Mitochondrial phylogenies in the light of pseudogenes and Wolbachia: re-assessment of a bark beetle dataset

Wolfgang Arthofer¹, Dimitrios N. Avtzis², Markus Riegler³, Christian Stauffer⁴

¹ Molecular Ecology Group, Institute of Ecology, University of Innsbruck, Technikerstrasse 25, 6020 Innsbruck, Austria ² NAGREF-Forest Research Institute, 570 06 Vassilika-Thessaloniki, Greece ³ Centre for Plants and the Environment, School of Natural Sciences, University of Western Sydney, Locked Bag 1797, Penrith South DC NSW 1797, Australia ⁴ Institute of Forest Entomology, Forest Pathology and Forest Protection Department of Forest & Soil Sciences, Boku, University of Natural Resources and Applied Life Sciences, Hasenauerstrasse 38, 1190 Vienna, Austria

Corresponding author: Christian Stauffer (christian.stauffer@boku.ac.at)

Academic editor: B.H. Jordal | Received 10 September 2009 | Accepted 11 January 2010 | Published 17 September 2010

Citation: Arthofer W, Avtzis DN, Riegler M, Stauffer C (2010) Mitochondrial phylogenies in the light of pseudogenes and Wolbachia: re-assessment of a bark beetle dataset. In: Cognato AI, Knížek M (Eds) Sixty years of discovering scolytine and platypodine diversity: A tribute to Stephen L. Wood. ZooKeys 56: 269–280. doi: 10.3897/zookeys.56.531

Abstract
Phylogenetic studies based on mtDNA become increasingly questioned because of potential pitfalls due to mitochondrial pseudogenes and mitochondrial selective sweeps. While the inclusion of nuclear markers should preferentially be considered for future studies, there is no need to abandon mtDNA as long as tests for the known mtDNA artefacts are performed. In this study we present additional data and test previous phylogeographical studies of Pityogenes chalcographus. We did not detect nuclear copies (numts) of the previously used mitochondrial markers by performing a combined long range/nested PCR of the COI gene and by an in silico analysis of the COI sequence data. This confirms the robustness of our previous phylogenetic study of P. chalcographus. We did not detect nuclear copies (numts) of the previously used mitochondrial markers by performing a combined long range/nested PCR of the COI gene and by an in silico analysis of the COI sequence data. This confirms the robustness of our previous phylogenetic study of P. chalcographus. Results of an in-situ hybridization of Wolbachia in P. chalcographus confirm the presence of this endosymbiont in this species. However, we did not detect a correlation between infection status, geographical region and mtDNA haplotypes. The hybridisation data also support a previous hypothesis that infections do not result from parasitoids or parasitic nematodes, insect surface or laboratory contaminations and are hence a true infection of P. chalcographus. We conclude that the deep structure found in mitochondrial populations of P. chalcographus indeed represents the evolutionary history of European populations.
Keywords
Wolbachia, Scolytinae, pseudogenes, numts, mtDNA, phylogeny, phylogeography

Introduction

In the last two decades several phylogeographic (e.g. Stauffer et al. 1999) and phylogenetic (e.g. Cognato and Sun 2007) studies on scolytines were presented and most of them used mitochondrial DNA (mtDNA) as one of, or the only genetic marker. Analyses of the mitochondrial genome pioneered the era of molecular ecology due to its small size, uniparental mode of inheritance, ease of isolation, and conserved simple structure, allowing the development of universal primers spanning several classes of Metazoa (e.g. Lunt et al. 1996). However, its potential for resolving the evolutionary history of organisms was gradually questioned when factors influencing the reliability of mtDNA derived phylogenies were identified, namely (i) nuclear non-functional copies of mitochondrial genes (e.g. Bensasson et al. 2001), (ii) maternally inherited endosymbionts (Hurst and Jiggins 2005), (iii) positive selection on mitochondrial genomes (Meiklejohn et al. 2007) and (iv) mitochondrial introgression as a consequence of hybridisation (Petit and Excoffier 2009).

Mitochondria originated from the endosymbiosis of α-proteobacteria in ancestral eukaryotic cells. Mitochondrial genomes contain fewer genes than those of free-living α-proteobacteria, due to a loss of genes during their evolutionary history. This gene loss is explained by (1) the functional redundancy of mitochondrial genes with pre-existing nuclear genes and (2) the functional transfer of mitochondrial genes to the nucleus. The transfer of mtDNA derived sequences to the nucleus is an ongoing process in eukaryotes and mitochondrial pseudogenes have been identified in the nuclear genome of many species (Timmis et al. 2004). Such nuclear mitochondrial (numt) pseudogenes can derive from any part of the mtDNA and occur typically as single copies at dispersed genomic locations. Numts are usually less than 1 kb in size (Richy and Leister 2004). Larger fragments as well as tandemly repeated numts have been reported in mammals (e.g. Bensasson et al. 2001). Phylogenies derived solely from mtDNA sequences may hence be erroneous due to numts being co-amplified by universal mitochondrial primers.

A set of strategies is available in order to avoid numt based errors, including in silico analysis of sequences to detect an eventual increased number of non-synonymous base substitutions, frameshifts, additional stop codons and reduced transition/transversion ratios (Bensasson et al. 2001). Positive results should raise doubt on the mitochondrial origin of the retrieved sequences. Furthermore, long PCR techniques can be utilized because most numt sequences are shorter than 1000 base pairs (Richy and Leister 2004).

A specific feature of mtDNA is its strict maternal inheritance in most insects. Due to this asymmetrical inheritance within a species the marker only reflects the female
part of the species’ genealogy. Hence, mtDNA transmission will be influenced by any selection for maternally transmitted genes or other maternally selective traits. Several maternally transmitted endosymbionts are well known in invertebrates, with *Wolbachia* as the most prominent one (Warren et al. 2008). *Wolbachia* was also detected in *Ips typographus* (Stauffer et al. 1997), *Hypothenemus hampei* (Vega et al. 2002), *Xylosandrus germanus* (Peer and Taborsky 2005) and *Coccytopus dactyliperda* (Zchori-Fein et al. 2006). Recently, *P. chalcographus* was found infected with two *Wolbachia* strains *w*CHA1 and *w*CHA2 (Arthofer et al. 2009a). Both strains occur in low titres not accessible by conventional PCR detection methods.

While some *Wolbachia* infections do not alter host physiology and reproduction, such effects have been found in others. Reproductive fitness traits range from cytoplasmic incompatibility (CI) to male-killing, feminisation and the induction of thelytokous parthenogenesis (see Warren et al. 2008 for a review). In a population infected with CI-inducing *Wolbachia*, the mtDNA associated with the initially infected females will hitchhike through the population and replace the original haplotypes (Hurst and Jiggins 2005). From a phylogenetic point of view this selective sweep may easily be mistaken for a population bottleneck or a founder effect. On the other hand, old and established *Wolbachia* infections within a population might maintain mitochondrial isolation in spite of nuclear gene flow. In such cases, deep mtDNA structure may contradict homogenous nuclear phylogenies. Thus, the presence of *Wolbachia* must be checked when mtDNA based phylogenies and phylogeographies are established. This is usual done by conventional PCR using the *Wolbachia* specific primers for *wsp* (Zhou et al. 1998) or 16S rDNA (O’Neill et al. 1992). More sophisticated methods include high sensitivity detection (Arthofer et al. 2009a, b) or *in situ* hybridization which offers a possibility to detect *Wolbachia* directly in infected tissues (Chen et al. 2005). The latter method reduces the risk of false positive results due to contamination with infected parasitoids, parasitic nematodes or prey in the gut content of predators.

In this study we show that numts do not influence the phylogenetic pattern of *P. chalcographus* (Avtzis et al. 2008) by performing a combined long/range nested PCR of the COI gene and by an *in silico* analysis of the COI sequence data. Furthermore, we present results of an *in-situ* hybridization of *Wolbachia* in *P. chalcographus* confirming the presence of the endosymbiont in tissues of this species.

**Material and methods**

**Numt search**

Mitogenomic sequences of the coleopteran species *Pyrocoelia rufa* (Lampyridae), *Tri-bolium castaneum* (Tenebrionidae) and *Crioceris duodecimpunctata* (Chrysomelidae) were obtained from GeneBank (for accession numbers see table 1) and aligned using
Clustal X (Thompson et al. 1997). To facilitate identification of conserved regions sequences of *Apis mellifera* (Apidae), *Bombyx mori* (Bombycidae) and *Drosophila simulans* (Drosophilidae) were included in the alignment. Conserved regions were selected for primer design (Table 1). Occasional variable nucleotide positions within the conserved regions required the selection of primer sequences characteristic for coleopterans. Developed primers were Met/F 5’ gctwhtgggttcataccc 3’ located in the methionin tRNA region and CO2/R 5’ caaatttctgaacattg 3’ located in CO2. This primer pair amplifies a stretch of about 3463bp.

Fourteen DNA extracts of *P. chalcographus* representing all clades were selected for analysis. Thermocycling was performed in a Primus 25 advanced thermocycler (peqlab, Germany). Full length 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, USA), 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 1:10,000 with sterile distilled water and 1 μl diluted amplicon was used as template for the nested PCR. Dilution series were carried out to prove that the carry over of genomic DNA from the full length to the nested PCR reaction was small enough to avoid detectable amounts of amplicon. Nested PCR was done in 25 μl reactions containing 3.75 mM magnesium chloride, 125 μM dNTPs (Fermentas, Lithuania), 0.5 μM of each K698 (Caterino and Sperling 1999) and UEA10 (Lunt et al. 1996) primer and 1U Taq polymerase (Sigma, USA). Cycling conditions contained an initial denaturation step of 3 min at 94° C followed by 33 cycles of 94° C (30 sec), 48° C (60 sec) and 68° C (1.5 min) and a final extension step at 68° C (10 min).

### Table 1.

| Table 1. Primer sequences of Met/F and CO2/R for *Pityogenes chalcographus* amplifying 3463bp: alignments and GenBank accession numbers. |

| Met/F                     | 5’ gctwhtgggttcataccc 3’ |
|---------------------------|--------------------------|
| *Criocerus duodecimpunctata* | NC_003372...at............ | |
| *Pyrocoelia rufa*          | NC_003970...tt............ | |
| *Tribolium castaneum*      | NC_003081...at.a........... | |
| *Apis mellifera ligustica* | NC_001566...aaca........... | |
| *Bombyx mori*              | NC_002355...at..c.......... | |
| *Drosophila simulans*      | NC_005781...ac............ | |

| CO2/R                     | 5’ caaatttctgaacattg 3’ |
|---------------------------|------------------------|
| *Criocerus duodecimpunctata* | NC_003372............... | |
| *Pyrocoelia rufa*          | NC_003970...g............. | |
| *Tribolium castaneum*      | NC_003081............... | |
| *Apis mellifera ligustica* | NC_001566............... | |
| *Bombyx mori*              | NC_002355............... | |
| *Drosophila simulans*      | NC_005781............... | |
Amplicon size was checked by gel electrophoresis, products were purified with the QiaQuick PCR purification kit (Qiagen, USA) and Sanger sequencing was performed using nested PCR primers by a commercial provider.

An in-silico analysis was performed on 262 sequences of the original study (Avtzis et al. 2008) representing 58 European haplotypes of *P. chalcographus* (DQ515997-DQ516054) to identify non-synonymous base substitutions, additional stop codons, insertions and deletions, frameshifts and the transition:transversion ratio. Eleven molecular traits listed in table 2 were selected to discriminate numt and mtDNA which are extensively discussed in the results section.

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

*In situ* hybridization followed a slightly modified protocol of Chen et al. (2005). Insects from locations with elevated *Wolbachia* prevalence were dissected under a stereo microscope using sterile forceps and scalpel blades. Ovarial tissue was recovered, transferred onto 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

**Table 2. In silico analysis of CO1 mutations of data presented in Avtzis et al. (2008). 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.**

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

*a* expected relative values as given in reference ± χ² confidence interval at α=0.05 (Sachs 1999), *b* Blouin et al. (1998), *c* Shoemaker et al. (2004), data of *Drosophila subquinaria*, *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
5 min. Slides were washed twice by pipetting 2 ml buffer 1 (100 mM Tris.HCl, 150 mM sodium chloride, pH=7.4) on the tissue. The buffer was kept on the tissue for 30 sec and was then decanted. 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 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 colour 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 Drosophila simulans strains were used.

Results and discussion

Phylogeographic analysis of European P. chalcographus populations revealed a deep genetic structure between the most diverged haplotypes with three major clades and an estimated divergence time of 100,000 years before present (Avtzis et al. 2008). Recently, low titre infections of two Wolbachia strains were detected in more than 30% of the analysed specimens (Arthofer et al. 2009a). Thus, tests for integrity of the mtDNA based phylogeny in the light of numts and endosymbiont infection were mandatory. Here we present a data set demonstrating that the phylogeny of Avtzis et al. (2008) is not influenced by numt pseudogenes. Arthofer et al. (2009a) have detected Wolbachia in all major P. chalcographus clades in a pattern that is unlikely to be caused by CI inducing strains. Here we prove the presence of the endosymbiont directly in ovarial cells of the beetle, excluding positive Wolbachia detection by PCR due to contamination.

Long/nested PCR and in silico analysis for presence of numts

Alignment of mitochondrial genomes of three coleopteran and three non-coleopteran insect species resulted in six candidate primers (data not shown), of which one primer pair (Table 1), after extensive optimization of PCR conditions, amplified a clear band
from *P. chalcographus* DNA extracts. Dilution series of genomic DNA gave no visible bands in dilutions of more than 1:1,000, ensuring that all amplicons produced in the nested PCR originated solely from the full length PCR product and not from genomic carry-over (data not shown). After nested PCR extensive products of the expected size could be obtained from almost all haplotypes of *P. chalcographus* examined. Even templates without visible amplification in the full length PCR had formed enough product to be amplified in the subsequent nested reaction. Comparison of the NJ trees derived from direct PCR sequences (Avtzis et al. 2008) and from nested PCR sequences of 14 representative haplotypes of the major clades showed identical topologies (data not shown).

PCR conditions were chosen to remove any numt shorter than 3.4 kb, i.e. three times longer than the largest numts ever observed in insects. Both direct and long/nested PCR sequences were identical, and so were the phylogenetic trees. With our test, co-amplification of numts in the direct PCR approach would have led to discrepancies in tree topology between direct and long PCR sequences.

In order to extend numt screening to 262 individual sequences representing 58 different haplotypes, an *in silico* analysis was performed targeting characteristic differences between mtDNA and numt sequence composition. Eleven numerical traits were analyzed independently and all of them resulted in values within 5% confidence intervals for authentic mtDNA (Table 2). Thus, presence of numts in the analyzed populations of *P. chalcographus* can be excluded.

Several strategies to avoid numt co-amplifications are known. The purification of mtDNA by caesium chloride gradient centrifugation (Nishiguchi et al. 2002) prevents the isolation of numts but is inapplicable when the amounts of source DNA are limited. Beside this, the procedure is slow and laboursome and therefore not suitable for the screening of large populations. Other enrichment techniques 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). The long PCR approach utilized in this study should exclude any amplicons derived from nuclear DNA. Furthermore, mtDNA shows some characteristics in base composition and mutational patterns that are different from the nuclear genome. Most obvious, mtDNA is strongly AT biased (Lewis et al. 1995) and evolves faster than single copy nuclear genes (Galtier et al. 2009). Most probably this fast evolution is explained by inefficient repair mechanisms at the mitochondrial replication complex. More recent studies have shown substantial rate heterogeneity between different species and mitochondrial genes (e.g. Mueller 2006). After transfer into the nucleus, a mitochondrial sequence will 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). Transition-transversion ratio is significantly higher in mtDNA than in corresponding pseudogenes (Arctander 1995). The GC dinucleotide is often methylated in nuclear DNA and 5-methylcytosine mutates abnormally often to T (Bird
1980). Therefore the rate of GC \rightarrow GT mutations among the four possible nC \rightarrow nT combinations is highly overrepresented in the nucleus but not in mtDNA where methylation does not occur (Bulmer 1986).

While we consider the long/nested PCR approach as very reliable to exclude any numt from a genetic analysis, it requires additional handling time, costs for PCR consumables and high quality DNA allowing the amplification of \textgreater 3kb products. Especially the latter condition will not be given when long term stored specimens have to be analyzed that might have degraded DNA. The \textit{in silico} approach presented here can be readily applied to individual haplotypes within any mtDNA alignment and does not require additional manipulations in the laboratory. It is thus suitable for a re-check of existing mtDNA based phylogenies.

Detection of \textit{Wolbachia} by \textit{in situ} hybridization

The principal functionality of a modified protocol for \textit{Wolbachia} detection by \textit{in situ} hybridization with DIG labelled probes was tested using ovarial tissue of \textit{Wolbachia} free \textit{D. simulans} STC and \textit{D. simulans} flies infected with \textit{wRi}. Differences in colouration were clearly distinguishable between infected and uninfected \textit{D. simulans} (Fig. 1 A, B).

Compared to \textit{wRi} in \textit{D. simulans}, \textit{Wolbachia} titre in \textit{P. chalcographus} was low, and in average only 35.5% of the individuals were infected (Arthofer et al. 2009a). The ovarial tissue of several individuals analysed showed staining patterns at different intensities, comparable to the \textit{D. simulans} positive controls (Fig. 1C).

Conclusion

Evidence of a range of selective forces on mtDNA markers make phylogenetic studies that are purely based on mtDNA less reliable. While the inclusion of nuclear markers like microsatellites or AFLP should preferentially be considered for future studies, there is no need to completely abandon mtDNA as long as tests for the potential manipulation of mtDNA sequences are performed. Such tests should also be included in ongoing efforts to barcode the tree of life based on mtDNA (Song et al. 2008). Here, we confirm that the data of the previous phylogeographic analysis by Avtzis et al. (2008) are not caused by numts. It can be concluded that the deep structure found in mtDNA populations of \textit{P. chalcographus} indeed represents the evolutionary history at least of the female branch of European populations

Furthermore, we have detected \textit{Wolbachia} in \textit{P. chalcographus} cells in low titre by \textit{in situ} hybridisation. Our results confirm earlier work that used a highly sensitive PCR method (Arthofer et al. 2009a). Such an approach can be prone to false positive results due to contamination, as it was found in one extract that carried a uniquely isolated \textit{Wolbachia} sequence, that most likely derived from co-isolated DNA of a parasitoid (Arthofer et al. 2009a). The previous work showed that two strains are present in this
beetle in low titre and low frequency, without any correlation between infection status, geographical region and mtDNA haplotype. Despite the inability to differentiate both strains with the presented hybridisation technique, the new data support that infections do not result from parasitoids, parasitic nematodes or laboratory contaminations and are hence true Wolbachia infections of *P. chalcographus*. In general, additional tests for presence of numts and endosymbionts are laborious and time consuming. However they are required for species that exhibit deep mtDNA divergences in order to exclude potential misinterpretation of mtDNA sequence data.

**Acknowledgements**

We wish to thank Wolfgang Miller for providing *Drosophila* strains, Andrea Stradner for her help in the Wolbachia work and the Austrian Science Foundation (FWF) for financial support.
References

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

Arthofer W., Avtizis DN, Riegler M, Stauffer C (2009a) Low titer Wolbachia infection of mitochondrial defined European Pityogenes chalcographus (Coleoptera, Scolytinae) populations. Environmental Microbiology 11: 1923–1933.

Arthofer W., Riegler M, Schneider D, Miller WJ, Stauffer C (2009b) Hidden Wolbachia diversity in field populations of the European cherry fruit fly, Rhagoletis cerasi (Diptera, Tephritidae). Molecular Ecology, 18: 3816–3839.

Avtizis DN, Arthofer W., Stauffer C (2008) Sympatric occurrence of diverged mtDNA lineages of Pityogenes chalcographus (Coleoptera, Scolytinae) in Europe. Biological Journal of the Linnaean Society 94: 331–340.

Bensasson D, Zhang DX, Hartl DL, Hewitt GM (2001) Mitochondrial pseudogenes: evolution’s misplaced witnesses. Trends in Ecology and Evolution 16: 314–321.

Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Research 8: 1499–1504.

Blouin MS, Yowell CA, Courtney CH, Dame JB (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. Molecular Biology and Evolution 15: 1719–1727.

Bulmer M (1986) Neighboring base effects on substitution rates in pseudogenes. Molecular Biology and Evolution 3: 322–329.

Caterino MS, Sperling FAH (1999) Papilio phylogeny based on mitochondrial cytochrome oxidase I and II genes. Molecular Phylogenetics and Evolution 11: 122–137.

Chen WJ, Tsai KH, Cheng SL, Huang CG, Wu WJ (2005) Using in situ hybridization to detect endosymbiont Wolbachia in dissected tissues of mosquito host. Journal of Medical Entomology 42: 120–124.

Cognato AI, Sun JH (2007) DNA based cladograms augment the discovery of a new Ips species from China (Coleoptera : Curculionidae : Scolytinae). Cladistics 23: 539–551.

Galtier N, Jobson RW, Nahholz B, Glémin S, Blier PU (2009) Mitochondrial whims: metabolic rate, longevity and the rate of molecular evolution. Biology Letters 5: 413–416.

Hurst GDD, Jiggins FM (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.

Lewis DL, Farr CL, Kaguni LS (1995) Drosophila melanogaster mitochondrial DNA: completion of the nucleotide sequence and evolutionary comparisons. Insect Molecular Biology 4: 263–278.

Lin C-P, Danforth BN (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.

Lunt DH, Hutchinson WF, Carvalho GR (1999) An efficient method for PCR-based isolation of microsatellite arrays (PIMA). Molecular Ecology 1999: 891–894.
Meiklejohn CD, Montooth KL, Rand DM (2007) Positive and negative selection on the mitochondrial genome. Trends in Genetics 23: 259–263.

Mueller RL (2006) Evolutionary rates, divergence dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. Systematic Biology 55: 289–300.

Nishiguchi MK, Doukakis P, Egan M, Kizirian D, Phillips A, Prendini L, Rosenbaum HC, Torres E, Wyner Y, DeSalle R, Giribet G (2002) DNA isolation procedures. In: DeSalle R, Giribet G, Wheeler WC (Eds) Methods and Tools in Biosciences and Medicine. Techniques in Molecular Evolution and Systematics, 249–287.

O’Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proceedings of the National Academy of Sciences 89: 72699–2702

Peer K, Taborsky M (2005) Outbreeding depression, but no inbreeding depression in haplodiploid ambrosia beetles with regular sibling mating. Evolution 59: 317–323.

Petit RJ, Excoffier L (2009) Gene flow and species delimitation. Trends in Ecology and Evolution 24: 386–393.

Richy E, Leister D (2004) NUMTs in Sequenced Eukaryotic Genomes. Molecular Biology and Evolution 21:1081–1084.

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

Shoemaker DDW, Dyer KA, Ahrens M, McAbee K, Jaenike J (2004) Decreased diversity but increased substitution rate in host mtDNA as a consequence of Wolbachia endosymbiont infection. Genetics 168: 2049–2058.

Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proceedings of the National Academy of Sciences 105: 13486–13491.

Stauffer C, van Meer MMM, Riegler M (1997) The presence of the proteobacteria Wolbachia in European Ips typographus (Col., Scolytidae) populations and the consequences for genetic data. Proceedings of the German Society for General and Applied Entomology 11: 709–711.

Stauffer C, Lakatos F, Hewitt GM (1999) Phylogeography and postglacial colonization routes of Ips typographus (Col., Scolytidae). Molecular Ecology 8: 763–774.

Sunnucks P, Hales DF (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.

Tamura K (1992) The rate and pattern of nucleotide substitution in Drosophila mitochondrial DNA. Molecular Biology and Evolution 9: 814–825.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882.

Timmis JN, Ayliffe MA, Huang CY, Martin W (2004) Endosymbiotic gene transfer: Organelle genomes forge eucaryotic chromosomes. Nature Reviews: Genetics 5: 123–135.

Vega FE, Benavides P, Stuart JA, O’Neill S (2002) Wolbachia Infection in the Coffee Berry Borer (Coleoptera: Scolytidae). Annals of the Entomological Society of America 95: 374–378.
Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nature Reviews: Microbiology 6: 741–751.

Zchori-Fein E, Borad C, Harari AR (2006) Oogenesis in the date stone beetle, *Coccotrypes dac-tyliperda*, depends on symbiotic bacteria. Physiological Entomology 31: 164–169.

Zhang DX, Hewitt GM (1996) Nuclear integrations: challenges for mitochondrial DNA markers. Trends in Ecology and Evolution 11: 247–251.

Zhou W, Rousset F, 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.