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Host Tree and Insect Genetic Diversity on the Borderline of Natural Distribution: a Case Study of *Picea abies* and *Pityogenes chalcographus* (Coleoptera, Scolytinae) in Greece

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Picea abies (L.) Karst. and *Pityogenes chalcographus* constitute a commonly observed host tree–insect association in Eurasia, with the natural distribution of the bark beetle overlapping that of Norway spruce. The southernmost borderline of their distributions occurs in the Elatia forest (Mt. Rodopi, Greece), where these interacting organisms may experience severe conditions due to the effects of climate change. In order to assess the dynamics of this host tree–insect association, the genetic diversity of both organisms was studied. In contrast to previous studies, the assessment of molecular diversity was based on the same mitochondrial gene (Cytochrome Oxidase One) sequence for both host and pest. This analysis revealed a remarkably higher genetic diversity of *P. chalcographus* compared to that of *P. abies*, something that renders the insect capable not only of adapting to novel environmental conditions, but even of shifting to other host species. On the contrary, *P. abies* presented a narrow genetic base, a potential drawback at the southern-most region of the species natural distribution. Synthesizing the preliminary outcome for both organisms, it appears that *P. chalcographus* exhibits an evolutionary advantage over *P. abies*, something that should be considered when planning conservation strategies for the relict forest of Elatia.

Keywords bark beetles, genetic bases, marginal populations, northern Greece, Norway spruce
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1 Introduction

Genetic variation of natural populations plays a crucial role in the adaptation and survival of a species, since “the greater the number of genetic types within a population, the greater the probability that the population will include genotypes that will survive seasonal and temporal changes, particularly those of a violent nature” (Mayr 1963). Since neo-Darwinian evolutionary synthesis (Huxley 1942) became the current paradigm in evolutionary biology many studies have investigated the genetic diversity of natural populations with a recent focus on the molecular level (Dvornyk et al. 2002, Hill et al. 2006, Cognato et al. 2005, Miller et al. 2009). At present genetic diversity has become a very important component of conservation policies and sustainable management. Experts evaluate the effects of climate change on genetic diversity, emphasizing its significance in maintaining the resilience of ecosystems to exogenous disturbances, which offers a competitive advantage to species and genotypes in the novel environments that climate change may shape (Groom et al. 2006, Secretariat of the Convention on Biological Diversity 2003).

Norway spruce *(Picea abies* (L.) Karst.) is one of the most important coniferous species in Europe both ecologically and commercially. Its natural distribution extends northwards to the forests of Scandinavia, reaching its southernmost limit at the Mt. Rodopi forest of Elatia (Drama, Northern Greece) (Schmidt-Vogt 1974). Due to the importance of this species, both the biodiversity and biogeography of Norway spruce have been extensively investigated by approaches ranging from palinography (Huntley and Birks 1983), fossil pollen-macroremains investigation (Giesecke and Bennett 2004) and morphological features analysis (Krutzsch 1974, Kleinschmitt et al. 1981, Kleinschmitt 1982), to DNA approaches (Lagercrantz and Ryman 1990, Scotti et al. 2000, Vendramin et al. 2000, Sperisen et al. 2001). In the latest and most comprehensive study that incorporated almost all available information, combining fossil pollen data with novel molecular markers, Tollefsrud et al. (2008) confirmed the occurrence of glacial refugia from where Norway spruce re-colonized post-glacially the European terrain, giving rise to the current pattern of diversity. Even though samples from the southernmost area of the natural distribution of *Picea abies* were not included in this analysis, results from the southwest Bulgarian mountains showed surprisingly low genetic diversity. The focus area of this study, Mt. Ropodi, is considered a glacial refugium that has contributed to the species mainly by a westward expansion that occurred in the early and middle Holocene (9000–5000 ybp) (Latalowa and van der Knaap 2006).

The six-toothed spruce bark beetle *Pityogenes chalcographus* (Coleoptera, Scolytinae) is one of the most commonly found pests of Norway spruce in the Palearctic region (Schwerdtfeger 1929, Postner 1974, Pfeffer 1995) that in concert with *Ips typographus* (Coleoptera, Scolytinae) are responsible for severe damages in the conifer forests of Central Europe, especially after tree health has been disturbed by exogenous factors e.g. drought, storm, etc. (Lekander 1972, Ehnström et al. 1974, Christiansen et al. 1987, Christiansen and Bakke1988, Eidmann 1992, Schroeder 2001, Bouget and Duelli 2004). The natural distribution of this bark beetle overlaps with the distribution of its main host tree, Norway spruce (Knizek et al. 2005), a fact supporting the apparent close association between host tree and insect. Phylogeographic studies based on mtDNA molecular markers (Avtzis et al. 2008) provided a further indication of this connection, suggesting not only that during the last glacial did *P. chalcographus* and *P. abies* share the same refugial areas but also that postglacially, they followed similar migration routes as they expanded once again in the European massif.

The genetic diversity of each organism within this dual interactive system of host tree and insect species has been insofar studied separately. A combined comparative analysis of genetic diversity in the *P. abies–P. chalcographus* complex using the same molecular marker at a common genomic region has never been attempted before. For this reason, a part of the mitochondrial DNA Cytochrome Oxidase One (COI) gene has been sequenced and analysed for both organisms, as this region aggregates many important features that render it useful in similar studies (Demesure et al. 1995, Lunt et al. 1996, Dumolin-Lapègue et al. 1997, Duminil et al. 2002, Jeandroz et al. 2002, Tollesfrud et al. 2008). The current study aims particularly at the comparison of genetic diversity within a host tree–insect species complex at the geographic location where both species meet the southernmost border of their natural distribution (Elatia, Greece). Ultimately, this analysis targets at sampling from the extant genetic diversity of these species which may reflect their co-evolutionary pathways. Based on the present situation, a projection in the future under the light of climate change is attempted.

2 Materials and Methods

Plant and insect samples were collected during the summer of 2008 from the forest of Elatia located at the southern Rodopi mountain range (Latitude from 41º16'49'' to 41º18'16'' / Longitude: from 24º11'07'' to 24º12'24'') which corresponds to the southernmost border of the *Picea abies* natural distribution (Schmidt-Vogt 1974). Since the maternally inherited mtDNA was used, sampling was conducted in a way to avoid any haplotypic bias. *P. abies* needles from 25 trees were sampled every 100 m, whereas only one bark beetle per mother gallery was collected reaching a total sample of also 25 individuals. In this preliminary investigation, one transect was used to study the dynamics of the host-insect system. The transect consisted of a straight line 2,5 km long and about 100 m wide, following the inclination of the mountainous forest slope terrain (0–10%). The transect was vertical to topographic contour lines, starting at an altitude of 1800 m and ending at an altitude of 1900 m. The sampling area covered about 15% of the Elatia forest. Needles as well as beetles were collected from twigs at a height of 2 meters. Where possible needles and beetles were collected from the same individual, but this was possible in only 10% of the total sample. Norway spruce trees were sampled at a minimum distance of 100 m in order to avoid sampling filial structures. When a Norway spruce individual was selected for sampling and it was not possible to collect an insect from the same tree, the closest mother gallery was sampled.

Insect DNA was extracted from 25 individuals of *P. chalcographus* separately using the GenElute^{TM} Kit (Sigma) following the protocol of the manufacturer. A DNA sequence of about 800 bp from the 5' end mitochondrial COI gene was amplified using the universal primers UEA5 and UEA10 (Lunt et al. 1996). PCR conditions included an initial denaturation step at 97ºC for 4 m, followed by 35 cycles at 96ºC for 25 s, 48ºC for 25 s and 68ºC for 90 s with a final extension step that lasted 10 m at 68 ºC.

Plant DNA was extracted from needles using the PureLink™ Genomic Plant DNA Purification Kit (Invitrogen), following the protocol of the manufacturer as well. The mtDNA primers that were used in the amplification of a 1000 bp DNA fragment were CO1X-forward and CO1X-reverse (Duminil et al. 2002), which were designated as universal plant mtDNA amplifying primers. The PCR conditions were those indicated by Duminil et al. (2002), and included an initial denaturation step at 97 ºC for 4 m, followed by 35 cycles at 96ºC for 15 s, 62ºC for 15 s and 68ºC for 60 s with a final extension step that lasted 10 m at 68 ºC. PCR products were then purified with GenElute™ Gel Extraction Kit (Sigma) and sent for sequencing at a commercially available service. The sequences were initially visualized by ChromasLite® (Technelysium Pty Ltd.) and aligned by eye, whereas haplotypes were deposited in NCBI GenBank under the accession numbers HQ727683–HQ727690.

Population genetic indices were calculated, in order to assess the population genetic diversity at the Single Nucleotide Polymorphism (SNP) level of the bark beetle – Norway spruce complex. Nucleotide diversity (π; Nei 1987) and nucleotide frequency were calculated by DAMBE software (Xia and Xie 2001), whereas Tajima's neutrality test (Tajima 1989) and the transition – transversion bias R were estimated by MEGA version 4 (Tamura et al. 2007). Arlequin version 3.11 (Excoffier et al. 2005) was employed in order to conduct an additional neutrality test (Fu's test), calculate haplotype diversity (H), genetic distances among haplotypes and theta (θ) values based on the number of polymorphic sites (S) and the mean number of pairwise differences (π). Finally, mismatch distributions were calculated and their respective histograms were drawn.

Table 1. Molecular indices calculated for both *Picea abies* and *Pityogenes chalcographus*.

Index	*Picea abies*	*Pityogenes chalcographus*
No. individuals	25	25
bp	843	718
Polymorphic sites	1	20
Ts/Tv	1/0	16/4
Nucleotide diversity (π)	0.001186	0.027855
Haplotype diversity (*h*)	0.0800 ±0.0722	0.8633 ±0.0241
Theta S	0.26483	5.29667
Theta π	0.08000	7.16667
Tajima's neutrality test (Ds)	–1.15753 (p=0.13400)	1.27618 (p=0.91400)
Fu's neutrality test (Fs)	–1.06131 (p=0.06600)	5.46380 (p=0.97300)

3 Results

Insect and plant tissues were analysed by direct sequencing of about 800bp loci (718bp and 843bp respectively) located in the COI gene of the mitochondrial genome. The number of haplotypes retrieved in both cases differed significantly. Only two were found among all 25 *P. abies* trees, in contrast to *P. chalcographus* insects that incorporated six haplotypes. Taking into account the distribution of individuals in each haplotype, it is evident that *P. chalcographus* individuals are more uniformly distributed ($h = 0.8633 \pm 0.0241$) in comparison to *P. abies* ($h = 0.0800 \pm 0.0722$) (Table 1). The number of polymorphic sites was respectively unequal. In fact, the number of polymorphic sites, as well as π, was much higher in the beetle than in the host tree. Only one polymorphic site was detected among the 25 individuals of *P. abies* (ts/tv = 1/0), whereas 20 polymorphic sites were detected among the same sample size of *P. chalcographus* (ts/tv = 16/4). On the other hand, π was more than 10 times smaller for the *P. abies* loci analysed, compared to that of *P. chalcographus*. Theta indices provide an insight into the genetic structure of a population and thus, interesting conclusions can be drawn from the comparison of theta values calculated for a host plant and an insect. In our study, the theta index that is based on the number of polymorphic sites (S) was 20 times higher for the insect dataset compared to the dataset of the host plant, whereas the theta index that refers to the pairwise differences (π) was more than 80 times greater among the sequences of *Pityogenes chcalcographus* than for the sequences of *Picea abies*.

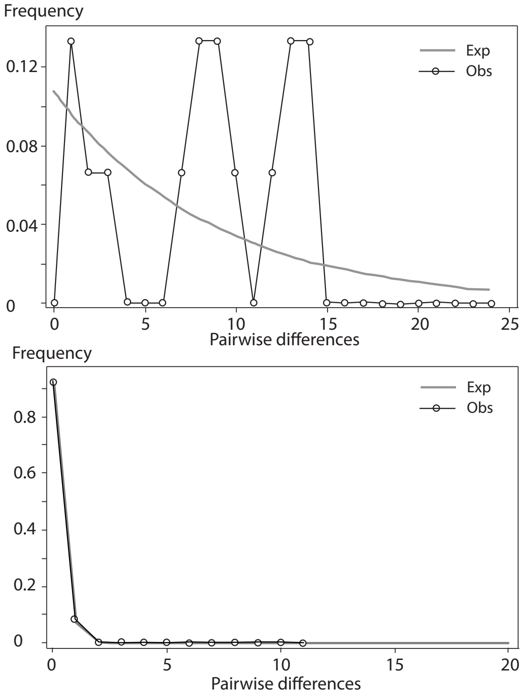

Fig. 1. Mismatch distribution histograms of *Pityogenes chalcographus* (above) and *Picea abies* (below).

Two different neutrality tests were conducted in order to study the nature of the signal that our results demonstrated. However, only Fu's neutrality test for plant data was marginally statistically significant ($F_S = -1.061$, $p = 0.066$), revealing a possible recent population expansion, or genetic hitchhiking. Mismatch distributions were additionally used for the study of demographic events that shaped the populations. The ragged histogram created when the number of pairwise mismatches among the samples of *P. chalcographus* is associated with their frequency (Fig. 1), indicates a steady-state population with a constant population size. In the case of the *P. abies* population however, the respective histogram was relatively smooth with a single peak, a common finding in populations that experienced a burst of growth.

4 Discussion

The low genetic diversity revealed for the *P. abies* population in the Elatia forest is congruent to the results of Tollefsrud et al. (2008). In this work, the southwest Bulgarian mountains were recognised as a refugial area for Norway spruce, nevertheless local populations exhibited low genetic diversity. This outcome contradicted the general notion that refugial areas demonstrate high genetic diversity due to the accumulation and conservation of rare alleles (Hewitt 2001, Turelli et al. 2001). However, in this case it was assumed that genetic drift induced by the small size of the refugial population in concert with the contraction of populations during the cold and dry Younger Dryas (11,000 ybp) can well account for the reduced genetic diversity (Tollefsrud et al. 2008). The population of Norway spruce sampled in our analysis originated from the southernmost part of the forest continuum sampled by Tollefsrud et al. (2008) in the south Balkan region. It can therefore be assumed that the low diversity revealed in the population of our investigation may be attributed to the same events that shaped the south Balkan *P. abies* population of Tollefsrud et al. (2008).

In contrast to *P. abies*, significantly higher genetic diversity was revealed for the same gene in the equivalent sample size of *P. chalcographus*. This result is congruent with the conclusion drawn from the phylogeographic analysis conducted among European populations of *P. chalcographus* by Avtzis et al. (2008). In this study, the population from the forest of Elatia harboured one of the highest genetic diversity indices among the populations studied (Avtzis et al. 2008). It was thus concluded, that this area acted as a refugial area that contributed significantly to the total diversity of *P. chalcographus* in Europe (Avtzis et al. 2008). Therefore, these two interacting organisms clearly demonstrated completely different population dynamics, with the insect likely showing a far greater potential for adaptation compared to Norway spruce.

A comparison between the diversity of the host tree and its pest reveals the role of biology in the evolutionary history of a species. Despite the fact that both organisms, as revealed by previous studies (Avtzis et al. 2008, Tollefsrud et al. 2008), shared and used the area of Mt. Rodopi as an Ice Age refugium, from where they expanded northwards postglacially, their evolution was not parallel. *Picea abies* on one hand, where one generation needs more than 30 years to reach sexual maturity in closed stands (Skrøppa 2003) in concert with an apparent lower migration rate, evolved at relatively slower rate, compared to that of *Pityogenes chalcographus*, which establishes two (or sometimes even three) generations per year (Postner 1974) depending on the environmental conditions. Consequently, *P. chalcographus* would obviously take the lead in an evolutionary "arms race" against *P. abies*. A result of this uneven evolutionary pace of these organisms is the current incongruity in molecular diversity levels of the COI gene that may portray an evolutionary advantage to the bark beetle against its host tree.

A synthesis of the genetic diversity analysis of *P. abies* and of one of its main pest species, namely *P. chalcographus,* in the southernmost area of the Norway spruce natural distribution demonstrates the opposing differences of these two interacting organisms. These results, especially when verified at the nDNA level, may indicate that the low genetic diversity of *P. abies* in that area could possibly threaten the viability of this forest, potentially rendering it less capable of adapting to novel environmental conditions, or rapid environmental changes. A low adaptation rate may have overwhelming results under the light of the ongoing climate change which is expected to be more severe and even abrupt at marginal environments (Diaz and Graham 1996, Easterling et al. 1997, Trenberth et al. 2003, Knutson and Tuleya 2004). In such areas, mal-adaptation inhibits tree regular growth causing trees to be more susceptible to pests (Kalkstein 1976, Mattson and Haack 1987, Boyer 1995). Especially for bark beetles, such as *P. chalcographus*, weakened trees constitute a major attraction by releasing volatile chemicals (monoterpenes) (Byers 2004). This feature when considered together with the higher genetic diversity that *P. chalcographus* exhibits in this region, may portray a considerably faster adaptation rate of the pest to changing environmental conditions by locating and exploiting potentially weakened host trees. These processes may apparently feed one another in the *P. abies–P. chalcographus* complex. Therefore, only by adopting specific forest management measures could this situation be inverted. Regular surveys of the area for infested individuals and/or clusters of trees and subsequent prompt removal of infested logs would reduce the risk of a dramatic population outbreak of *Pityogenes chalcographus*. In general, forest management practises and complex species interactions will be significant determinants of the ecosystems' future. Coevolution may eventually produce adaptive responses (Wade 2007), however at non-equilibrium stages the faster the rate of environmental change, the more important anthropogenic influence through careful forest management will become.

The high genetic diversity demonstrated by *P. chalcographus* in concert with the low diversity of its main host, *P. abies*, poses an additional threat for the pine flora of Greece as well. It has already been shown in the laboratory (Bertheau et al. 2009) that *P. chalcographus* prefers mainly Norway spruce for its development, however field observations (Avtzis et al. 2008) and literature reviews (Schwerdtfeger 1929, Postner 1974, Pfeffer 1995) report that *P. chalcographus* can also develop on several pine species (*Pinus* spp.) as well as on European larch, *Larix decidua*. As a result, a highly diversified population of *P. chalcographus*, after infesting the mostly preferred host tree, namely *P. abies* in Greece, could potentially expand on other host trees such as indigenous pine species through host shift. Host shifts are facilitated by a pest's large gene pool, a manifestation of high genetic diversity levels such as those detected in the present study.

The current investigation, which is affiliated with an on-going broader study of the Mt. Rodopi region underlines the significance of similar approaches that aim not only to the investigation of a single organism's diversity, but consider potential conspecific interactions in terms of population dynamics and coevolution. Comparing genetic indices of interacting organisms sheds light on the way that different biological features determine the evolutionary pathways that these species will ultimately follow. Moreover, such studies become more important in marginal environments that may be adversely affected by climatic change, such as in the case of *P. abies* in Europe. A low genetic diversity in the host coupled with a high diversity demonstrated by one of its main pests, as this study indicates, could lead to the gradual infestation of the Norway spruce forest whereas *P. chalcographus*, due to its high genetic diversity, could potentially even experience a host shift to other indigenous pine species. The outcome presented in the current research note, points toward the importance of further investigation aiming at a larger scale sampling of both host tree and pest and targeting both nuclear and cytoplasmic genomes in order to achieve an even more representative depiction of genetic diversity in this region.

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