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Phylogenetic relationships of *Dysaphis pyri* (Boyer de Fonscolombe) and *Dysaphis reaumuri* (Mordvilko) (Hemiptera, Sternorrhyncha: Aphididae): COI and EF-1α evidence

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Abstract *Dysaphis* (*Pomaphis*) *pyri* (Boyer de Fonscolombe, 1841) and *Dysaphis* (*Pomaphis*) *reaumuri* (Mordvilko, 1928) are two holocyclic aphid species alternating between *Pyrus* (Rosaceae) and *Galium* (Rubiaceae). Comparative phylogenetic analysis was performed using partial mitochondrial cytochrome oxidase subunit I (COI) and nuclear elongation factor 1 alpha (EF-1α) sequences. Partial COI data indicate the possibility of the early divergence in the *D. pyri–D. reaumuri* stem, which might have occurred even before the splitting of the common ancestral species of the *D. reaumuri–D. plantaginea* complex. Such a conclusion seems to be compatible with the available data on host specificity, life cycles and distribution of both species. This introductory phylogenetic analysis based on partial COI and EF-1α sequences indicates the need for reconsideration of the subgeneric structure in the genus *Dysaphis*.

Keywords *Dysaphis pyri* · *Dysaphis reaumuri* · Host plants · Mitochondrial COI · Morphology · Nuclear EF-1α

Introduction

The palaearctic aphid genus *Dysaphis* Börner, 1931 belongs to the subtribe Anuraphidina of the tribe Macrosiphini (Aphididae: Aphidinae). *Pomaphis* Börner, 1939, one of its subgenera, comprises 17 species (Remaudiére and Remaudiére 1997; Holman 2009). These aphids cause deformations of *Crataegus*, *Malus*, *Pyrus*, *Cotoneaster* and *Sorbus* leaves in spring, and then migrate to herbaceous plants of various families, e.g. Apiaceae (Umbelliferae), Asteraceae (Compositae), Campanulaceae, Plantaginaceae, Polygonaceae, Ranunculaceae, Rubiaceae, Valerianaceae (Blackman and Eastop 2000; Holman 2009). The so-called *Dysaphis* (*Pomaphis*) *pyri* species group (Stroyan 1985) comprise four species exploiting *Pyrus* and Rubiaceae/Linaceae as their winter and summer hosts, respectively. Of these four, *Dysaphis* (*Pomaphis*) *pyri* (Boyer de Fonscolombe, 1841) and *Dysaphis* (*Pomaphis*) *reaumuri* (Mordvilko, 1928) are the most widely distributed and predominant representatives of this species group (Holman 2009). Due to their economic importance (Barbagallo et al. 1997; Blackman and Eastop 2000) these species are also the most studied. Stroyan (1985) discussed the possible evolution of this group indicating host association, with pear being the primary host, and the evolutionary centre presumably of Asian origin. The hypothesis that members of this species group "probably diverged from each other rather long ago" (Stroyan 1985: 385) was based on morphological and host specificity data. The application of molecular techniques and phylogenetic analyses has revealed the relationships among congeneric taxa and the identity of particular species (Zhang et al. 2008; Carletto et al. 2009; Ellis et al. 2009; Puillandre et al. 2011). The case of *D. pyri* and *D. reaumuri* appears relevant from both theoretical and practical approaches. First, the DNA sequences of these species are not available in the GenBank at present (http://www.ncbi.nlm.nih.gov/genbank/, accessed 19 January 2012), so phylogenetic reconstruction and evaluation of species DNA sequences polymorphism is still not possible. Second, these species might also be important for the pest management, because harmfulness, pesticide resistance and natural enemy complexes of closely related pest aphid species are expected

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Table 1 Samples of *Dysaphis pyri* and *D. reaumuri* examined in this study. Sample numbering is the same in all figures and text. *COI* Mitochondrial cytochrome oxidase subunit I, *EF-1α* nuclear elongation factor 1 alpha

GenBank accession no.		Sample information and no.
COI	EF-1α	
Dysaphis pyri		
JQ437444	JQ437460	Skirgiškės, Vilnius reg., Lithuania, 22 June 2004, *Pyrus* sp. cult., 04-25
JQ437445	JQ437461	Nida, Neringa, Lithuania, 8 July 2004, *Pyrus* sp. cult., 04-107
JQ437446	–	Pervalka, Neringa, Lithuania, 10 July 2004, *Pyrus* sp. cult., 04-133
JQ437448	JQ437463	Nemunaitis, Utena reg., Lithuania, 19 July 2005, *Pyrus* sp. cult., 05-104
JQ437453	JQ437468	Tauras hill, Vilnius, Lithuania, 14 June 2011, *Pyrus communis*, 11-43
JQ437455	JQ437470	Skirgiškės, Vilnius reg., Lithuania, 29 June2011, *Pyrus communis*, 11-58
JQ437450	JQ437465	Afşin, Kahramanmaraş prov., Turkey, 31 May 2011, *Pyrus communis*, 11-32
JQ437451	JQ437466	Afşin, Kahramanmaraş prov., Turkey, 31 May 2011, *Pyrus communis*, 11-33
JQ437452	JQ437467	Afşin, Kahramanmaraş prov., Turkey, 31 May 2011, *Pyrus communis*, 11-37
JQ437447	JQ437462	Česky Krumlov, Czech Republic, 19 June 2005, *Pyrus* sp. cult., 05-43
JQ437449	JQ437464	Randazzo, Sicily, Italy, 10 June 2009, *Pyrus* sp. cult., 09-20
JQ437454	JQ437469	Bouville, France, 19 June 2011, *Pyrus* sp. cult., J11-05
Dysaphis reaumuri		
JQ437459	JQ437474	Çataloluk, Kahramanmaraş, Turkey, 31 May 2011, *Pyrus* sp. cult., 11-23
JQ437456	JQ437471	Tbilisi, Norio, Georgia, 8 June 2011, *Pyrus* sp., J11-12
JQ437457	JQ437472	Tbilisi, Norio, Georgia, 8 June 2011, *Pyrus* sp., J11-13
JQ437458	JQ437473	Tbilisi, Sartichala, Georgia, 9 June 2011, *Pyrus* sp., J11-23

to be much more similar when compared to more distantly related species.

The aim of this study was to evaluate evolutionary relationships of *D. pyri* and *D. reaumuri* based on the partial sequences of mitochondrial cytochrome oxidase subunit I (COI) and nuclear elongation factor 1 alpha (EF-1α) together with the available reference data concerning their host specificity, life cycles and distribution.

Materials and methods

Aphid material was collected in 2004–2011 and included samples from four European countries and also Georgia and Turkey (Table 1). Microscope slides in Canada balsam were prepared according to Blackman and Eastop (2000). Identification keys for pear-inhabiting aphid species compiled by Kolesova (1975), Shaposhnikov (1988) and Blackman and Eastop (2000) were used for morphological identification of samples. The key morphological characters used are illustrated in Fig. 1. Ethanol-preserved and mounted specimens are stored at the Department of Zoology, Vilnius University. Photographs of microscope slides were taken using an Olympus BX40 microscope equipped with MicroImage software (Olympus Optical, Hamburg, Germany). Images were edited with Adobe Photoshop CS5.

For molecular analysis, a single aphid individual from one sampled plant was considered as a unique sample. Total genomic DNA was extracted from a single aphid using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), which involved at least a 2-h digestion of tissue with proteinase K. Partial sequences of COI and EF-1α were PCR-amplified using previously published primers (Turčinavičienė et al. 2006). PCR amplification was carried out in a

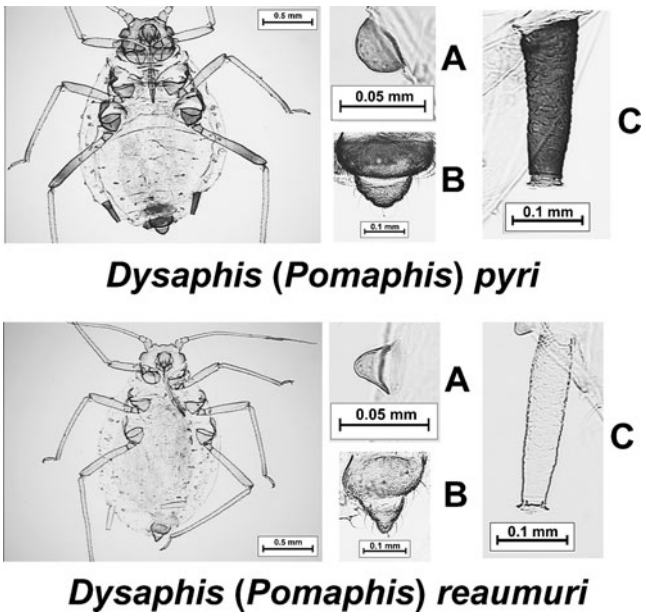

Fig. 1 **a–c** Apterous viviparous females of *Dysaphis* (*Pomaphis*) *pyri* (top, specimen from sample 11–32) and *Dysaphis* (*Pomaphis*) *reaumuri* (bottom, specimen from sample J11–12) showing the characters used to discriminate between species: **a** Lateral tubercle, **b** cauda, **c** siphunculus. Sample information is given in Table 1

thermal cycler (Eppendorf, Germany) in 50 μl volumes containing 1–2 μl genomic DNA, 5 μl of each primer (10 μM), 5 μl PCR-reaction buffer, 5 μl dNTP mix (2 mM each), 4–8 μl 25 mM $MgCl_2$ and 1.25 U AmpliTaq Gold 360 polymerase (5U/μl) and ddH_2O to 50 μl. The cycling parameters were as follows: denaturation at 95°C for 10 min (1 cycle), denaturation at 95°C for 30 s, annealing at 49°C (for COI) and 57°C (for EF-1α) for 30 s and extension at 72°C for 30 s (32–37 cycles in total), and a final extension for 5 min (1 cycle). PCR products were subjected to electrophoresis on 2 % TopVision agarose (Fermentas, Vilnius, Lithuania), stained with ethidium bromide and sized against a MassRuler Low Range DNA ladder (Fermentas) under UV light. PCR products were cleaned using the QIAquick PCR purification kit (Qiagen). Cyclic sequencing was performed at the Institute of Biotechnology (Vilnius, Lithuania) using a BigDye® Terminator v3.1 Cycle Sequencing Kit and products sequenced using a 3130xl Automated Sequencer (Applied Biosystems, Foster City, CA). The amplification primers were also used as sequencing primers. DNA sequences for each specimen were confirmed with both sense and anti-sense strands and aligned in the BioEdit Sequence Alignment Editor (Hall 1999). Sequences of the COI gene were tested for stop codons and none were found. The sequence data for all species have been submitted to GenBank (for accession numbers, see Table 1).

MEGA 5 (Tamura et al. 2011) was used to calculate uncorrected pairwise sequence distances (p-distances). To test the phylogenetic relationships of *D. pyri* and *D. reaumuri*, available sequences of other *Dysaphis* species were downloaded from GenBank (Table 2). Sequences of *Aphis gossypii* Glover, 1877 (Aphidini: Aphidina) and *Toxoptera citricida* (Kirkaldy, 1907) (Aphidini: Aphidina) were selected as outgroups (Table 2) for the phylogenetic analyses, which included maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference in phylogeny (BI). MP and ML analyses were performed using MEGA 5 (Tamura et al. 2011). Bootstrap values for MP tree were generated from 1,000 replicates. For ML analysis, the Tamura-Nei model with Gamma distribution (TN93 +G) and Tamura 3-parameter model (T92) were selected by MEGA 5 model selection option (Tamura et al. 2011) for COI and EF-1α fragments, respectively. To estimate the node support 1,000 bootstrap replicates were performed. Bayesian analysis was conducted in MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) using the general time reversible model with Gamma distribution (GTR +G), which was selected by jModeltest (Posada 2008) for both data sets. One run for 2,000,000 generations with tree sampling every 1,000 generations was performed using the coalescence model of the molecular clock. In this model, the tree generating process is looked at from the opposite perspective, backward in time, and, instead of lineages branching, it sees them as coalescing into fewer and fewer ancestral lineages (Ronquist et al. 2005). The topologies obtained by MP, ML and BI were similar, so only BI trees are shown with values of MP/ML bootstrap support indicated above branches. The COI and EF-1α alignments, together with dendrograms resulting from the various analyses, were submitted to the TreeBase website (study accession number S12354: http://purl.org/phylo/treebase/phylows/study/TB2:S12354).

Results and discussion

Analyzed partial sequences of COI contained 581 positions in the final set. Within-species mean p-distances were

Table 2 Partial sequences of COI and EF-1α from the GenBank used in the present study for comparison

Aphid species	GenBank accession no.	
	COI	EF-1α
Dysaphis (*Dysaphis*)		
Dysaphis rumecicola (Hori, 1927)	GU978795	HM117785
Dysaphis newskyi ossiannilssoni Stroyan, 1961	JF776568	
Dysaphis apiifolia petroselini (Börner, 1950)	JF776569	
Dysaphis (*Pomaphis*)		
Dysaphis plantaginea (Passerini, 1860)	JN546629 JN546628 JN546627 EU701636 JF521490	DQ005143
Outgroup species		
Aphis gossypii Glover, 1877	AY227082	EU019867
Toxoptera citricida (Kirkaldy, 1907)	EF591607	AY219728

0.03 % for *D. pyri*, ranging from 0.00 to 0.17 %, whilst all *D. reaumuri* samples appeared identical in their partial *COI* sequences. Between-species mean p-distances were 4.63 %, ranging from 4.48 to 4.65 %. The values of between-species sequence divergence for the COI fragment observed in this study appeared similar to those reported for other genera of the subtribe Anuraphidina, such as *Brachycaudus* van der Goot, 1914 (from 3.92 to 5.59 %), and *Muscaphis* Börner, 1933 (from 4.09 to 5.94 %) (Foottit et al. 2008). On the other hand, p-distance values of partial COI sequences for *D. pyri* and *D. reaumuri* turned out to be high enough to call into question the allocation of both species in the same subgenus. In the aphid genus *Brachycaudus*, between-species sequence divergence of partial COI sequences was reported to be from 2 to 3.5 % among species belonging to the same subgenus (Coeur d'acier et al. 2008), whilst our study showed p-distances between *D. pyri* and *D. reaumuri* ranging from 4.48 to 4.65 %. This level of sequence divergence is characteristic for species representing different subgenera of the genus *Brachycaudus* (Coeur d'acier et al. 2008). To clarify the subgeneric position of *D. pyri* and *D. reaumuri*, more representatives of different subgenera of the genus *Dysaphis* were included. Partial COI sequences of four *Dysaphis* species from GenBank (Table 2) were added to the data set analysed in this study, and the final alignment contained 217 positions. The results of distance analysis are presented in Table 3. The values of p-distances within species proved to be comparable with those calculated for the dataset containing 581 positions (see above). A similar trend was observed for between-species sequence divergence. In the phylogenetic tree based on partial COI sequences and representing relationships among *Dysaphis* species (Fig. 2), *D. reaumuri* seems to be closer to *Dysaphis plantaginea* (Passerini, 1860) than to *D. pyri*, thus corroborating the idea of the early divergence of *D. reaumuri* and *D. pyri* (Stroyan 1985). The present data suggest that divergence probably occurred between *D. pyri* and the common ancestor of *D. reaumuri–D. plantaginea* (Fig. 2). Separation of two subgenera of the genus *Dysaphis* is only poorly supported by this analysis (Fig. 2). In conclusion, distance (Table 3) and phylogenetic analyses (Fig. 2) of partial COI sequences indicate the need for reconsideration of the subgeneric structure of the genus *Dysaphis*.

Analyzed partial sequences of EF-1α contained 478 positions in the final set. Within-species mean p-distances were 0.32 % (0.00–0.84 %) for *D. pyri*, and 0.63 % (0.00–1.26 %) for *D. reaumuri*. Between-species mean p-distances were 1.95 %, ranging from 1.47 to 3.14 %. The values of between-species sequence divergence for the EF-1α fragment observed in this study appeared similar to those reported for other genera of the tribe Macrosiphini, and were close to 2 % (Kim and Lee 2008; Lee et al. 2011). Partial EF-1α sequences of two *Dysaphis* species available from GenBank (Table 2) were added to the data set analyzed in this study; the final alignment contained 489 positions, including gaps. The results of distance analysis are presented in Table 3. The values of p-distances within *D. pyri* and *D. reaumuri* seem to be comparable with those calculated for the dataset containing 478 positions (see above). Contrary to the COI data, distance (Table 3) and phylogenetic analyses (Fig. 3) of partial EF-1α sequences show closer relationships of *D. reaumuri* with *D. pyri* than with *D. plantaginea*, and definitely support the present subgeneric subdivision of the genus *Dysaphis*.

In general, available molecular data tend to support the opinion of Stroyan (1985) on the early divergence in the *D. pyri–D. reaumuri* stem. Partial COI sequence data indicate that separation might have occurred even before the splitting of the common ancestral species of the *D. plantaginea–D. reaumuri* complex (Fig. 2). Similarity in host specificity and life-cycles of *D. pyri* and *D. reaumuri* is suggested to be of secondary character, gained independently by allopatric vicariance. If winter host association with *Pyrus* is taken as a primary and primitive feature of the Anuraphidina (Shaposhnikov 1956; Stroyan 1985), then association with this host plant in the *D. pyri–D. reaumuri* stem should be treated as ancestral homology. Available reference data show *D. pyri* and *D. reaumuri* being almost identical in their winter host specificity and the single reference on the finding of *D. pyri* on *Malus*

Table 3 Within- and between-species divergences (p-distances, %) of the analyzed partial COI (217 positions in final set, bottom left) and EF-1α (489 positions in final set, top right) sequences among species of the genus *Dysaphis*

EF-1α COI	*D. rumecicola*; *n* =1	*D. plantaginea*; *n* =1	*D. newskii*	*D. apiifolia*	*D. pyri*; *n* =11 (0.00-1.05)	*D. reaumuri*; *n* =4 (0.00-1.26)
D. rumecicola; *n*=1		6.37	–	–	5.83 (5.53-6.37)	6.46 (6.14-7.42)
D. plantaginea; *n* =5 (0.00 %)	6.45		–	–	4,17 (3.99-4.62)	4.30 (3.98-5.24)
D. newskii; *n* =1	5.99	7.37		–	–	–
D. apiifolia; *n* =1	5.53	5.53	4.15		–	–
D. pyri; *n* =12 (0.00-0.46)	6.87 (6.45-6.91)	6.41 (5.99-6.45)	8.72 (8.29-8.76)	9.18 (8.76-9.22)		1.95 (1.47-3.35)
D. reaumuri; *n* =4 (0.00)	4.15	2.77	6.45	5.99	5.49 (5.07-5.53)	

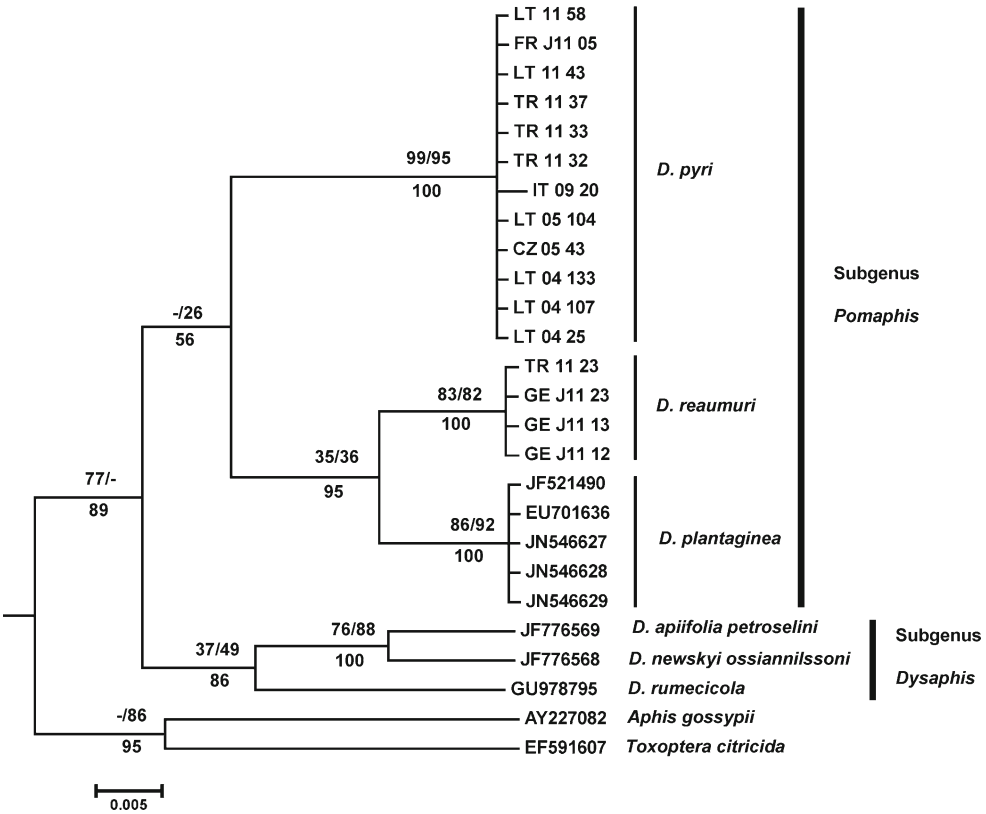

Fig. 2 Bayesian inference (BI) tree showing phylogenetic relationships among *Dysaphis pyri*, *Dysaphis reaumuri* and other congeneric species with two outgroup species, *Aphis gossypii* and *Toxoptera citricida*, based on partial sequences of mitochondrial cytochrome oxidase subunit I (COI; 217 positions in final set). Numbers above branches indicate support of maximum parsimony (MP; *left*) and maximum likelihood (ML; *right*) bootstrap test with 1,000 replicates, and numbers below branches indicate posterior probabilities of BI analysis. Sample acronyms as in Table 1

and *Cydonia* (Wood-Baker 1979) may be taken as an accidental case (Table 4). Nonetheless, experimental host specificity studies indicate *D. pyri* being associated mostly with cultivated pear varieties (Kolesova 1974; Rakauskas 1996), which has also been confirmed by other reference data (summarized by Blackman and Eastop 2000; Holman 2009). Conversely, *D. reaumuri* do not avoid wild pears (Kolesova 1974; Grigorov 1977; Blackman and Eastop 2000). The taxonomic status of taxa in the genus *Pyrus* is rather complicated due to frequent

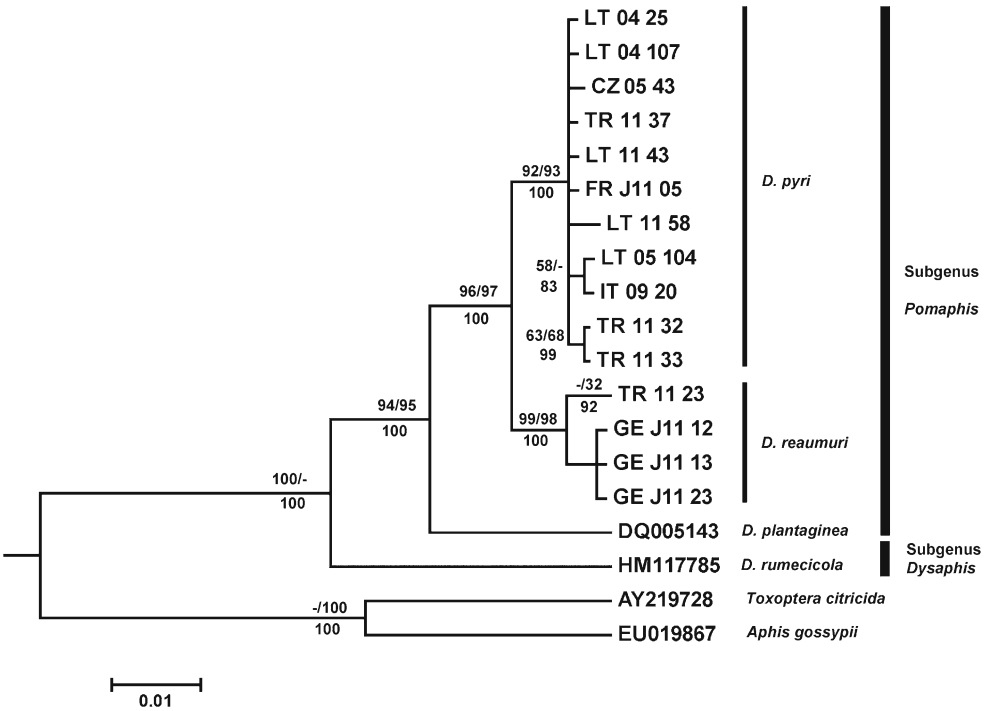

Fig. 3 BI tree showing phylogenetic relationships among *D. pyri*, *D. reaumuri* and other congeneric species with two outgroup species, *A. gossypii* and *T. citricida*, based on partial sequences of nuclear elongation factor 1 alpha (EF-1α; 489 positions in final set). Numbers above branches indicate support of MP (*left*) and ML (*right*) bootstrap test with 1,000 replicates, and numbers below branches indicate posterior probabilities of BI analysis. Sample acronyms as in Table 1

interspecific crosses (Yamamoto and Chevreau 2009). Whatever the current taxonomic status of wild and cultivated *Pyrus* taxa (species, subspecies or varieties), they differ in their characters, including molecular ones (Volk et al. 2006). Therefore, different winter host specificity of aphids, even at the pear subspecies or variety level, might demand different evolutionary strategies when adapting for feeding on different *Pyrus* taxa available in different areas. This might explain coexistence of both ecologically similar species in their overlapping distribution areas. The centre of origin of the genus *Pyrus* is thought to be around the foothills of the Tian Shan—a mountain range of Central Asia—whilst varieties of the cultivated European pear (*Pyrus communis* subsp. *communis*) are undoubtedly derived from one or two wild subspecies (*P. communis* subsp. *pyraster* and *P. communis* subsp. *caucasica*) in Asia minor (Transcaucasia, Iran, Turkmenistan) (Volk et al. 2006; Yamamoto and Chevreau 2009). *D. pyri* is reported to have Holarctic distribution, associated originally with southern regions of the Palaearctic area. *D. reaumuri* has a more local distribution area, being associated with the Ponto-Caspian region (Blackman and Eastop 2000; Holman 2009; Nieto Nafria et al. 2010). When the known natural history of genus *Pyrus* is compared with the known distribution areas of both aphid species, the slight difference in their winter host specificity may be explained by their pre-existence in different regions of the distribution area of *Pyrus*.

D. pyri has been reported as having a broader summer host species list, containing 14 species belonging to two plant families, when compared with *D. reaumuri*, which was detected on 3 species of one family (Table 4). Experimental transfers showed *Galium mollugo* being the preferred summer host both for *D. pyri* and *D. reaumuri* (Kolesova 1974, 1975; Grigorov 1977). In addition to similar host specificity, both species exhibit identical life-cycles: they are obligatorily heteroecious. Although viviparous females can thrive throughout the entire season on pears producing oviparae at the end of the season, males emerge on summer hosts only (Kolesova 1974, 1975; Grigorov 1977). Such similarity in host specificity and life-cycles of both species corroborates the allopatric vicariance speciation model rather than sympatric divergence. The niche fragmentation model, which is based on separation of the life-cycle and/or host specificity in diverging sympatric aphid populations, is considered the most reliable model of sympatric speciation in aphids. A substantial study of the sympatric speciation of *Cryptomyzus* Oestlund, 1922 aphids has demonstrated that specialization to exploit different summer hosts has led to the emergence of sympatric siblings, *Cryptomyzus galeopsidis* (Kaltenbach, 1843) and *Cryptomyzus maudamanti* Guldemond, 1990 (Guldemond 1990; Guldemond and Dixon 1994). The same process has been shown to take place in the experimentally induced host specificity changes in the anuraphidine aphid species *Dysaphis anthrisci* Börner, 1950 (Shaposhnikov 1965). The study of the sympatric currant-inhabiting *Aphis* Linnaeus species, *Aphis grossulariae* Kaltenbach, 1843 and *Aphis schneideri* (Börner, 1940), also supports the niche fragmentation model: both sympatric species have slightly different

Table 4 Reference data concerning the host plants of *Dysaphis pyri* and *D. reaumuri* (Kolesova, 1974, 1975; Grigorov 1977; Wood-Baker 1979; Heie 1992; Blackman & Eastop 2000; Holman 2009; Blackman 2010). Host plants are grouped by the descending reference numbers

Dysaphis pyri	*Dysaphis reaumuri*
Winter hosts	
Rosaceae	Rosaceae
Pyrus communis L.	*Pyrus communis* L.
Pyrus pyraster Burgsd.	*Pyrus pyraster* Burgsd.
Pyrus amygdaliformis Vill.	*Pyrus amygdaliformis* Vill.
Pyrus pashia Buch.-Ham.ex D.Don.	*Pyrus salicifolia* Pall.
Pyrus elaeagnifolia Pall.	*Pyrus korshinskyi* Litv.
Pyrus ussuriensis Maxim.	*Sorbus domestica* L.
Pyrus nivalis Jacq.	*Pyrus georgica* Kuth.
Sorbus domestica L.	*Pyrus*
Sorbus aucuparia L.	*Pyrus syriaca* Boiss.
Pyrus sargentii Bean.	*Pyrus boissieriana* Buhse
Pyrus khasyana Hook f.	
Pyrus	
Malus domestica Borkh.	
Cydonia oblonga Mill.	
Summer hosts	
Rubiaceae	Rubiaceae
Galium mollugo L.	*Galium aparine* L.
Galium verum L.	*Galium mollugo* L.
Galium sylvaticum L.	*Cruciata taurica* (Pallas ex Willd.)
Rubia peregrina L.	*Galium tauricum* = *Cruciata taurica*
Rubia agostinhoi Dansereau	
Galium spurium L.	
Galium schultesii Vest.	
Galium pseudoaristatum Schur.	
Galium odoratum (L.) Scop.	
Galium album L.	
Linaceae	
Linum	
Linum austriacum L.	
Primulaceae	
Primula	
Galium aparine L.	
Galium	
Asperula cynanchica L.	

host spectra yet remain closely related in their morphological features (Rakauskas 1998). Moreover, they are inseparable in their partial EF-1α gene sequences and very similar in their partial COI sequences with between-species partial COI sequence divergences ranging from 0.48 to 0.81 % (Turčinavičienė et al. 2006; Rakauskas et al. 2011).

The above molecular data suggest that the similarity in host specificity and life cycles of *D. pyri* and *D. reaumuri* can be explained by the early divergence of both species, with the subsequent allopatric vicariance mode explaining their evolutionary history. This introductory phylogenetic analysis based on partial COI and EF-1α sequences indicates that the subgeneric structure in the genus *Dysaphis* should be reconsidered.

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Ethical standards The experiments comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

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