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Relationships between aphid species of the family Adelgidae (Hemiptera Adelgoidea) and their endosymbiotic bacteria: a case study in Lithuania

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Abstract

Partial sequences of COI and EF-1α of nine aphid species of the family Adelgidae and partial sequences of 16S rRNA of their endosymbiotic bacteria were used to reveal co-evolutionary relationships between endosymbiotic bacteria and their aphid hosts in Lithuania. In addition to currently recognized taxa of gamma- and beta-proteobacterial endosymbionts ("*Candidatus* Vallotia", "*Candidatus* Profftia", "*Candidatus* Annandia", "*Candidatus* Hartigia", "*Candidatus* Ecksteinia", "*Candidatus* Steffania" and "*Candidatus* Gillettellia") of Adelgidae, new gamma-proteobacterial (*Sodalis*-allied) endosymbionts were detected in *Adelges* (*Aphrastasia*) *pectinatae* and *Pineus cembrae*. Cophylogenetic analyses based on aphid partial COI and EF-1α sequences and 16S rRNA gene fragment of their endosymbiotic bacteriae showed reliable cophylogenetic events confirming the importance of host aphid species relatedness in structuring symbiont communities of adelgid aphid species. Molecular aphid species delimitation analyses based on Bayesian phylogenies of aphid COI and EF-1α and bacterial 16S rRNA fragments indicate adelgid species complexes *Adelges* (*Adelges*) *laricis* - *Adelges* (*Adelges*) *tardus*, *Adelges* (*Gilleteella*) *cooleyi* - *Adelges* (*Gilleteella*) *coweni*, *Adelges* (*Dreyfusia*) *nordmannianae* - *Adelges* (*Dreyfusia*) *piceae*, *Adelges* (*Sacchiphantes*) *abietis* - *Adelges* (*Sacchiphantes*) *viridis* and *Pineus pini* - *Pineus orientalis* representing single species each.

Key words: Adelgidae, endosymbiotic bacteria, phylogeny, coevolution.

Introduction

Species of the family Adelgidae are oviparous aphids endemic in the boreal and temperate zones of the Northern Hemisphere (Havill and Foottit, 2007). They feed by phloem sap sucking on host plant species of the Gymnosperm plant family Pinaceae. Adelgids are cyclically parthenogenetic, and have complex life cycles that can be either holocyclic or anholocyclic. Holocycle is characterized by sequence of sexual and asexual morphs, when initial generations develop on primary hosts (*Picea* spp.) and then migrate to secondary hosts belonging to genera *Abies*, *Larix*, *Pinus*, *Tsuga* or *Pseudotsuga*. Anholocycle is based on asexual reproduction without host alternation. Both holocyclic and anholocyclic adelgid lineages are strictly host specific and might cause substantial damage to their host plants (McManamay *et al*., 2011; Ravn *et al*., 2013; Brockerhoff and Liebhold, 2017). Due to parthenogenetic reproduction, gall production and dispersive winged morphs, adelgids show high invasive capacity (Havill *et al*., 2016; Csoka *et al*., 2017). All this explains long lasting research efforts concerning Adelgidae species (Annand, 1928; Foottit *et al*., 2009; Toenshoff *et al*., 2012a; 2012b; 2014; Sano and Ozaki, 2012; Havill *et al*., 2006; 2007; 2016). Nonetheless, the diversity of life cycle modes and uncertainty of morphological diagnoses of species caused controversy in taxonomy of Adelgidae (Steffan, 1968; Mantovani *et al*., 2001; Havill *et al*., 2007; Favret *et al*., 2015, Albrecht, 2017; Blackman and Eastop, 2020). Bacterial DNA sequences appeared a promising tool to clarify the phylogeny in aphid family Aphididae (Clark *et al*., 2000; Martinez-Torres *et al*., 2001; Jousselin *et al*., 2009; Liu *et al*., 2013; Nováková *et al*., 2013). Application of this method for Adelgidae still requires further research efforts (Toenshoff *et al*., 2012a; Michalik *et al*., 2013; von Dohlen *et al*., 2017). Phylogenetic analysis of Adelgidae based on partial sequences of mitochondrial COI, COII, cytb and the nuclear EF-1α gene revealed evolutionary history of adelgid species being closely associated with that of their secondary host plants (Havill *et al*., 2007). Because adelgids relay on the nutritional provisioning by endosymbionts, it might influence opportunities for colonization of new alternate conifer hosts by adelgids (Toenshoff *et al*., 2014; von Dohlen *et al*., 2017; Weglarz *et al*., 2018; Mech *et al*., 2019). Consequently, analysis of evolutionary history of endosymbiotic bacteria might help to resolve the relationships between species of Adelgidae (Toenshoff *et al*., 2014; von Dohlen *et al*., 2017).

Recognition of the bacteriome as an organ keeping microorganisms inside the insect body emerged at the beginning of the past century bringing understanding of this type of relationship being widespread in many insect groups (for broader review see Gil and Latorre, 2019; Thiery *et al*., 2019; Zytynska, 2019). Aphids have established close symbiotic associations with bacteria that contribute to many of their functionalities. *Buchnera aphidicola* is the principal primary endosymbiont of viviparous aphids occupying specialized aphid cells (bacteriocytes) and supplying essential nutrients to its host (Douglas, 1998). *B. aphidicola* is strictly maternally transmitted and exhibits pattern of co-diversification with aphid hosts during long-term evolution (Jousselin *et al*., 2009; Liu *et al*., 2013). Aphids also host various facultative symbionts, which undergo vertical and horizontal transmission. Facultative symbionts can influence important features of aphids: defence against parasitic wasps, fungal pathogens, thermal resistance, host plant

specificity and modification of body colour (Łukasik *et al*., 2013; Wagner *et al*., 2015; Frago *et al*., 2017; Nikoh *et al*., 2018). Communities of microbial symbionts differ across aphid species, influenced by aphid geographical distribution, aphid host plant, aphid parasitoid community, temperature and other ecological characteristics (Gauthier *et al*., 2015; Zytynska and Weisser, 2016; Guo *et al*., 2019; Xu *et al*., 2020). There still remains controversy concerning principal role of host relatedness and host ecology in structuring symbiont communities of diverse aphid species (Henry *et al*., 2015; McLean *et al*., 2019). Bacterial communities of oviparous aphid family Adelgidae are less studied when compared with those of Aphididae aphids despite rather long period of studies (see broader review by von Dohlen *et al*., 2017). Nonetheless, some data on the taxonomy, structural details, functional peculiarities and possible adaptive meaning of bacterial endosymbionts of adelgids are already available (Toenshoff *et al*., 2012a; 2012b; 2014; Michalik *et al*., 2013; von Dohlen *et al*., 2013; 2017). Bacteriome of Adelgidae aphid species is unique in having two different obligate (primary) endosymbionts differing in major adelgid lineages, thus presenting an unusual case of multiple replacements of both the senior symbiont and the more recent junior symbionts (Toenshoff *et al*., 2012b; 2014; von Dohlen *et al*., 2017). For the present, the following associations were reported: "*Ca.* Vallotia spp." and "*Ca.* Profftia spp.", "*Ca.* Ecksteinia adelgidicola" and "*Ca.* Steffania adelgidicola", "*Ca.* Gillettelia cooleyi" and "*Ca.* Vallotia cooleyi", "*Ca.* Hartigia pinicola" and "*Ca.* Annandia pinicola", "*Ca.* Pseudomonas adelgestsugae" and "*Ca.* Annandia adelgestsugae" for adelgids inhabiting *Larix*, *Abies*, *Pseudotsuga*, *Pinus* and *Tsuga* as secondary hosts, respectively. Molecular marker selection is one of the important steps in studying endosymbiont diversity.16S ribosomal DNA (rDNA) sequencing approach (Větrovský and Baldrian, 2013) enabled investigation of microbial diversity and ecology without time-consuming cultivation of bacterial lineages. The 16S rRNA is suitable for this purpose for several reasons. The gene allows the analysis of distant taxa due to its wide distribution, the presence of both conserved and variable regions and it is also expected to be weakly affected by horizontal gene transfer (Větrovský and Baldrian, 2013). Another important step is to ensure confidence in the validity of aphid species definition and endosymbiotic bacteria host specificity. For this purpose, samples for DNA extraction should be represented by the material from the same source - single aphid specimen or aphids from the same colony. Actually, a non-destructive DNA extraction method has to be used so that each aphid specimen could be subjected for the subsequent morphological identification. Revealing coevolution and cospeciation of aphids and their endosymbiontic bacteria also requires the application of species delimitation methods for both hosts and their endosymbionts.

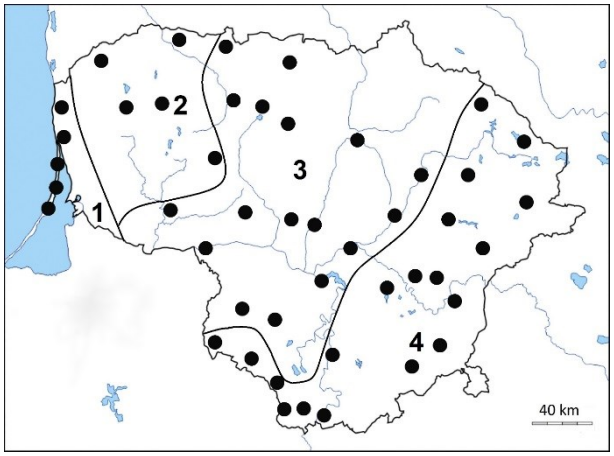

Figure 1. Sites of aphid collection in 2017-2018 in four climatic regions of Lithuania. 1 - Coastal; 2 - Samogitian; 3 - Middle Lithuanian lowland; 4 - Southeastern highlands.

Lithuania is at the northernmost part of the Central European floristic province (Frey and Lösch, 2010). Four species and five species complexes of the aphid family Adelgidae are listed for Lithuanian fauna (Havelka *et al*., 2020), without any information on their symbiotic bacteria. The aim of this study is to investigate possible coevolutionary relationships of Adelgidae and their endosymbiotic bacteria exploiting partial sequences of aphid hosts (COI and EF-1α) and their endosymbionts (16S rRNA) based on material collected in Lithuania.

Materials and methods

Sample collection and identification

Aphid material has been collected in 2017-2018 in all four climatic regions of Lithuania (figure 1, supplemental material table S1). Microscope slides of sampled aphids in Canada balsam were prepared according to Blackman and Eastop (1984). For morphology-based identification of aphid species, keys of Binazzi (2000), Blackman and Eastop (2020) and Albrecht (2017) were exploited. Aphid material is deposited at the Life Sciences Centre of the Vilnius University (Lithuania). Additional verification of aphid species was made by comparison of mitochondrial COI and nuclear EF-1α fragments of our samples with those available in the GenBank (see Havelka *et al*., 2020 for details). We follow classification of Adelgidae by Favret *et al*. (2015) in the present study. Based on earlier reference data (Mantovani *et al*., 2001; Foottit *et al*., 2009; Žurovcová *et al*., 2010; Havelka *et al*., 2020), we take species complexes *Adelges* (*Adelges*) *laricis* - *Adelges* (*Adelges*) *tardus*, *Adelges* (*Gilletteella*) *cooleyi* - *Adelges* (*Gilletteella*) *coweni*, *Adelges* (*Dreyfusia*) *nordmannianae* - *Adelges* (*Dreyfusia*) *piceae*, *Adelges* (*Sacchiphantes*) *abietis* - *Adelges* (*Sacchiphantes*) *viridis* and *Pineus pini* - *Pineus orientalis* as a single species for the aims of this study. Aphid specimens used for the DNA extraction followed by the amplification of 16S rRNA fragment of endosymbiotic bacteria were those described by Havelka *et al*. (2020). The species of endosymbiotic bacteria were identified by using species-specific primers and further comparison of aligned sequences with BLAST. In other cases, we use the designation "*Candidatus* endosymbiont species" of particular aphid species, for example "*Candidatus* Profftia sp." of *Adelges* (*Cholodkovskya*) *viridanus*.

Table 1. Primers for the amplification of selected DNA fragments.

Fragment	Primer name	Primer sequence	Primer annealing temperature, °C
Adelgidae (Toenshoff *et al*., 2012a)			
COI	911-F	TTT CTA CAA ATC ATA AAG ATA TTG G	50
COI	912-R	TAA ACT TCA GGG TGA CCA AAA AAT CA	50
EF-1α	AdelEF1-F	GTA CAT CCC AAG CCG ATT GT	61
EF-1α	AdelEF1-R	CTC CAG CTA CAA AAC CAC GA	61
Other endosymbiotic bacteria (Toenshoff *et al*., 2012a)			
16S rRNA	Bac-F	AGA GTT TGA TYM TGG CTC	52
16S rRNA	Bac-R	GGY TAC CTT GTT ACG ACT T	52
"*Candidatus* Steffania adelgidicola" (Toenshoff *et al*., 2012b)			
16S rRNA	SteAd-F	CAT CGG AAA GGA GTT TAC TTC	58
16S rRNA	SteAd-R	GAG GTC CGC TGA CCC TCA	58
"*Candidatus* Ecksteinia adelgidicola" (Toenshoff *et al*., 2012b)			
16S rRNA	EckAd-F	GGA CGG GTG AGT AAT ATT	58
16S rRNA	EckAd-R	GTA AGT GCC CTC CAA TAC	58
"*Candidatus* Profftia virida" (Toenshoff *et al*., 2012a)			
16S rRNA	ProVi-F	ATG TCT GGG GAA CTG CCT	55
16S rRNA	ProVi-R	CGA GGG TTA AGC TAC TTG	55
"*Candidatus* Profftia tarda" (Toenshoff *et al*., 2012a)			
16S rRNA	ProTa-F	ATG TCT GGG AAA CTG CCT	61
16S rRNA	ProTa-R	CGA AGG TTA AGC TAC CTG	61
"*Candidatus* Vallotia spp." (Toenshoff *et al*., 2012a)			
16S rRNA	Vallotia-F	CGT RTC TTA GAG TGG GGG	62
16S rRNA	Vallotia-R	ATC CTA CCG TGG TAA CCG	62
"*Candidatus* Annandia pinicola" (Toenshoff *et al*., 2014)			
16S rRNA	AnnPi-R	TGG AAA CAT ATT CAC CGT G	60
16S rRNA	AnnPi-F	TAC GGT CCA GAC TCT TAC	60

DNA extraction, PCR amplification and sequencing

For molecular analysis, several aphid individuals sampled from one colony were considered as a unique sample. Total DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). Primers for the amplification of Adelgidae COI and EF-1α fragment, and partial sequences of bacterial 16S rRNA and their annealing temperatures are given in table 1. PCR amplification was carried out in a thermal cycler (Eppendorf) in 50 µl volumes containing 2 µl genomic DNA, 5 µl of each primer (1 µM), 25 µl of PCR master mix (Thermo Scientific) and 13 µl of nuclease free water (Thermo Scientific). The cycling parameters were the following: denaturizing at 95 °C for 4 minutes (1 cycle), denaturizing at 95 °C for 45 seconds, annealing at 50 °C - 62 °C (see table 1 for details) for 45 seconds, extension at 72 °C for 1 minute 30 seconds (35 cycles in total), and final extension at 72 °C for 10 minutes (1 cycle). PCR products were purified using Gene Jet PCR purification kit (Thermo Scientific) and sequenced at Macrogen Europe (Amsterdam, the Netherlands). The amplification primers were also used as sequencing primers. DNA sequences for each sample were confirmed with both sense and anti-sense strands and aligned in the BioEdit Sequence Alignment Editor (Hall, 1999). The GenBank Accession numbers are MT460428 - MT460439, MT460444 - MT460446, MT460495, MT460497 - MT460502 and MT465176 - MT465267. Additional sequences of aphids (HQ668155, HQ668157, JN810887 - JN810896, KC784363 - KC784363 for COI fragment and HQ668164 - HQ668167, JN810898 - JN810908, KC784365 - KC784366 for EF-1α fragment) and endosymbiotic bacteria (HQ668158 - HQ668162, JN810865 - JN810869, JN810871 - JN810886, KC764415 - KC764418, KC961956) were downloaded from GenBank.

Evaluation of the diversity of aphids and their primary endosymbionts

Species delimitation method of Pons *et al*. (2006) was used to identify relevant entities for this study, i.e. genetic clusters of specimens potentially subject to selection and genetic drift. It identifies clusters representing independently evolving entities by means of the generalized mixed Yule coalescent model (GMYC). This model optimizes the maximum likelihood value of a threshold, such that the nodes before the threshold are identified as species diversification events, while the branches beyond the threshold are clusters following coalescent processes. One ultrametric tree was constructed from the alignment of partial 16S rRNA sequences from endosymbiotic bacteria and two ultrametric trees based on partial COI and EF-1α sequences from their aphid host species. Substitution models were selected with jmodeltest 2.1.7 (Darriba *et al*., 2012) and were GTR+G for COI, GTR+I+G for EF-1α and HKY+I+G for 16S rRNA fragment. BEAST v1.7.4 (Drummond and Rambaut, 2007) was used for tree construction with uncorrelated lognormal relaxed clock, assuming a Yule tree prior without partition between three codon positions. One run of 70 million generations with sampling every 7000 generations was performed for Adelgidae and one run for 50 million generations with

sampling every 5000 - for endosymbiotic bacteria. The convergence was checked using Tracer 1.5 (Drummond and Rambaut, 2007). Sampled posterior trees were summarized using TreeAnnotator 1.7.4 to generate a maximum clade credibility (MCC) tree without the removal of burn-in. The GMYC method as implemented in the R package SPLITS (https://splits.r-forge.r-project.org/), was then applied to the MCC tree that best fitted our data, and species list was derived from this phylogenetic tree. These results were used for grouping of samples to evaluate within- and between group diversity of partial COI, EF-1α and 16S rRNA sequences. The calculation of p-distances was performed with MEGA 7 (Kumar *et al*., 2017).

Cophylogenetic analyses

For the analysis of cophylogeny, species trees for Adelgidae and their endosimbiotic bacteria were constructed using StarBEAST (Heled and Drummond, 2010) under the conditions described above. We utilized both event-based method and distance-based methods. Event-based method Jane 4 (Conow *et al*., 2010) was used to map onto the host and ensymbiont phylogenies such events as co-speciation (the host and parasite speciate simultaneously), duplication (a parasite speciates and both of the new species remain on the same host), duplication with host switch (a parasite speciates and one of the new species switches onto a different host), loss (an edge of the parasite tree passes through a node of the host tree) and failure to diverge (a host speciates and the parasite remains on both new host species). Jane 4 uses a polynomial time dynamic programming algorithm paired with a genetic algorithm to compare the host and parasite tree topologies (Conow *et al*., 2010). The program optimally maps the parasite tree onto the host tree using different user-defined event cost models to reconcile the two trees. We used default parameters of Jane 4 for the genetic algorithm and the test of significance. Distance-based cophylogenetic method PACo (Balbuena *et al*., 2013) was used to perform a simple test of independence between phylogenetic trees and generate statistics allowing the assessment of the congruence between the Adelgidae species tree and their endosymbiotic bacteria species tree. PACo uses a Procrustean superimposition that scales and rotates the parasite tree to fit the host tree topology, resulting in a global test statistic (residual sum of squares, m^2) that explicitly tests the dependence of the parasite tree on the host tree. All goodness-of-fit statistics in PACo were performed with 100000 permutations.

Results

Diversity of Adelgidae species and their primary endosymbionts

Species delimitation procedures based on COI and EF-1α sequences gave similar numbers of candidate species coinciding with respective Adelgidae morphospecies (table 3, supplemental material figures S1-S2). Average within-species p-distances ranged from 0 to 0.91% for COI and from 0 to 0.52% for EF-1α fragment (table 3). Between-species average p-distances were from 6.52% (between *P. strobi* and *P. orientalis* - *P. pini*) to 13.05% [*P. strobi* and *A.* (*S.*) *viridis* - *A.* (*S.*) *abietis*] for COI and from 2.23% (*P. strobi* and *P. orientalis* - *P. pini*) to 10.13% [*A.* (*C.*) *viridanus* and *P. orientalis* - *P. pini*] for EF-1α (table 2). Overall mean p-distances were 7.85% for COI fragment (85 samples, 657 bp) and 6.1% for EF-1α fragment (94 samples, 690 bp).

During this study *A.* (*A.*) *pectinatae*, *A.* (*C.*) *viridanus* and *P. cembrae* were checked for the presence of endosymbiotic bacteria for the first time by using partial 16S rRNA sequences. BLAST search showed, that bacterial 16S rRNA fragment from *A.* (*A.*) *pectinatae* had the percent of identity from 95.76% to 96.68% with sequences of "*Ca.* Steffania adelgidicola" (FR872579) and 96.19-96.82% of identity with sequences of endosymbiotic bacteria of the genus *Sodalis* (AB604872, AB604873). In case of *Larix* inhabiting *A.* (*C.*) *viridanus*, partial sequences of 16S rRNA were the most similar to the sequences of "*Ca.* Profftia japonica" (MF108836) and "*Ca.* Vallotia japonica" (MF063344) and reached 99.05-99.91% and 99.25-99.83% respectively. Partial 16S rRNA sequences with 98.74-99.06% of similarity to "*Ca.* Annandia pinicola" from *P. pini* (MF077640) were detected in samples of *P. cembrae*. This Adelgidae species also seems to harbour other than "*Ca.* Hartigia pinicola" species of endosymbiotic bacteria, which had the sequence identity with *Sodalis* (AB507712, AB517595, AB54010, AB915782) from 97.01 to 97.52%.

Species delimitation procedure for the primary endosymbionts yielded 17 groups, which included partial 16S rRNA sequences of bacteria from particular host species (table 3, supplemental material figure S3). Average within-

Table 2. Average between-species p-distances (%) for nine groups of Adelgidae. Lower left - COI fragment, upper right - EF-1α fragment.

	1	2	3	4	5	6	7	8	9
Adelges (*Adelges*) *laricis* - *A.* (*A.*) *tardus*		7.23	5.13	7.01	6.77	8.25	10.09	9.58	6.93
Adelges (*Aphrastasia*) *pectinatae*	8.49		6.61	3.72	6.57	7.07	7.11	7.41	6.61
Adelges (*Cholodkovskya*) *viridanus*	7.91	9.55		6.14	6.34	9.53	10.13	9.15	3.76
Adelges (*Dreyfusia*) *nordmannianae* - *A.* (*D.*) *piceae*	10.54	7.98	8.81		5.70	7.68	7.59	7.93	6.99
Adelges (*Gilletteella*) *cooleyi* - *A.* (*G.*) *coweni*	9.83	7.22	8.25	8.32		8.33	8.82	9.49	6.87
Pineus cembrae	10.92	10.27	9.17	10.14	11.13		2.95	3.18	7.49
Pineus orientalis - *P. pini*	9.63	10.94	10.07	11.28	12.51	7.14		2.23	8.86
Pineus strobi	11.51	10.42	9.72	11.16	11.72	6.69	6.52		8.68
Adelges (*Sacchiphantes*) *viridis* - *A.* (*S.*) *abietis*	8.05	9.41	7.89	10.94	9.58	11.78	12.09	13.05	

Table 3. Species delimitation based on endosymbiont partial 16S rRNA sequences and their Adelgidae hosts COI and EF-1α fragment data using General Mixed Yule Coalescent (GMYC) model showing intrataxonal average and range of p-distances (%). n = number of sequences used.

Adelgidae morphospecies	COI	EF-1α	Endosimbiotic bacteria	16S rRNA	p-distances
Adelges (*Adelges*) *laricis* - *A*. (*A*.) *tardus* n = 20 / 20	0.46, 0 - 1.22	0.40, 0 - 1.03	"*Ca*. Profftia tarda" n = 23	1	0.17, 0 - 0.69
			"*Ca*. Vallotia tarda" n = 19	1	0.23, 0 - 1.33
Adelges (*Aphrastasia*) *pectinatae* n = 5 / 6	0.30, 0 - 0.76	0	Endosymbiont of *A*. (*A*.) *pectinatae* n = 5	1	0.92, 0 - 1.76
Adelges (*Cholodkovskya*) *viridanus* n = 4 / 4	0.63, 0 - 1.07	0	"*Ca*. Profftia sp." n = 4	1	0.64, 0.17 - 1.04
			"*Ca*. Vallotia sp." n = 3	1	0.33, 0.08 - 0.50
Adelges (*Dreyfusia*) *nordmannianae* - *A*. (*D*.) *piceae* n = 5 / 7	0.15, 0 - 0.30	0.52, 0 - 0.96	"*Ca*. Ecksteinia adelgidicola" n = 4	1	0.34, 0.08 - 0.59
			"*Ca*. Steffania adelgidicola" n = 5	1	0.09, 0 - 0.19
Adelges (*Gilletteella*) *cooleyi* - *A*. (*G*.) *coweni* n = 9 / 10	0.20, 0 - 0.76	0.42, 0 - 1.14	"*Ca*. Gillettellia cooleyia" n = 2	1	0.14
			"*Ca*. Vallotia cooleyia" n = 9	1	0.19, 0 - 0.50
Pineus cembrae n = 2 / 4	0.91	0.30, 0 - 0.61	"*Ca*. Annandia pinicola" n = 3	1	0.49, 0.10 - 0.73
			Endosymbiont of *P*. *cembrae* n = 3	1	3.36, 2.04 - 4.09
Pineus orientalis + *P*. *pini* n = 9 / 10	0.23, 0 - 0.46	0	"*Ca*. Annandia pinicola" n = 8	1	0
			"*Ca*. Hartigia pinicola" n = 5	1	0.12, 0 - 0.22
Pineus strobi n = 2 / 4	0	0	"*Ca*. Annandia pinicola" n = 3	1	1.17, 0.15 - 1.75
			"*Ca*. Hartigia pinicola" n = 3	1	0.07, 0 - 0.11
Adelges (*Sacchiphantes*) *viridis* - *A*. (*S*.) *abietis* n = 29 / 29	0.40, 0 - 3.27	0.47, 0 - 2.09	"*Ca*. Profftia virida" n = 23	1	0.18, 0 - 0.61
			"*Ca*. Vallotia virida" n = 26	1	0.21, 0 - 0.67

Table 4. Average interspecific p-distances (%) for Adelgidae endosymbionts from different species of their hosts.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
"*Ca*. Vallotia cooleyia"																	
"*Ca*. Vallotia virida"	4.86																
"*Ca*. Vallotia tarda"	4.59	2.39															
"*Ca*. Vallotia sp." from *A*. (*Cholodkovskya*) *viridanus*	4.92	2.23	2.05														
"*Ca*. Profftia virida"	19.09	19.44	19.50	19.60													
"*Ca*. Profftia tarda"	19.22	19.65	19.71	19.96	4.69												
"*Ca*. Profftia sp." from *A*. (*Cholodkovskya*) *viridanus*	18.66	19.17	19.40	19.59	3.25	4.39											
"*Ca*. Hartigia pinicola" from *P*. *strobi*	19.09	19.04	19.42	19.51	9.84	10.17	9.32										
"*Ca*. Hartigia pinicola" from *P*. *orientalis*	19.55	19.30	19.65	19.77	9.76	9.77	9.39	3.17									
Endosymbiont of *P* .*cembrae*	18.11	18.58	18.78	18.98	9.99	10.44	9.93	10.35	10.15								
"*Ca*. Annandia pinicola" from *P*. *strobi*	21.99	21.97	21.65	21.74	15.06	14.75	14.99	14.72	15.50	16.00							
"*Ca*. Annandia pinicola" from *P*. *orientalis*	21.53	21.73	21.61	21.49	14.67	14.25	14.80	14.29	15.15	15.70	2.35						
"*Ca*. Annandia pinicola" from *P*. *cembrae*	22.28	22.05	21.72	21.85	14.86	14.44	14.89	14.41	15.32	15.45	1.82	1.50					
"*Ca*. Ecksteinia adelgicola"	18.61	19.68	19.80	19.84	9.59	8.76	9.40	10.19	10.08	9.91	14.80	14.18	14.52				
"*Ca*. Steffania adelgidicola"	18.14	18.07	18.03	17.96	10.33	9.79	9.15	12.23	12.18	8.63	14.96	14.85	14.48	9.25			
"*Ca*. Gillettellia cooleyia"	20.44	20.38	21.25	21.02	8.82	7.96	8.55	16.37	16.12	14.31	15.37	15.16	15.11	7.10	8.09		
Endosymbiont of *A*. (*Aphrastasia*) *pectinatae*	20.67	21.30	21.43	21.21	9.88	10.12	9.57	15.21	15.31	10.98	15.90	15.90	15.96	9.33	3.79	9.70	

group p-distances for the sequences from the same host aphid species were lower than 1% in most cases (table 3), except the endosymbiont of *P. cembrae* (average 3.36%, range 2.04-4.09%). Average between-group p-distances (%) for Adelgidae endosymbionts from different species of their hosts were from 1.50% ("*Ca*. A. pinicola" from *P. cembrae* and "*Ca*. A. pinicola" from *P. orientalis* - *P. pini*) to 22.28% ["*Ca*. A. pinicola" from *P. cembrae* and "*Ca*. Vallotia sp." from *A*. (*C*.) *viridanus*] (table 4). Groups of bacterial species level taxa mostly coincided with currently recognized seven genera of Adelgidae primary endosymbionts: "*Ca*. Vallotia" (beta-proteobacteria), "*Ca*. Profftia", "*Ca*. Annandia", "*Ca*. Hartigia", "*Ca*. Ecksteinia", "*Ca*. Steffania" and "*Ca*. Gillettellia" (gamma-proteobacteriae). The values of average intrageneric diversity of partial 16S rRNA were the following: 2.28% for "*Ca*. Vallotia", 2.68% for "*Ca*. Profftia", 1.86% for "*Ca*. Hartigia", 1.21% for "*Ca*. Annandia", 0.34% for "*Ca*. Ecksteinia", 0.09% for "*Ca*. Steffania", 0.14% for "*Ca*. Gillettellia", 3.36% for endosymbiont from *P. cembrae* and 0.92% for endosymbiont from *A*. (*A*.) *pectinatae*. The values lower than 1% were detected for those genera, where only one species was previously described. Average between-group sequence diversities for different genera of Adelgidae endosymbionts were from 3.79 to 21.76% (table 5). Average intergeneric p-distances were the highest between "*Ca*. Vallotia" and the representatives of gamma-proteobacteria and ranged from 18.06% to 21.76%. The values of intergenetic diversity between the remaining genera of gamma-proteobacteria were from 3.79% to 16.21%, see table 4 for details. Overall mean distances were 13.14% for 16S rRNA fragment (145 samples, 1486 bp).

Cophylogeny of Adelgidae and their endosymbiotic bacteria

Species trees for Adelgidae and their endosymbiotic bacteria coincided in their topology. The pattern of endosymbiotic bacteriae and respective adelgid host specificity is shown in figure 2. Cophylogeny map (Jane 4.0) based on Bayesian species trees showed a strong cophylogenetic signal: 10 cospeciations, 1 duplication, 5 duplications with host switch, 3 losses, and no failures to diverge (figure 3). Eight of ten cospeciation events are indicated as cases, when all other placements are worse, and only two are marked as cases, when equally good placements exist. Cospeciation events are present in all clades of Adelgidae endosymbionts species tree and correspond with host insect species feeding on the same genus of secondary host (figure 3). Similar pattern is observed for loss events: they are present in each clade representing Adelgidae endosymbionts detected in insects inhabiting the same secondary host genus. Three duplications followed by host switch are mapped near the ter-

Table 5. Average intergeneric p-distances (%) for Adelgidae endosymbionts.

	1	2	3	4	5	6	7	8	9
"*Ca*. Vallotia"									
"*Ca*. Profftia"	19.48								
"*Ca*. Hartigia"	19.37	9.81							
Endosymbiont of *P. cembrae*	18.73	10.25	10.19						
"*Ca*. Annandia"	21.76	14.63	14.94	15.97					
"*Ca*. Ecksteinia"	19.56	9.22	10.12	10.08	14.39				
"*Ca*. Steffania"	18.06	10.00	12.20	8.71	14.80	9.25			
"*Ca*. Gillettellia"	20.72	8.43	16.21	14.60	15.20	7.10	8.09		
Endosymbiont of *A*. (*Aphrastasia*) *pectinatae*	21.24	9.96	15.27	11.07	15.91	9.33	3.79	9.70	

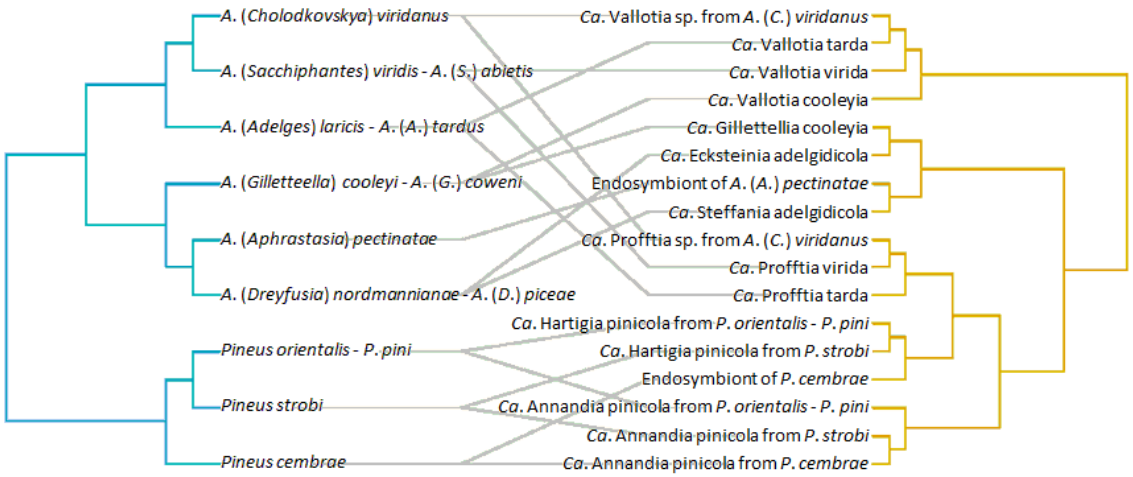

Figure 2. Tanglegram (TreeMap 3b1243) between endosymbiotic bacteria (right) and Adelgidae (left) reconstructed from 16S rRNA (bacteria) and COI and EF-1α DNA (Adelgidae) data. Lines connecting Adelgidae and endosymbiotic bacteria indicate patterns of host specificity. For details, see supplemental material figures S1, S2, S3.

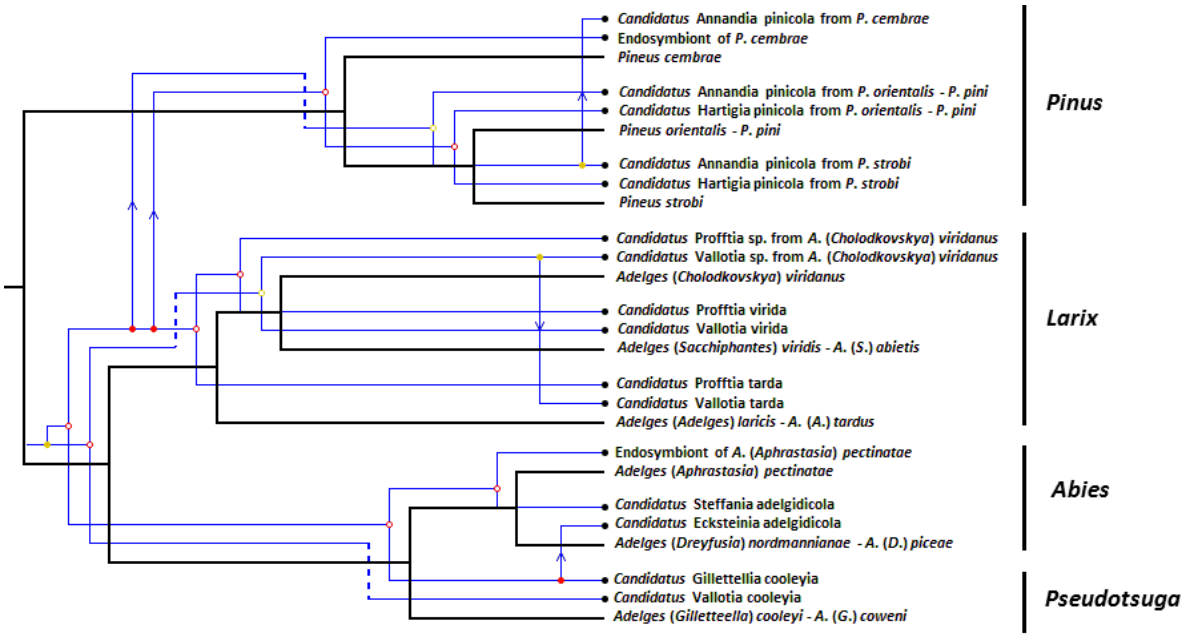

Figure 3. Cophylogeny of Adelgidae and their endosymbiotic bacteria from Jane 4 (Conow *et al*., 2010) with the reconciled trees based on the species trees of Adelgidae and bacteria. Blue lines - phylogeny of Adelgidae endosymbionts; black lines - phylogeny of Adelgidae; hollow red circles - cospeciation events, when all other placements worse; hollow yellow circles - cospeciation events, when equally good placement exists; solid red circles - duplication, when all other placements worse; yellow circles - duplication, when equally good placement exists; arrows - host switch events; dotted lines - loss events.

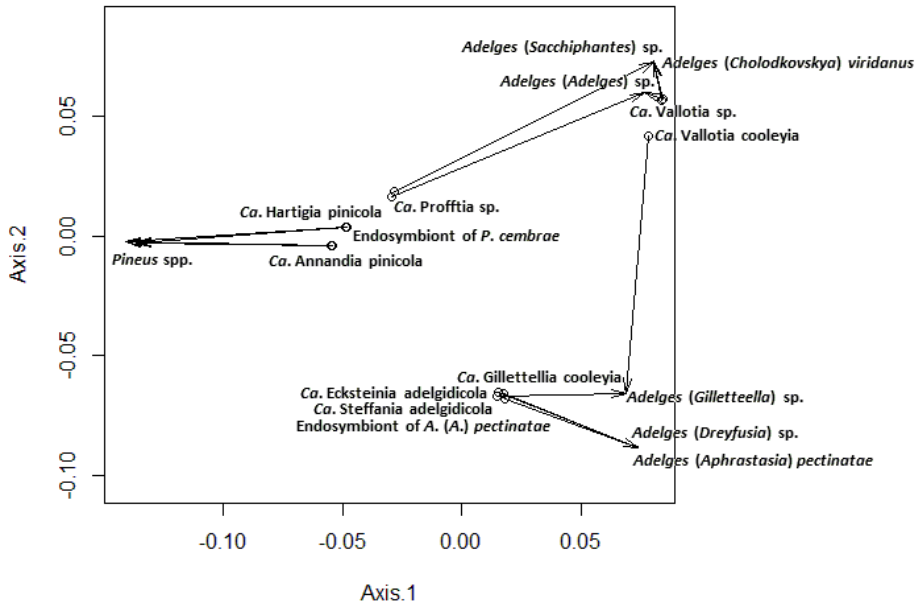

Figure 4. Procrustean superimposition plot of Adelgidae and their endosymbiotic bacteria. The ordinations of Adelgidae and endosymbionts are Principal Correspondence Coordinates of species trees based on COI and EF-1α fragments for Adelgidae and on 16S rRNA partial sequences for endosymbiotic bacteria. The configuration of endosymbionts (dots) has been rotated and scaled to fit the ordination of Adelgidae (arrow tips).

minal branches of species trees and three duplications are at the basal part, two of them are with host switch and are associated with *Pinus*-inhabiting Adelgidae and their endosymbionts. Distance-based analyses also showed a strong cophylogenetic signal for Adelgidae and their endosymbionts (figure 4). Host species grouped according to their secondary host plant species. Endosymbiont species belonging to gamma-proteobacteria from *Adelges* (*Dreyfusia*) and *Adelges* (*Aphrastasia*) inhabiting fir (*Abies*) and *Adelges* (*Gilletteella*) inhabiting *Pseudotsuga* clustered together. Gamma-proteobacteria endosymbionts from larch (*Larix*) inhabiting *Adelges* (*Adelges*), *Adelges* (*Sacchiphantes*) and *Adelges* (*Cholodkovskya*) formed a distinct cluster similarly as gamma-proteobacteriae from

Pinus inhabiting adelgids of the genus *Pineus*. Beta-proteobacteria represented by "*Ca*. Vallotia spp." also showed the correlation with secondary host plant of Adelgidae: *Larix* and *Pseudotsuga* inhabiting species were situated more distantly from each other. PACo analysis provided evidence for significant cospeciation (residual sum of squares m^2 global value = 0.20, P = 0.0042 based on 100000 permutations).

Discussion

Congruence between phylogenies of Adelgidae and their endosymbiotic bacteria was reported by Toenshoff *et al*. (2012a) based on aphid (COI and EF-1α) and bacterial (16S rRNA) sequences of *A.* (*D.) nordmannianae* - *A*. (*D.*) *piceae*, *A.* (*A.*) *laricis* - *A*. (*A.*) *tardus*, *A.* (*S.*) *viridis* - *A*. (*S*). *abietis* and *A.* (*G.*) *cooleyi* - *A*. (*G.*) *coweni* species complexes and their endosymbiotic bacteriae "*Ca*. Steffania adelgidicola", "*Ca*. Ecksteinia adelgidicola", "*Ca*. Profftia spp. ", "*Ca*. Vallotia spp. " and "*Ca*. Gillettellia cooleyia". Subsequent studies based on new adelgid species and several new populations of previously sampled species, including geographically distinct populations, and populations on alternate conifer hosts added more evidence concerning cospeciation of endosymbionts and adelgid hosts (von Dohlen *et al*., 2017). Our cophylogenetic analyses based on partial aphid COI and EF-1α sequences of 77 aphid samples from Lithuania representing 9 aphid taxa and bacterial 16S rRNA of their respective 17 endosymbiotic bacteria taxa also indicate reliable cophylogenetic relations between adelgid species and their endosymbionts (figures 3-4). One might consider that evidence on the coevolutionary relationships between adelgids and their endosymbionts support the viewpoint that relatedness between aphid species is the key factor explaining the microbiome composition (McLean *et al*., 2019). Such understanding is taken as an alternative to the one suggesting that ecological conditions (aphid host plant specificity) is the main reason facilitating relationships between endosymbionts and aphids (Henry *et al*., 2015). On the other hand, evolutionary history of Adelgidae is strictly dependent on acquisitions of new alternate-conifer hosts and formation of host-alternating life cycles (Steffan, 1968; Havill *et al*., 2007; 2016; Sano and Ozaki, 2012). Most of sap-feeding insects are adapted to feed on a single plant tissue - phloem, xylem, or parenchyma, however, adelgids are capable to exploit both phloem and parenchyma during their life cycle: generations on spruce (primary host) branches and inside galls tap nitrogen-rich parenchyma cells, whilst those on secondary host exploit nitrogen-poor phloem of conifer needles (von Dohlen *et al*., 2017). Such an alternation between phases of high nutrition and phases of low nutrition during the life cycles of Adelgidae was suggested to be the principal explanation of fluctuations in selection for nutritional provisioning by symbionts, consequent with acquisitions of new secondary conifer hosts (von Dohlen *et al*., 2017). Therefore, one might consider controversy concerning principal role of host aphid species relatedness versus host aphid ecology as an unjustified one. Namely, once bacteriome is important when acquiring new aphid host plant species (Havill *et al*., 2016), host plant mediated evolution of aphids is expected to be dependent also on the respective formation of effective microbiome. Both processes are mutually dependent.

The present data also contribute to the long lasting discussion on the taxonomic status of anholocyclic lineages in Adelgidae. For example, *Adelges* (*Adelges*) *laricis* Vallot and *Adelges* (*Adelges*) *tardus* (Dreyfus) differ in their life cycles. The former is holocyclic alternating between *Picea* and *Larix*, whilst the latter is anholocyclic monoecious on *Picea.* Apart from different life cycle, galls of *A.* (*A.*) *tardus* open later than those of *A.* (*A.*) *laricis* (August - September against June - July in Lithuania). Yet both species are reported to have little (if any) differences in their morphology (Albrecht, 2017; Blackman and Eastop, 2020), their interspecific distances in COI and EF-1α gene fragments are on the intraspecific level (Foottit *et al*., 2009; Žurovcová *et al*., 2010; Havelka *et al*., 2020). Our current data add new evidence supporting close similarity of both species: seventeen Lithuanian samples of *A.* (*A.*) *laricis* - *A*. (*A.*) *tardus* species complex grouped together by molecular species delimitation analysis based on Adelgidae partial COI and EF-1α sequences and bacterial16S rRNA fragment (table 3, supplemental material figures S1, S2, S3). The same holds for species complexes *A.* (*G.*) *cooleyi* - *A*. (*G.*) *coweni*, *A.* (*D.) nordmannianae* - *A*. (*D.*) *piceae*, *A.* (*S.*) *abietis* - *A*. (*S.*) *viridis* and *P. pini* - *P. orientalis*. This supports the opinion that anholocyclic aphid lineages of the above-mentioned adelgid species complexes should be taken for intraspecific units (life-cycle forms, host races or subspecies) rather than separate species (Foottit, 1997; Havill and Foottit, 2007; Sano and Ozaki, 2012; Ravn *et al*., 2013).

The level of 16S rRNA fragment diversity in Adelgidae endosymbiotic bacteria is similar to that of *Buchnera aphidicola* in Aphididae aphid species. Intraspecific divergences of 16S rRNA fragment of *B. aphidicola* from *Mollitrichosiphum tenuicorpus* (Hemiptera Aphididae Greenideinae) samples was reported to be 0-1.4%, and 0-0.4% from other six species of the same aphid genus (Liu *et al*., 2013). Our data for Adelgidae fall within this range (0-0.92%, except for endosymbiont from *P. cembrae*, which is 2.04-4.09%). According to Liu *et al*. (2013), interspecific genetic divergences of 16S rDNA for *Buchnera* from individual aphid species ranged from 0 to 5.8% and the average values of divergence within genera of Adelgidae endosymbiotic bacteria also were similar - from 0.09% in "*Ca*. S. adelgidicola" to 3.36% for endosymbiont from *P. cembrae*.

Endosymbionts of *A.* (*A.*) *pectinatae* and *P. cembrae*, which were detected with universal bacterial primers of 16S rRNA fragment (table 1), were the most similar to gamma-proteobacterial endosymbiotic bacteria of the genus *Sodalis*, 96.19-96.82% and 97.01-97.52% of sequence similarity, respectively. *Sodalis*-allied bacteria were reported as both presumable bacteriocyte-associated obligate, or primary, symbionts and facultative, or secondary, symbionts in a diverse array of insects (Hosokawa *et al*., 2015). These bacteria are also known as facultative endosymbionts of aphids that are restricted to

feeding on trees, mostly in the subfamily Lachninae (McLean *et al*., 2019), and some *Pinus* inhabiting species of the genus *Cinara* in particular (Meseguer *et al*., 2017). Noticeably, the *Sodalis*-allied symbiont has been reported as having replaced an ancient beta-proteobacterial symbiont in Cercopoidea (Hemiptera Auchenorrhyncha), potentially relaxing the severe energy limitations of the xylem feeding hosts (Koga and Moran, 2014). Additional analysis of endosymbionts harboured by other fir (*Abies*) and pine (*Pinus*) inhabiting Adelgidae species will provide further insights into the diversity and structure of bacterial communities and the co-evolution of bacteria and their aphid hosts.

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