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HOKKAIDO UNIVERSITY
Multiple host shifts between distantly related plants, Juglandaceae and Ericaceae, in the leaf-mining moth *Acrocercops leucophaea* complex (Lepidoptera: Gracillariidae)

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Abstract

Insect herbivores such as gall formers and leaf miners are often highly specialized and adapted to their respective natal host plants. Due to the specialization and adaptation, it is presumed that host-shifts readily occur among closely related plant species. Leaf-mining moths, the *Acrocercops leucophaea* complex, consist of three species, *A. leucophaea*, *A. defigurata* and *A. transecta*. Larvae of all the species of the complex feed on Juglandaceae plants, but *A. leucophaea* and *A. transecta* are also associated with an Ericaceae plant, which is quite distantly related to Juglandaceae. Such a host utilization as in this species complex is very rare among phytophagous
insects. In the present study, we estimate the history of host shifts by reconstructing the phylogeny of the *A. leucophaea* complex using molecular data (partial sequence of mitochondrial COI, 12S rDNA and ND5). Parsimony and maximum likelihood analyses indicated that the common ancestor of the *A. leucophaea* complex used Juglandaceae only, and that the association with Ericaceae has evolved in *A. leucophaea* and *A. transecta* independently. Parametric bootstrap analysis also supported multiple origins of the association with Ericaceae in this complex. These results imply that there are ecological and biochemical factors that promote host shifting between Juglandaceae and Ericaceae despite the two families being not closely related.

**Key words:** Phytophagous insects; Insect-plant interactions; Host shift; Host race; Speciation; Mitochondrial DNA; Phylogeny

1. Introduction

The majority of phytophagous insects are associated with one or a few closely related host plants (Strong et al., 1984; Schoonhoven et al., 1998; Symons and Beccaloni, 1999). Such mono- and oligophagous insect herbivores are highly specialized to their natal host plant species via physiological and behavioral adaptations. These adaptations to the host plants have led to differentiation in life-history traits between populations using different host plant species (Thompson, 1998; Vanbergen et al., 2003). Recent studies have suggested that there are more examples of host race formation in herbivorous insects than has previously been recognized (Abrahamson et al., 2001). Studies on host race formation in phytophagous insects have postulated that
such a host specialization via host shift leads to ecological speciation (Howard and Patrick, 2005) without geographic isolation (Bush, 1969, 1994; Wood, 1980; Wood and Guttman, 1983; Craig et al., 1997, 2001; Feder, 1998; Berlocher and Feder, 2002; Drès and Mallet, 2002). Thus, host shifts may contribute as a first step to the formation of host races and subsequently to sympatric speciation.

The mechanisms and process of the host shift in phytophagous insects include numerous genetic and ecological factors (Thompson, 1991, 1999). Ehrlich and Raven (1964) particularly stressed the importance of the similarities in the plants' secondary metabolic compounds between the novel and old hosts. Closely related host plants (e.g. the same genus or family) often meet these conditions. Therefore, host shifts in phytophagous insects are generally considered to occur more readily between closely related host plants than between distantly related ones (Futuyma et al., 1995; Janz and Nylin, 1998, Lopez-Vaamonde et al., 2003).

A leaf-mining moth, the *Acrocercops leucophaea* complex, consists of three species, *A. leucophaea*, *A. defigurata* and *A. transecta*. *A. leucophaea* and *A. defigurata* are distributed in Nepal and Northern India, while *A. transecta* is distributed in the Russian Far East, South Korea, Japan, and Taiwan. *Acrocercops leucophaea* and *A. transecta* are associated with distantly related plants, several Juglandaceae species and *Lyonia ovalifolia* (Ericaceae), while *A. defigurata* is associated with Juglandaceae species only (Kumata et al., 1988; Ohshima, pers. obs.). Among Juglandaceae, each of *A. leucophaea* and *A. defigurata* uses only one plant species (*Engelhardtia spicata* and *Juglans regia*, respectively), but *A. transecta* is associated with *Pterocarya rhoifolia*, *Platycarya strobilacea* and several *Juglans* plants (Kumata, et al., 1988; Ohshima, pers. obs.). There are no morphological differences between the population feeding on
Ericaceae and that feeding on Juglandaceae in each of *A. leucophaea* and *A. transecta* (Kumata et al. 1988). However, females exclusively prefer to oviposit on their natal host plant in *A. transecta* (Ohshima, 2005), and oviposition preference of *A. leucophaea* also differs between Juglandaceae-associated population and Ericaceae-associated population (Ohshima, unpubl. data). The Juglandaceae and Ericaceae belong to different orders, Fagales and Ericales, and the two orders are affiliated with distantly related clades, rosids and asterids, respectively (Soltis et al., 1999; Soltis et al., 2000). These unique host associations, a single species using only two distantly related host plants, in this species complex are remarkable cases in phytophagous insects.

An increasing number of studies using molecular markers have documented genetic differentiation between host-associated populations within a single phytophagous species (Feder et al., 1988; McPheron et al., 1988; Brown, 1996; Lopez-Vaamonde et al., 2003; Brunner et al., 2004). These examples suggest that the two host-feeding populations in each of *A. leucophaea* and *A. transecta* could be genetically differentiated. However, they are morphologically indistinguishable, and thus molecular phylogenetic analyses are needed to evaluate genetic differentiation. Furthermore, if monophyly in each of *A. leucophaea* and *A. transecta* is supported, this extreme host association between such distantly related host plants should have evolved in the two distinct lineages independently. The alternative possibility is that *A. leucophaea* and *A. transecta* are not monophyletic. If monophyly of Juglandaceae-associated and/or Ericaceae-associated populations is supported beyond the present nominal species, this unique host association should have been formed at a single evolutionary event, indicating the paraphyly of at least one of the morpho-species.
The present study aims to test if the unique host utilization patterns shared by *A. leucophaea* and *A. transecta* have evolved independently or not by inferring the molecular phylogeny of the *A. leucophaea* complex. Determining the direction of the host shift between Juglandaceae and Ericaceae plants in this species complex is crucial for understanding the evolutionary process of the host-association in this complex. This case thus provides a unique opportunity for studying the evolutionary dynamics of host-plant usage in phytophagous insects.

2. Materials and Methods

2.1. Taxon sampling

This study used specimens stored in 99.5% ethanol and dried specimens (more than 20 years old). Larvae were collected with the host plant and were reared in the laboratory. Emerged adults were used for analysis except for eight larvae (voucher number IO-058, 059, 062, 064 - 066, 068 and 069). A total of 37 specimens were selected from among the *Acrocercops leucophaea* complex as ingroups, including 16 *A. leucophaea* (including seven samples from the Juglandaceae associated population and nine samples from the Ericaceae associated population), four *A. defigurata*, and 17 *A. transecta* (including 12 samples from the Juglandaceae associated population and five samples from the Ericaceae associated population). Three *Acrocercops* species not belonging to the *leucophaea* complex were selected as outgroups (Table 1).

2.2. Sequence determination
Total DNA was extracted using DNeasy Tissue Kit (Qiagen) following Cruickshank et al. (2001). Vouchers were slide mounted and stored in Hokkaido University. For molecular phylogenetic inference, three mitochondrial DNA regions, cytochrome oxidase subunit I (COI), NADH dehydrogenase subunit 5 (ND5) and 12S rDNA were chosen. Mitochondrial COI gene is known to be useful for inferring relationships among closely related moth species and populations (Brown et al., 1994), and NADH dehydrogenase subunit genes are more rapidly evolving than COI (Simon et al., 1994). In contrast, mitochondrial 12S rDNA gene is suitable for analyzing deeper clades (Moritz et al., 1987; Simon et al., 1994). Primer sets 12Sai + 12Sbi (Simon et al., 1994), L6625 + H7005 (Hafner et al. 1994), and F7081 + R7495 (Yoshizawa, 2004) were used to amplify 335 bp fragment of mitochondrial 12S rDNA, 344 bp of COI, and 413 bp of ND5, respectively. Additional primer sets L6625 + R2352 (5'-GTA TCA ATA TCT ATT CCT AC-3') and F2314 (5'-GCT ATA ATA GCA ATT GGA TT-3') + H7005 were used to amplify 160 bp and 221 bp fragments of COI, and F7081 + R7345 (5'-GCT AAT TAT GAG TTT GAT CTA AA-3') and F7259 (5'-ATA TAG CAT GAG TTA ATA AAT G-3') + R7495 were used to amplify 263 bp and 235 bp fragments of ND5 for the old specimens. Reaction cycle was 94 C for 3’ followed by 40 cycles of 94 C for 30", 45 C (12S and COI) or 42 C (ND5) for 30", and 65 C for 45". For the old specimens, 2nd PCR reaction was conducted using 1st PCR products as template. Reaction cycle of 2nd PCR was the same as the 1st PCR (12S), or 94 C for 3’ followed by 5 cycles of 94 C for 30", 48 C (COI) or 47 C (ND5) for 30", and 72 C (COI) or 65 C (ND5) for 45", and then followed by 35 cycles with annealing temperatures of 45 C (COI) or 42 C (ND5). Amplified products were purified using PCR Purification Kit
(Qiagen), and then were performed a single-stranded PCR for sequencing following manufacture's protocol. PCR products were sequenced by CEQ2000 DNA Analysis System (Beckman Coulter). Obtained sequences were aligned manually.

NEXUS files of the aligned sequences are available from the URL at <http://insect3.agr.hokudai.ac.jp/psoco-web/data/index.html> or by requested to the first author.

2.3. Phylogenetic analysis

Parsimony (MP) and maximum likelihood (ML) analyses were conducted by PAUP 4.0b10 PPC (Swofford, 2002). For MP analysis, all characters were equally weighted. MP trees were searched with 100 random addition replication using TBR branch swapping. Parameters for ML analysis were chosen based on the Akaike information Criterion (AIC) (Akaike, 1973, 1974; Sakamoto et al., 1986) as implemented in Modeltest ver 3.06 (Posada and Crandall, 1998) (Posada and Buckley, 2004). As a result of Modeltest, the TVM + I + G model was selected (unequal base frequencies: A = 0.4354, C = 0.0990, G = 0.1022, T = 0.3634; six substitution categories: A-C = 3.0362, A-G = 11.2902, A-T = 1.4384, C-G = 0.6908, C-T = 11.2902, G-T = 1.0000; gamma distribution shape parameter = 1.1336; proportion of invariable sites = 0.7200; four rate categories). The ML tree was searched with TBR branch swapping using neighbour-joining tree as a starting point. To assess confidence in clades, nonparametric bootstrap tests (Felsenstein, 1985) were performed using 100 replicates with TBR branch swapping. History of host shift was parsimoniously reconstructed on the estimated MP trees using Mac Clade 4.03 (Maddison and
2.4. Testing alternative hypotheses

We tested alternative hypotheses using parametric bootstrapping (Swofford et al., 1996), which was referred to as SOWH test by Goldman et al. (2000). We chose parametric bootstrap tests because other non-parametric methods are subject to type II statistical error (Goldman et al. 2000) and to serious biases (Shimodaira, 2002). Due to the computational complexity of the SOWH test, we subsampled twelve samples from the clades (asterisked in Figure 1). The substitution model and the parameters were re-calculated for the twelve samples. Estimated parameters and constrained ML trees were used to generate 100 simulation data using Seq-Gen 1.5.3 (Rambaut and Grassly, 1997).

3. Results

3.1. Phylogenetic analysis

No insertion or deletion were observed in 12S rDNA, COI and ND5 gene sequence. Of a total of 1092bp aligned nucleotide sites, 81 were variable and 70 of the 81 bp were parsimony-informative for the ingroup (including the outgroup185 and 101, respectively). A heuristic search detected three MP trees (length 287 steps, RI = 0.88). Of the three MP trees, two placed *A. defigurata* at the most basal position of the *A. leucophaea* complex and the two trees differed in the branching within *A. defigurata*.
The remaining MP tree placed *A. defigurata* as the sister of *A. transecta + A. leucophaea*-clade1 subgroup. The tree obtained by ML analysis (Fig. 1) was identical with one of the former MP trees. Monophyly of *A. transecta + A. leucophaea* was recovered, although bootstrap support was low (bootstrap support < 50%). Hence, we postulate that the tree shown in Figure 1 as the best phylogenetic hypothesis because both analyses supported it.

Both analyses strongly supported monophyly of the *A. leucophaea* complex (MP 100%; ML 96%) and monophyly of *A. defigurata* (MP 100%; ML 87%). Monophyly of *A. transecta* was also fairly well supported (MP 85%; ML 62%). However, neither of the analyses supported monophyly of *A. leucophaea*, which was divided into two clearly distinguished clade. Three samples of *A. leucophaea* composed a monophyletic group with 100% bootstrap support for both analyses (clade1), and the clade was placed as the sister group of *A. transecta* (MP 84%; ML < 50%). The remaining members of *A. leucophaea* also composed a monophyletic group with high bootstrap supports (clade2: MP 99%; ML 83%). The clade1 consisted of the Pharping population infesting Ericaceae only, and the clade2 contained the moths from Godawari, Nagarjun and Pharping, which are associated with Juglandaceae or Ericaceae.

Two host-associated populations in *A. transecta* were clearly distinguished phylogenetically and showed a rather large intraspecific variation (uncorrected pairwise distance: 1.01 - 1.83%). Monophyly of the Juglandaceae population was strongly supported by MP analysis (79%), while ML analysis supported the monophyly less strongly (56%). Monophyly of the Ericaceae population was also well supported by both analyses (MP 74%; ML 72%). Variation in the sequences within the
Juglandaceae-associated population or the Ericaceae-associated population was also rather large (0.09 - 1.10%, respectively). In contrast, two host-associated populations in *A. leucophaea*-clade2 were less clearly distinguished.

We conducted the most parsimonious reconstruction (MPR) of evolutionary history of the host-association on the MP tree that was selected as the best phylogenetic hypothesis (Fig. 1). Figure 2a, b shows the results of the MPR on the MP tree. Two MPRs, ACCTRAN and DELTRAN, were possible on each tree, but both showed that host shift has occurred three times within the *A. leucophaea* complex. ACCTRAN optimization indicated two host-shifts from Juglandaceae to Ericaceae and one host-shift in the opposite direction (Fig. 2a, c). In contrast, DELTRAN optimization suggested that host-shift from Juglandaceae to Ericaceae has evolved three times (Fig. 2b, d). The results of MPR indicated that the Juglandaceae-association was the most ancestral condition for this species complex. The results of MP analysis also supported alternative phylogenetic hypothesis, *A. defigurata* as the sister of *A. leucophaea*-clade1 + *A. transecta*, but the MP tree basically did not alter the interpretation on the history of host shifts in the *A. leucophaea* complex (Fig. 2c, d).

3.2. *Tests of alternative hypotheses*

To evaluate the above-mentioned results statistically, we tested the following alternative hypotheses using the SOWH test. We inferred trees using ML analysis in which either monophyly of the Juglandaceae-populations or that of the Ericaceae-populations was constrained. However, the single origin of Juglandaceae- and Ericaceae-association was rejected (Δ-ln L = 58.72319, p < 0.01, Fig. 3a; Δ-ln L =
A. leucophaea was clearly divided into two separated clades in the estimated trees (Figs. 1, 3). In the tree, A. leucophaea-clade1 was the sister group of A. transecta, indicating that A. leucophaea is a paraphyletic species. We inferred a ML tree with monophyly of A. leucophaea being constrained, but this was rejected ($\Delta-Ln L = 1.38428, p < 0.05$, Fig. 3c).

Both MP and ML trees indicated that A. leucophaea-clade1 was the sister group of A. transecta (Fig. 1). This result suggests that A. leucophaea-clade1 may compose a monophyletic group with the Ericaceae-population of A. transecta. SOWH test also rejected this possibility ($\Delta-Ln L = 2.71142, p < 0.05$, Fig. 3d).

4. Discussion

4.1. Phylogenetic inferences

The present analyses are based only on mitochondrial genes and thus the result represent gene tree. Phylogenetic hypotheses inferred from mitochondrial genes sometimes provide inaccurate estimates of species phylogeny due to mitochondrial introgression (Shaw, 2002). However, mitochondrial DNA is inherited maternally, so that the mitochondrial gene phylogeny directly reflects evolutionary history of female host preference and is useful for estimation of the history of host shifts (Diegisser et al. 2004).

Both MP and ML analyses based on the combined sequences provided well resolved trees. Four major clades, A. leucophaea-clade1 and 2, A. defigurata and A.
*transecta* were clearly detected. The present analyses also clearly distinguished two host-associated populations in *A. transecta*. However, the relative position of *A. defigurata* and *A. leucophaea*-clade2 in *A. leucophaea* complex are rather unstable by MP analysis, although it does not influence the estimation of the history of host shifts.

*A. transecta* is monophyletic, and each of the Juglandaceae-population and the Ericaceae-population is also monophyletic. A preliminary study on oviposition preference revealed that females from different hosts exclusively oviposited on their natal host plant (Ohshima, unpubl. data). In this regard, genetic differentiation may be suggested between the two populations. However, the two populations mated freely in the laboratory, and their hybrids are viable and fertile (Ohshima, unpubl. data). This mating compatibility indicates that these host races are maintained only by the differential host preferences of the females. Nevertheless, uncorrected pairwise distance between the two populations is rather large (1.01 - 1.83 %). Avise (2000) pointed out that intraspecific genetic divergences are rarely greater than 2% and most are less than 1%. In particular, uncorrected pairwise distance for COI gene between the two populations is 0.87 - 2.91 %, which is equal to interspecies divergence (Hebert et al., 2003b). Even within each host-associated population, the maximum COI genetic distance for the Juglandaceae-population is 2.62 % and that for the Ericaceae-population is 1.16 %. These intra-population divergences are larger than the intraspecies divergence reported in several Lepidopteran families, 0.17 - 0.36 % (Hebert et al., 2003a). An accelerated substitution rate in mitochondrial genes has been reported (Page et al., 1998; Johnson et al., 2003; Yoshizawa and Johnson, 2003). This possibility should be tested for these moths.

The two host-associated populations in *A. leucophaea*-clade2 are not clearly
distinguished in the present analyses. These results might imply that there is no host-specialization in *A. leucophaea*-clade2. However, two host-associated populations in the same locality (Godawari and Pharping) do not share the same haplotype, implying that host specialization has already developed within this clade. Another possibility is mitochondrial introgression from *A. leucophaea*-clade2 to *A. leucophaea*-clade1. Ericaceae-associated individuals in clade2 may actually belong to clade1. However, in *A. leucophaea*-clade2 the Ericaceae-population in Godawari or Nagarjun have a unique haplotype. Available evidence indicates that the present results were not due to mitochondrial gene introgression from *A. leucophaea*-clade2 to clade1. SOWH test also clearly distinguished clade1 from clade2 (Fig. 3c). Therefore, we conclude that the Ericaceae-association has evolved in the two distinct *A. leucophaea* lineages independently.

The MPR of host plant associations suggests that the Juglandaceae-association is the ancestral state for the complex. Outgroup taxa are associated with Fagaceae, which is phylogenetically closer to Juglandaceae rather than to Ericaceae (Soltis et al., 1999; Soltis et al., 2000). Therefore, the result of MPR concerning ancestral host association in *A. leucophaea* complex is reasonable from the viewpoint of plant phylogeny. The MPR suggested two alternative hypotheses by ACCTRAN and DELTRAN optimizations (Fig. 2). Several studies on host race formation have revealed that derived races often retain adaptation for each ancestral host plant (Ikonen et al., 2003; Vanbergen et al., 2003; Gross et al., 2004). In *A. transecta*, preliminary studies on larval performance revealed that larvae from the Ericaceae-population can survive on Juglandaceae plants but that larvae from the Juglandaceae-population cannot survive on the Ericaceae plant (Ohshima, unpubl. data).
The potential adaptation to Juglandaceae in the Ericaceae population may indicate that the population has derived from Juglandaceae-population. Therefore, if above mentioned relationships between phylogeny and host adaptation could be applicable for the case of *A. transecta*, three host shifts from Juglandaceae to Ericaceae is more plausible.

The present results also provide biogeographical information for the host shift in *A. transecta*. MP and ML analyses indicated that the Juglandaceae-associated population in Vladivostok (Far East Russia) composed a monophyletic group with other Juglandaceae-associated populations in Japan, and that the Ericaceae-associated population in Taiwan also formed a monophyletic group with other Ericaceae-associated populations in Japan. These results suggest that the host shift in *A. transecta* had completed before its colonization of Japan.

4. 2. *Evolution of host associations*

Becerra and Venable (1999) summarized three models to explain the macroevolution of insect-plant associations: (1) host shift to a new host plant that is chemically similar to the old host (Ehrlich and Raven, 1964; Futuyma and McCafferty, 1990; Becerra, 1997); (2) A parallel cladogenesis of plants and herbivorous insects (Farrell and Mitter, 1990; Mitter et al., 1991); (3) shift to new hosts that are present in the same geographic location as the range of their ancestral hosts (Bernays and Chapman, 1994; Dobler et al., 1996).

The host plants of the *A. leucophaea* complex (Juglandaceae and Ericaceae) belong to different plant orders (Fagales and Ericales, respectively), and these orders are
not phylogenetically closely related (Soltis et al., 1999; Soltis et al., 2000). Therefore, the parallel cladogenesis hypothesis between these moths and host plants is rejected. In contrast, Juglandaceae plants and *Lyonia ovalifolia* often grow side by side in the natural habitats of the *A. leucophaea* complex in Japan and Nepal (Ohshima, pers. obs.). This observation might support the same geographic location hypothesis. However, it is difficult to explain why *A. leucophaea* and *A. transecta* exclusively associated with *Lyonia ovalifolia* and why host shift from Juglandaceae plants to *Lyonia ovalifolia* has occurred at least twice in different lineage. For example, several plant species, e.g. *Quercus glauca* (Fagaceae) that are closely related to Juglandaceae (Soltis et al., 1999; Soltis et al., 2000) and some species of *Pieris* and *Vaccinium* (Ericaceae) that are closely related to *Lyonia ovalifolia* (Kron et al., 2002), often grow sympatrically with Juglandaceae plants and *Lyonia ovalifolia* in Japan and Nepal (Ohshima, pers. obs.). Nevertheless, the member of *A. leucophaea* species are not associated with these plants. Therefore, we hypothesize that chemical similarity (e.g. sharing of some oviposition attractant compounds) between Juglandaceae host plants and *Lyonia ovalifolia* may have contributed to the unique host-association in this species complex. Future studies should assess chemical similarity between Juglandaceae and *Lyonia ovalifolia* to test this hypothesis.

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References


**Figure Legends**

**Fig. 1.** A tree estimated by the ML analysis (-ln = 2901.30358). Branch length are proportional to ML estimated genetic distances. The numbers associated with branches
indicate bootstrap values higher than 50% (MP: upper, ML: lower). '≤' indicates bootstrap support lower than 50%. Major groups are indicated with bold bars. Within sample names, the former alphabet of <> refers to the generic name of each host plant as follows: J: *Juglans*, Pt: *Pterocarya*, Pl: *Platycarya*, E: *Engelhardtia*, L: *Lyonia*, C: *Castanopsis*, Q: *Quercus*. The alphabet included in the <> refer to the family name to which each host plant belong as follows: J: Juglandaceae, E: Ericaceae, F: Fagaceae. Asterisks indicate samples used in the SOWH test.

Fig. 2. Most parsimonious reconstruction (MPR) of host-associations onto the two parsimony (MP) trees. These two trees differ in position of *A. defigurata*. (a) and (b) place *A. defigurata* at the most basal position of the *A. leucophaea* complex, and (c) and (d) place *A. defigurata* as the sister of *A. transecta + A. leucophaea*-clade1 subgroup. (a) and (c) show ACCTRAN optimization, indicating two independent origins of Ericaceae-association and one reversal from Ericaceae- to Juglandaceae-association. (b) and (d) show DELTRAN optimization, suggesting that Ericaceae-association has evolved three times independently.

Fig. 3. Results of the parametric bootstrap analysis. The distribution of the differences in likelihood scores between the constrained (null hypothesis) and unconstrained trees for 100 replications are shown. (a) Test of monophyly of all Juglandaceae-associated populations. (b) Test of monophyly of all Ericaceae-associated populations. (c) Test of monophyly of *Acrocercops leucophaea*. (b) Test of monophyly of *A. leucophaea*-clade1 and Ericaceae-population of *A. transecta*. The former two hypotheses was rejected at $p < 0.01$, and the later two
hypotheses was rejected at $p < 0.05$. 
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Table 1
Sampling Information and GenBank Accession Numbers
a. Monophyly of all Juglandaceae-associated population

- *A. leucophaea* (IO-077)
- *A. defigurata* (IO-059, 060)
- *A. transecta* (IO-007, 043)

\[ p < 0.01 \]

b. Monophyly of all Ericaceae-associated population

- *A. leucophaea* (IO-071, 073)
- *A. transecta* (IO-001, 045)

\[ p < 0.01 \]

c. Monophyly of *A. leucophaea*

- *A. leucophaea* (IO-071, 073, 077)

\[ p < 0.05 \]

d. Monophyly of *A. leucophaea*-clade1 and Ericaceae-associated populations of *A. transecta*

- *A. leucophaea* (IO-071)
- *A. transecta* (IO-001, 045)

\[ p < 0.05 \]