGAT
ACGAGCGAGTGGAACAGCGGGTAAAAATGAAAAGAACAGGAAG**GAGAGAGAGAGAGAGAGAGA**GCGAGGGAGAAA
GGAGGAAAAA**AGAGAGAGAGAGAGAG**TGGGAGGGAGAGAGAGGGACTGCTTGTGTAAGGAAGACGCACTACAAAA
ACGGAGATGATGACTATAGCTACGAGTCAAAAGACGAGAATCACCATGTACAATGTGAACGTT

1B

TAAGGGCGAGAGCGCGGACCGCGGGCGAAATTATGCAAAGTGAATGAAGATAAAAATACACAATGGATAGGGCGC
ACGCGATATTACGTATTACTCGATGAAATCTTATTTATGGACGCGACGGTGGACCCACCCCGGTGGATGCGAGCG
ACTCCAATATTTTACCTCATCGGTAAAATAAGAATTCATATCCGCGTGCGCGGTTCCTTTGTATGACCGGCATTC
TCCTGCCCCCCCC**TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC**TTCCTCTCCCTTCTCTCTCCCTTTCTGA
CCGAGTTCCGGCGAGTCTGGAAAACTGCTCGAGCATTTTCGTAACGACGTCGTTGGACTTTCGTGCATCCGACGC
CAAATTGACGCTTTCTTACGACCGTGATGTAGACCACGAAGAATTTAGACCGAAACATGCGATGCTGTGCCGGCC
AGCGGCGGCGGCGGTCGCCGTTCTTCCAAGAACGCGCCGCAAGGTTCAT

1C

TAAGGCGAAGCTGCATAACCTTACGTCGTACTTCGTTATTGCGAGTCTGTGGATTATCGATAACTGGTGATGGGA
AATTTCCT**TGTGTGTGTG**TAAAAGATGGTAAACGTATCTCATCTTCTAGGAAGAGAAAGCGTGTCATATTGGATC
GGCTAAATGAGAAGCTGCCTTGCATATGGCTTGCAGGGCGTCGCGCGAATCCAGACAGACGGAAAATTGAAAACT
ATAAATTTCTTTGCTTTTCCACCGT

1E

TAAGGAAAATCGCAAATTATGTCATAAGATATTGTGGGTATTGTCCTGAGCTAGATAGAAACACGCAGATACGTT
GCAGTAAACAATGTAACGTCACGAGAACTGCAATTTTCTGGATCTTCTAGATTACCTATGCTAGTAGATTTTTTT
GTATAATAACGATGAACCGTGCAAGTCGACAAATGTTACATTTGCCTCTTCCTCTTGAACCAGTTTTTCCTCTTG
AAAATCAATATTGACTTCGTCTATCGAAAACTAAATTAGTAACAGAAATTGATACATTTCTATTTGGCTTTTTGC
AGTTTCGTATTTTTAGAGATATATAGGT**CGCGCGCG**CAACATCTTC**TGTGTGTGTGTGTGTGTGTGTGTGTAT**
ATATATATATGTGCGCGCGGCAGAACAATTCTAGAGATAAATGTAAAAGACAAAAAAAAGCAAAATGTACTACGC
GATACTACTTTGAAGTCGCCGAGCTCAGTAGAGAAAGAGAGTTTCGAAGAGCCTGGCAGAGATATCGTCGTGCTG
TTCGACTAAGCGCTTCCAAGAGCCAAACCCCTCGAGCGTCCGGCAAAGAAATTCCGCTCGTATTTTCGTGCAAGA
ACCCGCGCGACGTCGCTCTTGTACGATAT

1F

TAATATAGCGCTCGTTAGTGGTCGATACTATGACATATATACACCAAGTAACATCGTTTACTCCTATATATATGA
GCACACACACGCGTGCGCGCGCGCACG**CACACACACACACACACACACACACACACACACA**TG**CGCGCGCGCG**CA
CACACAGACTCAACATAAGTGAATTTAGCTGAAGCTAGAGAACGGTGAGAAATGCAAAATATTGGTGAATCTCTG
TCCCTTTTTGCGCGCATATTGATAATAATGGCCGGCAACATGCCAGGATTCCGAAAAGCTCATAGATAGGTCTAA
TCTTATCTAGTAAACAAAGTAAAAGCACTTGCCGGCAGATTGGAAGCAATTATATATTGTAGCACGTAATTGCCT
TGTGGTGTTTGT

2A

TAACACACGTAGGACGAACGTTGAACGATGAACTTGACACTGCACGTGTGAGAGAGAAGA**AGAGAGAGAGAGAGA**
GAGAGAGAGAGAGAGAGAGAGAGAGAAAGAAGGAGAGAAAGAGTGTGCTCGAGCAAGAGCTGTAGAGAGAAAAGA
AAGAGAAAGGAAGATGTGAGCCTGCAGCCTCACGAACGCCTACCCATTTCTATCTCGCCTTTTTCATCCTCAACG
AAACAAATGACGTCCATAAGTCTATCGATCCCCTTAGATTACCCTTCT

2C

TAAGGAGGAAGAGACAGACCGATAGAGGAAAAGAGAGGAAGAGAAGCTACGGGTTCAAGTAGCAAACCGATTTAG
CAGGCGCACACGTGCTTTGAGGAAGGGATAAAAGAAGGAAGGGTTTGTTGAAGTAACAAGGAAAGAATTTGAAGA
TAGAGGAAAGGAGACGAAACTATAGATAAAAAAGAAAGACGAGAGAAAGAGAGGG**AGAGAGAGAGAGAGAGAGAG**
AGACGGAGATAAGAGAACAAGACAGAGAGGCTAAGAT

2D

TAATACTCGCAAAAAATGACGTTGTACATTGGCGCGAACGCGTTGTAAAAAATCCAATTGTACTCGCAGTTGAAT
CAATTTCCACCGAGACGGATCGGCGCTTTTTGCGGCACGGAGACAATACTTCATGGTAGGAATTAGCCAGGCTCG
ATTTCGTGAAGCGATCTCAG**TCTCTCTC**TTT**TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT**
CTCTCTTCCTTTNTGTG**TCTCTCTC**GCCTTCTGTATCTCTTTCCCGCGACCGCTTCACGGCGTTTTACACGGAGA
TAGTTTTCGCCGAACAGCCGCGTTATTACATGCT

2E
AAATCGGTTTCCGCGGCCGCTTTGAGCCAGGCCGGATAGACTGATTTACGACGTGTATATCCCAACAGGGACCAT
AACTCTCAAAACTACTGCGTACAGCTGTAGGCCGTCACGCGTTCGGCAAATCCCAATTTCCAACTACATGGTTCC
TACAATCGAGCCGAATTCTATTGCATACAACGTACACGTGTCGAAGAGAGAGAA**AGAGAGAGAGAGAGAG**GGGGA
GG**GAGAGAGA**GTGACGAATCATCGTAACGT

3A
TAAAATAAAAAATAAAATACGCGTGTCACGCGATGGATTGCCTGAAATCTAAGTTTGAAAAATTGAGTAAATTGA
AATGCGCGTAGAATTTTTAACAGCGTCTGGCTATAGAATTATTGGTCGCGACATT**TATATATA**TGTCGACACCCT
CGATGAAATGGCACAAAAAAAGTTAAATTGAAGCGTTAGCATAGGAAGTGAAGCAGGGAAGTGAGCAGAATGAAG
GAAAATTGTCGGGTAAACGGAGGACGAGGATTTCTATTTTCTTCAAGTCTACTATGAAGCGGATCAATTTCATTT
TCCCTCAAGAGTAGAAGAAAAACAAGCTTTATCTTTCTCTT**TCTCTCTCTCTCTC**TTCCCTGTCTCGCGTCGAGA
TGAGGAGACGAGCGAAAAGTGTCGACGGCAGAATACCT

3B
TAAGATCCTAGAGAAAG**AGAGAGAG**GG**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**TTTATC
TAGTACACCGTGTCTTATAATAGGCCGATATATATTAGTAACAAAGGGAGCCATGTTTACGTGGTTCTGTGTACG
TTCAGAAATTATTGATCTTACTCCAGAGCACATCTCGGACAGATTCATTATCGAGCTTTATATACCGATAAGCTC
AT

3E
TAAGGGATGGTTACTAACTAAGACTGTGTGTAAATAATTGCA**TGTGTGTGTGTGTGTGTGTGTGTG**TTTCGCGGG
TGTTACATTGGTTCGGTAAGAATAAATTTACAATATGGTTTGACTAGC**TGTGTGTGTGTGTGTGTGTGTG**TATAA
ATTTCGAACGCGTGGGTGGACTAGAGAATAAGATATACATTCTAAATCAAATTTCTTCAATTTCTACGGTTGACA
TCGGGAATATTCTGTAAAACGAGCTGACTGGGATAAAAAGGGGTAATGTTCCCCTGGAATATACACAGGTATTGC
ATCGCGAGGGCGAAGCCGCGTTACTTCCGCCCTGAGCTCGGT

6A
TAACAAGGAAGAACAGGGCAATGGGNAAAAGAATAGACGCGCAAAAGGAGAAGTAAAACGGCANCATAGAAGCAA
ACAGGAT**AGAGAGAGAGAGAGAGAAAGAGAGAGAGA**GAAATGTGCGAGAATACCGTGGTAATTAGC

8J
TAATTAGGGTACTAGGAGGAGAAGGAGAAGGGGAGGAATGGATGAAGGAGGTGGAGAGGTCGAGGAGAGAGGGGA
AACAGGAGGAGGAGAATGGGCAGGAGGCAGAGAGGGGGACGGAGAATGAGTGGGAGAGTGTATGAGAGAGTGAAG
AATGAGAGAGGAGTATAAAATAAATTGTAAACGGAAACGGAAGTCCAGAGGCGGGTAGGCTAGATTTGGGATTGC
ATGCGGTCAAAGAGC**GAGAGAGAGAGAGAGAGAGAGAGTGTGTGTGTGTGTGTGTGTGT**GAATAGGCAGGGAACAAA
CGAAATGTAACAGGAGGAGGAGGGAGACGGAGGGAGATGAAGAGTATANACTATGGT

12C
TAACAAGGTACTTTTTACATTCATACACCATAAACGAGTGTTGGAAACTGTGCTGCTGTTTATCACCGCTAGTGG
AATGCGCTTCGTGGGATTATTGTAGCTGCACGATTTCCGGCCGAATGAACTGCATCTCGCTGAATTTTCAATGAC
AGAAGTGTGCACACATCAATCCCGGGCTTTGCATAATGATGCGCAGAGCATTAGTGTGTAATCGTTGATGGCTGA
TAACA**AGAGAGAGAGAGAGGGAGAGAGAGGGAGAGAGAGAGAGAGGGAGAGAGAGGAGAGAGAGAGAGAGAG**
AGACGCATAAAAGATAAATGACCATAAATGAAATAAGATTCCAAGAGCGTTTGCTCAAATGAATTGCNACTCANA
TTACGGAATACGGAAATGCTAT

12E
AAGGATTATAACGTAAAAATTTTATAATAACGACGGACTTATTGTTTATAGAATATTGTTTATAGAAAGAAGAAG
ACGGAGGGAGATAGAAACAGGAGGGGGG**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**
AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAATTTGTCGTCGTGTTTTCATTGCACGTAATTATCGGACTTAG
GAAGTTTCGCCAGTTGGAAACTTCGTTAGAAGATCTTTACTGTAAGGTGCTTTGCCTAAGCTTTTATCCTCCGCA

12I
TAAGGCAACACGTTTGCCTTTGTAAATTTTACGAATCACAGAACAACGTTCGCCTTATGGCGCCGCTACAAGAGT
CG**ACACACACACACACACACAC**ATACA**CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT**TTCTTTCTCTCTCT
NGAGCTTT

12O
TAAACCAGGAGACCAGGTATTCTTATTGAAAGGGCCTAAACCAGGTAAATTCGGAGACCAATATACGATTGGACA
CCGCACGATTGGACACCGCACGATTGGACACCGCACGATTGGACACAATACGATTGGACACCGCACAGTCTTCAT
ACACACACACACACACACACACACACACACACACACACACACACACACACACACTCTAACATA

13D
TAATGGAAAGATGATATTTTGTGCCACGTTGTGCCGTCGAAAAAAAGGGCGTATCCCGATTGGACACGTAACGAT
TGGACACAACTCGATTGGACACGCACGATTGGACACCGCACGATTGGACACCGCACAGTCTTCAT**ACACACACAC**
ACTAACATAGGTTAGGTTGAATTAGATTTCTTGGCAGGGGGGTCTGGGGAGGGCGCANCCCCCCCCCCCAGTACA
CTCAAGCATGTACT

13J
TAAGGGCGCGGGCATAATCTACATTTACAGCGGGGACTCGATGAGCATCTGCTTCTACCCCTCTCGCCCTCGCCC
GTCCCCCGTTCACCCGTCTCCCTAAACTCGATCCCTTTCCCCGCCAATGAGCATCCTCTCCCAACCGCGCATTCT
CCCAATCAGGATCGTTCCCTCTTCGTTGATTGGCCTCGCTATCCTACTCTCTCGCTCTCGTCCCCCC**CTCTCTCT**
CTCTCTCTCTCTCTTCTTCGTCGCTCGCTTCCTCTCGTTCTCTCGCAAACACATGAGCGAACGCGTGCAGTGGTG
TCGATAGGTCACGCAAATCCCTGTTCGAATCGACTGCCTTATCTTGATAT

13K
TAAGACCCGCCAACAAGAATGTGTGCGAAACGTTTACTTCACACATTCACCTCGACTTTGCGTAATATTCATCTT
CGATAAAATC**GAGAGAGAAAGAGAGAGA**AAGGGGGAGGGGAGGAGAGGGAGAGAGGGAAGGAGGGAAGAGGAAGA
GAGGGAAAGAATTGTTGAAAAGCCATTCACAGATGGAAAATGAGACGAAGGATAACCGAGGCGGAATCCGCGAGA
TATTTGCGCGGACTCGTATATTTGCAGGGAAAATATTTTTTAGCGCATATCAATTCGCGAGGTCCTACGAGTCTG
CGATAACAATTACGTTCCATATTTCAACCGTATCTCGAATTGAATTTGCAT

21C
TAATACCGAGCACGAGAACGTGCAGGAGACACGACGCATGTACCGAGATCCGCTATAAGTGATATCCTCCGTCTT
ACTCTCTCCGGGGGAACGCCGCGCTACGCCGACAGTCCATGTATTGTAATTTAGTGTTTAGCTCTCATTGTTTGT
TCGTGCCATTAGTATTTGTGTAACGGCGGTGTACCGGGCCGTCCGTCAATAAGTAGGGAGAAGTCCCTCGTGTCG
CGATTTCGCACCTAAAGCCACCGCTAGAAGGTCGTATATCGCGATATTGGTTTGTGCGATTCCCGAGAAGAAATT
GTACACGGAGGTACGGCTGTCCCCGTGAGACTTAGTTGGGGATCGCCGTGCGACCGAACCTTACTACGAA**ATATA**
TATATCCATTTTATTATTCTAAGTGTTGCGTTATTCTTTTGGACTCTCCTCCCCTCCGACACCTCAGTCGTTATT
CCACCCT

24B
TAAGTGCATCGCAAGGTGGCAGTTGAGGTATATTCCAGTGGGCTAGCGGCAGCAGTAATAACAACTGGCGAAATA
AGAAAGAAAACGAAATAAGAAAGAAAAAAGACCACGATGTAACGAGATAAGGCTTCTGTTGTGTAATCGGCTGTA
TGAGCAAAAAGAAATCGAGCGCGTACGTTGCTCCCCAAACAGCTCTCCTTTGCCGTGAAAATCTCAAATGAGTTT
CCGACGTGTTTGCCAAAAATTTCTGTCGATTGTTTCAGACGCCTTGAAGTACTCAGTATTCGTATTCTTCTTCTC
TCCCTCCCTCCCCTCTCG**CTCTCTCTCTCTCTCTCTCTCTCTGTCTCTCT**TTCTGAACAAATATCCAAAGCTCGA
CTATAGCGAGACACGTCCGCGTGAAAAGCTTTATGCTCAGGCGATAATCGATTGTTCGATGTAATATCGAGTGTA
TACTATCCGAACGACAAACATATCTGTATGCTCCGTTTGCAACGTGCTAATCTCCGGCTGCACGTCCCTTCGGTT
T

24E
TAAGGGAGGGAGGGGAAGAA**AGAGAGAGAGAGAGAGAG**CGCAATGCCTACGATGTTATACTTACAAGTGTTTACTAA
GCTTTTGCTCGAGTTGATACGATGCTTTCTCACGGATGCTTTCCATTTTTGCGATTTTATGAGACATTTCTGTTG
AATTGATTTAGTTTGAGTTGATAATTCCAATATAATAAATAGAAACCATTTATCCAGAAGTTGAGTCATATAATT
ACAGGATGTCCCAAAACACTTTGCTCAACGCTTGTGAGGATGCAGAACGGGTCAAGATAAACAAAAATGTCCTGT
ACTATTTTGCGATATTCGCAATGGT

7.2.2 *Lasius austriacus*

29F
TAAAGAGAA**AGAGAGAGAGAGAGAGAG**ACAAAGAGAGAATAAGAGCTTTCTCCTCGCACATAAAACCGCATGCGA
TTTACGCTTGCTCACGCAAGCTGCGTGTGTTTTCATATGGGAACGTCGAAAATATGCGGGCCGAGATCAAAGCTT
GAGACCGACCTAACCAGAAAGGATGAACGCGTACACATGCTCCGTGATAATGGT

30P
TAAGGTAATGGTG**AGGGTGAGGGTG**ATTGTG**AGGGTG**TGAGTGAGGTT**AGGGTG**TGGCTGAGGTG**AGGGTG**AGGT
AAGAGTGAGGAGTGGTG**AGGGTGAGGGTG**AGGTTG**AGGGTGAGGGTGAGGGTG**ATGGTG**AGGGTG**ATAGTG**AGGG**
TGATGGTGATGGTGCGGGTGAGCGTG**AGGGTGAGGGTGAGGGTG**AGTTTGAAGGTG**AGGGTGAGGGTG**AGTATGA
GTGTG**AGGGTG**AGTGTGTGGGTG**AGGGTGAGGGTG**AGGTGAGGT

32A

TAAACTTTTCAAATCGTAATATGTGTAAAATTATGAGTGAGAATATTTGATATAATAACAGATATAATAAAAATA
TCTTATTT**TGTGTGTGTGTGTGTGTGTGTGTATGTGTGTG**GGAAATTACAAATTTATAATCACAACAATAATCAT
AGCACGCGCCTTTCAAAGAAAGGATCCTAGAATCTTAGGGAAGATTTATATTTCCTTCCTCTTTCCCCTCAGTCG
GTTTTCGTCGTTCTTTTCTCCATTGCGGGTGGATATCTCGTGTTCTTTATGATTCTTTGAAAGGGACAATCTTAG
TAAAAGCATGCAATTTCAAGGTGCATAGCTTGTGTCATTGTCCT

32B

TAATATCAGATACGTCGCAGGTTGCGATATATGTATATCTGTGTATGTATGTGTGTGTAAAATTCCGAAACCGCG
GGCGTAGACTCGTCGCTCGCTACGTCTGCGCGCGA**GAAAGAAAGAAAGAAAGAAAGAAAGAAA**GTAAAAGAGAAC
AAGACGAAGCT**AGAGAGAGAAAGAGAGAGAGAGAGAGAGAGAGAGGGGGGGGGG**AGCACACATGTTGCGCCGGCGGA
CGCGAATCGACGTCACAGCACGGCGATAACGGCAGGTAAAGATGGCGGGCGCGAGGGAAAATTAGCGGCTCGTGG
ACGCGCGCGTCGCAGTATACATTCGTCTAGAATATACATACATACATCGCCGGACAGAGTGAAAGGGATCTCTTG
CGAGGCGACGGTCGCGAGAAGACAGATTCCTCCGATCGCGCCT

32E

TAATGTGTAACGTGCCACCTTTCGAGTACGGCTCGAACGTGCTCAGTCGGGATCAAGGTTCGAAAGGGGTACGAG
AGAACCAGTCGTGTGTTTATGATGTATAGTTGTTTATTGAATAAATGTCAGAATGCATGTACAAAATGAGAATGT
GTCGATGAACGC**TGTGTGTGCGTGGGTGTGTGTGTGTGT**ATATAATGTGTATATTTACAGTGTGTAAGAATATGT
GCGCGAACGGTACGGGTGTGCACGTGACTGTAAGCGGTATGTCGGTTTACAGTGTGCGTGTGTGTCTGCGTGT
ATATAATGTGTATATTTACAGTGTGTAAGAATATGTGCGCGAACGGTACGGATGTGCACGTGAATGTAAGCGGTA
TGTCGGTTTACAGTGTGCGCGAATATGCGCGTGAGCGGAACGCAGGCATACGTGGATGCAAGCGCTATATTCAAT
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ATCGAGCTCACATAAATGCAGTCAGTATTTCGCGCTT

32F

TAATGTACAAATTACGGTCAAGAATTTATGTAAAATGGCGAGCACTTGTATATCGCACTTCGGAAAGTGTTCCTC
TTCTCGAGACAAGAAGGCGCGCAGGGAGAGAAAGAGGAAAGAG**GAGAGAGAGAGAGAGAGA**AAGTTGCGTGATGATA
TGCACGATCGCGTAATCTGCCGGTGCGTTTGGGTCATCTAATCGGTCCTCGCGACGAGCAAAGTGACGAAATGTA
CGATAGTTTCAATTCGAAGGAACCATCCTCTCGTCCGACGGTCTCTCTATGACTCTCTCGCGGATTTGCGTTGTC
CGGAGCGGAAAAAATCCGAGGGGAAATAATCCGATCGTCATTTCGTCCTCCGATTCGACGAAGTAACGTACAAAG
**TGTGTATGTGTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TGTGTG**TCGAAATTCCCTGTACATACTTTCCGTTATTCACTGATCCGNCGACCAGTTTGGAGCCGACTTTTCCTC
GGCGATAGAACTCGAGATGCCGAGTCTCTCTCCAGT

32G

TAAAATAACTCTGTCATTCGTGATATCCAAGCATTGCGAATTGTCTTCTCCGGAGTGTAAGAGGAAAATTGGTGA
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GTCAACGAGACGATGCTCTGTCCGATTACTCGGATTCTGCTCTCCGCCAACAAAACTCGCTCTGCTCTTTTAGCC
AAACGGACCCGCAAATGCAAGCGTCGGATAATCTCGTCAGTTTCCACATATATGTATACAACCGAAGATTTTCGT
AGGCGCGCGATATATTTCCGTTGAGGGAAATGTACGCTAAGAAA**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
AGAGAGAGAGAGAGAGAGAGA**ACGGATACGTGGTACACCTGCTATTTTAT

32I

TAAAGTGAAACCGATATCGGTGTAGACACGCGTGTGCTTAGATTGCGTACAGGCGTAAGAAGCAGCCTCTTGCGC
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CCGACGAACTATCTCACGCTGTTCTATGTTCACGAGAACGCGAGCGTAGCTCTCTCGCTACCTGTGATAAATACT
TTGTCATTACCTCGTTTATATTGAACACATCAATCATCGCTGCGCGAAATAGATGCGCCAGGCTACGTCCCATAA
AACTTTATGTTGCAACGATTTTCTCATCCCCCG**CTCTCTCTCTCTCTCTCTCTCTCT**TGAAAAGAGTAAAAAGAA
CAATAAATAACAATCTAAATAACAAAGTCCTCGAT

33A

TAAAGCATCGTCTTTGTCCCTGTTATCGCAGTTACAAAGACACCCCTGTAAAACCTTATTCTACATACAAGTATA
TTATATTTTCAGTAAAAGCGAAAACAGATTTACTCATATGCACAATAATTGCACAATAATTGCGCATATATGCGC
GAGCGA**TGTGTGTGTGTGTGTATGTATGTGTGTG**TATGTGTATATAAACCTTGTTCCAGCTCCTCTCCGAAACTG
AGGAGCGTCTGCTTTGGCTTATCCTTCTTCTTCGTCT

33B

TAATATCGCTGAGGAGAGGATCTGCGTAGTGCCATAACTGACCGTTACGCGAAGTATGCACAACGGTGCATTCCG
CGTGTGCGTGCTAGCGCGTTCTCACGTGGTTGACCACCACACACGTACGTGTATAGGAAAACACGTCGTGAACGT
GGGCGTGCATTCCTGCCTTCGCATTATCTCTTCTTGGCTCTTTCTCTCTCTTCTTCTTCTTTTCTCGTTTCTCTT
GC**CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT**TTCTCTCTCTCGCGCCCGTCGTCGACC
GACCACGTACGAGAAGGAATCTTCTCCTCTTTTTATTCCTTATCCAGACCCGTTATCGTCGATACCATGGGCCGA
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TCGATGACCGCCCCACCCCGCT

33C

TAAGAATTTATCTAACAATATTACTCAATCGATCATTCATCGACGNTATCTGTATTTTGCGCCATGCATCTGCCC
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TCTTGTCAAGAAGTAGATTTTCTCTTGAGTTCGTGCACATTCTACATTTCAGATGGAGCGCTGTTGAGGGGACAA
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AGGATAGGATGAAATACTGTACGGGGAATAATATTGGGAAGTGTGCAGCTGTCACAGCAGCTGCAAACT**TCTCTC**
TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCACACACACACACACACACACACACACACACACACCA
CTTCCNCCCTCTCTCTTTCGCCCTCTCCTTTTCNCNCTCNTNNNCTCCTTCCCTCCTCTCTATCTATCTATCTAT
CTCTATCTTTTTCTCTTATTGATTGGCAATCTTTCTTTTGTATCTTCAGCCTCACGGTTCCTGTGCGCCATT

33E

TAATTGATGTTACAGGTACGAGAGAGGGTAGCTACCCCCGGGAAAGGGATCGTTATTGCACGCTCGCA**AGAGAGA**
GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATGTGTGCCAAGATTTCGTTCC
ATTATAACATTACACAACATTCATCGCTATATAATTTATTTCATTTTATTTATGTCTCATTTTATATAACGATGA
CGCAAAATATGACGATAAAATGAAATGAAATTTTCCGGGTCCCGATGGATATACATTCAGAAGAAACGCGTGAAG
CGAAGTGGTACATTAATTAAAGAAATCTCCGTCGAAAGAAAAATCTCTCGGCAATCTTTTGTGAGATTTAACTTT
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34A

TAAGCAAAGAGAAAGCAGAAAAAACAGATTGGAAACATAGGAAGGTTGAAGGAAAGCAAGAAGAAAGAACAAAAA
GGAGAGATGATAGAATCAAGAAATTGCGATCGAGGTGACTGGATAACAATGAAAGAAAAGATGAGAGATTGAGGT
TAGACTCGGAACAGACTAGAGGCTAGGGAGAAGTGCG**AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**TGAGTG
AGTGAGTGAGTGAGAGGGAGCAGGAGAGAAGGAAGGAAGGAGGTAGGTGTTGGATATCGGACACTTGGATAATAA
ATGTCTGATTATTCTTATAAAAACATTCAATTTCATCTCATTCTCATT

34C

TAACACTTTGACAAGTTACAAAAACGTAATGATATACGAAATGTTAGGAAATCTGTCATAACAAAGATGTGCAAG
GAAAAGATGC**AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**ATGATATTATGCTTATTTATC
AACGAACAGTAACATACTAACAAAAGGCAGAAATATATACTTGCTTCCAAAGCGGTTTAGCACATTTTTTTTTCC
AATAAAGTTACTACTAAGAATCTTTCTTCCGACTCAAAATACACAAAAAATAT

34D

TAAGTCGCCTGATAACTCCCTCTCGAGCTCAGATCTTGCTCGCGTACCGCCAGCCGGCGCCATAACTCCCTAGTT
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GGACATCACAGCCGCTTTTTCAAGCCGTGGATTTTTTTTATCTCTGCAACGC**GTGTGTGTGTGTGTGT**CGCGAAATT
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AGGGTTGTCGTTGCTCCAAACGTAAACTTCAT

34E

TAAGGGCTCAGGTGCCCCGATAAAGCGGTCGATAAGGCTCTCATATCTTTGCTCCTATCTCTGATGCGCGCGAGC
GCGTCTCTCTGTAAAGGGTGGCAC**GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT**TTGTGTGGAAACCAGCGTATT
TTCTGCGAGACGCGAGAACACATTTATAGACAGAGCCTAAGAGAGAACAAAGGATCCCGGACGTGCCTTCGAAAT
TTGTTATCAGATGGCGGAGAATGTTTCTCTATGCGACGTGCCAATATCGTCGAATGATGATTGGAAATTAGGAGA
GTAAT

34F

TAAGGATAAAGAGATAGCACCAATAAAGAAGAAAAAGCACTTAGTGGGACATTCAAAGGACCCCGCACAAACCTT
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CATTGCACCAACTTGATTCATTGAGCCGTTTGAGCTCTAAGGACCGTCGAAAGTATGAAATAGGCGTTTTACCGT
CGGCATTAGCGTTACCCAATGTGCACCACGGGGAGGTTGCGCGATCGGAATTGCACATCATGTGACTCTGAAAAG
AGCCGTTTGTATGAA**TGTGTGTGTGTGTGTGTG**TCAGAAAAGATAAGGACCCTACGATTCGTCACTCCCGTAGTATT

35E

TAAAGTGAAACGAGGGGGGGGAGAAAATGAAAGACAGAGAGAGATAGAAGACAGAGTGACAAGCGATTTGCGATA
GGTGACGAGAGCCACGTGTGCACGTGAAATTCGATGTAAATAGGTTACAATAAAATCTCACTCTAATGTCCGTCT
CTCGATGAGCTTGATGTGCAGCTAAGCGTTACGTTTTATGTGTGCTTCGGCGAGGAAACGCGGGCGGGAGAAAAG
AAGCGAACAACCGCTTCTTTTCGAGCGGGAGAGGATATAGAGGGGGAGGATATAGGGGGGTGGAAGGGGGGTGGA
TGGTGAGGTAGTCTTGCGTTTGCCTCCTTGTTTTGTGTCGCCCGTCTGTGAAAGTCCGCCCTGATTTTACTCGAG
CTGAAATCTCGCCGAA**TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC**TTTGTCAAATTCG
CGAANGCGGCTTCNTTCGCACACAAAGGGATAACGATAGTTGCCGGCCGTTTGAGTTCTCCTACCTTTTTCGGGC
CGACTCGGCCTTGTCGTCCTTGAAGCTTCNATCTATTCCTCGGAACCGTCNATGATCGAAAAACCGAANGGTCTC
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35G

TAATATTTTATTTACTACCTATCAAAACTTATGGATTTTTTTGCGGTTTGTAAACTAGCTGTCGGCCCGGACTCG
TCCCGCGCTTCTTTTAGTTTCGGCGATTTGAGATAATGCGC**AAAAAAAAAAAAAAAAAAA**GCGCGCGATTTATACG
AATCGCACACTTCTATAAAATATTTTTCTAATTTTCTAATTTATCATCGCGCCGCCGTCTCGGGGATAAAGGCGC
AAGGAAATAAAGAATTGTAATGTTACAAGGAAAAGAAAGACTGTAATGTCGAGTGCTTTATCGTAGCCTTCCCTC
TTCTTCGGCTCTCAGAGGCAGCAGGGATAGTTGGTGAAGGGCCAGGGGAGGGGGAAGGGAGAAGTGAAGTCGAGA
GGACTGTCGTAAGAGGAAGATGGAATAGGAGAGGGAC**AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**
AGAGAGAGAGAGAGAGAAAAAGGAGAAACGTAATGTCAAGTGCACGAAATTATTCCCTCGAGTGCTGTAGATCGGCA
AGACTAAGAACTTTCCGGATTATACCGCCTTGACGGGAGTTTGAATACGT

35H

TAACGGTAGCCGGGACATCGCCGCTATTCTAGAATCAATATACGAAATGAGAACGATAGGTCAGAATGGATTTCG
AATAAAATAATATACATGTATGTATGTATATGTATATA**TGTGTCTGTGTCTGTGTGTGTGTGTGTGTGTGTG**TAT
ACACACGATGTACGACAATATAAAATTTTCTCTTATGATGTGCCATACCATACGAGACTTACCTTCCACCAGACA
TATTGTAAAGGAATCTCCAATAAGATGATCTCAAGTTTTGTGGTTCTATTATAGCTGCGTGAAAGAGAGTGGCCG
TACTAAAGCAATATAAAAAGATCGCGAACCATTTTATTTCTGGTACTTTTTCATTTTCTGCTGCATCATTCCACA
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35K

TAAATGGACAACTGCGAATTACCGGGGAATAAGGACGTGGGATTATTCATTTAGCGCAATAAATTACATTCAAGT
AACGTGGGGGTGTTTATTCGTATTACGAGTAAGCCGATTAGACGGGACAGCGGAAAAACGTAGAGTCGAAAAAAG
ATTGAACGTCGGTCGGGAGAAAAGCGCTTCGCGAAAGATACATTGCCGAATGAACAAGCATA**CACACACACACAGCA**
CACACACACACACACACACACACACACACACACACACACAGAATTATCGATCGATGAT

35L

TAAATCCCGAGAGGAGATTTAGAGAGACTCGGAGTTTGGAAAAAAATGCACCGCGATATAGAGGGAACTAAAATT
TTCCATATACCACGCGGTTTGCAAATATCAAGATTGAAAGCTTTTAGTGCGCGATGAAGTGTCGCGCAGAGGGAT
CGGAACGTTATTCGCGAACACAACACAC**GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT**GCAATATAACACC
TTCTCTGCGCGAGAGCGCAAAAATAAAGCCGTTATGTGTAAACGCTAAATACCGGCGATATGAAATTGCGGCTCT
AGAACGTCGGGAGCGAGGATCCAAAGTTTAGTTTGACGGTGAGCTTATGAATTCGCGGGCGTTATCGTTCGCATA
TACGCGTATCGCCATCATTCAGGATCTCACGGCAAATCCTCGCGAAAGGCGTCTGTGCATGCTGCGTGCACAATA
CGTGCGCAACACGTGGGTTCCATCGCGCTGCTACTGAACTCGTATAAAAACGCGATGACAGAATTATCAGGTGCA
CCGTGACGCT

36A

TAAAACGACTGCGTGAATGCTAGCAGCGTGAGTGCTAT**ACACACACACACACACACACACACACACACACACACACA**
CAGAGAGAGAGAGAGAGAGAAAGAGAGAGAGAGAGAGATACCGCTCACTATTTTACTCGCAATGGCCGGGACTGATA
ATTTTTCGCTCACGTGTCTGGATCGTGATCCTCCCTTGGTAGTATAATCCTTTTTTTATATAACATCATTCTGTA
ATAAAGTCTAGTCACGTGGCGCGTATTCTCAGACAAATACCAGAGAAATATTGCGTGTTGACAAGCGACGACTAG
CGTTACAAGAACGGGGCTTACACACATATCAACATTATATACGAGTATATATAATCCTTTGATCATAATATATTC
AGCAATAATACGTACATTATTATCAT

36C

TAACTTTACAATAATTGATATGGAAACTTTGACGATGAAACAATCATTATCTTTAGTATCTGTACCCGAAAAGAT
ATTCAGTGGGTCCAGGACACCCGCTGTGAGGACGAAATTTATTTGTTGCAGTGATCTTTCCTTGCTTTGCTCGTG
CTCTCGCAATGAGTCTACTTATGTAGGTATCTAGACAACAAAACAAGATCCGTGTGAAAGCCGCACGGCGCTTTC
CTGAATGGTTGCTGACGTCAGAGAGCGAGGAAAAATTGCGCAATTTCACCATTCCGGCCGAGGCGGAATTTGTTC
GCGCAGCTTTCTTCCGGAATTGGATCCGCTCGAGGAGACGGAACGG**GAGAGAGAAAGAGAGAGAGAGAGAGAGAG**
AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGGGAGAGCAAGAGAGAAGGAGAAGAGGG

AAAAAAAAAACGAGGGTGGAATGGCGATGATTCGTTTTGTAAAACACGCGGCGTATTGTTTTGTCTGGCGATGCC
TACAAAGCTTTCAAT

36D
TAACACACAAGGAAGCAGCGAGAAGCGTGTGCGAATCCGATTGATCTGATTATCGCGAGATGTCCGAAACAAGCG
GCAGACGAAAGTGCGAATCGGTCAAGCGGTTGACTCGATGACAGACCGGTCGCTGCGCGAGATGAGATGACGGCG
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TGTAGCGAC**CACACACACACACACACACACACACACACACACACACATATATATATATATA**GAGGTACACACGCAAAATCA
CACATCCGTTGTAATCATCAAAGTTGGGATCGAGTCGAGCGTGGGTTCGGGCCGCATCGTCATGTGAGACAAGGT
TTATCCAAAGTGCGTCTACCGATGAAATTACCCCGGAAATTGACGTTCTCTACGAT

36G
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GGAGAGATGATAGAATCAAGAAATTGCGATCGGGGTGACTGGATAACAATGAAAGAAAAGATGAGAGATTGAGGT
TAGACTCGGAACAGACTAGAGGCTAGGGAGAAGTGC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**
AGAGAGAGAGAGAGAGAGAGAGAGAGAGTGAGAGGAAGCAGGAGAGAAGGAAGGAAGGCGGTAGGTGTTGGATAT
CGGACACTTGGATAATAAATGTCTGATTATTCTTATAAAAACATTCAATTTCATTTCATTCTCATT

36H
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ACGATTGCTCCTCGCGATAACTGTCGCAGTGGAACTTATAAATTGTGTTGTTTTTCTCCAACAATGTGACGTGCG
TCACATCCAAGCCATAGTTGATAAATAAATATTATCGACAACCAG**TCTCTCTCTCTCTCTCTCTCTCTCTCTCTC**
TCTCTCTCTCTCTCTCTCTCTCTCTCTCCTAGATATAAATATAGACGAGACATTCTAGATATATTGATTCCTGTT
CAGTGATTCAATGTTCTTCTGTAGATAATTATTGTAACTATGATATATACAGAGAGAGAAAGAGGAAATTTACT

7.2.3 *Pityogenes chalcographus*

11L
TAAGACAATCGCGTTGCTTATGTGCTGGGATTATATCCATTCTAGGTCGACACAAATCAGGTACATTTTAGAGAA
AAA**TGTGTGATGTGTG**TTGGCCACGTCCCAACCCAGACACAATTTGGAGAGTATTATGTTGAACGGTTGAACCTA
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TCAAAGATGCAGAGTCCGTGCCTCATAT

41E
TAATATCCAAGACGGGNCCGTTAACGNAAAGTAAACAACTGATTCTCAAGTATTTTGGACACGNTCAGCAGGAGG
AAAGAAGATCTCGAAAAACTTATGATCCAAGGGAAAGTAGAAGAAACACGACCTAGAGGACGTTCTCCGAAAGCA
GTTTACAGGAAAATCTCTTCAGGACAGCNTTAGAGGCACGTCAGATAGAAGGGAATAGAGGGAAACTGCTAAAAC
CACAACATGAGACAAACATAGGATCACGACGCTTCANGCATGAAGNTGACGACTG**GAGAGAGAGAGAGAGAGAGA**
GAAGGCGGCGATTGCCAAAATTGATGTCGCTAACTCTCANAATTATGAAAATACATTTATCTTTTGATGTTGNAT
ATATTTGAAGTTTCT

41F
TAATTTGGTAGGTCTTTNCCCACAAACTGGAAACGTCTGCTTTACGGCGAGGTTACGAGTCCGGAAATCGGATTC
GGAAGCGCGTACCTGACCTGATACCGCCGCCGGCTGGAAAAACAACGAAAAGTGGAAAGTATACCGTCGGTTCAC
GATAAAAATTGCCAACCCA**TGTGTGTGCGTGTGTGT**ACATGTATTCGACAAAATACGCGAGTTCAGACGCTGACT
ATTGCCCATTGACGTCATTATCGCACCGGCACGTGGACGAATTCGTACACGGGTGCT

41G (= 37C-type)
TAAGAAGGGTAATCTAACNCTTANNNATAGACTTATGGACGTCATTTGTTTCGTTGAGGATGAAAAAGGCGAGAT
AGAAATGGGTAGGCGTTCGTGAGGCTGCAGGCTCACATCTTCCTTTCTCTTTCTTTTCTCTCTACAGCTCTTGCT
CGAGCACACTCTTTCTCTCCTTCTTT**CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT**TCTTCTCTC
TCACACGTGCAGTGTCAAGTTCATCGTTCAACGTTCGTCCTACGTGTGT

41H
TAATTTGTTGTTTTTT**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**GCGCGTTCGCGTTTCGC
GTTTATTTTAGTCAATCAGGGCTGAACTGAGTAGGTTCTATTGGCTAAACGTTTCGTTTCGTTCATCATGTCCGA

TGAGGAGGTTCTACCTATGGTCACCCAGGTCATAATGACCCCAGACATGTTGCATACCCTTCTACAAGAACTACG
AGTTGATCATACACCTACCGCAGTCTCAAACCATGGCAATTTTTCCCAATGCACATCTAGATTTTCAGGAGGCAG
GGATGATGTTAGTGCATTTATTGATGGTGTCTCTGTGTACAAAGAATGTT

41M

TAAGGAAATTGGTATATGNCCCCTAANCACACAAAAAACATTAGAACCGAACTGATAAAGAGAACAAAACACTTC
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CTGTTGAAAAATTTTCACCTTCCATAAAAAACTCTTTTTATCAACACACTTTACCTAAATTCATACTCCGACTAA
TCAAACATAAAAGGGAACTATACAGACTTT**ACACACACACACAC**GATGACGATCTGAAAAGAAAACTAAACGCAC
TAAATAGAAACGTCAAAACACTGAT

43A

TAAGCCACTAGAATAATACCAATATACACATTTTACATTCAATCGAAAGCAGGATGCGTATGGCACTTACTTCCC
AGATAACATTTGATATTCCGTGAATATACCCGAACGTGCAAAATATATTCATAATATAACTGGAATATTCCCCTA
ATATACACGAATGGAATGTTCAAAGAATATCCCTGATATGAATTTTATTTCAAAGCCATAAAGGGTATATTGCCG
GTATATACCTATAAAGTATATTCGCTCTGCAAACATGTATGGTACATTCATCAGGAATGTATGCGTAATATATAT
ACATTTACATGTGGAGTATTCACTCACTGTTGTTGGAAGCGCAACAGTATATCATGCGTTATATATTTATCTTGA
AAAATGCAATTATTAAAATTATTATTCTTATTATTCTA**TGTGTGTGTGTGTGTGTG**TAGATAGACACTCCTATTT
GTACTACTTATACCGATATTATAGTACCGGTAGCGTAGCACTAGCCACTCAGCCACTGCGTATGTTTTTT

44A

TAATAATAACTTACGTTTTACATGATACAAATACCACTTCTACTCTTTGATTAGAGAACGTTATTTATAACCGTT
A**TGTGTGTGTGTGTGTGTGTGTGTGTGTG**GAATTGGCTCTGTCCTTTCCGAACAATCTGCCAGCACTATTGGT
TTGAGGGCGAGGGGTTTGTCTGGCTCTGGACCATATCCCCACCCCCGATTCTTCAATTGGAGCGATTGACCACTA
GCTATTTTATTGGTTTTGTGCTAGTTCGAGTAGTTCGCGTGTTCGTGCTATTTTACTATTTCGGGTCAAGGAGTT
AGTATTTGGACGTCACAACACAGGAGTCCTACCAATTTATTCTTTTCGGAACTTCACAGGGACACAAAAGTTGAT
ATCTTGCTTCAGTTATATTATCCTACCTTTTCTTATAATTGTATTTTGTTTTTAGATAAAAAGGATGTAATAATT
ATTATAATTTGTTTAGATGGATTCTGCAAGACCGAGGCAACACTCAATGGCAACACCACCCCCACCTTGGACAGT
TCCAAGTATAAACAGAAATTTGGCAATTTATTTATCGGGCACTCAAATAAAACATGTTCAATATTCTCTATTTGT
CCGCATTCACACAGAGGGTTATCCCTAATGCCCATCCTATAAAGATGTTCTCCCGTACAGCCAATGACCCGTCCT

44D

TAAACATAAGTTTGGGCCCGGGCAAAGAGGCGAATGAATTGATGCGAGAGTA**TGTGTGTGAGAGAGAGAGAGAGA**
GAGAGATGGATTGATATAGTGATAGTGAAGCTACATTAGACCACAGGTCGCAGTGGAGGACGGAGAGTCCACTCA
CCGTAATAAATAGTGATAGTGACGGAACCGTATAAAAGGAAAGTGAAATGGGAAGGATATAAAAAGTATGGTAAA
TACGAGGCAAAAGTAGTGATATGGACGAAAGAAGGAGTGAAAGCTTGGCAGGATGACAATGAGACATCCGCCAAT
ATATTTAGTAAGTGTGATTATGGAGAGTGGTAGCAGAGTGATTAGGATGTATGGGATATATGATGAACCGAATGG
CAGGATAAACAAAAGGATGAGAAACTGCGAGGGTGTATGGGAAGCGTTGGAGGGACCCGTGCTATTGGCCGGAGA
CTTCAATGCAAAAATTTAGGCATGGGAGGCGATGTTACGGATAAGAGGAGAGAGGAATTGCTTGCGATGGCAATA
TTGCATGCATGAGAACTGAGAAACGATAGAAT

44I

TAAAACCAATACGAGGACCAGGCCGACCTAAACTAGATCGACACAGAGAACCACCTGAAATTGAGACTAGAAGAG
TTGGAATGACAGAAGACTCAAACCACCTTACAGAGCGAGAATATGAGGCGGAAGGAGAGATAGACCGAACAAGAA
ACCAGAGAGAGGAGAATCTATGTGAAAGACAAAGTAGGAAAGGCACCCGGAAAAGGAAGAAAGCTACTAGGTGTC
CCTGTTGTCCAAATGAAGTTTCCTCTTGCAACACAGACGAGGACGTAACTTGTGTTGCAAAGGGGGAGTATGTAA
CAGACCGACCGCCCCGTTGGGAAGAGGAGAAGAGTGAATGACAGAAGTGACAGTGACAGTGACAGT**GAGAGAGAG**
AGAGAGAGAACGAGAAGTAGGTAGGGATGTTCGATCCTCGGAAAAGTATCNATNTTTTGTATCGATCCGGATCGA
TCGCTNTTGTATCGATACCACTNAATATCGTTGTTGAAAAGATCGA

44K

TAATCCACAATGGTGTACCC**GTGTGTGTGTGT**GATAGTTGGTGTAGTGCCAACTCGGAATGAGTGCGGCTAATCT
AAGCAGTCAAGATAGATCAAGGTCTCGGGTACAGGACTTCAACTTTCTCTGGTTCCCACGTCACAGGCTAGAGTA
ATGGATATACCCAAGTTGGCAGGAGCTGAACACGCTAGCTGGTAGTGAGGTCTCGGTTGGCCAAAATGACAACCA
CAAAAGGTGGTGGAAAACGACTTCCAAATTAGAAGTACCGATATTAGAAGGCCTAAGATGAAATGCGGCCTTGTC
GGTTTCTTCTTGAATCTCTCAGTGTTTAGTTTACTGCTGAATTTTCCAATAAATCCCCACAAATGGGTAAAAGAC
AACGGTCTTCAAACAAAACGTGGAATAATTTCAGAGTTCACTGAGTTTTTTTT

44N

TAAAAGTAGTAAAAGTNCCCCNTTNNNTGGAGGTCGAGTAGTAACTGCCCGCGAATGCGCGTGTGACAGACCTTG
TGGTTCTTTCTCTAAAAGGGCCGACATGCGCCGTTACCGCAAAAATCACACTGGTCTTCGAGCTGGTCAGTGTGC
AACCGCCCTCAACTTGTAGTGTCCGGGGCGGCCCACTACCTTGTGCGGATCCCAGGCTGATATGAGAAGGGTTCA
GCACAACTTGTAGTGGCTGTTTGAATCTCTCAGCATTATTAGTTCAGTCAGTTTACACCGACTCCTTCACACACC
TAG**ACACACACACACCCACACACA**CCCGCAATCTCCCCTTGCAGTGACCTATTGTTACCATCTAAGGACCTATTG
CCAATAAACGAAATATTACCTTATGACTACCATTGTTTCCCTGACCTGCGCACCCCT

45B

TAAAACCAATACGAGGACCAGNCCGACCTGAGCTAGATCGACACAGAGAACCACCTGAAATTGAGACTAGAAGAG
TTGAAATGACAGAAGACTCAAACCACCTTACAGAGCAAGAATATGAGGTGGAAGGAGAGATAGACCGAACAAGAA
ACCAGAGAGAGGATAATCCATGTGAAGGAGAAAGTAGGAAAGGCACCCGGAAAAGGAAGAAAGCTACTAGGTGTC
CCTGTTGTCCAAATGAAGTTTCCTCTTGCAACACAGACGAGGACGTAACTTGTGTTGCAAAGGGGGAGTATGTAA
CAGACTGACCGCCCCGTTGGGAAGAGGAGAAGAGTGAATGACAGAAGTGACAGTGACAGTGACAGT**GAGAGAGAG**
AGAGAGAGAGAGAGAGAGAGAGAGAGAGGGAGAGAGAGAGAGAGAGAACGAGAAGTAGGTGAGTAGAATGTGGTT
AGATAAGATGACTAAGCTTTTCTTGGATTCTTTCGGGAATATAATNATTTTCGAGTTACGACCTGTGTGTGCTTT
TCCTTGTCCAGAACTACTAATGGCTTCTTACCGAACCCCT

46B

TAAGGGCTCAGGTGCCCCGCATAAAGCGGTCGATAAGGCTCTCATATCTTTGCTCCTATCTCTGATGCGCGCGAG
CGCGTCTCTCTGTAAAGGGTGGCAC**GTGTGTGTGTGTGTGTGTGTGTGTGTGTTTGTGTG**GAAACCAGCGTAT
TTTCTGCGAAACGCGAGAACACATTTATAGACAGAGCCTAAGAGAGAACAAAGGATCCCGGACGTGCCTTCGAAA
TTTGTTATCAGATGGCGGAGAATGTTTCTCTATGCGACGTGCCAATATCGTCGAATGATGATTGGAAATTAGGAG
AGTAAT

46C

TAAGTGCATTCACGCTTGGAACACTTCGTAGTACCGTAGCTACCTCAGGCACAAGTACGCCAAAACGAAGTGTGA
TGTCTACATTAGCAAAGTACGTGCCATATAAGCAGAAAATAACATCAAAAAAAGGAGTCGACGTGGAAAGACAAA
ATGAGATTATATTTGCAACAAGACCTAAAATTTTTTTTCCAATAAACGCAGGTTATTGTCCCT**ACACACACACAC**
GCACACACGCACATTCATGCAATATTACACAAAATAATTTTTTTGATAAAAAAAAACCAATTGAACGAGAAAAAC
TCTCGGAATTTTTTTTCTTCAGAAT

46F

TAATTACTCTCCTAATTTCCAATCATCATTCGACGATATTGGCACGTCGCATAGAGAAACATTCTCCGCCATCTG
ATAACAAATTTCGAAGGCACGTCCGGGATCCTTTGTTCTCTCTTAGGCTCTGTCTATAAATGTGTTTTCGCGTCT
CGCAGAAAATACGCTGGTTTCCACACAAACG**CACACACACACACACACACACACACACA**CGTGCCACCCTTTACAGA
GAGACGCGCTCGCGCGCATCAGAGATAGGAGCAAAGATATGAGAGCCTTATCGACCGCTTTATCGGGGCACCTGA
GCCCT

46J

TAAGGGATGGTTACTAACTAAGACTGTGTGTAAATAATTGCAT**GTGTGTGTGTGTGTGTGTGTGTGTG**TTTCGCGGG
CGTTACATTGGTTCGGTAAGAATAAATTTACAATATGGTTTGACTAGC**TGTGTGTGTGTGTGTGTGTGTG**TATAA
ATTTCGAACGCGTGGGTGGACTAGAGAATAAGATATACATTCTAAATCAAATTTCTTCAATTTCTACGGTTGACA
TCGGGAATATTCTGTAAAACGAGCTGACTGGGATAAAAAGGGGTAATGTTCCCCTGGAATATACACAGGTATTGC
ATCGCGAGGGCGAAGCCGCGTTACTTCCGCCCTGAGCTCGGT

47H
TAACCACAAGTAACTAGCTTACATGGTTACCCTCGCGGAAATTTGCTTCTTGTTCCTAACCCAAGATTTTTTTGT
CGTTGATTGTTGATGTCAATTTATTGCTTCATCATGACTTATTACGTCAATAATGTACTAGAGACTGCTTCAAAA
CTCACCACCAGAGGACGTATCGCTTCTCTTGAATCGCCAATACTGAGGGTAAATCAAAAATAAACTCACAATCAA
AGAGCTCAAGCTCGAAAAGCAGACGAGTCAAAAATAACCGTATCAATTTGTAGGCCGCCATTACGAGATGTTTTT
CACACCCACACGCACACACACACACTTTTTGGACCAAAATTCCAATAAGGAAACACTTTCCCGACCGGACCGAAA
CGCTCAACGCGTAGTAGACACTTTGGGCTACACTT

48B
TAAAACAGGATATATGCTATTGTAACATAGTTAGTGTTCGGGGCGAACACTTTGCAGGCGGGGGAGATGTCACCA
ATGTAGATAAATACATTCACCATCGCACAGAATTGTATGTTACACCGGGCAAATCGCTGACACAACAAAACACGC
CAGAATCCGGGTCACTCGACGGATATAAAAGCAGTACGGTTTGCCATCTAAAGTTACTGGTCTCCGAACTGGTCA
GTGTGCAACCTCCCTCAACTTGTAGTGACAGGGTGGTCCACTGCCTTTTCAGAGCCCAGGCTGATCAGAAAGGCG
TTCGGCACAACTTGTAGTGTCCTGTCAAACCCCTCAGCACTGTCAGTACCAACCTAGACCTTTGGGCACGGCGAC
CGAGTGTACCCAGCTTGTAGCGAAGAGTTTCACTCGGCACGCTCCTAAAAGGGCTAGAAAACACCTAG**ACACACA**
CACACACCTAACCGCCTTCTGCAGGGTCGGATGCCGACCAAAGACGTACGTACTACTTGCGGCCCTCTTGTTGTG
GGGGAGAATCCAATTGTTACCACT

48E
TAACTGAGACGAGTAAGTTTCAAAAATGCCTATAGATAGAAATAGAAAAAAATTTCTTCTCTATGAGCCTACGGA
GTTCGCCTTCTGCTATCTCCTTTGATAATTGTGTGTGCCATGGCACTGCTTCACCCAGTTTTATCCTTACTAGCT
TTACTTGCTCTACATACGAACCCTTCCTGTCAGTCCCTGTTCCCATTCATC**ACACACACACACAC**CCTGGGTCCT
TATTCTTGAAACTAGGGAAAGGAGTTATCAGTAGAACTGTGCACAGAATATGACCGACACCTGCCAATGTCATGT
AGTTTTCTGACATTCTGAAAACACTTCTCTTGGCAATATTCAAGAATACAGCACCAGCGGTCTT

48L
TAAGGCAACACGCTTTGCCTTTGTAAATTTTACGAATCACAGAACAACGTTCGCCTTATGGCGCCGCTACAAGAG
TCG**ACACACACACACACACACACATACACTCTCTCTCTCTCTCTCTCTCTCTCCCTCTCTCTTTCTTTCTCTCTC**
TACGAGCTTTACAATCTGTGCAATAAATAAAAATTTTACGCTGCGCGCTACAAAATATATCGCTGTTACATAAAA
TATGGCACAAGCTGTCCAGTCATCGCGCGTAATGATGGCTATGTTTTTCAGTTGTAGATTCTTTGCGGAACTTCG
AGGATCACTATGCATATT

7.3 Transposon sequences

mar2a
GCGAAGTCGGGACAGTAAGCATGTACGAGCCCTGGTGTCGTCCTTTAAGTAGCCTCTGCAAGCCGGGTTCTCATG
GATTTCCCAACAGTGACCCGTCATTAAACCAGTTTGCTCCTGTCAAGATTTAATAGAAATCTTTTGTCCGTTTAG
GACAGGGACCACGGCGTACTGGTTTCTTTGCCTGTCTCATACCGGGAGCAGTTGCTCAGCGTTGCTGAAAAAGTT
TAGCAGTCATTGCATTCATTGTCAGGACGGCAGTGTTGTAGATGATCCCTACAATATGTTTCGGCTTCGCGAGCA
TCCAGAATGGCGAGTCAGCTTCAACGTTGCCACGAACACCCATGTGCCTCGGTACCCACGCGAGGGTAACCCTAC
CAAACCGAGA

mar2b
GCGAGATCGGGACAGTAAGCATGTACGAGCCCTGGTGTCGTCCTTTAAGTAGCCTCTGCAAGCCGGGTTCTCATG
GATTTCCCAACAGTGACCCGTCATTAAACCAGTTTGCTCCTGTCAAGATTTAATAGAAATCTTTTGTCCGTTTAG
GACAGGGACCACGGCGTACTGGTTTCTTTGCCTGTCTCATACCGGGAGCAGTTGCTCAGCGTTGCTGAAAAAGTT
TAGCAGTCATTGCATTCATTGTCAGGACGGCAGTGTTGTAGATGATCCCTACAATATGTTTCGGCTTCGCGAGCA
TCCAGAATGGCGAGTCAGCTTCAACGTTGCCACGAACACCCATGTGCCTCGGTACCCACACGAGGGTAACCCTAC
CAAACCGAGACAGGGCAGGGTTCGGTTTTGGCTCCCTTGCTGTTCAACCTATATATTGCTGACTTGCCCGCCACC
ACTTCTAAGAAATTCTGCTACGCAGATGATATTGCCCTCGCTACTAAACATACTGCACTTGCCAAAATGGAAGA

mar3a
GCGAGGTCGGGAGAGTAGGAGATTTTAGGCTCATCAGCCATTTCAGTGGTTGATGATCACTTTTTATAGTAACTA
TAGCTCCTTCGAGGTGACCACGAAATTTGTCAGCAACTGCCCACACGACGGCTAGGGCTTCTCTTTCAGTGGTGG
TGTAGTTCTTCTCAGCGTCGGTTAACAGGCGACTGGCGTACTCTCCCGATCTCGC

7.4 Protocols

7.4.1 Microsatellite isolation by agarose bound avidine

Equipment and consumables

Thermocycler
Hybridization oven
Centrifuge for Eppendorf tubes, cooled
Waterbath, thermoregulated
Sealer for plasic bags
4 pcs plastic trays (VWR 216-2103 Q)

Sterile toothpicks and forceps
Nylon-membrane neutral, 82 mm diameter (Roche 1 699 075)
Whatman 3MM paper
X-ray film
Test tubes suitable for bacteria culture

Oligos

MseI AFLP adaptor 10µM: 5' – TAC TCA GGA CTC AT – 3'
5' – GAC GAT GAG TCC TGA G – 3'

MseI-A primer 10 µM: 5' – GAT GAG TCC TGA GTA AA – 3'
MseI-T primer 10 µM: 5' – GAT GAG TCC TGA GTA AT – 3'
MseI-C primer 10 µM: 5' – GAT GAG TCC TGA GTA AC – 3'
MseI-G primer 10 µM: 5' – GAT GAG TCC TGA GTA AG – 3'

biotinylated microsatellite probes, 10 µM

SP6 promotor primer: 5' – ACG ATT TAG GTG ACA CTA TAG – 3'
T7 promotor primer: 5' – TAA TAC GAC TCA CTA TAG GG – 3'

Kits and premade substances

Invitrogen REact 1 Buffer 10x
Qiagen QIAquick PCR purification kit
Promega pGEM-T vector system I (Promega A3600)
Biotin Luminescent Detection Kit (Roche 1 811 592)
Avidin D beads (Vector Labs A-2010)
Invitrogen *MseI* 5 U / µl
T4 DNA Ligase 1 U / µl
Biotherm 10x PCR Buffer
Biotherm *Taq* Polymerase

Stock solutions

DTT 100 mM
BSA 10 mg/ml
ATP 10 mM
dNTPs 2,5 mM
20x SSC
10% SDS
EtOH absolute
EtOH 70%
Xgal (0.2 g in 10 ml DMSO)
IPTG 100 mg/ml sterile
Ampicillin 100 mg/ml sterile

DNA, from salmon sperm, denatured, 10 mg/ml
Proteinase K (600 U/ml)
EDTA 10 mM sterile

Cells and media

LB agar
LB broth
SOC broth
competent JM109 cells

Buffers

1x TBT (100 mM Tris pH 7.5, 0.1% Tween 20)
1x TBST (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20)
1x TLE (10 mM Tris pH 8.0, 0.1 mM EDTA)

Denaturation solution (0.5 N NaOH, 1.5 M NaCl)
Neutralization solution (1.5 M NaCl, 0.5 M Tris.Cl pH=7.4)

Church Buffer (0.25 M $NaHPO_4$ pH=7.2, 1 mM EDTA, 1% BSA, 7% SDS)
2x SSC / 0.1% SDS
0.5x SSC / 0.1% SDS
0.1x SSC / 0.1% SDS

Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH=7.5 set with NaOH pellets)
Washing buffer (Maleic acid buffer supplemented with 0.3% Tween 20, pH=7.5)
10 x Blocking Solution: 10% w/v Blocking Reagent (Roche 1 811 592) in Maleic acid buffer
Detection buffer (0.1 M Tris.HCl, 0.1 M NaCl, pH=9.5)

Day 1 ----------

DNA preparation

1. For one reaction, prepare the following mix:

Genomic DNA	25 – 250 ng
10 x REact 1 buffer	2,50 µl
DTT 100 mM	1,25 µl
BSA 10 mg/ml	0,15 µl
MseI AFLP adaptor	2,50 µl each
ATP 10 mM	0,50 µl
MseI	0,50 µl
T4 DNA Ligase	1,00 µl
H_2O	ad 25,00 µl

2. Incubate 3 h at 37° C, inactivate 5-10 min at 70° C
3. Dilute 1:10 by adding 225 µl H_2O
4. Prepare the following PCR mastermix:

Biotherm 10x PCR buffer	6,0 µl
MseI-N primers	1,2 µl each
dNTPs	1,2 µl
Biotherm *Taq*	0,5 µl
diluted mix from (3)	15,0 µl
H_2O	32,5 µl

5. Aliquot into 6 tubes, 10 µl each. PCR conditions: 94°-30sec / 53°-60sec / 72°-60sec; 29 cycles, no hot start. Remove one eppi after cycle 14/17/20/23/26/29
6. Load 5 µl to a minigel; the cycle number where a faint smear >200 bp becomes visible is sufficient for production PCR; avoid over-amplifications
7. Repeat PCR with optimized cycle number in 50 µl reactions until several hundred ng product are yielded

Day 2 ----------

8. Pool the products, clean them with the QIAquick PCR purification kit, elute DNA in 30 µl EB
9. Prepare the following hybridization mix:

20x SSC	21,00 µl
H_2O	49,00 µl
10% SDS	0,70 µl
biotinylated probe, 10 µM	2,00 µl each
DNA from (8)	28,00 µl

10. Denature 5 min at 95°, allow to cool at RT for 15 min
11. Pellet 50 µl Avidin D beads in the meantime (2 min, 15000 rpm, 4° C), wash them twice with 400 µl TBST, resuspend in 350 µl TBT
12. Add the hybridization mix from (10) to the Avidin D beads, incubate 60 min at 50° in a hybridization oven under constant agitation
13. Centrifuge (2 min, 15000 rpm, 4° C), remove supernatant; supernatant may be collected for later troubleshooting purposes
14. Wash the pellet 3x with 400 µl TBST, each time completely resuspending it; collect the last washing solution
15. Prepare 1500 µl fresh washing solution for step (16): 15 µl 20x SSC, 15 µl 10% SDS, 1470 µl H_2O
16. Wash 3x with the fresh solution; collect the last washing solution
17. Dissolve pellet in 100 µl TLE, vortex vigorously, incubate 5 min at 95° C and immediately cool on ice
18. Vortex, centrifuge (2 min, 15000 rpm, 4° C) and transfer supernatant into a new eppi
19. For alkaline elution, add 12 µl 0.15M NaOH, vortex, incubate 5 min at RT, neutralize with 38 µl 0.1M Tris pH=7.2. Centrifuge and recover supernatant like (18)
20. Precipitate DNA from the washing solutions (14) and (16) and the eluate(s) (18) and (19) with 2 Vol EtOH o/n at -20° C

Day 3 ----------

21. Pellet the DNAs (15 min, 13000 rpm, 4° C), discard the supernatant, wash in 70% EtOH, air-dry and dissolve the pellet in 50 µl H_2O
22. For each DNA precipitation prepare a PCR reaction as follows:

Biotherm 10x PCR buffer	1,00 µl
MseI-N primers	0,20 µl each
dNTPs	0,20 µl
Biotherm *Taq*	0,08 µl
Templates (21)	1,00 µl
H_2O	6,92 µl

perform a PCR like in (5) with 22 cycles. Expect faint smear for the last non-stringency wash, almost no product for last stringency wash, strong smear for eluates

Construction of the enriched library

23. In case results of (22) are convenient, scale up PCR reaction of the eluate to >5 ng amplicon. Clean PCR product with QIAquick kit.
24. Prepare the following ligation reaction:

2x rapid ligation buffer	5,0 µl
pGEM-T vector	0,5 µl
DNA aus (23)	5,0 ng
T4 DNA Ligase	0,5 µl
H_2O	ad 10,0 µl

25. mix gently, incubate at 4° C o/n

Day 4 ----------

26. Prepare 10 petri dishes with LB agar supplemented with ampicillin (250 µl/250 ml media), Xgal (400 µl/250 ml media) and IPTG (120 µl/250 ml media)
27. Aliquot 2 µl of the liagtion mix (25) into eppis, keep on ice
28. Put competent cells into an ice bath until they are thawed; mix gently
29. Add 50 µl cells to each eppi from (27), incubate 20 min on ice
30. Heat shock cells 50 sec at 42° C (water bath) and put immediately back on ice; incubate 2 min
31. Add 950 µl SOC broth (at RT), incubate 90 min at 37° under constant smooth agitation (hybridization oven)
32. Concentrate cells by centrifugation (5 min, 3000 rpm, 4° C), pour off supernatant and resuspend pellet in 200 µl SOC broth
33. Plate out 100 µl aliquots to the prepared petri dishes, incubate o/n at 37°

Day 5 ----------

34. In case white and blue colonies are not yet differentiated, incubate plates at 4° C until colors are clearly distinguishable
35. Use sterile toothpicks to transfer white colonies onto masterplates; grow masterplates until colonies have ~2 mm diameter

Day 6 ----------

Library screening

36. Place a sterile nylon filter on the masterplates until the filter is completely soaked (~1 min); for positioning, stitch 3 holes into the filter using a hypodermic needle (2, 6 and 12 o'clock position) and mark the positions on the petridish
37. Remove the filter; seal masterplates with parafilm and store at 4° C
38. Place the filters on 3MM paper soaked with following buffers (colony side up; liquid should not reach the upper side of the filters):
    a. 10 % SDS 3 min
    b. Denaturation solution 15 min
    c. Neutralization solution 5 min
    d. 2x SSC 5 min
39. Put the filters on dry 3MM paper and allow to air-dry for 30 min
40. Bake filters 60 min at 80° C or UV-crosslink for 2 min
41. For each filter diltue 100 µl Proteinase-K solution with 900 µl 2x SSC
42. Spread the solution on the filters, cover with saran wrap and incubate 1 h at 37°
43. In the meantime denature 150 µl salmon sperm DNA 5 min in a boiling water bath and cool on ice
44. Remove cell debris from the filters by pressing wet 3MM paper against them; use a clean plastic ruler for the purpose
45. Add denatured salmon sperm DNA to 15 ml Church-Buffer; prewarm to 65° C (this amount is sufficient for 4 filters)
46. Put the filters into a hybridization tube, separated from each other with nylon mesh
47. Pre-hybridize 60 min at 65° C
48. Decant the pre-hybridzing solution, add fresh Church-Buffer (15 ml + 150 µl denatured salmon sperm DNA + 15 µl of each biotinylated probe); hybridize o/n at 65°.

Day 7 ----------

49. Decant the hybridizing solution and put each filter into a single plastic petri dish
50. Wash filter 5 min in 2xSSC/0.1%SDS at RT
51. Wash filter 15 min in 0.5xSSC/0.1%SDS at 65° C
52. Wash filter 15 min in 0.1xSSC/0.1%SDS at 65° C

Biotin Luminescent Detection

53. Dilute a 40 ml aliquot of the 10x Blocking Solution 1:1 with Maleic acid buffer (sufficient for 4-5 filters); split into two 40 ml aliquots
54. Wash filters 1-5 min in washing buffer, incubate 30 min in diluted blocking solution (~10 ml/filter)
55. In the meantime add 2 µl Biotin-AP antibody (Kit) to the remaining 40 ml Blocking Solution.
56. Incubate filter 30 min in the antibody solution
57. Wash filter 2x 15 min in 20 ml washing buffer
58. Equilibrate filter 5 min in 10 ml detection buffer
59. Place the filters in a plastic bag (DNA side up) and spread ~500 µl CSPD solution (Kit) above. Immediately close and seal the bag. Avoid trapping of air bubbles.
60. Incubate 10 min at 37° C
61. Place filter with DNA side towards x-ray film; the chemiluminescent signal will last for at least 48 h, so exposition time may be determined empirically (30 min to o/n)

Day 8 ----------

Retrieval of candidate sequences

62. Develop the x-ray film. Select dark colonies for further investigation and mark colony positions on the masterplates
63. Pick the colony with a sterile toothpick and transfer it to 2 ml LB broth in a test tube; at this step, part of the bacterial cell mass may be used as template for direct colony PCR
64. Incubate bacteria o/n at 37° C. Use them for immediate plasmid extraction or store at 4° C for later processing

Day 9 ----------

65. Extract plasmid DNA. Use the DNA for PCR-pretesting and sequencing

7.4.2 Microsatellite isolation by PMPs

Equipment and consumables

Thermocycler
Hybridization oven
Centrifuge for Eppendorf tubes, cooled
Waterbath, thermoregulated
Sealer for plasic bags
4 pcs plastic trays (VWR 216-2103 Q)
MagneSphere Separation Stand (Promega Z5332)

Sterile toothpicks and forceps
Nylon-membrane neutral, 82 mm diameter (Roche 1 699 075)
Whatman 3MM paper
X-ray film
Test tubes suitable for bacteria culture

Oligos

MseI AFLP adaptor 10µM: 5' – TAC TCA GGA CTC AT – 3'
5' – GAC GAT GAG TCC TGA G – 3'

MseI-A primer 10 µM: 5' – GAT GAG TCC TGA GTA AA – 3'
MseI-T primer 10 µM: 5' – GAT GAG TCC TGA GTA AT – 3'
MseI-C primer 10 µM: 5' – GAT GAG TCC TGA GTA AC – 3'
MseI-G primer 10 µM: 5' – GAT GAG TCC TGA GTA AG – 3'

biotinylated microsatellite probes, 10 µM

SP6 promotor primer: 5' – ACG ATT TAG GTG ACA CTA TAG – 3'
T7 promotor primer: 5' – TAA TAC GAC TCA CTA TAG GG – 3'

Kits and premade substances

Invitrogen REact 1 buffer 10x
Qiagen QIAquick PCR purification kit
Promega pGEM-T vector system I (Promega A3600)
DIG Luminescent Detection Kit (Roche 1 363 514)
Streptavidin MagneSphere Paramagnetic Particles (= PMPs) (Promega Z5481)
Invitrogen *MseI* 5 U / µl
T4 DNA Ligase 1 U / µl
Biotherm 10x PCR Buffer
Biotherm *Taq* Polymerase

Stock solutions

DTT 100 mM
BSA 10 mg/ml
ATP 10 mM
dNTPs 2,5 mM
20x SSC
0.5x SSC
0.1x SSC
10% SDS
EtOH absolute
EtOH 70%
Xgal (0.2 g in 10 ml DMSO)
IPTG 100 mg/ml sterile
Ampicillin 100 mg/ml sterile
DNA, from salmon sperm, denatured, 10 mg/ml
Proteinase K (600 U/ml)
EDTA 10 mM sterile

Cells and media

LB agar
LB broth
SOC broth
competent JM109 cells

Buffer

Denaturation solution (0.5 N NaOH, 1.5 M NaCl)
Neutralization solution (1.5 M NaCl, 0.5 M Tris.Cl pH=7.4)

Church Buffer (0.25 M $NaHPO_4$ pH=7.2, 1 mM EDTA, 1% BSA, 7% SDS)
2x SSC / 0.1% SDS
0.5x SSC / 0.1% SDS
0.1x SSC / 0.1% SDS

Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH=7.5 set with NaOH pellets)
Washing buffer (Maleic acid buffer supplemented with 0.3% Tween 20, pH=7.5)
10 x Blocking Solution: 10% w/v Blocking Reagent (Roche 1 811 592) in Maleic acid buffer
Detection buffer (0.1 M Tris.HCl, 0.1 M NaCl, pH=9.5)

Day 1 ----------

DNA preparation

1. For one reaction, prepare the following mix:

Genomic DNA	25 – 250 ng
10 x REact 1 Buffer	2,50 µl
DTT 100 mM	1,25 µl
BSA 10 mg/ml	0,15 µl
MseI AFLP adaptor	2,50 µl each
ATP 10 mM	0,50 µl
MseI	0,50 µl
T4 DNA Ligase	1,00 µl
H_2O	ad 25,00 µl

2. Incubate 3 h at 37° C, inactivate 5-10 min at 70° C
3. Dilute 1:10 by adding 225 µl H_2O

4. Prepare the following PCR mastermix:

Biotherm 10x PCR buffer	6,0 µl
MseI-N primers	1,2 µl each
dNTPs	1,2 µl
Biotherm *Taq*	0,5 µl
diluted mix from (3)	15,0 µl
H_2O	32,5 µl

5. Aliquot into 6 tubes, 10 µl each. PCR conditions: 94°-30sec / 53°-60sec / 72°-60sec; 29 cycles, no hot start. Remove one eppi after cycle 14/17/20/23/26/29
6. Load 5 µl to a minigel; the cycle number where a faint smear >200 bp becomes visible is sufficient for production PCR; avoid over-amplifications
7. Repeat PCR with optimized cycle number in 50 µl reactions until several hundred ng product are yielded

Day 2 ----------

8. Pool the products, clean them with the QIAquick PCR purification kit, elute DNA in 30 µl EB
9. With H_2O, set 1 µg DNA to a total Vol of 250 µl, denature 5 min in a boiling water bath and immediately cool on ice
10. Add 5 µl of each biotinylated capture probe, 13 µl 20x SSC and fill up to 500 µl final Vol with H_2O
11. Hybridize 1-2 h at 50° C under constant agitation
12. In the meantime, transfer 300 µl PMPs from the stock vials to an eppi and separate 30 sec on the magnetic stand. Remove free liquid with a pipette
13. Wash PMPs 3x with 300 µl 0.5x SSC, each time completely resuspending the pellet
14. Resuspend PMPs in 100 µl 0.5x SSC, add the solution (11) and incubate 20 min at RT. Gently mix by tipping the eppi every 2 min
15. Separate on the magnetic stand and recover the supernatant
16. Wash PMPs 4x with 300 µl 0.1x SSC; recover the last washing solution. For lower stringency, wash instead 2x with 0.5x SSC and 2x with 0.2x SSC.
17. Add 100 µl pre-warmed H_2O to the beads and incubate 3 min at 50° C. Mix gently and immediately separate on the magnetic stand. Remove the supernatant which contains the enriched DNA fraction. Be careful not to remove any PMPs together with the supernatant
18. Precipitate the DNA of the unbound fragments (15), the last washing step (16) and the eluate (17) with 2 Vol EtOH at -20° C o/n

Day 3 ----------

19. Pellet the DNAs (15 min, 13000 rpm, 4° C), discard the supernatant, wash in 70% EtOH, air-dry and dissolve the pellet in 50 µl H_2O
20. For each DNA precipitation prepare a PCR reaction as follows:

Biotherm 10x PCR buffer	1,00 µl
MseI-N primers	0,20 µl each
dNTPs	0,20 µl
Biotherm *Taq*	0,08 µl
Templates aus (21)	1,00 µl
H_2O	6,92 µl

Perform a PCR like in (5) with 22 cycles. Expect strong smear for unbound fraction, almost no product for last wash, some smear for eluates

Construction of the enriched library

21. In case results of (20) are convenient, scale up PCR reaction to yield >5 ng amplicon. Clean PCR product with QIAquick kit.
22. Prepare the following ligation reaction:

2x rapid ligation buffer	5,0 µl
pGEM-T vector	0,5 µl
DNA (23)	5,0 ng
T4 DNA ligase	0,5 µl
H_2O	ad 10,0 µl

23. mix gently, incubate at 4° C o/n

Day 4 ----------

24. Prepare 10 petri dishes with LB Agar supplemented with ampicillin (250 µl/250 ml media), Xgal (400 µl/250 ml media) and IPTG (120 µl/250 ml media)
25. Aliquot 2 µl of the liagtion mix (22) into eppis, keep on ice
26. Put competent cells into an ice bath until they are thawed; mix gently
27. Add 50 µl cells to each eppi from (25), incubate 20 min on ice
28. Heat shock cells 50 sec at 42° C (water bath) and put immediately back on ice; incubate 2 min
29. Add 950 µl SOC broth (at RT), incubate 90 min at 37° under constant smooth agitation (hybridization oven)
30. Concentrate cells by centrifugation (5 min, 3000 rpm, 4° C), pour off supernatant and resuspend pellet in 200 µl SOC broth
31. Plate out 100 µl aliquots to the prepared petri dishes, incubate o/n at 37°

Day 5 ----------

32. In case white and blue colonies are not yet differentiated, incubate plates at 4° C until colors are clearly distinguishable
33. Use sterile toothpicks to transfer white colonies onto masterplates; grow masterplates until colonies have ~2 mm diameter

Day 6 ----------

Library screening

34. Place a sterile nylon filter on the masterplates until the filter is completely soaked (~1 min); for positioning, stitch 3 holes into the filter using a hypodermic needle (2, 6 and 12 o'clock position) and mark the positions on the petridish
35. Remove the filter; seal masterplates with parafilm and store at 4° C
36. Place the filters on 3MM paper soaked with following buffers (colony side up; liquid should not reach the upper side of the filters):
    a. 10 % SDS 3 min
    b. Denaturation solution 15 min
    c. Neutralization solution 5 min
    d. 2x SSC 5 min
37. Put the filters on dry 3MM paper and allow to air-dry for 30 min
38. Bake filters 60 min at 80° C or UV-crosslink for 2 min
39. For each filter diltue 100 µl Proteinase-K solution with 900 µl 2x SSC
40. Spread the solution on the filters, cover with saran wrap and incubate 1 h at 37°
41. In the meantime denature 150 µl salmon sperm DNA 5 min in a boiling water bath and cool on ice
42. Remove cell debris from the filters by pressing wet 3MM paper against them; use a clean plastic ruler for the purpose
43. Add denatured salmon sperm DNA to 15 ml Church-Buffer; prewarm to 65° (this amount is sufficient for 4 filters)
44. Put the filters into a hybridization tube, separated from each other with nylon mesh
45. Pre-hybridize 60 min at 65°
46. Decant the pre-hybridzing solution, add frsh Church-Buffer (15 ml + 150 µl denatured salmon sperm DNA) 15 µl of each biotinylated probe; hybridize o/n at 65°.

Day 7 ----------

47. Decant the hybridizing solution and put each filter into a single plastic petri dish; the hybridizing solution may be reused several times when stored at -20° C.
48. Wash filter 5 min in 2xSSC/0.1%SDS at RT
49. Wash filter 15 min in 0.5xSSC/0.1%SDS at 65° C
50. Wash filter 15 min in 0.1xSSC/0.1%SDS at 65° C

DIG Luminescent Detection

51. Dilute a 40 ml aliquot of the 10x Blocking Solution 1:1 with Maleic acid buffer (sufficient for 4-5 filters); split into two 40 ml aliquots
52. Wash filters 1-5 min in washing buffer, incubate 30 min in diluted blocking solution (~10 ml/filter)
53. In the meantime add 2 µl DIG-AP antibody (Kit) to the remaining 40 ml Blocking Solution.
54. Incubate filter 30 min in the antibody solution

55. Wash filter 2x 15 min in 20 ml washing buffer
56. Equilibrate filter 5 min in 10 ml detection buffer
57. Place the filters in a plastic bag (DNA side up) and spread ~500 µl CSPD solution (Kit) above. Immediately close and seal the bag. Avoid trapping of air bubbles.
58. Incubate 10 min at 37° C
59. Place filter with DNA side towards x-ray film; The chemiluminescent signal will last for at least 48 h, so exposition time may be determined empirically (30 min to o/n)

Day 8 ----------

Retrieval of candidate sequences

60. Develop the x-ray film. Select dark colonies for further investigation and mark colony positions on the masterplates
61. Pick the colony with a sterile toothpick and transfer it to 2 ml LB broth in a test tube; at this step, part of the bacterial cell mass may be used as template for direct colony PCR
62. Incubate bacteria o/n at 37° C. Use them for immediate plasmid extraction or store at 4° C for later processing

Day 9 ----------

63. Extract plasmid DNA. Use the DNA for PCR-pretesting and sequencing

7.4.3 Microsatellite isolation by vectorette PCR

Equipment

Thermocycler
Centrifuge for Eppendorf tubes. cooled

Oligos (10 µM)

Vectorette Adaptor V1:	5' GAT CTC CGG TAC ATG ATC GAG GGG ACT GAC AAC GAA CGA ACG GTT GAG AAG GGA GAG 3'
Vectorette Adaptor V2:	5' CGC TCT CCC TTC TCC TAG CGG TAA AAC GAC GGC CAG TCC TCG ATC ATG TAC CGG A 3'
VFor2:	5' TAG CGG TAA AAC GAC GGC CAG 3'
VRev2:	5' GAC TGA CAA CGA ACG AAC GGT 3'
AC8:	5' GTG TGT GTG TGT GTG 3'
GA8:	5' GAG AGA GAG AGA GAG 3'

Buffers and other chemicals

T4 DNA ligase 1 U / µl
Biotherm 10 x PCR buffer
Biotherm *Taq* polymerase
Gibco *MboI*
Gibco 10x REact 2 Buffer
CI 24 : 1
EtOH absolut
EtOH 70 %
TE
1M Tris.HCl pH 7.5
100 mM $MgCl_2$
T4 DNA ligase, 4U / µl
10 mM ATP
100 mM DTT

1. Extract high-quality genomic DNA and perform an RNase digest. Validate DNA integrity by agarose gel electrophoresis, quantify on a spectrophotometer and adjust DNA content to 50 ng/µl
2. Prepare the following mixture:

genomic DNA	10,00 µl
SauIIIA (= *MboI*)	1,00 µl
10 x REact buffer 2	5,00 µl
H_2O	34,00 µl

   Digest 3 h at 37° C, inactivate enzyme by 5-10 min heating to 70° C
   Alternatively the digest may be performed with *TaqI*, *HinPI* oder *HpaII* in an appropriate buffer.
3. *steps 3-5 are optional:* Add 1 Vol CI to digested DNA, vortex, centrifuge 5 min at 13000 rpm and RT
4. Transfer aquaeous (upper) phase to a new eppi, add 1 Vol $EtOH_{abs.}$, incubate 1 h at RT
5. Centrifuge 20 min at 15000 rpm and 4° C, wash pellet with 70 % EtOH, air dry and dissolve DNA in 50 µl TE; quantify on a spectrophotometer
6. Prepare vectorette adaptor in the meantime:

1 M Tris.HCl pH 7.5	10 µl
100 mM $MgCl_2$	10 µl
H_2O	40 µl
Vectorette V1	20 µl
Vectorette V2	20 µl

   Incubate the mixture 5 min at 100° C and ramp the temperature down to 4° C over 90 min. Ramping is best perfromed on a thermocycler. Stored at 4° C the adaptor mix ist stable for several days. For longer periods storage at -20° C is recommended. Repeat ramping if adapter was frozen.
7. Ligation of adatpors and target DNA:

Digested DNA		100,0 ng
Vectorette adaptor mix (6)		5,0 µl
T4 DNA ligase		1,0 µl
ATP 10 mM		1,0 µl
DTT 100 mM		1,0 µl
H_2O	ad	20,0 µl

   Incubate o/n at 4° C, , inactivate enzyme by 5-10 min heating to 70° C
8. Set a total Vol of 100 µl with sterile water
9. Perform 1st vectorette PCR:

Biotherm 10x PCR Buffer	2,5 µl
SSR specific primer	0,5 µl
Vectorette primer	0,5 µl
dNTPs	0,5 µl
Biotherm *Taq*	0,2 µl
Template (8)	1,0 µl
H_2O	19,8 µl

   Use vectorette primer VFor2 if digest was performed with *SauIIIA* and VRev2 if digest was performed with *TaqI*, *HinPI* or *HpaII*. When AC8 or GC8 are used as SSCR specific primers, set $T_{anneal.}$ ~59° C. PCR conditions: 94° - 3' / [94° - 45" / $T_{anneal.}$ – 60" / 72° - 90"] x 32 / 72° - 15'
10. Check PCR result on a minigel; clone and sequence products to develop site specific primer for 2nd vectorette

7.5 Publications in frame of this thesis

7.5.1 Articles in SCI journals

Arthofer W.[+], Schlick-Steiner B.C.[+], Steiner F.M.[+], Konrad H., Espadaler X., and Stauffer C. (2005) Isolation of polymorphic microsatellite loci for the study of habitat fragmentation in the harvester ant Messor structor. Conservation genetics, online first, DOI: 10.1007/s10592-005-9033-5

Arthofer W., Steiner F.M., Schlick-Steiner B.C., Konrad H., and Stauffer C. (in prep.) Microsatellite loci in the sociobiologically enigmatic ant, *Lasius austriacus* (Hymenoptera: Formicidae). Molecular Ecology Notes.

Arthofer W., Avtzis D.N., and Stauffer C. (in prep.) Targeted primer development and SSCP as an alternative to direct sequencing in phylogenetic analysis of *Pityogenes chalcographus* (Coleoptera, Scolytidae). Electrophoresis.

Avtzis D.N.[+], Arthofer W.[+], and Stauffer C. (in prep.) Phylogeography of *Pityogenes chalcographus* (Coleoptera, Scolytidae) based on mitochondrial and microsatellite markers. Molecular Ecology.

Arthofer W., Avtzis D.N., Riegler M., Miller W., Koivisto R.K.K., and Stauffer C. (in prep.) Detection of a B-strain *Wolbachia* in *Pityogenes chalcographus* (Coleoptera, Scolytidae). European Journal of Entomology.

Arthofer W., Avtzis D.N., and Stauffer C. (in prep.) Two library enrichment methods for microsatellite isolation: differences in applicability between hymenopteran and coleopteran insect species. Biotechniques.

7.5.2 Other Articles

Avtzis D.N., Arthofer W., and Stauffer C. (2005): Identifying haplotypes of Pityogenes chalcographus (Col., Scolytidae) by Single Strand Conformation Polymorphism (SSCP). Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie, in press.

7.5.3 Congress contributions and posters

Arthofer W., Avtzis D.N. and Stauffer C. (2005) Validating mtDNA derived phylogenetic data of *Pityogenes chalcographus* in the light of nuclear pseudogenes and *Wolbachia* endosymbionts. in: X. Congress of the European Society for Evolutionary Biology. Krakow, Poland.

Arthofer W., Avtzis D.N., and Stauffer C. (2005) Phylogeographische Analyse der europäischen *Pityogenes chalcographus* (Coleoptera, Scolytidae) Populationen. in: Entomologentagung DGaaE. Dresden, Germany.

Avtzis D.N., Arthofer W., and Stauffer C. (2005) Identifying haplotypes of *Pityogenes chalcographus* (Col., Scolytidae) by Single Strand Conformation Polymorphism (SSCP). in: Entomologentagung DGaaE. Dresden, Germany.

Riegler M., Arthofer W., Daxböck S., and Stauffer C. (2005) Hitchhiking of the mitochondrial DNA in *Wolbachia* superinfected *Rhagoletis cerasi*. in: Entomologentagung DGaaE. Dresden, Germany.

[+] authors contributed equally to this publication

7.6 Curriculum vitae

Professional experience

2003 – present	Ph. D. student at the Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU, University of Natural Ressources and Applied Life Sciences, Vienna, Austria.
1996 - present	Volunteer at the Vienna Red Cross. Emergency paramedic and responsible team leader of the NBC (nuclear, biological, chemical threats) recovery group.
2003	Six months project assistant at the Austrian Red Cross head office, Department of Disaster Relief. Evaluation of structural damages after the 2002 floods in Austria and development of mathematical models for the distribution of relief funds.
1996 – 2003	Staff scientist at the Plant Biotechnology Group, Institute of Applied Microbiology, BOKU. Establishment and maintainance of plant tissue cultures. In vitro, greenhouse and field trials in plant virology, detection of viruses and mycoplasmas by ELISA, tissue print and PCR. Virus elimination by meristem culture. Development and implementation of the working group's web presentation.
1995 – 1996	11 months alternative civilian service at the Austrian Red Cross
1988 - 1996	Freelancing team member at the Plant Biotech Lab, Department of Life Sciences, Austrian Research Center Seibersdorf. Virus detection and elimination in potato, study of plant-pathogen interactions using the chestnut blight fungus - chestnut tree - system. Training and applied work with different radioisotopes.

Education, training, others

1993 - 1999	Diplomstudium (MSc equivalent) in landscape ecology, BOKU, Vienna. Master thesis ´Investigations on biocontrol of chestnut blight´; practical work performed at the Austrian Research Center Seibersdorf under supervision of Dr. Eva Wilhelm
1994	Applied radiation safety training according to Austrian law §28 and §29 BGBl 47/1972
1988	Trainee at the Federal Biological Research Centre, Braunschweig, Germany
1987	Trainee at the Federal Agency for Viticulture, Eisenstadt, Austria
1986	General qualification for university entrance, Bundesrealgymnasium Eisenstadt, Austria
May 18th, 1968	Born in Eisenstadt, Austria

7.7 List of abbreviations

° C	degree Celsius
µl	microliter
µM	micromolar
€	Euro
Acc. Nr.	accession number
AFLP	amplified fragment length polymorphism
ATP	adenosinetriphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumine
CI	cytoplasmic incompatibility
cm^2	square centimeter
CO1	cytochrome oxidase 1
D.	*Drosophila*
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
DGaaE	Deutsche Gesellschaft für allgemeine und angewandte Entomologie
dGTP	deoxyguaninetriphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleosides
DTT	dithiothreitol
dTTP	deoxythyminetriphosphate
dUTP	deoxyuraciletriphosphate
E.	*Escherichia*
EDTA	ethylenediamineteraacetic acid
et al.	et altera
EtOH	ethanol
FIASCO	Fast Isolation by AFLP of Sequences COntaining Repeats
Fig.	Figure
h	hour
HCl	hydrochloric acid
ITS	internal transcribed spacer
kb	kilobases
L.	Linnaeus
LB	Luria Bertani
LINE	long interspersed nuclear element
LTR	long terminal repeat
M	molar
min	minute
ml	milliliter
mm	millimeters
mM	millimolar
mtDNA	mitochondrial DNA
myr	million years
NBT	nitro blue tetrazolium
ncDNA	nuclear DNA
ng	nanogram
NJ	neighbour joining
nr	number
numt	nuclear mitochondrial DNA
OD	optical density
o/n	over night
P.	*Pityogenes*

PCR	polymerase chain reaction
PMP	paramagnetic particle
RAPD	random amplified polymorphic DNA
RBIP	retrotransposon based insertional polymorphism
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RT	room temperature
RTase	reverse transcriptase
SDS	sodiumdodecylsulphate
sec	second
SINE	short interspersed nuclear element
SNP	single nucleotide polymorphism
sp.	*speciae*
SSAP	sequence-specific amplified polymorphism
SSC	standard citrate buffer
SSCP	single strand conformation polymorphism
SSR	simple sequence repeat
STR	short tandem repeat
TAE	Tris-acetic acid-EDTA buffer
TBE	Tris-boric acid-EDTA buffer
TE	transposable element
TE	Tris-ETDA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
TSS	transformation and storage solution
U	unit
UV	ultraviolet
V	Volt
Vol	Volume
v/v	volume per volume
VNTR	variable number tandem repeats
w/v	weight per volume