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Species delimitation and invasion history of the balsam woolly adelgid, *Adelges* (*Dreyfusia*) *piceae* (Hemiptera: Aphidoidea: Adelgidae), species complex

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Abstract. The *Adelges* (*Dreyfusia*) *piceae* (Ratzeburg) species complex is a taxonomically unstable group of six species. Three of the species are cyclically parthenogenetic [*Ad. nordmannianae* (Eckstein), *Ad. prelli* (Grossmann), and *Ad. merkeri* (Eichhorn)] and three are obligately asexual [*Ad. piceae*, *Ad. schneideri* (Börner), and *Ad. nebrodensis* (Binazzi & Covassi)]. Some species are high-impact pests of fir (*Abies*) trees, so stable species names are needed to communicate effectively about management. Therefore, to refine species delimitation, guided by a reconstruction of their biogeographic history, we genotyped adelgids from Europe, North America, and the Caucasus Mountains region with 19 microsatellite loci, sequenced the COI DNA barcoding region, and compared morphology. Discriminant analysis of principal components of microsatellite genotypes revealed four distinct genetic clusters. Two clusters were morphologically consistent with *Ad. nordmannianae*. One of these clusters consisted of samples from the Caucasus Mountains and northern Turkey, and the other included samples from this region as well as from Europe and North America, where *Ad. nordmannianae* is invasive. A third cluster was morphologically consistent with *Ad. piceae*, and included individuals from Europe, where it is native, and North America, where it is invasive. In North America, the majority of *Ad. piceae* individuals were assigned to two geographically widespread clones, suggesting multiple introductions. The fourth cluster included individuals morphologically consistent with *Ad. prelli* or *Ad. merkeri*. However, based on genetic assignments, hybrid simulations, and approximate Bayesian computation, we find it likely that these are contemporary hybrids between *Ad. nordmannianae* and *Ad. piceae* that arose independently in Europe and North America, so we propose that *Ad. prelli* and *Ad. merkeri* are invalid. Finally, we synonymise *Ad. schneideri* **(syn.n.)** with *Ad. nordmannianae* and designate *Ad. nebrodensis* as subspecies *Ad. piceae nebrodensis* **(stat.n.)**. Our revised taxonomy therefore recognises two species: *Ad. nordmannianae* and *Ad. piceae*, which we estimate to have diverged recently, during one of the last two interglacial periods. Finally, we comment on this species complex being in the midst of transition between sexual and asexual reproduction, a pattern that is probably common in Adelgidae.

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Introduction

Taxonomy of Adelgidae (Hemiptera: Aphidoidea) has been unstable since their discovery, due in large part to the complexity of their facultatively parthenogenetic life cycles (Havill & Foottit, 2007). Prior to the turn of the 20th century, when the extraordinary details of adelgid life cycles were first being worked out (e.g. Cholodkovsky, 1888; Dreyfus, 1889; Eckstein, 1890), there were accompanying disagreements about how to delimit species. For example, Cholodkovsky (1888, 1902, 1915) tended to describe separate species based on whether they had holocyclic (sexual) or anholocyclic (asexual) life cycles, even if there were no morphological characters to distinguish them, because different life cycles indicated reproductive isolation. On the other hand, Börner (1907, 1908a,b) emphasized that most groups of morphologically indistinguishable holocyclic and anholocyclic adelgids were probably reproductively connected by cryptic facultative production of sexual forms. He therefore took an integrative approach to species delimitation that required information about the insects' biology, morphology, and a working theory of life cycle evolution. Similar disagreement about the validity of life cycle differences for species delimitation continued on through the mid-20th century (e.g. Marchal, 1913; Annand, 1928; Balch, 1952; Varty, 1956; Pschorn-Walcher & Zwölfer, 1958; Eichhorn, 1967; Steffan, 1970). This confusion remains to the present day, and as a result, some authors have chosen to refer to closely related holocyclic and anholocyclic adelgids as species complexes without clear boundaries (e.g. Toenshoff *et al.*, 2011; Ravn *et al.*, 2013; Havelka *et al.*, 2020).

One of these problematic species complexes is composed of *Adelges* (*Dreyfusia*) *piceae* (Ratzeburg) and its relatives. The cyclically parthenogenetic life cycle in this group involves alternation of generations between *Picea* (spruce) and *Abies* (fir) host plants (Marchal, 1913; Havill & Foottit, 2007; Fig. 1). *Picea orientalis* (L.) Link, which is endemic to the Caucasus Mountains, is their main primary host species (i.e., where the sexual generation occurs) (Pschorn-Walcher & Zwolfer, 1960; Eichhorn, 1975). They have also been reported to occasionally utilize *P. omorika* (Pančić) Purk, which is endemic to western Serbia and eastern Bosnia and Herzegovina (Eichhorn, 1975). The offspring of the sexual generation, called fundatrices (singular = fundatrix), settle at the base of *Picea* buds where they induce new shoots to become galls in the spring. The asexual offspring of the fundatrix settle inside the developing galls, and are called gallicolae (singular = gallicola). They emerge from the galls as winged adults that migrate to a variety of *Abies* secondary host species, where only asexual generations occur. These asexual generations can be a continuous series of wingless aestivating forms, called sistentes (singular = sistens), or during the summer, some individuals can develop into non-aestivating forms, called progredientes (singular = progrediens). Some progredientes remain wingless and stay on *Abies*, while others, called sexuparae (singular = sexupara), develop wings and migrate back to *P. orientalis* (or *P. omorikia*) to produce the sexual generation. If primary host species are not available, asexual reproduction can continue on the secondary hosts. Species that can complete the entire host-alternating, cyclically parthenogenetic life cycle are called holocyclic, and those that produce only the asexual generations on *Abies* are called anholocyclic.

The *Ad. piceae* species complex contains six named species (Favret *et al.*, 2015) that are distinguished by differences in life cycle and morphology. Three species are holocyclic: *Ad. nordmannianae* (Eckstein), *Ad. prelli* (Grosmann), and *Ad. merkeri* (Eichhorn); and three are believed to be anholocyclic: *Ad. piceae*, *Ad. schneideri* (Börner), and *Ad. nebrodensis* (Binazzi & Covassi). While difference in life cycle mode has historically been used as a species diagnostic character in this group, scoring this is complicated because it requires observing populations for several generations in the presence of different host species. To date, studies that have examined the anholocyclic species in this complex have found that sexuparae are rare or have not been observed, and the forms that follow the sexual generation have not been observed (Annand, 1928; Balch, 1952; Bryant, 1971; Binazzi & Covassi, 1991; Binazzi, 2000).

Within the *Adelges* (*Dreyfusia*) *piceae* complex, two species have received the most attention because they are invasive pests of fir trees: *Ad. piceae* is invasive in North America, and *Ad. nordmannianae* (Eckstein) is invasive in Europe. *Adelges piceae*, the balsam woolly adelgid, is anholocyclic and considered native to Europe where it feeds on *Ab. alba* Mill. (Balch, 1952). It was first recorded in North America in Maine in 1908 (Kotinsky, 1916). Over the next 40 years, it spread to most of the Maritime Provinces in Canada and throughout New England in the United States (Balch, 1952). In western North America, it was first reported in California in 1928 (Annand, 1928), and then spread north along the Pacific coast. It was found in Oregon around 1930 (Keen, 1938), in southwestern Washington in 1954 (Johnson & Wright, 1957), and in southwestern British Columbia in 1958 (Zilahi-Balogh *et al.*, 2016). It then spread into the interior west. In 1983, it was reported in Idaho (Livingston *et al.*, 2000) and its range has since expanded to Montana, Utah (Davis *et al.*, 2020), and interior British Columbia (Zilahi-Balogh *et al.*, 2016). In the southeastern United States, it was first detected in Virginia in 1956 and North Carolina in 1957 (Speers, 1958), and has since spread throughout the range of *Ab. fraseri* (Pursh) Poir. in the southern Appalachians (Hollingsworth & Hain, 1991). Throughout its introduced range in North America, it has caused extensive damage and death of fir trees (Montgomery & Havill, 2014) due to atypical cell division in xylem tissue caused by its feeding, and the subsequent formation of large parenchyma cells, restricting water transport (Balch *et al.*, 1964).

The other invasive species, *Adelges nordmannianae*, is holocyclic and considered native to the Caucasus Mountains, where it alternates between *Ab. nordmanniana* (Stev.) Spach, and *P. orientalis* (Eichhorn, 1967). It is thought to have been introduced to Europe during the mid- to late-1800's on imported *Ab. nordmanniana*, and it can readily feed on the European *Ab. alba* (Schneider-Orelli *et al.*, 1929; Varty, 1956). It has since spread throughout Europe where it is considered a pest, especially in Christmas tree plantations (Nierhaus-Wunderwald & Forster, 1999; Ravn *et al.*, 2013), because it causes curling of the needles and can kill young trees (Pschorn-Walcher &

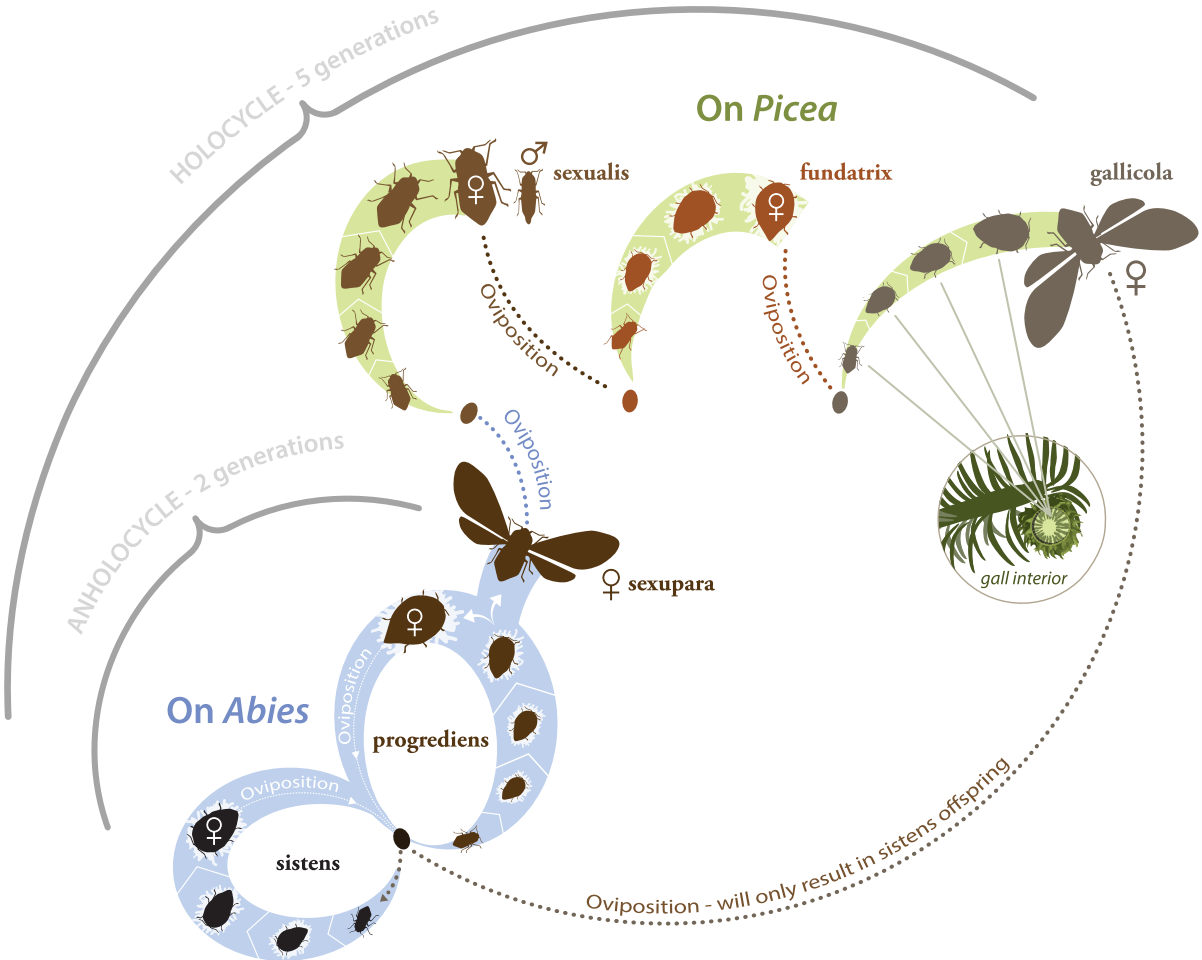

Fig. 1. Multi-generation life cycles in the *Adelges piceae* species complex. [Colour figure can be viewed at wileyonlinelibrary.com].

Zwölfer, 1958). In Europe, the main primary host (*P. orientalis*) is rarely planted as an ornamental tree; therefore, most populations experience continuous asexual reproduction (Ravn *et al.*, 2013).

Adelges nordmannianae has also been introduced to North America; however, the invasion history of this species has been complicated by the difficulty in distinguishing it from *Ad. piceae*. For example, in eastern North America, Felt (1910) reported *Ad. piceae* on imported *Ab. nordmanniana* nursery stock. Kotinsky (1916) examined this same material and determined that it was 'probably' *Ad. nordmannianae*, but Balch (1952) later called Kotinsky's identification 'dubious'. McCambridge & Kowal (1957) reported that *Ad. nordmannianae* had been killing fir trees 'for some years' near Luray, Virginia but this was later determined to be *Ad. piceae* (Amman, 1962). In western North America, the presence of *Ad. nordmannianae* has been more definitive, with the first report on ornamental *Ab. alba* and *Ab. procera* Rehder in California (Annand, 1928). Balch (1952) later reported it in Vancouver, British Columbia, and Harris (1966) reported it in on imported *Ab. nordmanniana* in tree nurseries in Vancouver and Victoria, British Columbia, and at a private residence in Burnaby, British Columbia.

Two other species in the complex, *Ad. prelli* and *Ad. merkeri*, are holocyclic, with *Ab. nordmanniana* as a secondary host, and they are rarely found on *Ab. alba* (Francke-Grosmann, 1937; Pschorn-Walcher & Zwolfer, 1960; Eichhorn *et al.*, 1968). Because of their holocyclic life cycles, they are both considered native to the Caucasus Mountains and introduced to central Europe (Eichhorn, 1967, 1968) and Italy (Binazzi & Covassi, 1991). The morphology is only slightly different between these two species, but the phenology of their life cycles is described as being distinct (Eichhorn, 1967, 1975).

The final two species, *Ad. schneideri* and *Ad. nebrodensis*, are both anholocyclic and are morphologically similar to *Ad. nordmannianae* and *Ad. piceae*, respectively. *Adelges schneideri* is morphologically indistinguishable from *Ad. nordmannianae* (Steffan, 1972), but differs in that it tends to settle on trunks of fir trees, rather than on the branches, young shoots and needles (Pschorn-Walcher & Zwolfer, 1960; Steffan, 1972). *Adelges nebrodensis* is morphologically similar to *Ad. piceae* and is described from a single secondary host species, *Ab.*

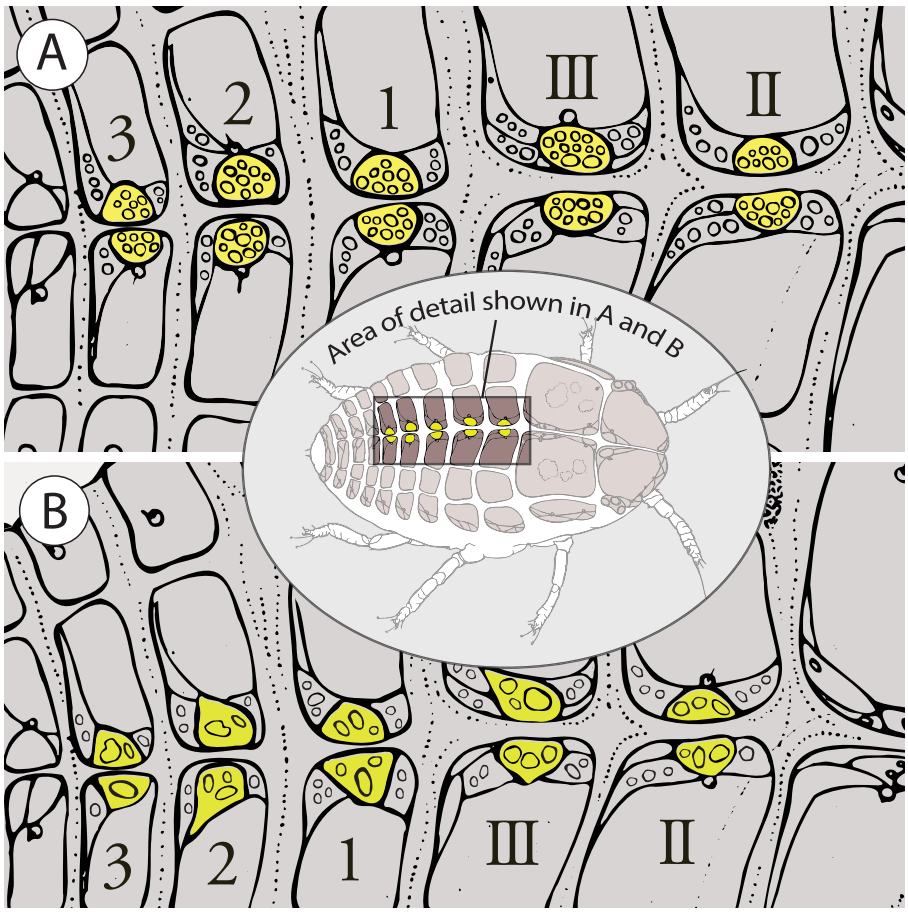

Fig. 2. The character (highlighted in yellow) that distinguishes species in the *Adelges* (*Dreyfusia*) *piceae* species complex: The number of facets on the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and meta-thorax and first three abdominal segments of first instar sistentes. (A) *Ad. nordmannianae*; (B) *Ad. piceae*. Some figure elements modified from Schneider-Orelli (1945). [Colour figure can be viewed at wileyonlinelibrary.com].

nebrodensis (Lojac.) Mattei, which exists as a relict group of fewer than 30 trees in northern Sicily, Italy (Binazzi & Covassi, 1991).

The complex life cycles of these six species result in the production of many morphological forms, but only the first instar sistens stage is consistently used for morphological species delimitation (Binazzi & Covassi, 1991; Blackman & Eastop, 1994). Taxonomic characters in other stages have been noted, but they are impractical for diagnostic purposes because they either broadly overlap between species or vary according to environmental and host plant factors. For example, the behaviour of settling on the needles, shoots, and twigs of younger trees, rather than the trunks of older trees, was used in the past to distinguish *Ad. nordmannianae* from *Ad. piceae* (e.g. Börner, 1908b). However, both behaviours were later observed in both species (Peirson & Gillespie, 1934; Schneider-Orelli, 1950; Balch, 1952; Eichhorn & Pschorn-Walcher, 1972). As another example, overall body size has been cited as a discriminating character (e.g. Binazzi & Covassi, 1991), but Eichhorn *et al.* (1968) found that size varies within species based on *Abies* host plant species, and whether they were feeding on twigs versus on stems.

The single character that has consistently been used to distinguish species is the number of facets in the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and meta-thorax and first three abdominal segments on first instar sistentes (Marchal, 1913; Schneider-Orelli *et al.*, 1929; Varty, 1956; Eichhorn, 1967; Steffan, 1972; Binazzi & Covassi, 1991; Fig. 2). Note, that first instar progredientes lack wax gland facets on the spinal sclerites, so cannot be used for species determination (Marchal, 1913). A complication with using the number of sistens wax facets is that they are variable between individuals and can overlap among species, so determination involves calculating a mean from numerous individuals at a locality (Varty, 1956; Eichhorn, 1967; Steffan, 1972; Binazzi & Covassi, 1991; Table 1). This makes species determination exceedingly difficult both in terms of the need to collect large numbers of individuals of the correct life stage and in the labour required to slide mount and score them. It also confounds identification of groups that might contain multiple species.

Table 1. Behavioural and morphological characters used to distinguish the species in the *Adelges* (*Dreyfusia*) *piceae* species complex. The number of facets on the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and meta-thorax and first three abdominal segments of first instar sistens nymphs, averaged across multiple individuals. Different columns show values from different sources.

		Mean number of facets (range in parentheses)		
Species	Life cycle	Eichhorn (1967)	Steffan (1972)	Binazzi & Covassi (1991)
Adelges piceae	Anholocyclic	26.5 (18–42)	27 (18–42)	26 (18–41)
Adelges nebrodensis	Anholocyclic	N/A	N/A	33 (26–42)
Adelges prelli	Holocyclic	34.4 (19–61)	29 (21–47)	34 (23–52)
Adelges merkeri	Holocyclic	46.4 (23–79)	46 (23–79)	41 (26–57)
Adelges schneideri	Anholocyclic	N/A	73 (57–104)	N/A
Adelges nordmannianae	Holocyclic	79.4 (57–104)	79 (72–94)	80 (62–101)

For many other taxonomic groups, DNA data have illuminated species boundaries where morphological and behavioural differences were unclear. Unfortunately, this has not been the case in the *Ad. piceae* species complex. Gene regions that have been explored include the mitochondrial cytochrome c oxidase I (COI) DNA barcoding region (Foottit *et al.*, 2009; Žurovcová *et al.*, 2010; Ravn *et al.*, 2013; Havelka *et al.*, 2020), combined mitochondrial cytochrome b and NDH4 sequences (Mantovani *et al.*, 2001), and the nuclear elongation-factor 1 alpha gene (Havill *et al.*, 2007; Žurovcová *et al.*, 2010; Havelka *et al.*, 2020). While these regions have successfully been used to uncover variation among other closely related insect species (e.g. Cho *et al.*, 1995; Hebert *et al.*, 2004), they likely lack the resolution required for actively speciating insects (Funk & Omland, 2003). Therefore, to explore the extent of genetic differentiation between species in the *Ad. piceae* species complex, reconstruct their biogeographic and invasion histories, and offer clarity to their taxonomy, we developed 19 new microsatellite markers, compared the genotype results to DNA barcode sequences, and performed morphological examinations.

Methods

Insect sampling and DNA extraction

Adelgid samples were collected from 160 locations in the Caucasus Mountains, Europe, and North America between 2002 and 2019 (File S1; Figs 3 and 4). Adelgids were separated from host plant material and preserved in 95% ethanol and stored at −80°C until they were processed. Host plant species were determined by the collectors, and if there was adequate host material with the sample, were confirmed by us using morphological characters (Farjon, 2010). For trees growing in arboreta, host plant accession numbers were recorded and this information was included with the adelgid vouchers. For sampling locations in the Caucasus Mountains (N = 22), where *P. orientalis* naturally occurs, the likelihood of sexual reproduction and clonal diversity is high, so we genotyped more individuals per location (range = 1–16). All samples from the Caucasus Mountains were collected from *Ab. nordmanniana*. For sampling locations in Europe (N = 32) and North America (N = 94), where *P. orientalis* is planted only rarely as an ornamental, the likelihood of sexual reproduction is lower, so we genotyped fewer individuals per location (range = 1–4). Most samples from Europe and North America were collected from *Abies* spp. hosts. Exceptions were: (i) at the CABI Centre in *Delémont*, Switzerland, where we genotyped 16 individuals collected on *Ab. alba*, and one individual from a gall on *P. orientalis*; and (ii) at Arnold Arboretum in Jamaica Plain, Massachusetts, where we genotyped five individuals from *Abies* spp. and 39 individuals from several galls on *P. orientalis*.

DNA was extracted from individual adult adelgids using the Promega DNA IQ kit (Promega, Madison, WI, U.S.A.) or the Mag-Bind Blood and Tissue Kit (Omega Bio-Tek, Norcross, GA, U.S.A.) using the manufacturer's protocol. For most samples collected prior to 2018, DNA was destructively extracted. For samples collected in 2018 and 2019, each adelgid was pierced with a sterile insect pin, and the cuticle was retained after the proteinase K incubation step and slide mounted. For all collections, additional insects were slide mounted as vouchers, targeting first-instar sistens nymphs for morphological analysis. Specimens were cleared with 10% potassium hydroxide or proteinase K, then mounted in Canada balsam and deposited at the Canadian National Collection (CNC) of Insects, Arachnids and Nematodes, or the Yale Peabody Museum (YPM) of Natural History. Accession numbers are indicated in Supplementary File S1.

Microsatellite development

Genomic reads for microsatellite enrichment were generated from 50 pooled adult *Ad. piceae* individuals collected in December 2011 in Fort Bragg, California on *Ab. grandis* Douglas. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen; Germantown, MD, U.S.A.). The genomic library was prepared with Ion Xpress Plus Library and PGM Sequencing 400 kits (Thermo Fisher; Waltham, MA, U.S.A.) at the Functional Genomics Laboratory at the University of California Berkeley. Sequencing was performed using an Ion 318 sequencing chip at Yale University's DNA Analysis Facility on Science Hill. The raw sequence reads are available from NCBI BioProject PRJNA625730.

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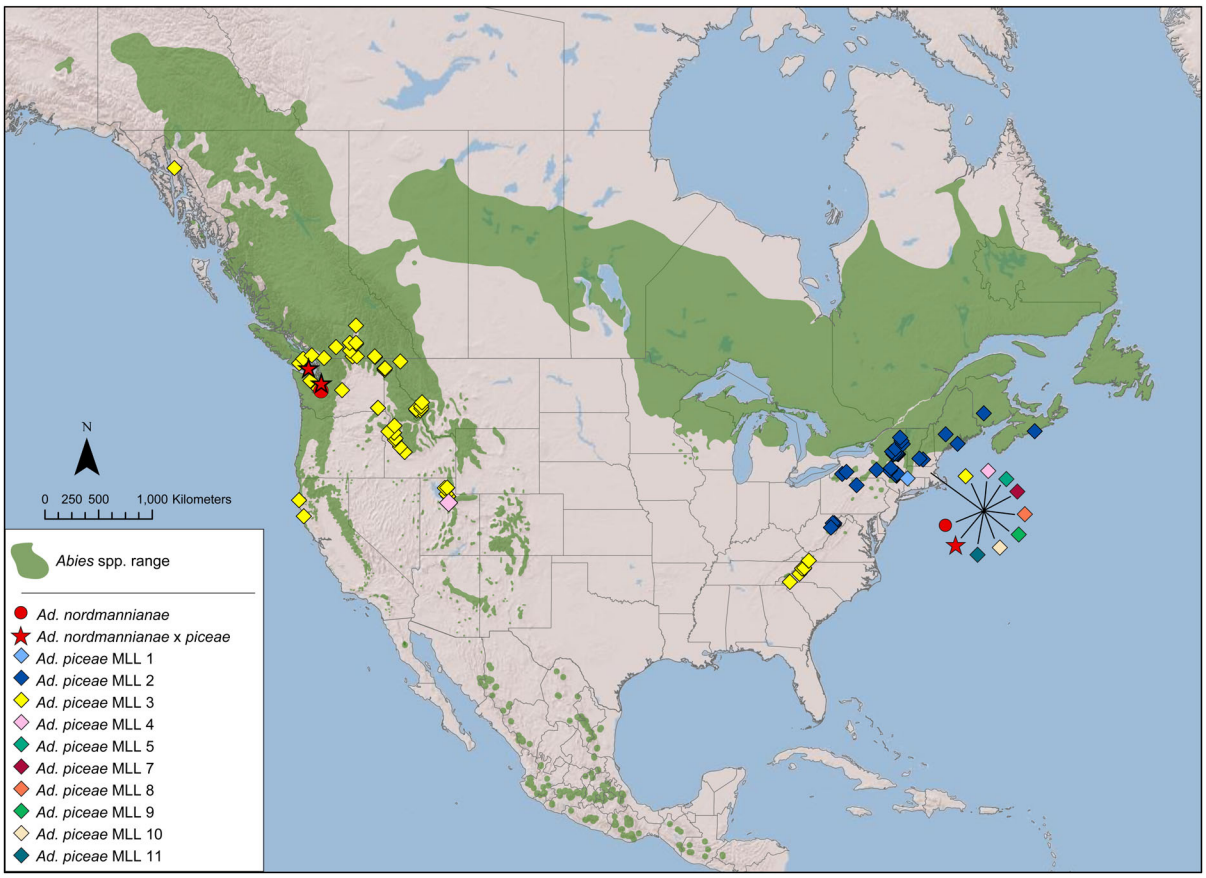

Fig. 3. *Adelges* (*Dreyfusia*) collection locations in North America. The geographic range of *Abies* spp. hosts, shown in green, is from Little (1971), Alizoti *et al.* (2011), and Jaramillo Correa (2018). Adelgid species are indicated with different shapes and multilocus lineages (MLLs) within the predominantly asexual species, *Ad. piceae*, are indicated with different colours to show clonal spread. Different MLLs within *Ad. normannianae* and hybrids are not shown. [Colour figure can be viewed at wileyonlinelibrary.com].

Microsatellite discovery and primer design from genomic reads were performed using QDD 3.1.2 (Meglecz *et al.*, 2014) with default parameters. The resulting loci were filtered to select those with pure (i.e., not compound) microsatellites and at least eight motif repeats. Primer pairs for 40 arbitrarily selected loci were first assessed for amplification success in a test panel of four *Ad. piceae* individuals from: (i) Delémont, Switzerland; (ii) Mt. Mitchell State Park, North Carolina, U.S.A.; (iii) Olympic National Park, Washington, U.S.A.; and (iv) Priest River, Idaho, U.S.A. It was not possible to test for deviation from Hardy–Weinberg equilibrium or linkage disequilibrium, as is often done when developing microsatellite markers, because these samples were from asexual populations. Reverse primers were modified with a 5′ GTTT 'pig-tail' to promote complete adenylation during polymerase chain reaction (PCR) and reduce stutter (Brownstein *et al.*, 1996). Forward primers were modified with a 5′ TCCCAGTCAC-GACGT M13 tail to allow incorporation of an M13 oligo labelled with 6-FAM (Schuelke, 2000). PCRs were performed in 10 μL volumes containing: 1X PCR Buffer, 1.0 μL dNTPs (10 mM each; New England Biolabs, Ipswich, MA, U.S.A.), 0.8 μL MgCl2 (25 mM), 0.025 μL of forward primer (10 mM), 0.25 μL of reverse primer (10 mM), 0.05 μL of 6-FAM labelled M13 primer (100 mM; Thermo Fisher), 0.10 μL Go Taq DNA polymerase (Promega, Madison, WI) and 1.0 μL template DNA. A touchdown thermocycler program was used: 95°C for 2 min (1 cycle), 95°C for 45 s, 61°C decreasing 2°C for each cycle for 30 s, and 72°C for 45 s (5 cycles), 95°C for 45 s, 51°C for 30 s, and 72°C for 45 s (30 cycles), and final extension of 72°C for 2 min (1 cycle). For these, and all subsequent microsatellite analyses, PCR products were combined with a LIZ 500 internal size standard (Gel Company; San Francisco, CA, U.S.A.) and run on an ABI 3730 sequencer (Thermo Fisher) at Yale University's DNA Analysis Facility on Science Hill. Alleles were scored using the microsatellite plugin in Geneious 10.0.5 (Kearse *et al.*, 2012).

Population genetic structure

For each adelgid, the 19 loci were amplified individually in 12.5 μL volumes containing: 1X PCR Buffer, 1.25 μL dNTPs (10 mM each), 1.0 μL MgCl2 (25 mM), 0.25 μL of

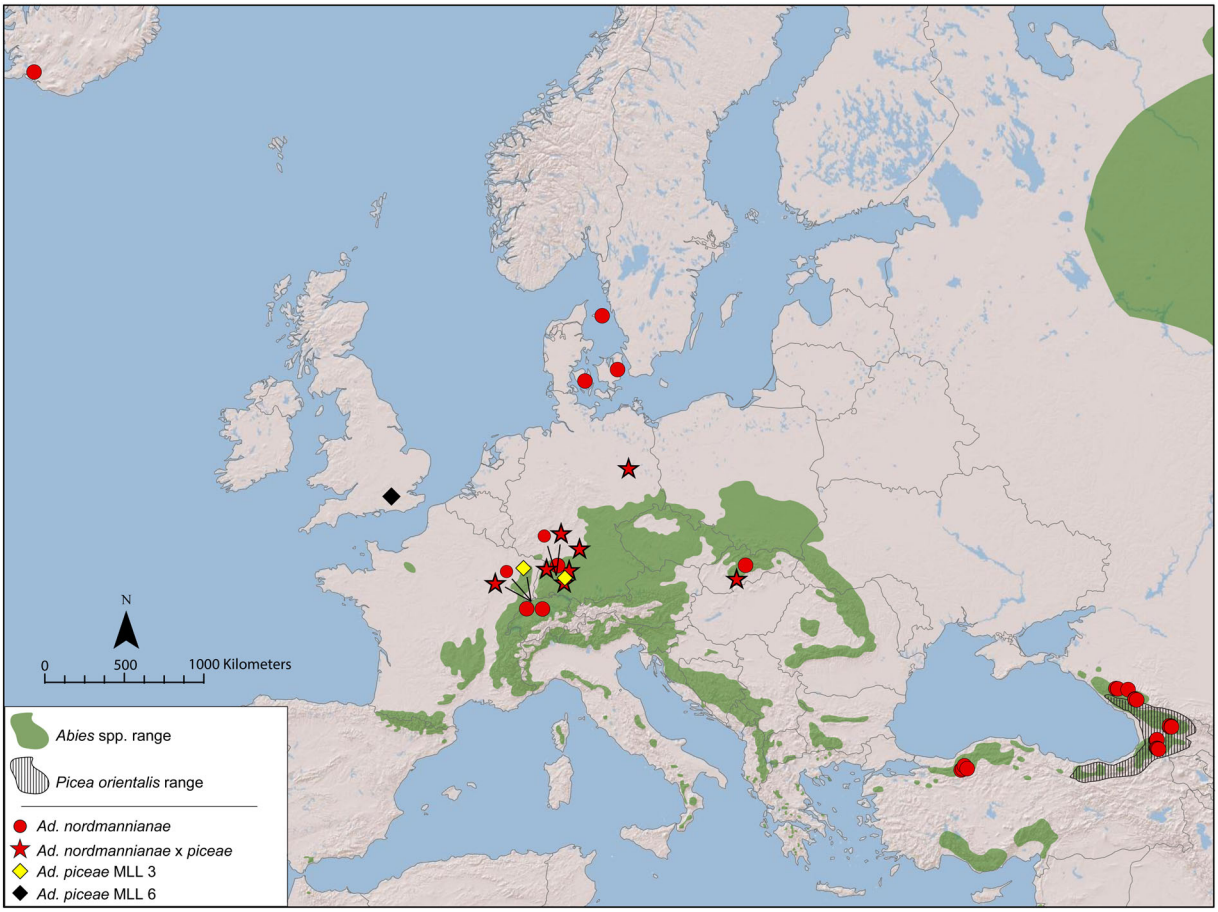

Fig. 4. *Adelges* (*Dreyfusia*) collection locations in Europe and the Caucasus Mountains. The geographic range of *Abies* spp. hosts, shown in green, is from Malyshev (2008) and Caudullo *et al.* (2017). The geographic range of *Picea orientalis* in the Caucasus Mountains, shown with hatch marks, is from Kayacik (1955). Adelgid species are indicated with different shapes and *Ad. piceae* clones are indicated with different colors. Different MLLs within *Ad. normannianae* and hybrids are not shown. [Colour figure can be viewed at wileyonlinelibrary.com].

dye-labeled forward primer (10 mM), 0.25 µL of reverse primer (10 mM), 0.10 µL Go Taq G2 DNA polymerase, and 1.0 µL template DNA. Forward primers were directly dye-labelled with 6-FAM, NED, PET, or VIC (Thermo Fisher), and reverse primers were modified with a 5′ GTTT 'pig-tail'. PCRs were performed using the same touchdown thermocycler protocol as above. For fragment analysis, equal volumes of each PCR product were combined into groups of 4 or 5 loci, separated by different dyes and/or allelic size ranges (Table S1).

Only genotypes with 17 or more successfully scored loci were used for analyses. These genotype data are provided in Supplementary File S1. Microsatellite genotypes were assigned to clonal multilocus lineages (MLLs; after Arnaud-Haond *et al.*, 2007) using GenoDive v 2.0b25 (Meirmans & Van Tiernderen, 2004) with an infinite allele model. Species with a mix of sexual and asexual individuals are expected to have genotype frequencies with a multimodal distribution, such that the peak nearest zero is due to clonal individuals and scoring errors, while successive peaks could represent somatic mutations, sibling crosses, or population structure (Douhovnikoff & Dodd, 2003; Meirmans & Van Tiernderen, 2004). Therefore, based on genotype frequency plot (Fig. S1), a threshold value of two steps was chosen for assigning MLL membership.

To explore clustering among genotypes, discriminant analysis of principal components (DAPC; Jombart *et al.*, 2010) was performed using Adegenet v 1.3.9 (Jombart, 2008) in R 3.5.0 (R Core Team, 2014) with one genotype per unique MLL included in the data set. Forty principal components and three discriminant functions were retained for this analysis. Arlequin 3.5.1.3 (Excoffier *et al.*, 2005) was used to calculate the mean number of alleles per locus, expected and observed heterozygosity, perform exact tests for Hardy-Weinburg equilibrium (HWE), and linkage disequilibrium (LD) within the resulting clusters, and to calculate genetic differentiation (F_{ST}) among clusters using the infinite allele model and 1000 permutations. For HWE and LD comparisons, the potential for false positives was accounted for using the method of Benjamini & Hochberg (1995) with a false discovery rate of 0.05.

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Testing for hybridisation

Preliminary results suggested that individuals assigned to one of the clusters recovered from the DAPC analysis could be hybrids between two other recovered clusters. Therefore, to evaluate this possibility, we calculated the probability of assignment of each MLL as a pure species, F1 hybrid, F2 hybrid, or a backcross, using NewHybrids 1.1 (Anderson & Thompson, 2002) with 10 000 burn-in iterations followed by 100 000 sample iterations. In addition, we simulated thirty F1 hybrid genotypes using the genotypes from DAPC Cluster 1 and DAPC Cluster 4, as *Ad. nordmannianae* and *Ad. piceae* parent populations, respectively, using HYBRIDLAB 1.0 (Nielsen *et al.*, 2006). We chose Cluster 1 as the parent population for *Ad. nordmannianae* because it included individuals collected in Europe and North America (see Results, below), and would therefore be more likely to represent the parents of hybrids in these regions. The simulated hybrids were then included in an additional DAPC analysis to test whether the they clustered with the putative field-collected hybrids.

Morphology

First instar sistentes, which were collected from the same tree as genotyped adults, were slide mounted and examined for morphological differences. Specifically, the number of facets in the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and meta-thorax and first three abdominal segments were counted (Fig. 2). Difference in facet counts among associated DAPC clusters was analysed as one-way analysis of variance (ANOVA) using R. Residuals were first examined for normality and facet count data were log transformed to achieve homogeneity of variances. Pairwise differences among means were tested using Tukey's HSD with a critical value of 0.05.

DNA barcoding

For most of the adelgid individuals that we genotyped, we also sequenced the 651 base-pair DNA barcoding portion of the mitochondrial cytochrome oxidase subunit I (COI) gene using primers LepF1 and LepR1 (Hebert *et al.*, 2004), using standard protocols (de Waard *et al.*, 2008). Sequencing was performed at the Yale University DNA Analysis Facility on Science Hill on an ABI 3730 sequencer. All sequences generated for this study were deposited in GenBank with accession numbers indicated in File S1. A network of relationships among COI haplotypes was reconstructed with the parsimony method of Templeton *et al.* (1992) using TCS v 1.21 (Clement *et al.*, 2000) with a 95% confidence limit.

Testing historical scenarios

We used the software diyABC v 2.0 (Cornuet *et al.*, 2014) to perform approximate Bayesian computation (ABC; Beaumont *et al.*, 2002) to test which historical scenario of divergence and hybridisation best fits our microsatellite and COI sequence data. Since DiyABC assumes sexual reproduction in each generation (Cornuet *et al.*, 2010), we included a single individual per unique microsatellite MLL to focus on the sexual generation in the life cycle. We compared three scenarios (Fig. 5) using the four clusters recovered from DAPC analysis (see Results, below). Scenario 1 hypothesized that DAPC Cluster 3 resulted from hybridisation between *Ad. piceae* (DAPC Cluster 4) and the *Ad. nordmannianae* population present in Europe and North America (DAPC Cluster 1). Scenarios 2 and 3 hypothesized two different direct divergence origins for Cluster 3. Prior distributions for microsatellite and COI mutation rates and demographic parameters are presented in Table S2. A reference table of 3 000 000 simulated data sets was generated. Summary statistics that we chose to compare simulated and observed data sets were: for microsatellites, mean number of alleles (one-sample), mean genetic diversity (one-sample), mean size variance (one-sample), and F_{ST} (two-sample); for mitochondrial sequences, number of haplotypes (one- and two-sample), number of segregating sites (one- and two-sample), and mean within-sample pairwise differences (one-sample). The fit of each scenario to the empirical data was compared using the direct and the logistic regression tests in DiyABC (Cornuet *et al.*, 2010). Confidence in model choice (fit of simulated to observed data) and confidence in scenario choice (type I and type II error rates) were evaluated using the analyses provided in DiyABC.

Results

Microsatellite development

Genomic sequencing for microsatellite discovery resulted in 4 737 354 reads with median length of 384-bp, of which, 166 785 contained microsatellite repeats. Of these, 11 586 occurred a single time in the library and the rest were aligned into 28 314 unique consensus sequences. From these, primer design was possible for 24 989 putative loci, and strict filtering retained 144 loci. Of the 40 loci that were selected for preliminary testing, 19 produced strong amplification products and were polymorphic among the four test panel individuals. These 19 were used for this study. These loci included three with tri-nucleotide and 16 with di-nucleotide repeat motifs. Primer sequences and repeat motifs are shown in Table S1.

Population genetic structure

Microsatellite genotypes were generated for 375 individuals, assigned to 94 different clonal MLLs. The Caucasus Mountains region, where sexual reproduction is most likely to occur and where we genotyped more individuals per location, had the highest clonal diversity, with 55 unique MLLs. In North America, where sexual reproduction is less likely, there were 17 unique MLLs. Two of these, which were determined to be *Ad. piceae* (described below), were geographically widespread

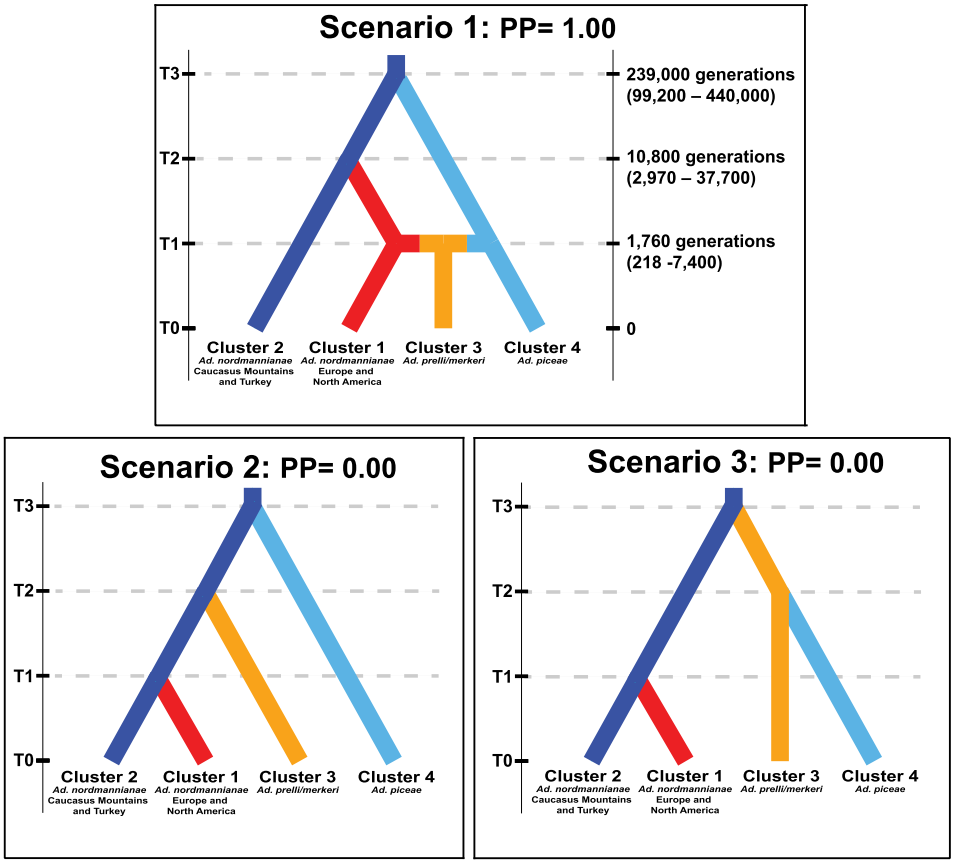

Fig. 5. Scenarios of lineage divergence and hybridisation for the *Adelges* (*Dreyfusia*) *piceae* species complex, tested with approximate Bayesian computation (ABC) using microsatellite and mitochondrial COI sequence data. Posterior probabilities (PP) are shown for each scenario. Scenario 1 had the best fit. [Colour figure can be viewed at wileyonlinelibrary.com].

(Fig. 3). *Adelges piceae* MLL 2 was found throughout north-eastern North America, and *Adelges piceae* MLL 3 was found throughout the southern Appalachian region and western North America (Fig. 3). The latter MLL was also found in Europe, in Switzerland and Germany (Fig. 4).

DAPC analysis of all unique MLLs separated the genotypes into four clusters (Figs 6 and S2). All MLLs were assigned to clusters with high probabilities ($P > 0.96$). Cluster 1 contained 31 MLLs (88 individuals), Cluster 2 contained 41 MLLs (118 individuals), Cluster 3 contained 11 MLLs (31 individuals), and Cluster 4 contained 11 MLLs (138 individuals). Cluster 1 included samples collected in the Caucasus Mountains, Europe, and North America. Cluster 2 included samples collected only in the Caucasus Mountains and northern Turkey. All the samples from a small area in northern Turkey, outside the natural range of the primary host, were assigned to a single MLL. Clusters 3 and 4 included samples found only in Europe and North America. Cluster 3 was intermediate along the major principal component axis, between Clusters 1 + 2 and Cluster 4. Samples collected from galls on *P. orientalis*, from a generation following the sexuales (Fig. 1), fell into Clusters 1, 3 and 4, indicating that individuals in these clusters are all capable of sexual reproduction.

DAPC Cluster 2 had the highest allelic diversity with a mean of 6.2 alleles per locus, followed by Cluster 1 with 4.2, Cluster 4 with 3.6, and Cluster 3 with 3.5 alleles per locus. None of the loci in Clusters 3 or 4 deviated from HWE. Two loci in Cluster 1 (BWA01007 and BWA00227), and one locus in Cluster 2 (BWA00815) deviated from HWE after controlling for false discovery rate. One of these (BWA00227) had observed heterozygosity lower than expected, suggesting the presence of null alleles or a Wahlund effect due to population subdivision within clusters, and the other two (BWA01007 and BWA00815) had observed heterozygosity higher than expected, which could signal of the mixing of previously isolated populations. Out of the 171 pairwise combinations of loci for each cluster tested for linkage disequilibrium, only Cluster 3 had a pair of loci (BWA03753 and BWA01451) that were significantly linked. The few incidences of deviation from HWE and LD show that our data are suitable for downstream analyses.

Genetic differentiation among DAPC clusters, measured with F_{ST}, mirrored their placement along the major principal component axis (Fig. 6). Clusters 1 and 2 were the least differentiated (F_{ST} = 0.071) from each other, while Clusters 1 and 2 were most differentiated from Cluster 4 (F_{ST} = 0.386 and 0.349, respectively). Cluster 3 was intermediate between Clusters 1

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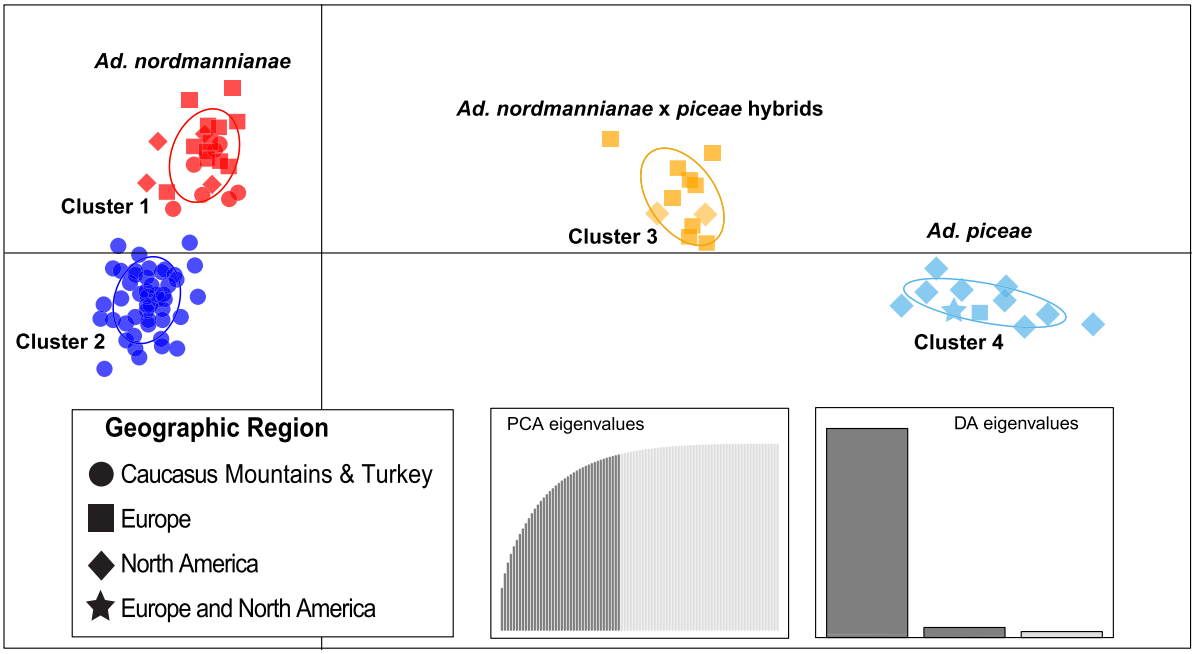

Fig. 6. Scatterplot showing the first two principal components from discriminant analysis of principal components (DAPC) of all unique microsatellite MLLs in the *Adelges* (*Dreyfusia*) *piceae* species complex. The geographic region from which the MLLs were sampled are indicated by different symbols. Clusters 1 and 2 included samples that were morphologically *Ad. nordmannianae*, Cluster 4 includes samples that were morphologically *Ad. piceae*, and Cluster 3 had intermediate morphology (Fig. 3), and were shown to be hybrids between *Ad. nordmannianae* and *Ad. piceae* (Fig. 7). Left inset is the cumulative variance explained by the eigenvalues of the principal components analysis (PCA) and right inset shown the eigenvalues for the discriminant analysis (DA). [Colour figure can be viewed at wileyonlinelibrary.com].

and 2 (F_{ST} = 0.164 and 0.186, respectively), and Cluster 4 (F_{ST} = 0.154).

Tests for hybridisation

NewHybrids analysis found that all the MLLs in DAPC Clusters 1 and 2 were classified as pure *Ad. nordmannianae* (species determined with morphology, described below) with high probability ($P > 0.99$) (Fig. S3). Of the 11 MLLs that were assigned to Cluster 4, seven were classified as pure *Ad. piceae* (species determined with morphology, described below) with high probability ($P > 0.99$), and the remaining four MLLs in Cluster 4 were classified as *Ad. piceae* backcrosses (P = 0.57, 0.82, 0.93, and 0.99). These backcrosses were all collected at Arnold Arboretum in Massachusetts, U.S.A. All 11 MLLs that were assigned to Cluster 3 were classified as hybrids. Seven were classified as F1 hybrids ($P > 0.96$), one was classified as an F2 hybrid (P = 0.65), and three were classified as *Ad. piceae* backcrosses ($P > 0.93$). One of these backcrosses was collected in Ziesar, Germany and the rest at Mlynany Arboretum, Slovakia. The F2 hybrid was also collected at Mlynany Arboretum.

DAPC analysis with field-collected *Ad. nordmannianae*, *Ad. piceae*, and putative hybrid MLLs, plus the simulated F1 hybrid genotypes (Fig. 7), resulted in a distinct cluster that consisted of all the putative hybrid MLLs assigned to Cluster 3 in the original DAPC (Fig. 6), with all the simulated F1 hybrid genotypes, and a single *Ad. nordmannianae* MLL from a sample collected in the Caucasus Mountains (File S1: MLL 84).

Morphology

The mean number of facets on first instar sistentes associated with each DAPC cluster (Fig. 6) were: Cluster 1 = 79.6 (n = 85, se = 0.86, range 64–101), Cluster 2 = 78.1 (n = 29, se = 1.80, range 61–101), Cluster 3 = 41.23 (n = 51, se = 2.14, range 21–88), and Cluster 4 = 28.0 (n = 146, se = 0.35, range 20–39) (Fig. 8). ANOVA indicated significant differences among Clusters ($P < 0.001$ df = 3, F = 714.1). Pairwise mean separations showed no differentiation between Clusters 1 and 2 ($P > 0.05$), but Clusters 1 and 2 were distinct from Cluster 3 and Cluster 4, which were distinct from each other. Based on existing species definitions (see Table 1), Clusters 1 and 2 are consistent with *Ad. nordmannianae*, Cluster 3 with *Ad. prelli* and *Ad. merkeri*, and Cluster 4 with *Ad. piceae*.

First instar sistentes associated with DAPC Cluster 3 included 8 individuals that were potential outliers (more than 1.5× the distance between the first and third quartiles), shown as bold points in Fig. 8. These data points came from just two of the 11 localities that had genotypes assigned to

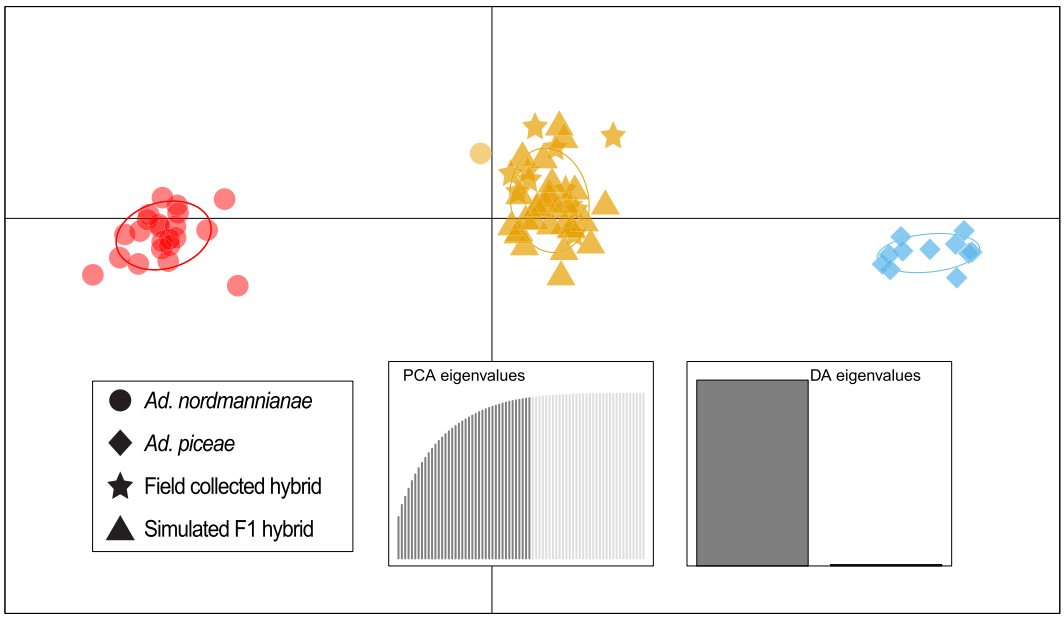

Fig. 7. Discriminant analysis of principal components (DAPC) showing clustering of field collected *Adelges* (*Dreyfusia*) MLLs with simulated F1 hybrids. Circles are *Ad. nordmannianae* MLLs that were assigned to Cluster 1 in Fig. 2, diamonds are *Ad. piceae* MLLs assigned to Cluster 4 in Fig. 2, stars are field-collected putative hybrid MLLs assigned to Cluster 3 in Fig. 2, and triangles are simulated F1 hybrid genotypes. Different colours represent different DAPC clusters resulting from this analysis. Field-collected and simulated F1 hybrids form a distinct cluster, with the exception of one *Ad. nordmannianae* MLL. Left inset is the cumulative variance explained by the eigenvalues of the principal components analysis (PCA) and right inset shown the eigenvalues for the discriminant analysis (DA). [Colour figure can be viewed at wileyonlinelibrary.com].

Cluster 3: (i) five individuals from Washington Park Arboretum, Seattle, WA, U.S.A. on *Abies* × *insignis* Carr. ex Bailly (= *Ab. nordmanniana* × *Ab. pinsapo* Boiss.); and (ii) three individuals from Mlynany Arboretum, Vieska nad Žitavou, Slovakia on *Abies balsamea* (L.) Miller. The gap of ten facets from these outliers to the rest of the points associated with Cluster 3, and their having similar values as those determined as *Ad. nordmannianae* (Clusters 1 and 2), suggest that these samples could consist of both hybrids and *Ad. nordmannianae*.

DNA barcoding

Sequences from the DNA barcode region of COI were obtained from 326 of the 375 adult adelgids that were genotyped with microsatellites. There were no indels or stop codons when translated to amino acids, suggesting that they were true mitochondrial DNA, not nuclear pseudogenes. Sequences could be assigned to 20 closely related haplotypes, with a maximum sequence divergence of 1.1% (six steps) between them (Fig. 9). Haplotype diversity was greatest in the Caucasus Mountains (n = 13), compared to Europe (n = 6) and North America (n = 7), and higher divergence was observed within *Ad. nordmannianae* in the Caucasus Mountains than between *Ad. nordmannianae* and *Ad. piceae*. Haplotypes did not cluster by species, with the most common haplotype (Haplotype 1) being recovered from samples identified as *Ad. nordmannianae*, *Ad. piceae*, and hybrids.

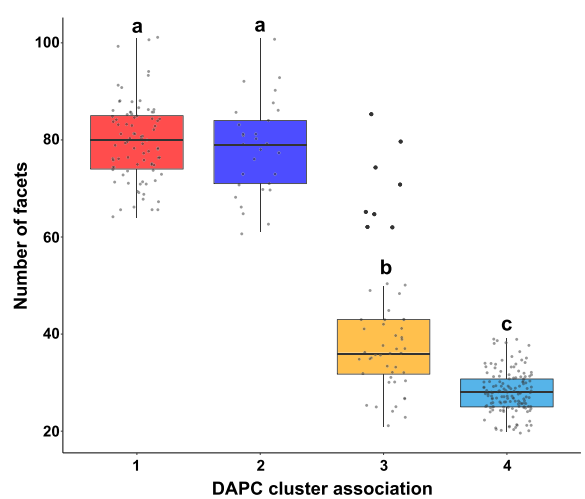

Fig. 8. Difference in facet counts (see Fig. 2) among first instar sistens adelgids sampled from the same tree as adults genotyped with microsatellite loci. DAPC cluster membership corresponds with those shown in Fig. 7. Boxes represent the first and third quartiles, and whiskers extend to 1.5× the distance between the first and third quartiles. Outlying points beyond the end of the whiskers are bold. [Colour figure can be viewed at wileyonlinelibrary.com].

Testing historical scenarios

The most likely scenario to explain the relationships among lineages in the *Adelges* (*Dreyfusia*) *piceae* species complex

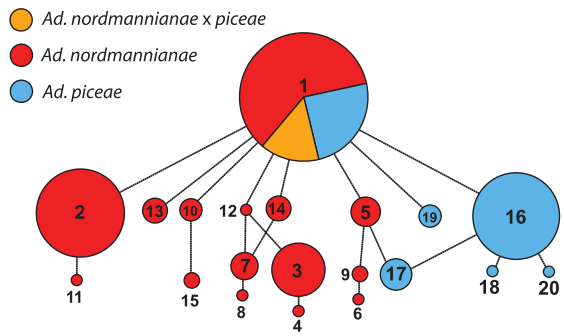

Fig. 9. Network of mitochondrial COI DNA barcode sequences from the *Adelges* (*Dreyfusia*) *piceae* species complex. Haplotype circles are coloured to indicate the proportion of individuals with that haplotype identified as a pure species or hybrid. The area of each circle is proportional to the number of individuals with that haplotype. [Colour figure can be viewed at wileyonlinelibrary.com].

(Fig. 5; Scenario 1) included an initial split between DAPC Clusters 1 + 2 (*Ad. nordmannianae*) and Cluster 4 (*Ad. piceae*), followed by a split between Cluster 1 (*Ad. nordmannianae* in the Caucasus Mountains, Europe, and North America) and Cluster 2 (*Ad. nordmannianae* in the Caucasus Mountains and Turkey), then hybridisation between Cluster 1 and Cluster 4 (*Ad. piceae*), producing Cluster 3 (*Ad. prelli* and *merkeri*). The posterior probability for Scenario 1 was 0.812 using the direct estimate method and 1.00 using the logistic regression method. Scenarios 2 and 3 had posterior probabilities of 0.062 and 0.126 for direct and 0.00 and 0.00 for logistic methods, respectively. A plot for evaluating model choice showed that the summary statistics generated using the simulated reference table were a good approximation of the observed data (Fig. S4). The Type I error rate for Scenario 1 (the probability of being rejected although it is the true scenario) was 0.074, and the Type II error rate (probability of accepting Scenario 1 when it is not the true scenario) was 0.031. The mutation rates estimated for the best-fit scenario were 2.30×10^{-6} per generation for microsatellites and 3.15×10^{-8} per generation for COI sequences. These values are within the ranges estimated for other insects (Zhang & Hewitt, 2003; Papadopoulou *et al.*, 2010). Posterior distributions of split and merge times, measured in generations, are shown in Fig. S5 and the median values for merge times are indicated in Fig. 5.

Discussion

Species delimitation between parthenogenetic and cyclically parthenogenetic lineages poses a challenge because it can be difficult to recognize meaningful morphological, genetic or biological discontinuities (Foottit, 1997). Delimitation of species that arise via hybridisation presents a similar challenge, because it requires evidence that the cluster of hybrid genotypes remains stable over time, even while in contact with the parent species (Mallot 2007). Practically speaking, if there is evidence that a parthenogenetic or hybrid lineage has experienced a unique and sustained evolutionary trajectory, that it occupies a different niche (e.g. different host plant preferences, phenology, etc.), and has clearly discernable characters to distinguish it, then it could be described as a separate species (Foottit, 1997). Species delimitation should therefore ideally be anchored by a robust reconstruction of evolutionary history and be based on reproducible biological and morphological differences.

Biogeographic history

In Adelgidae, anholocyclic lineages that are derived from holocyclic ancestors are sometimes called 'agamospecies' (Steffan, 1961; Foottit, 1997). The process of agamospecies formation in adelgids is hypothesised to have occurred during periods of rapid climate change, such as glacial periods, when primary and secondary host plants migrate at different rates or occupy separate glacial refugia, therefore, separating the hosts required to complete the holocycle (Mordvilko, 1923; Steffan, 1961; Havill *et al.*, 2016). Formation of agamospecies could also be initiated in the more recent past when holocyclic adelgids are transported by humans to a region where some novel hosts are suitable, but others are not (Steffan, 1961, 1964; Havill & Foottit, 2007; Havill *et al.*, 2016). The history of the *Ad. piceae* species complex was probably shaped by both of these processes.

Other authors have suggested that the ancestor of the *Ad. piceae* species complex likely originated in the Caucasus Mountains since the main primary host species, *Picea orientalis*, is endemic to this region (e.g. Varty, 1956; Eichhorn, 1967). Our results provide further evidence of this because there is higher diversity of microsatellite alleles and MLLs (Fig. 6), and COI haplotypes (Fig. 9) in this region, compared to Europe or North America. In addition, the effective population size of *Ad. nordmannianae* in the Caucasus Mountains and Turkey was estimated by ABC analysis to be an order of magnitude higher than for the other lineages (Table S2). Evolutionary theory also predicts that the ancestor is likely to have been holocyclic because anholoyclic lineages are not expected to persist in the long term because of a high extinction rate (Judson & Normark, 1996), caused by their inability to efficiently eliminate accumulated deleterious mutations (Muller, 1964) or effectively adapt to fend off antagonists (Kondrashov, 1993).

The ancestor of the *Ad. piceae* species complex might have arrived in the Caucasus region with the ancestor of its primary host, *P. orientalis*. Shao *et al.* (2019) hypothesized that the ancestors of *P. orientalis*, and other members of its clade of mostly east Asian spruce species, migrated south during the late Neogene (12–3 Ma), and the ancestor of *P. orientalis* survived in a Caucasus refugium while related *Picea* species (and perhaps their associated adelgids) survived in other refugia such as the Himalaya-Hengduan Mountains. Consistent with this scenario, the other species in *Adelges* (*Dreyfusia*) are endemic to the Himalayan region (Yaseen & Ghani, 1971), and Japan (Inouye, 1953).

The results of our ABC analysis (Fig. 5) show that the most likely historical scenario to explain relationships in this species complex involved an initial split between *Ad. nordmannianae*

and *Ad. piceae*, followed by a split within *Ad. nordmannianae*, followed by hybridisation between *Ad. nordmannianae* and *Ad. piceae*. The shallow differences among COI haplotypes that we observed (Fig. 9), consistent with other studies (Foottit *et al.*, 2009; Žurovcová *et al.*, 2010; Ravn *et al.*, 2013; Havelka *et al.*, 2020), strongly suggests that the initial split was recent. In our ABC analysis, this split was estimated to have occurred 239 000 sexual generations ago, with a 90% confidence limit of 99 200–440 000 generations (Table S2). However, this is difficult to accurately translate into years since the analysis assumes sexual reproduction each generation (Cornuet *et al.*, 2010) which is not the case for adelgids. We could place a cautious upper limit on the split time by using the highest rate of sexual reproduction that would occur in the species complex, for example, in *Ad. nordmannianae* in the Caucasus Mountains, where the primary host is most abundant. In this case, we would divide the number of generations by 2 years because there is a sexual generation every 2 years in the life cycle (Fig. 1). This would place an upper limit on the split of around 119 500 years ago, with a 90% confidence interval of 49 600 – 220 000.

Given this upper limit, the split between *Ad. nordmannianae* and *Ad. piceae* could be associated with one of the recent mild interglacial periods, MIS 5 (130–70 kya), or MIS 3 (60–27 kya) (Helmens, 2014). During these periods, the geographic ranges of *Abies* species expanded and they migrated to lower elevations in the Caucasus Mountains (Pakhomov, 2006) and the Balkan Peninsula (Liepelt *et al.*, 2009), possibly causing different lineages to come in contact with each other in the Mediterranean region, facilitating gene flow (Linares, 2011; Hrivnák *et al.*, 2017; Balao *et al.*, 2020). This contact could have allowed a bridge between *Abies* hosts in the Caucasus Mountains and Europe, thus initiating the European lineage that became *Ad. piceae*.

When this lineage moved into Europe, where it was isolated from suitable primary hosts, it probably started to lose the ability to form sexuparae which would be a reproductive dead end (Havill & Foottit, 2007). *Adelges piceae* rarely produces sexuparae and the subsequent generations, but our evidence of sexually reproducing *Ad. piceae* and hybrids in both Europe and North America show that a latent ability to do so was retained, despite the fitness cost, likely due to developmental or genetic constraints.

Invasion history

We find evidence for at least two independent introductions of *Ad. piceae* to North America, as evidenced by the two geographically widespread MLLs on the continent (Fig. 3). One of these (Fig. 3, MLL 3) was found in western North America plus the southern Appalachian region. This clone was also detected in Germany and Switzerland (Fig. 4), indicating that Central Europe was the likely source. The other widespread MLL (Fig. 3, MLL 2) was found in the maritime provinces in Canada and the northeastern United States, but was not detected among our European samples. We also did not detect matches to the other less abundant North American MLLs among our European samples, but given that our sampling was primarily focused on the Caucasus Region and central Europe, these MLLs could very well exist in unsampled parts of Europe.

For *Ad. nordmanniane*, the historical literature asserts that it was introduced into Europe in the mid to late 1800's on imported *Ab. nordmanniana* and then spread to European fir species (e.g. Schneider-Orelli *et al.*, 1929; Varty, 1956). This is consistent with our results. The DAPC analysis separated a cluster of samples collected only in the native range in the Caucasus Mountains and northern Turkey, from a cluster collected from the native range as well as in Europe and North America (Fig. 6). This pattern likely resulted from a serial invasion from the Caucasus Mountains or Turkey to Europe, then from Europe to North America. *Adelges nordmannianae* was previously confirmed in western North America, and our record from Arnold Arboretum confirms its presence in eastern North America.

Our samples of *Ad. piceae* from galls of *P. orientalis* provide evidence that *Ad. piceae* is capable of completing a holocycle with a sexual generation. *Adelges piceae* has been reported to rarely produce sexuparae in Scotland (Varty, 1956), Canada (Balch, 1952; Bryant, 1971), and Italy (Binazzi, 2000), but ours is the first report of subsequent generations on *Picea*, to our knowledge. Secondary contact between *Ad. nordmannianae* and *Ad. piceae* in Europe and North America where *P. orientalis* has been transplanted appears to have allowed occasional hybridisation between these divergent lineages. The hybrid MLLs that we detected in North America did not match those we collected in Europe, so there is no evidence that the introductions to North America were the parthenogenetic product of hybridisation in Europe, or *vice versa*. In fact, our hybrid samples from Arnold Arboretum were collected from the same *P. orientalis* tree where we collected holocyclic *Ad. piceae*, and pure *Ad. nordmannianae* was collected from three nearby trees. This strongly suggests that hybridisation occurred at that locality, independent of hybrid origins in Europe. The independent formation of hybrids in different geographic locations argues against treating them as a distinct species because they do not represent a monophyletic lineage with a sustained evolutionary trajectory. In this early stage of hybridisation between *Ad. piceae* and *Ad. nordmannianae*, it is not clear if the hybrid genotypes will remain distinct from their parent species. We also did not find evidence of hybrids in the Caucasus Mountains, contrary to the assumption that *Ad. prelli* and *Ad. merkeri* were native to that region based on their holocyclic life cycles.

In North America, we collected *Ad. nordmannianae* and hybrids only from ornamental non-native hosts, but there is some evidence that these groups could impact native *Abies* species. *Adelges nordmannianae* was collected from ornamental *Ab. nordmanniana*, *Ab. alba*, and *Ab. cilicica* (Antoine & Kotschy) Carrière. Hybrids were collected from *Ab. nordmanniana*, *Ab.* x *insignis*, and *P. orientalis* (File S1). This might suggest that *Ad. nordmannianae* and hybrids do not prefer North American *Abies* species. However, Chrystal (1925) reported that feeding by *Ad. nordmannianae* caused swelling of terminal buds of *Ab.*

grandis, and Oechssler (1962) reported that feeding by *Ad. nordmannianae* and hybrids (= *Ad. merkeri*) induced swelling of parenchyma cells in *Ab. procera*, *Ab. lasiocarpa* (Hooker) Nuttall, and *Ab. grandis*. Of the three fir species that were evaluated, *Ab. grandis* showed the least impact from both adelgids, *Ab. lasiocarpa* showed more of an impact and responded more to *Ad. nordmannianae* than to hybrids, and *Ab. procera* was highly reactive to both groups of adelgids. In addition, our study included hybrid samples from Mlynany Arboretum, Slovakia, collected from the eastern North American fir, *Ab. balsamea*, indicating that they can survive on this species as well.

About half of the 65 species in Adelgidae are described as being anholocyclic (Havill & Foottit, 2007; Favret *et al.*, 2015), but theory predicts that these asexual lineages will not persist (Muller, 1964; Kondrashov, 1993). As more adelgid species complexes are examined, we suspect that most, if not all, anholocyclic species will also be found to be in the midst of transition between sexual and asexual reproduction.

Revised taxonomy

Based on our results and previous taxonomic opinions, we propose that the number of species in the *Adelges* (*Dreyfusia*) *piceae piceae* species complex be reduced from six to two: *Ad. nordmannianae* and *Ad. piceae*. Two of the formerly recognized species, *Ad. prelli* and *Ad. merkeri*, we propose are invalid because these represent non-monophyletic hybrid crosses between *Ad. nordmannianae* and *Ad. piceae*. Finally, we propose that *Ad. schneideri* be synonymized with *Ad. nordmannianae*, and that *Ad. nebrodensis* be designated as subspecies *Ad. piceae nebrodensis*, joining the three other *Ad. piceae* subspecies described by Foottit & Mackauer (1983). Similar to Mayr (1969), we propose that subspecies designations should be reserved for morphologically recognizable geographic subdivisions. We include an entry for the hybrids to trace their taxonomic history, but this does not constitute a formal taxon.

Adelges (Dreyfusia) piceae piceae (Ratzeburg, 1843)

Chermes piceae Ratzeburg, 1844 – Die Forst-Insecten oder Abbildung und Beschreibung der in den wäldern Preussens und der Nachbarstaaten als schädlich oder nützlich bekannt gewordenen Insecten: Im systematischer Folge und mit besonderer Rücksicht auf die Vertilgung der Schädlichen 3: 204.

Chermes piceae 'var. Bouvieri' Cholodkovsky, 1903 – Zoologischer Anzeiger 26: 258–259, Figs 1–3.

Chermes (*Dreyfusia*) *piceae* Börner, 1908a – Arbeiten aus der Kaiserlichen Biologischen Anstalt für Land- und Forstwirtschaft 6: 138–147, figs. 26–34.

Dreyfusia piceae Börner, 1908b – Zoologischer Anzeiger 33:742–743, figs. 1b, 2a, 3a, b, f, h.

Adelges piceae Annand, 1928 – Stanford University Publication. University Series. Biological Sciences 6: 74–81, figs. 14–15.

Dreyfusia piceae forma *typica* Pschorn-Walcher & Zwölfer, 1956a – Zeitschrift für Angewandte Zoologie 37: 63, Fig. 1; 1956b – Anzeiger für Schädlingskunde 29: 116–118, Fig. 1.; 1958 – Zeitschrift für Angewandte Zoologie 42: 245–246.

Adelges piceae 'intermediate group' Foottit & Mackauer, 1980 – Canadian Journal of Zoology 58: 1494–1503.

Adelges piceae piceae Foottit & Mackauer, 1983 – Annals of the Entomological Society of America 76: 300–301, Fig. 1a.

Adelges (*Dreyfusia*) *piceae* Favret *et al.*, 2015 – ZooKeys 534: 40.

Diagnosis. *Adelges piceae* is distinguished from *Ad. nordmannianae* by first instar sistens nymphs having fewer than 45 facets in the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and metathorax and first three abdominal segments (Fig. 2b). Middle fields of these wax glands angular and individual facets polygonal (Eichhorn, 1967). See Foottit & Mackauer (1983) for a description of the nominal subspecies.

Distribution. **Europe**: Albania, Austria, Bosnia and Herzegovina, Bulgaria, Czech Republic, Denmark, France, Germany, Greece, Iceland, Ireland, Italy, Lithuania, Netherlands, North Macedonia, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovakia, Sweden, Switzerland, United Kingdom (England). **North America**: Canada (British Columbia, Yukon Territories), U.S.A. (Alaska, California, Idaho, Massachusetts, Montana, North Carolina, Oregon, Tennessee, Utah, Virginia, Washington, West Virginia).

Material examined. See File S1.

Comments. The previously reported distribution of subspecies *Ad. piceae piceae* (Foottit & Mackauer, 1983) corresponds with our *Ad. piceae* MLL 3 (Fig. 3).

Adelges (Dreyfusia) piceae canadensis (Merker & Eichhorn, 1956)

Dreyfus[*ia*] *pic*[*eae*] *canad*[*ensis*] Merker & Eichhorn, 1956 – Naturwissenschaften 43: 454.

Dreyfusia piceae forma canadensis Eichhorn, 1957 – Zeitschrift für Angewandte Zoologie 44: 310–312; Busby, 1962 – Scottish Forestry 4:243–254.

Adelges piceae 'Maritime group' Foottit and Mackauer, 1980 – Canadian Journal of Zoology 58: 1494–1503.

Adelges piceae canadensis Foottit & Mackauer, 1983 – Annals of the Entomological Society of America 76: 301–302, Fig. 1b.

Diagnosis. See Foottit & Mackauer (1983) for a description of this subspecies.

Distribution. **Europe**: United Kingdom (Scotland). **North America**: Canada (New Brunswick, Newfoundland and Labrador, Nova Scotia, Ontario, Prince Edward Island, Quebec), United States (Connecticut, Maine, Massachusetts, New Hampshire, New York, Pennsylvania, Vermont, West Virginia).

Comments. The previously reported distribution of this subspecies (Foottit & Mackauer, 1983) corresponds with our *Ad. piceae* MLL 2 (Fig. 3).

Adelges (Dreyfusia) piceae occidentalis Foottit & Mackauer, 1983

Adelges piceae "British Columbia group " Foottit and Mackauer, 1980. Can J. Zool. 58: 1494–1503.

Adelges piceae canadensis Foottit & Mackauer, 1983 – Annals of the Entomological Society of America 76: 302–303, Fig. 1c.

Diagnosis. See Foottit & Mackauer (1983) for a description of this subspecies.

Distribution. **North America**: Canada (British Columbia).

Comments. None of the *Ad. piceae* MLLs that we identified had geographic affinities to this subspecies, suggesting that individuals of *Ad. piceae occidentalis* were not included among our samples.

Adelges (Dreyfusia) piceae nebrodensis (Binazzi & Covassi, 1991), **stat.n.**

Diagnosis. See Binazzi & Covassi (1991) for a description of this subspecies.

Distribution. **Europe**: Italy (Sicily).

Comments. The slight, overlapping morphological distinction between *Ad. nebrodensis* and *Ad. piceae* as described by Binazzi and Covassi (1991), and its restricted geographic range justifies its designation as a subspecies.

Adelges (Dreyfusia) nordmannianae (Eckstein, 1890)

Chermes nordmannianae Eckstein, 1890. Zoologischer Anzeiger 13: 90.

Chermes piceae Nüsslin, 1903. Naturwissenschaftliche Zeitschrift für Land– und Forstwirtschaft 1: 25–33, figs 1–8.

Adelges (*Chermes*) *nordmannianae*, Schouteden, 1903. Annales de la Société Entomologique de Belgique 47: 168.

Dreyfusia nüsslini Börner, 1908b – Zoologischer Anzeiger 33: 739–742, figs 1a, 2b, 3c, d, e, g, 4c, d, e.

Dreyfusia nordmannianae Börner, 1932 – In Börner & Schilder. Aphidoidea, Blattläuse. In Reh [Ed.]. Handbuch der Pflanzenkrankheiten Begründet Volume 5: 682–686, figs 365, 367–369, 371.

Dreyfusia schneideri Börner, 1932 – In Börner & Schilder. Aphidoidea, Blattläuse. In Reh [Ed.]. Handbuch der Pflanzenkrankheiten Begründet Volume 5: 684–686, **syn. n.**

Dreyfusia nüsslini 'forma schneideri' Merker & Eichhorn, 1956 – Naturwissenschaften 43: 454.

Dreyfusia nüsslini 'forma typica' Merker & Eichhorn, 1956 – Naturwissenschaften 43: 454.

Adelges nüsslini Annand, 1928 – Stanford University Publication. University Series. Biological Sciences 6: 81–89, figs. 16–17.

Adelges (*Dreyfusia*) *nordmannianae* Favret *et al.*, 2015 – ZooKeys 534: 40.

Diagnosis. Greater than 55 facets in the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and metathorax and first three abdominal segments of first instar sistens nymphs (Fig. 2a). Middle fields of these wax glands round or oval, and facets round (Eichhorn, 1967).

Distribution. **Asia:** Georgia, Turkey. **Europe**: Austria, Czech Republic, Denmark, France, Germany, Hungary, Iceland, Ireland, Italy, Netherlands, Norway, Romania, Russia, Slovakia, Slovenia, Switzerland, Ukraine, United Kingdom (Scotland). **North America**: Canada (British Columbia), U.S.A. (California, Massachusetts, Washington). **Oceania**: Australia (Tasmania), New Zealand.

Material examined. See File S1.

Comments. Eichhorn & Pschorn-Walcher (1972) showed that the bark form (= *Ad. schneideri*) and stem forms of *Ad. nordmannianae*, along with their differences in phenology, stylet length, and fecundity, can be converted to one another depending on environmental or host factors. Börner & Schilder (1932), who first described *Ad. schneideri*, later considered it a synonym of *Ad. nordmannianae* (Börner & Heinze, 1957), while other authors have considered it a 'form' of *Ad. nordmannianae* (e.g. Eichhorn, 1957; Pschorn-Walcher & Zwölfer, 1958; Pschorn-Walcher & Zwolfer, 1960; Heinze, 1962). Therefore, similar to these authors, we propose that *Ad. schneideri* be synonymized with *Ad. nordmannianae*. It should not treated be as a subspecies because it is not a distinct morphological or geographic variant.

Adelges (Dreyfusia) nordmannianae x *piceae* hybrids

Dreyfusia prelli Grosmann, 1935 – Tharandter Forstliches Jahrbuch 86: 822–826, figs. 1–4.

Dreyfusia piceae 'forma aggressiva' Pschorn-Walcher & Zwölfer, 1956a – Anzeiger für Schädlingskunde 29: 117.

Dreyfusia piceae 'Freiburger Form' Merker & Eichhorn, 1956 – Naturwissenschaften 43: 453–454.

Dreyfusia merkeri Eichhorn, 1957 – Zeitschrift für Angewandte Zoologie 44: 312–346, figs. 6b, 7b, 8–13.

Adelges (*Dreyfusia*) *prelli* Favret *et al.*, 2015 – ZooKeys 534: 40.

Adelges (*Dreyfusia*) *merkeri* Favret *et al.*, 2015 – ZooKeys 534: 40.

Diagnosis. Offspring of hybridisation between *Ad. nordmannianae* and *Ad. piceae*. The number of facets in the ten middle fields of the wax glands on the inner margins of the spinal sclerites of the meso- and metathorax and first three abdominal segments of first instar sistens nymphs is variable and overlaps with the ranges in *Ad. nordmannianae* and *Ad. piceae*. Facet counts intermediate between these species (45–55) in a population may indicate the presence of hybrids.

Distribution. **Asia**: Turkey. **Europe**: Austria, France, Germany, Italy, Slovakia, Sweden, Switzerland. **North America**: Canada (British Columbia), U.S.A. (Massachusetts, Washington).

Material examined. See File S1.

Comments. The life cycles of these holocyclic hybrids are described as differing in phenology from *Ad. nordmannianae*, by developing and laying eggs on *Abies* in the winter versus early

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spring, breaking autumnal diapause later, and having two summer aestivating generations rather than one (Eichhorn, 1967). They can also have larger galls that open later, on average, than *Ad. nordmannianae* (Eichhorn, 1975). Alternately, they can lay eggs in the spring and break autumnal diapause in an intermediate period between *Ad. nordmannianae* and *Ad. piceae* (Eichhorn, 1957), and galls can open, on average, later than *Ad. nordmannianae* (Eichhorn, 1975). They have also been described as being less covered in wool and having a higher proportion of winged individuals developing from progredientes than *Ad. nordmannianae* (Pschorn-Walcher & Zwolfer, 1960).

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers for the microsatellite loci used in this study.

Table S2. Prior and posterior distribution of parameters for approximate Bayesian computation to test scenarios of divergence and hybridisation using diyABC.

Fig. S1. Histogram showing the distribution of pairwise comparisons of genetic distance under an infinite allele mutation model for *Adelges* (*Dreyfusia*) microsatellite genotypes.

Fig. S2. Bayesian information criterion versus the number of clusters for discriminant analysis of principal components for all unique *Adelges* (*Dreyfusia*) multilocus lineages (MLLs).

Fig. S3. Posterior probability of assignment of each unique microsatellite multilocus lineage (MLL) calculated as a pure species (*Ad. nordmannianae* or *Ad. piceae*), F1 hybrid, F2 hybrid, or a backcross.

Fig. S4. Model check plot showing the first two principal components of summary statistics generated using the prior distribution of model parameters, the posterior distribution of model parameters, and the observed data set.

Fig. S5. Prior and posterior distributions of effective population sizes, split times, and merge times for the most likely historical scenario of *Adelges* (*Dreyfusia*) *piceae* species complex resulting from approximate Bayesian computation (ABC) analysis.

File S1. Collection information, museum voucher accession numbers, COI DNA sequences, GenBank accession numbers, and microsatellite genotypes for all samples used in this study.

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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