Zootaxa 1688: 1–19 (2008)
www.mapress.com/zootaxa/

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

Molecular and morphological evaluation of the aphid genus *Hyalopterus* Koch (Insecta: Hemiptera: Aphididae), with a description of a new species

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Abstract

Aphids in the genus *Hyalopterus* Koch (Hemiptera: Aphididae) are pests of stone fruit trees in the genus *Prunus* globally, causing damage directly through feeding as well as transmission of plant viruses. Despite their status as cosmopolitan pests, the genus is poorly understood, with current taxonomy recognizing two, likely paraphyletic, species: *Hyalopterus pruni* (Koch) and *Hyalopterus amygdali* (Blanchard). Here we present a systematic study of *Hyalopterus* using a molecular phylogeny derived from mitochondrial, endosymbiont, and nuclear DNA sequences (1,320 bp) and analysis of 16 morphometric characters. The data provides strong evidence for three species within *Hyalopterus*, which confirms previous analyses of host plant usage patterns and suggests the need for revision of this genus. We describe a new species *H. persikonus* Miller, Lozier & Foottit n. sp., and present diagnostic identification keys for the genus.

Key words: Molecular, morphology, evaluation, *Hyalopterus*

Introduction

Entomologists have long been aware of the problems involved in identifying and describing species of closely related, morphologically similar groups of insects (Walsh 1864; Brown 1959; Hebert *et al*. 2004). The lack of informative morphological characters in many groups has led to difficulties in delineating species and determining their evolutionary relationships using traditional criteria, and such taxa have become appropriately known as 'cryptic species' (Brown 1959; Bickford *et al*. 2007). Cryptic species are especially common among the phytophagous insects, and careful research over the last several decades has revealed many morphologically similar complexes of reproductively isolated and previously unrecognized species with unique ecological characteristics (*e.g.* Guttman *et al*. 1981; Diehl & Bush 1984; Feder *et al*. 1998; Dres & Mallet 2002).

Accurate taxonomy that includes phylogenetic relationships is important for testing hypotheses regarding ecological and evolutionary patterns (Futuyma 1991; Nosil & Mooers 2005), and is also highly relevant for applied scientists striving to detect as well as prevent biological invasions and manage insect pests (Miller & Rossman 1995; Gordh & Beardsley 1999; Hoelmer & Kirk 2005). Rapid and accurate identification of a novel pest species allows access to a much wider store of biological data, which may include information on ecology, potential regions of origin, and interacting natural enemies. All such knowledge can assist in determining the most appropriate management strategies for a given species, including the need for quarantine or the development of a biological control program. For example, cryptic pest species of similar morphology may be

Accepted by L. Mound: 10 Dec. 2007; published: 23 Jan. 2008

found to have unique geographic distributions, life histories, and ecological relationships. Alternatively, groups of taxa with a diversity of ecological characteristics may be misclassified as 'good' species when, in reality, interbreeding among populations may be widespread and common. Both scenarios can have important implications, and an understanding of species level diversity in these groups is critical to avoid misidentification and potential mismanagement (Rosen 1986).

When considered in the context of the multitude of species concepts, defining and distinguishing among cryptic species can be a difficult task. For example, how can reproductive isolation be tested (as required for the 'biological species concept') if the appropriate taxa cannot be distinguished? The lack of readily distinguishable morphological characters has led to the use of alternative methods of species identification, more especially molecular markers which have assisted in the identification of diversity using DNA sequences (e.g., Hebert *et al*. 2004). These methods have their own pitfalls however, particularly if a single locus is relied upon (e.g., Moritz & Cicero 2004), or if few individuals per species are examined (Funk & Omland 2003), and their use and misuse in systematics is currently under scrutiny (Savolainen *et al*. 2005; Cameron *et al.* 2006). Perhaps the most important consideration deals with identifying new species: when a new DNA sequence is discovered, how can a novel species be distinguished from previously unrecognized intraspecific diversity (Mortiz & Cicero 2004; Meyer & Paulay 2005)? A preferable strategy uses a holistic approach, including data from morphological, genetic, and ecological characters. Once clear and consistent patterns of differentiation have been established with such an array of characteristics, simpler diagnostics may be used to identify species on a practical level (Meyer & Paulay 2005).

The genus *Hyalopterus* Koch contains two recognized species (i.e., Remaudière & Remaudière 1997): *H. pruni* (Geoffroy 1762) and *H. amygdali* (Blanchard 1840). Primary hosts of *Hyalopterus* are stone-fruit plants of the genus *Prunus* (Rosaceae), including the agriculturally important species plum (*P. domestica* L.), peach (*P. persica* (L.) Batsch), almond (*P. dulcis* Miller, syn. *P. amygdalus* Batsch), and apricot (*P. armeniaca* L.). *Hyalopterus* aphids are cyclically parthenogenetic and heteroecious throughout most of their range, having multiple asexual generations during the spring and summer, when winged forms migrate to secondary hosts, typically *Phragmites* reeds. In the autumn, aphids migrate back to the primary hosts, where a single sexual generation occurs, resulting in an overwintering egg stage. Because of their potential for rapid population growth and dispersal over great distances during migratory periods, *Hyalopterus* aphids are considered serious pests of stone-fruit crops (Blackman & Eastop, 2000). These aphids cause plant damage directly through feeding and indirectly due to the sooty mold which grows on their honeydew as well as through the transmission of plant viruses such as plum pox virus (Isac *et al*. 1998; Elibuyuk 2003). In California, particularly where *Hyalopterus* is adventive and found largely on plum and pluot (a plum-apricot cross), this pest has been recognized as a major factor inhibiting the reduction of pesticide use on these crops.

The exact nature of species within the genus *Hyalopterus* has been long debated (Smith 1936; Eastop 1966; Basky & Szalay-Marszo 1987; Mosco *et al*. 1997; Lozier *et al*.2007; Poulios *et al*. 2007). Identification has classically been based on host plant collection data (e.g., Barbagallo *et al*. 1997), with plum and apricot considered the primary hosts of *H. pruni*, and almond and peach utilized by *H. amygdali* (Mosco *et al*. 1997; Poulios *et al.* 2007). However, morphological studies have emphasized difficulties in distinguishing between *H. pruni* and *H. amygdali*, (Eastop 1966; Basky & Szalay-Marszo 1987). In contrast, genetic analyses using allozymes, mitochondrial and symbiont DNA sequences, and microsatellites have revealed three well-defined lineages within *Hyalopterus*, clearly highlighting problems with the taxonomy of this group (Mosco *et al*. 1997; Lozier *et al.* 2007). A recent study of morphometric characters in *Hyalopterus* from Greece revealed results similar to the genetic analyses, and demonstrated that morphological discrimination is possible using a sufficient number of characters (Poulios *et al.* 2007).

In this paper, we synthesize results from molecular, morphological, and ecological characters to identify cryptic species in *Hyalopterus*. First, we present a molecular phylogenetic analysis that extends the work of Lozier *et al*. (2007) using DNA sequences from 4 genes (2 mitochondrial, 1 endosymbiont, and 1 nuclear) to

increase our understanding of the molecular phylogeny and genetic diversity of the genus. In the context of this phylogeny, we also present a multivariate analysis of 16 morphological characters for aphids collected from the main *Prunus* host plants and from locations around the Mediterranean and in North America. The present study increases both the number of morphological characters examined and the geographic range sampled compared to earlier analyses (Poulios *et al.*2007), and supports previous findings suggesting the presence of three broadly distributed and host specific *Hyalopterus* species (Mosco *et al.* 1997; Lozier *et al.* 2007; Poulios *et al.* 2007). Lastly, we use these findings to redescribe members this genus and present diagnostic identification keys.

Materials and methods

Taxa studied

For molecular analyses we used aphids collected from primary host plants at various sites around the Mediterranean and in the USA (Table 1). We selected 24 *Hyalopterus* specimens from geographically disparate regions and from each of three previously identified host associated lineages (based on COI and microsatellite data; Lozier *et al*. 2007). Specimens were selected from an extensive part of *Hyalopterus*' range so as to investigate possible intraspecific genetic diversity and to ensure that species differences were maintained across a broad geographic area. We focused the analysis on aphids from plum, almond, and peach because these plants have been shown to be most strongly associated with *Hyalopterus* species; however, we also included three samples from Mediterranean apricot. One aphid from *Phragmites* was also sequenced so that eastern North America could be represented. Outgroups included *Aphis fabae* Scopoli which was selected as a represetnative of Aphidina (Aphidinae, Aphidini) and *Rhopalosiphum padi* (L.)and *Schizaphis graminum* (Rondani) which are representatives of Rhopalosiphina (Aphidinae, Aphidini), of which *Hyalopterus* is a member (Remaudière & Remaudière 1997). *Aphis fabae was* collected from laboratory colonies at UC Berkeley and *R. padi* and *S. graminum* sequences were taken from GENBANK (Table 1).

For morphometric analysis, we used aphids collected from 16 localities that were pooled into 10 groups as follows: Greece, plum (2 sites), almond (2 sites), peach (1 site); Italy, plum (1 site), almond (1 site), peach (2 sites); Republic of Georgia, plum (1 site); and Spain, plum (2 sites), peach (2 sites), almond (2 sites). We used adult apterous aphids, as this stage was the most readily available and the one upon which much of the previous taxonomy of the genus has been based. In total, the data set comprised up to 50 specimens representing each regional host plant group. Representative specimens were deposited in The Natural History Museum (BMNH), London, UK; Canadian National Collection of Insects (CNCI), Agriculture and Agri-Food Canada, Ottawa, the Essig Museum of Entomology (EMEC), University of California, Berkeley, and the National Museum of Natural History (USNM) Systematic Entomology Laboratory, USDA, ARS, Beltsville, Maryland.

Molecular methods

We used portions of the genes *Cytochrome Oxidase I* (COI; mtDNA), 12S rDNA (12S; mtDNA), *Buchnera* 16S rDNA (*Buch*16S; primary endosymbiont), and an intron from *Elongation Factor 1 alpha* (EF-1α, nuclear intron) to construct the molecular phylogeny. These genes were amplified in the 24 selected *Hyalopterus* individuals using polymerase chain reaction (PCR) with the primers as shown in Table 2. All PCR amplifications were performed in 10 µl volumes with 1.25 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl at pH 8.3, and 15 mM $MgCl_2$), 1.25 µl BSA (1.0 mg/mL), 0.6 mM dNTPs, 0.4–0.5 µM of each primer, and 0.35 U AmpliTaq® (Applied Biosystems, ABI) under the following thermocycling conditions: 2 min at 94 °C; 34–38 cycles of 30 s at 94 °C, 30 s at the appropriate annealing temperature (Table 2), and 30 s at 72 °C; with a final extension of 7 min at 72 °C. PCR products were purified using *ExoSAP-IT*® (USB Corporation). Using the appropriate PCR primers, we sequenced products in both directions for each locus in 10 µl

volumes under the following cycle sequencing conditions: 0.5 μl BigDye® v3.1 (ABI), 0.5 μl 5X sequencing buffer, 4.0–5.0 pmol primer, and 1.0–2.0 μl purified PCR product. Cycle sequencing products were purified using Sephadex® and sequenced on an ABI 3730 DNA sequencer.

Sequences were checked, edited, and aligned by eye in Sequencher™ 4.0 (Gene Codes Corporation). As expected for a nuclear gene, for *EF-1α* we detected a few instances of heterozygous nucleotide sites within individuals (based on equal or near equal chromatogram peaks), though no evidence of intra-individual or intraspecific length polymorphism. Heterozygous sites in *EF-1α* were coded as ambiguities using IUB nucleotide ambiguity code letters for the purposes of phylogenetic analysis.

TABLE 1. Collection information and sequence characterization for specimens used in the molecular analysis. Coordinates (latitude, longitude) are given in decimal degrees and the Clade identifier signifies the placement of each specimen in the full phylogeny (Figure 2).

Sample ID	Geographic region	Coordinates	Host plant	Clade	Genbank accession numbers			
					COI	12S	*Buch*16S	EF1
B12-1	Spain	37.77, -1.32	Almond	B	EF364008	EF363960	EF363984	EF363936
B17-1	Spain	36.99, -2.62	Plum	A	EF364009	EF363962	EF363986	EF363937
B22-1	Spain	38.14, -0.96	Peach	C	EF364010	EF363963	EF363987	EF363938
B32-1	Spain	39.27, -0.57	Peach	C	EF364019	EF363964	EF363988	EF363939
B5-1	Spain	39.85, -0.51	Peach	C	EF364020	EF363965	EF363989	EF363940
B7-1	Spain	39.85, -0.51	Apricot	B	EF364011	EF363966	EF363990	EF363941
D15-1	Greece-Mainland	36.88, 22.54	Almond	B	EF364013	EF363967	EF363992	EF363943
D29-1	Greece-Mainland	38.92, 22.60	Apricot	C	EF364014	EF363973	EF363993	EF363944
D1-1	Greece-Crete	35.52, 24.02	Plum	A	EF364012	EF363970	EF363991	EF363942
D7-1	Greece-Crete	35.07, 25.20	Apricot	C	EF364015	EF363974	EF363994	EF363945
K1-1	Greece-Crete	35.30, 25.25	Plum	A	EF364027	EF363972	EF364003	EF363955
K5-1	Greece-Crete	35.07, 25.20	Almond	B	EF364028	EF363982	EF364004	EF363956
K6-1	Greece-Crete	35.07, 25.20	Peach	C	EF364021	EF363979	EF364005	EF363957
E1-1	Tunisia	37.17, 10.03	Almond	B	EF364016	EF363968	EF363995	EF363946
F11-1	Italy	40.45, 17.51	Almond	B	EF364017	EF363975	EF363996	EF363947
F15-1	Italy	41.99, 14.98	Peach	C	EF364018	EF363977	EF363997	EF363948
F16-1	Italy	42.10, 14.71	Plum	A	EF364030	EF363971	EF363998	EF363949
F3-1	Italy	40.56, 17.81	Peach	C	EF364022	EF363978	EF363999	EF363950
J3-1	Israel	33.20, 35.59	Plum	A	EF364024	EF363981	EF364000	EF363952
J5-1	Israel	33.08, 35.24	Almond	B	EF364025	EF363976	EF364001	EF363953
J8-1	Israel	32.56, 34.97	Almond	B	EF364026	EF363969	EF364002	EF363954
HAMCA04-1	California, USA	39.74, -122.0	Plum	A	EF364023	EF363980	EF364006	EF363951
MD1-1	Maryland, USA	39.01, -76.9	Phragmites	A	EF364029	EF363961	EF364007	EF363958
RG1-1	Rep. of Georgia	41.92, 44.24	Plum	A	EF364031	EF363983	EF363985	EF363959
R. padi				Outgroup	AY594671	RPU36736	BUHRR16SE	AY219719
S. graminum				Outgroup	AY531391	AF275249	BUHRR16SC	AF068479
A. fabae				Outgroup	EF436580	EF436581	EF436582	EF436583

Phylogenetic analysis

Phylogenetic analysis was performed individually for each gene and for a concatenated data set (1,320 bp). While combined analysis of multiple loci can increase the precision and resolution of a phylogenetic tree,

incongruence in tree topologies can be problematic. To test for combinability, exhaustive pairwise incongruence length difference tests (ILD, Farris et al. 1994) were performed in PAUP* v4.0b10 (Swofford 2002) using all taxa, 100 replicates, and parameters at default settings (uninformative discarded sites, gaps treated as missing data, state changes weighted equally). Phylogenetic analyses were conducted using MrBAYES v3.1 (Huelsenbeck & Ronquist 2001). Models of evolution for each gene were chosen using MrMODELTEST (Nylander 2002; Table 2) and the appropriate code for each model was inserted in the MrBAYES input file. Contiguous gaps in *Buch*16S and 12S genes were treated as single characters using a separate partition coded as presence-absence restriction data. Gaps in *EF-1α* were excluded from the analysis as they were only present in outgroup taxa. The combined data set was partitioned by gene. All parameters were estimated independently except for topology and branch length, which were linked. For all analyses, uniform prior probabilities were used and 2 simultaneous runs with 4 heated chains for 5×10^6 'generations' each, sampling trees every 100 generations. The first 12,500 of the 50,000 sampled trees for each run were discarded, the remaining trees being summarized into a 50 % majority rule consensus tree using MrBAYES' 'sumt' command. Support for nodes was assessed by posterior probability (henceforth *PP*). Parsimony and neighbor joining methods for tree reconstruction was explored, but resulting relationships among taxa were essentially identical, and only trees estimated with the Bayesian method are presented.

TABLE 2. Loci used for phylogenetic analyses, with primers, reaction conditions, and the evolutionary model implemented in the MRBAYES analyses.

Locus (Genome)	Primer pair	Reference	Annealing Temp (ºC)	Sequence length (bp)	Evolutionary model
Cytochrome Oxidase I (mitochondrial)	C1-J-1718	Simon *et al.* 2006	52-53	343	GTR + I
	C1-N-2191				
12S (mitochondrial)	12Sai	Simon *et al.* 1991	52	359	F81
	12Sfi				
Elongation Factor Iα (nuclear)	EF1	Palumbi 1996	48-50	228	HKY
	EF2				
Buchnera aphidicola 16S rDNA (endosymbiont)	Buch16S1F	Tsuchida *et al.* 2002	52	393	GTR + I
	Buch16S1R				

Sequence diversity statistics for each locus—number of haplotypes, number of substitutions, number of segregating sites (*S*), transition: transversion ratio (*Ti*:*Tv*), nucleotide composition, and nucleotide diversity (π_n)— were calculated in ARLEQUIN 3.01 (Excoffier *et al.* 2005) using a pairwise difference distance model, with gaps and ambiguous sites excluded.

Morphometric measurement and analysis

Adult apterous viviparous specimens of *Hyalopterous* were cleared and individually mounted in Canada balsam on microscope slides using techniques as described by Maw & Foottit (1998). To determine differences in size and shape among the specimens, we measured characters that had been found useful in other studies of aphid morphometrics (Foottit & Mackauer 1990; Foottit 1992). We selected 16 continuous characters for analyses; other characters were measured and subsequently rejected because of high correlation with other characters, or because there were difficulties in precisely measuring them due to such factors as specimen mounting artifacts and specimen orientation (Table 3). Operational dimensions of the measurements are as shown in previous studies (Foottit & Mackauer 1990).

Morphological measurements were made by projecting each microscope slide specimen image onto a dig-

itizing tablet (SummaSketch II®, Summagraphics® Corporation, Seymour, Connecticut) using a Richert Polyvar compound microscope. Data capture was carried out using software written by E. Maw (Agriculture and Agri-Food Canada, Ottawa).

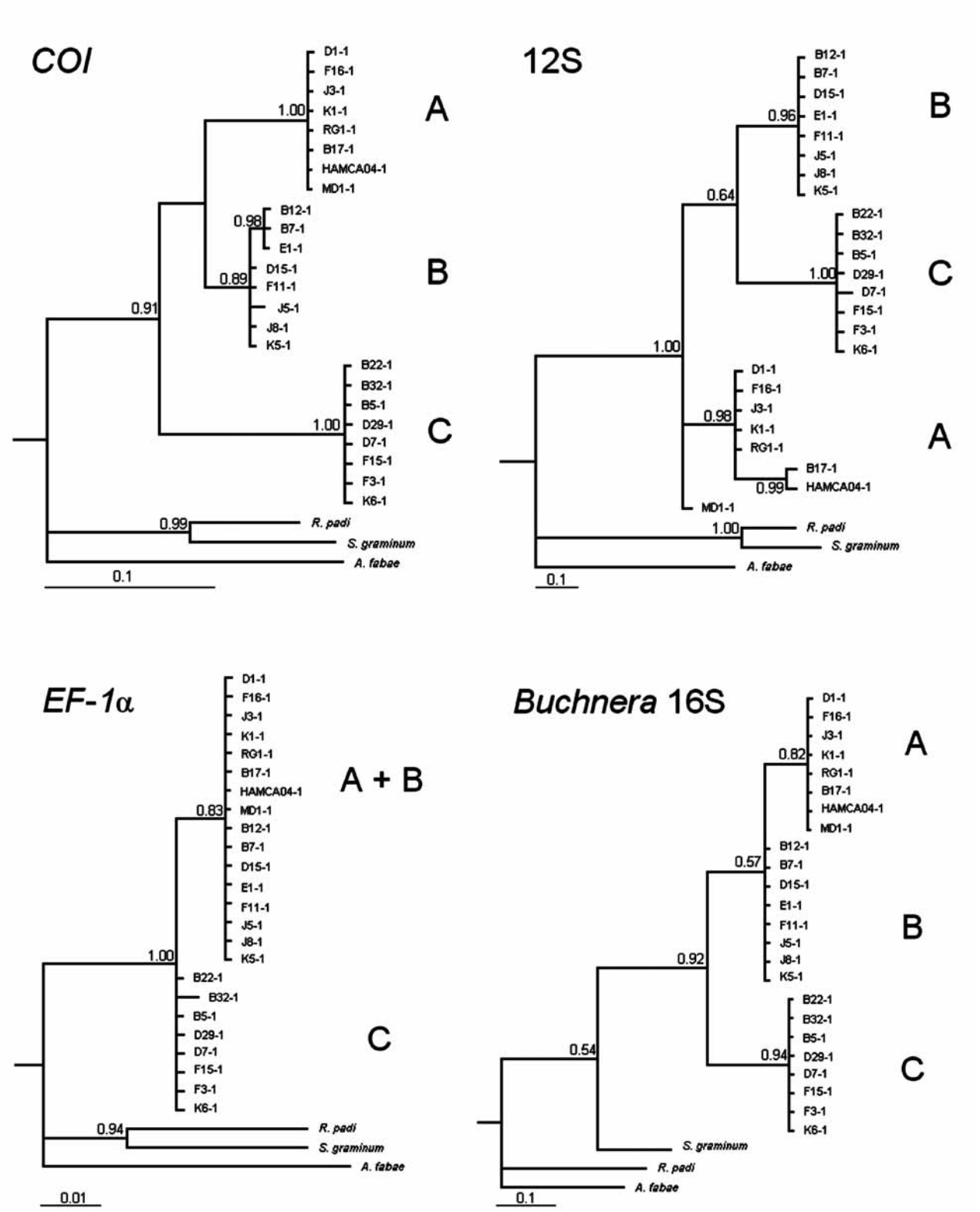

FIGURE 1. Locus specific phylogenies for *COI*, 12S, *EF-1α*, and *Buchnera* 16S. All trees are from analyses performed with MRBAYES, with node support given as posterior probabilities. Collection data for each specimen are provided in Table 1 and models of sequence evolution used for each gene are provided in Table 2.

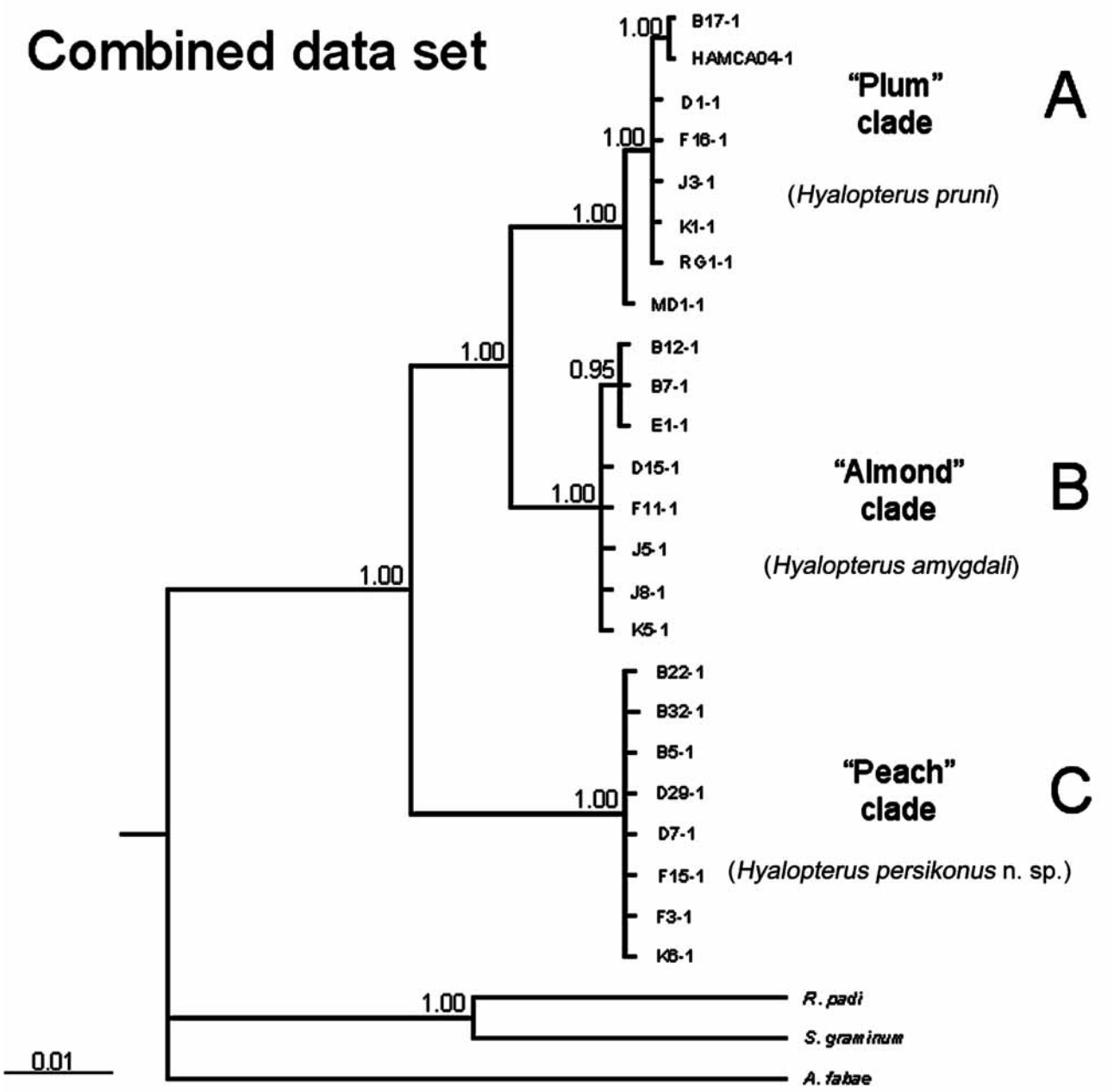

FIGURE 2. Phylogeny of the genus *Hyalopterus* constructed from 1322 concatenated base-pairs (plus two informative gaps) from all four genes. The data was partitioned by gene, using the models of evolution given in Table 2, in the program MrBAYES. Node support for each clade is given as a posterior probability value. See Table 1 for detailed sample information.

Multivariate morphometric analyses were used to represent subtle, multidimensional patterns of variation among specimens of *Hyalopterus* (Sorensen & Foottit 1992). Specifically, Principal Components Analysis (PCA; SAS Procedure PRINCOMP; SAS version 9.1.3; SAS Institute, Inc., Cary, North Carolina) was carried out on all specimens using the final 16 variables to determine the main components of variation in the morphological data. Canonical Discriminant Analysis (CDA; SAS Procedure CANDISC, SAS version 9.1.3) was then used to determine those variables which contributed most to separation of the host-based biological groups (Tabachnick & Fidell 2006).

TABLE 3. Continuous morphological variables used in multivariate morphometric analysis of *Hyalopterus* adult, apterous viviparous morphs. Variables measured but not included in analysis listed below.

Variable	Description
BL	Body length, front of head to base of cauda
A3L	Length of antennal segment 3
A4L	Length of antennal segment 4
A5L	Length of antennal segment 5
A6BL	Length of basal part of antennal segment 6
A2L	Length of antennal segment 2
A3SL	Length of longest seta on antennal segment 3
HW	Width of head across eyes
URW	Width of ultimate rostral article
URL	Length of ultimate rostral article
F3L	Length of hind femur
T3L	Length of hind tibia
DT3L	Length of hind distitarsus
SL	Length of siphunuclus
CL	Length of cauda
AT8SL	Length of submedian seta of abdominal tergite 8

Measured but not used	Reason
Basal width of antennal sement 3	Imprecise
Length of terminal process of antennal segment 8	Missing on significant proportion of specimens
Length of hind trochanteral seta	Imprecise measurement – many not perpendicular to optical axis
Length of longest seta on ventral surface of hind femur	Imprecise measurement – many not perpendicular to optical axis
Length of fore femur	Correlated with length of hind femur
Length of fore tibia	Correlated with length of hind tibia
Length of fore distitarsus	Correlated with length of fore distitarsus

Results

Sequence statistics

We used a total sequence data set of 1,320 bp plus one gap character in the 12S gene and one gap character in the *Buch*16S gene for analyses (Table 2). All individuals included in the analysis were full length, except for some of the outgroup sequences: *R. padi* COI (missing the first 76 bp), *R. padi* 12S (missing last 55 bp), *A. fabae* 12S (missing last 34 bp), *R. padi EF-1α* (missing last 18 bp), and *S. graminum* 12S (missing last 2 bp). The ingroup sample D7-1 was also missing the first 53 and last 3 bp of the12S sequence, though there was only a single polymorphic character in this region in all other samples. Analysis of pairwise differences and nucleotide diversity for the entire *Hyalopterus* data set reveals that COI was by far the most variable gene used in the study, followed by 12S, *Buch*16S, and *EF-1α*. The sequences were generally biased toward transitions and were A-T rich, though this pattern was only significant for the mitochondrial regions (COI and 12S; Table 4), in accordance with other studies of insects, including aphids (Lin & Danforth 2004).

TABLE 4. Sequence statistics for each locus, including the number of unique haplotypes, the transition to transversion ratio (*Ti*:*Tv*), percent adenine and guanine content (% AT content), number of total substitutions, number of segregating sites (*S*), nucleotide diversity (π_n), and average pairwise differences among sequences. Clade labels refer to those in Figures 1 and 2.

	No. sequences	No. unique haplotypes	*Ti*:*Tv*	% AT content	No. substitutions	S	π_n (s.d.)	Avg. pairwise differences (s.d.)
COI								
All	24	7	27:8	74.98	35	33	0.043 (0.022)	14.74 (6.84)
A	8	2	1:0	75.18	1	1	0.001 (0.001)	0.25 (0.31)
B	8	4	3:0	75.40	3	3	0.003 (0.003)	1.04 (0.77)
C	8	1	0:0	74.34	0	0	0	0
*Buch*16S								
All	24	3	2:2	52.89	4	4	0.005 (0.003)	1.86 (1.10)
A	8	1	0:0	52.80	0	0	0	0
B	8	1	0:0	52.81	0	0	0	0
C	8	1	0:0	53.06	0	0	0	0
12S								
All	24	5	4:3	83.92	7	7	0.008 (0.005)	2.91 (1.58)
A	8	3	1:1	83.79	2	2	0.002 (0.002)	0.68 (0.57)
B	8	1	0:0	84.04	0	0	0	0
C	8	1	0:0	84.04	0	0	0	0
EF1α								
All	24	2	1:0	61.64	1	1	0.002 (0.002)	0.47 (0.42)
A + B	16	1	0:0	61.40	0	0	0	0
C	8	1	0:0	62.12	0	0	0	0

Molecular phylogeny

The ILD tests indicated no significant evidence for incongruence among loci, suggesting no problems for combining data sets (all $P = 1.00$). In all phylogenetic trees, *Hyalopterus* was always recovered with high *PP* support as monophyletic relative to the outgroup and having, in general, three major monophyletic clades that always contained the same sets of individual specimens. For all genes except 12S, clades A and B were placed as sister taxa with C located basally. For 12S, B and C were instead recovered as sister groups, with A as basal, though these relationships were poorly supported in the *PP* distribution (*PP* = 0.64). For EF-1α, A and B were placed together in a single undifferentiated clade with moderate support (*PP* = 0.83). The combined analysis had 100% *PP* support for all three clades, with C basal to the A+B sister group. This topology was supported in the COI, *Buch*16S, and, more generally, in the *EF-1α* trees and we thus find it likely that it best represents the evolutionary history of *Hyalopterus.*

The differences between lineages were clearly maintained across the broad geographic range included in the analysis, and were, as previously determined from a smaller set of sequenced genes, microsatellites (Lozier *et al*. 2007) and allozymes (Mosco *et al.* 1997), structured by host plant, providing further evidence that *Hyalopterus* comprises geographically widespread, host adapted species. Based on the present and previous findings we assign clade A as the "plum-type" lineage, clade B as the "almond-type" lineage, and clade C as the "peach-type" lineage. The two aphids included from apricot were placed in clade B or C, supporting previous results suggesting that apricot supports different *Hyalopterus* lineages.

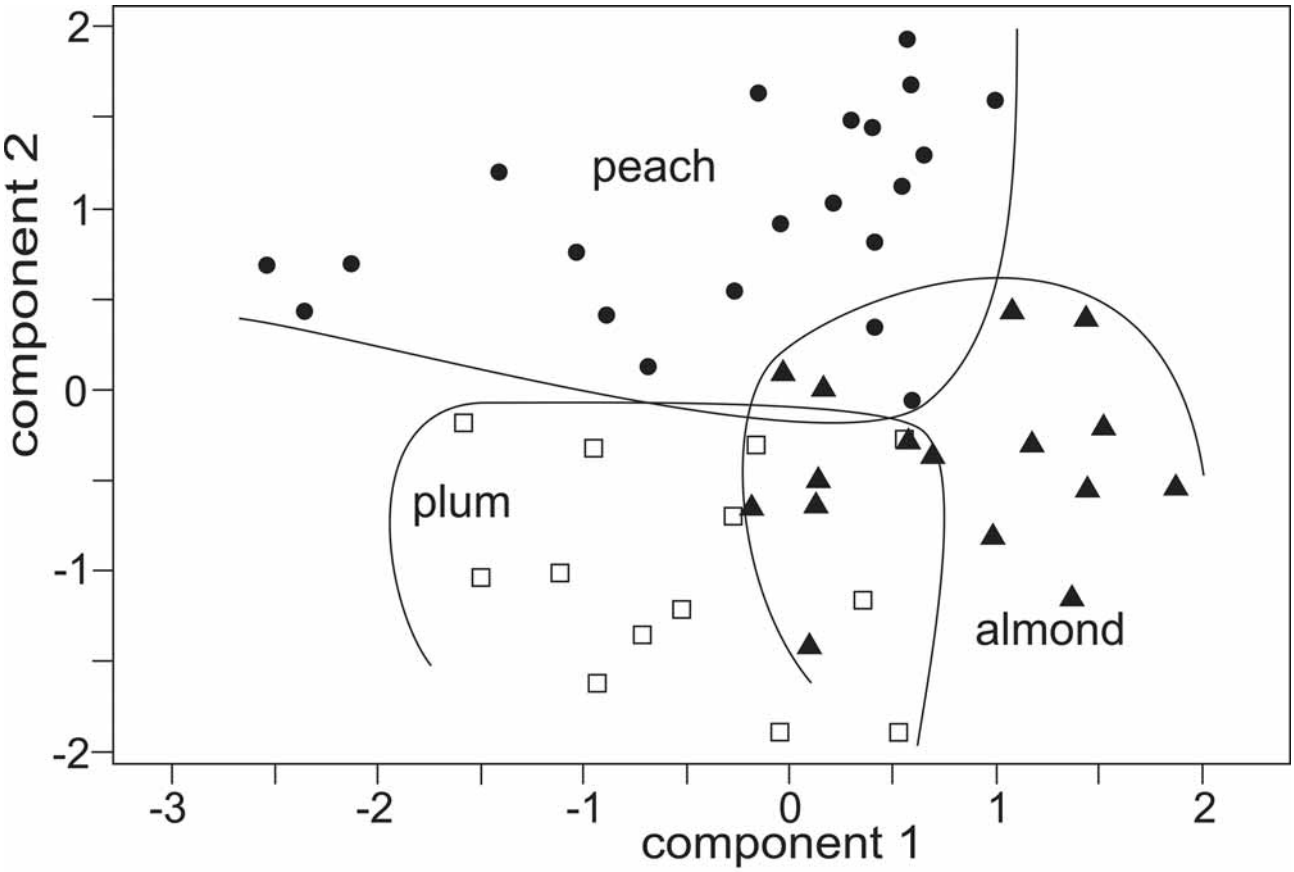

FIGURE 3. Principal component ordination of 49 specimens of *Hyalopterus* based on the analysis of 16 morphological variables onto the first and second principal axes (Table 5). Host plants of the specimens are indicated by the following symbols: peach (●), plum (□), almond, (▲)

The combined phylogeny and summary statistics demonstrate that while interclade variation is relatively high, there is little to no variation among individuals within each of the three major clades despite the geographically widespread sampling (Figure 2, Table 4), and only for clade A at the 12S gene did intraclade variation exceed 10 % of the total interclade variation (Table 4). However, the use of more loci did reveal a slightly greater level of substructure than previously observed in *Hyalopterus* (Lozier *et al* 2007). Intraclade variation for clade A came from contributions by the 12S and COI genes, and suggests a potential link between Spanish and Californian samples *(PP* = 1.00). Variation at COI in clade B places Spanish and Tunisian samples into a subclade compared to the rest of the Mediterranean (*PP* = 0.95). Clade C had no significant intraclade variation, though there were a few ambiguous sites for EF-1α in clade C taxa that were not included in the calculation of summary statistics. The only site that consistently varied in multiple individuals was position 148 of the EF-1α alignment, which was always a cytosine in clades A+B and was either a thymine or heterozygous for thymine and cytosine in clade C.

Morphometric analysis

Contributions of the 16 morphological variables to the first two principal components are given in Table 5 and the projections of the specimens onto the first two principal axes are shown in Figure 3. Mapping host associations of specimens onto a principal component ordination (Figure 3) shows good separation according to host plant, while mapping of geographic origin on the orientation showed substantial overlap of regions (Figure 4). Separation of specimens along principal axis 1 is due to general size differences, particularly due to antennal and hind leg dimensions. Separation of specimens along principal axis 2 represents variation due

to contributions from ultimate rostral article, caudal length, and length of submedian seta of abdominal tergite 8 (see also Table 5).

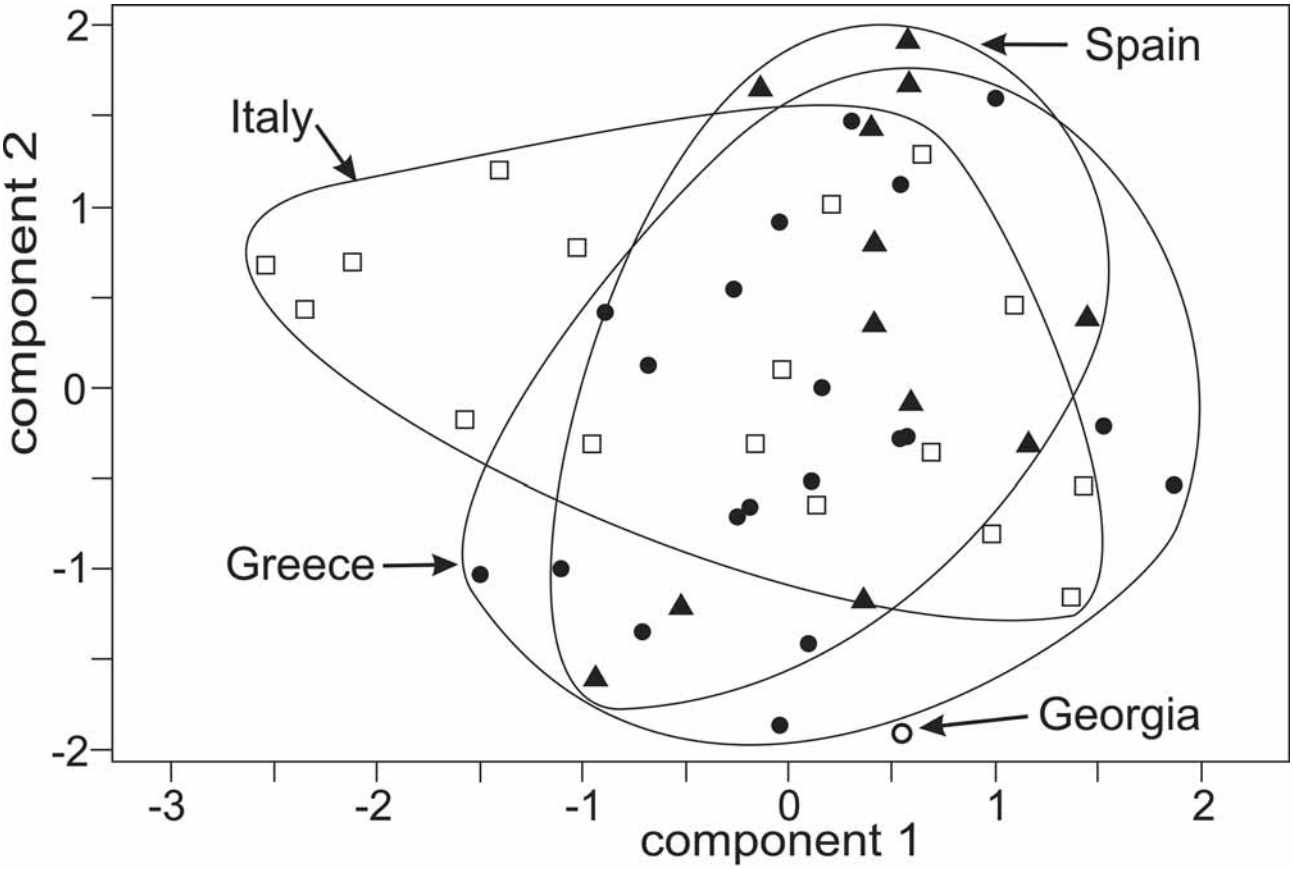

FIGURE 4. Principal component ordination of 49 specimens of *Hyalopterus* based on the analysis of 16 morphological variables onto the first and second principal axes (Table 5). Geographic origins of the specimens are indicated by the following symbols: Greece (●), Italy (□), Spain (▲), Republic of Georgia (○).

Since the principal component analysis showed a clear separation of the specimens from different hosts, a canonical discriminant analysis was carried out using host as the classification variable. Contributions of the 16 morphological variables to the first two canonical discriminant axes are given in Table 5 and the projections of the specimens onto the first two canonical discriminant axes are shown in Figure 5. The canonical discriminant analysis shows that the specimens clearly aligned by host plant group and not along geographic origin. Separation along discriminant axis 1 was generated by contrasts in the size of several variables, particularly the length of the third antennal segment, caudal length, the length of the hind tibia and the length of the ultimate rostral segment. Separation of the specimens along discriminant axis 2 is mainly the result of contributions from hind femur length, length of the cauda, length of the submedian seta of abdominal tergite eight, and contrasts in the length of the third and fourth antennal segments.

Hyalopterus persikonus Miller, Lozier, and Foottit new species
(Fig. 6)

Recognition characters. Apterous vivipara (Fig 6.1): Body length 1.89–2.85 (2.38); width through eyes, 0.32–0.48 (0.44). Head (Fig. 6.2) weakly sclerotized, smooth; dorsal head and frons setae filiform; longest frons seta (fs) nearly as long as width of antennal segment II; distance of the bases median dorsal head setae (mdhs) usually less than their lengths (Fig. 6.3B); antennal tubercle weakly to moderately developed. Antenna 6-seg-

mented, shorter than body without secondary sensoria, pale with segment VI darker and occasionally apex of segment V, setae on segment III 0.01–0.03 (0.02) long, more than half width of segment; segment III 0.30–0.46 (0.39) long; IV 0.21–0.40 (0.28) long; V 0.18–0.28 (0.24) long; base of VI 0.09–0.16 (0.12) long; terminal process, 0.32–0.44 (0.38) long. Rostrum extending to metacoxae, bluntly rounded apically; ultimate rostral article (Fig. 6.4) 0.07–0.11 (0.09) long, 0.06–0.09 (0.08) wide at base, with a pair of accessory setae. Hind femur 0.42–0.70 (0.59) long; hind tibia 0.84–1.23 (1.00) long; hind tarsus II 0.15–0.21 (0.17) long; basitarsi with 3 ventral setae on pro- and mesobasitarsi and 2 on metabasitarsi; Apex of tibia and tarsi darker than rest of leg. Abdomen without pigment, with faint fine reticulation and marginal tubercles on segments I–VI; abdominal setae pointed, longest seta on abdominal segment VIII 0.03–0.08 (0.06); anal plate entire, genital plate (Fig. 6.5) with several anterior and median setae and posterior row of setae. Siphunculus (Fig. 6.6) small, 0.07–0.14 (0.11) long, shape variable, ranging from cylindrical with slight tapering to slightly swollen on apical half, weakly scabrous, apically dark with paler base, apical flange absent. Cauda (Fig. 6.7) 0.14–0.26 (0.20) long, elongate, with 3–4 pairs of lateral setae and a subapical seta.

TABLE 5. Contributions of 16 morphological variables to the first two principal components calculated from 49 specimens of *Hyalopterus* (see also Figure 3). Contributions of 16 morphological variables to the first two canonical discriminate axes calculated using 50 specimens of *Hyalopterus* (see also Figure 4).

	Principal components analysis		Canonical discriminant analysis	
	Prin1	Prin2	Can1	Can2
BL	0.2455	-0.0804	-0.1416	-0.0592
A3L	0.3053	-0.2660	-1.4209	0.5329
A4L	0.2659	-0.0666	-0.1305	-0.5616
A5L	0.3394	0.0982	0.4112	0.0749
A6BL	0.2735	0.0075	0.1142	0.8568
A2L	0.2599	0.2607	0.5167	0.1318
A3SL	0.0460	0.0238	0.4091	0.0547
HW	0.2334	0.2348	-0.2333	0.1710
URW	0.0222	0.5495	0.5071	0.0359
URL	0.1796	-0.2977	-0.8097	0.0910
F3L	0.3507	0.0766	0.7402	-1.2791
T3L	0.3406	-0.1240	-0.8455	0.3514
DT3L	0.2979	0.1138	0.1746	0.2968
SL	0.2335	-0.1978	-0.1611	-0.0070
CL	0.1667	0.4527	0.9656	1.1911
AT8SL	-0.1376	0.3409	-0.3556	-0.8547

Embryo: Antenna 5-segmented; setae pointed; disc with 2 pair of anterior and 2 pair of posterior setae; pronotum with 1 anterior, 1 posterior lateral, and 1 posterior submedian seta on each side; abdominal segments each with 4 setae medially and a dorsolateral seta on each side of I–VI ; siphunculus short, poriform; basistarsi with 2 ventral setae.

Etymology of specific epithet. The name *persikonus*, a variant of the Greek “Persikon malum” or Persia apple for early reference to supposed origins of the “peach,” refers to one of the primary hosts for this aphid species.

Specimens examined. Holotype: **GREECE**: Kala Nera, Peliou, 12-V-2004, on *Prunus persica*, N. Mills coll. (apterous vivipara) USNM; with slide label stating “*Hyalopterus persikonus* Miller, Lozier, and Foottit

HOLOTYPE"

Paratypes: **ITALY**: Squinzano, Gemini, 15-VI-2004, on *Prunus persica*, N. Mills coll.(9 apterous viviparae on 9 slides) BMNH, CNCI, EMEC, USNM; **GREECE**: Kala Nera, Peliou, 12-V-2004, on *P. persica*, N. Mills coll. (8 apterous viviparae on 8 slides) CNCI, EMEC, USNM; **SPAIN**: Benferi, Muro d'Alcoi, summer 2002, on *P. persica*, N. Mills coll. (8 apterous viviparae on 8 slides) CNCI, EMEC, USNM; all slides with label stating "*Hyalopterus persikonus* Miller, Lozier, and Foottit PARATYPE".

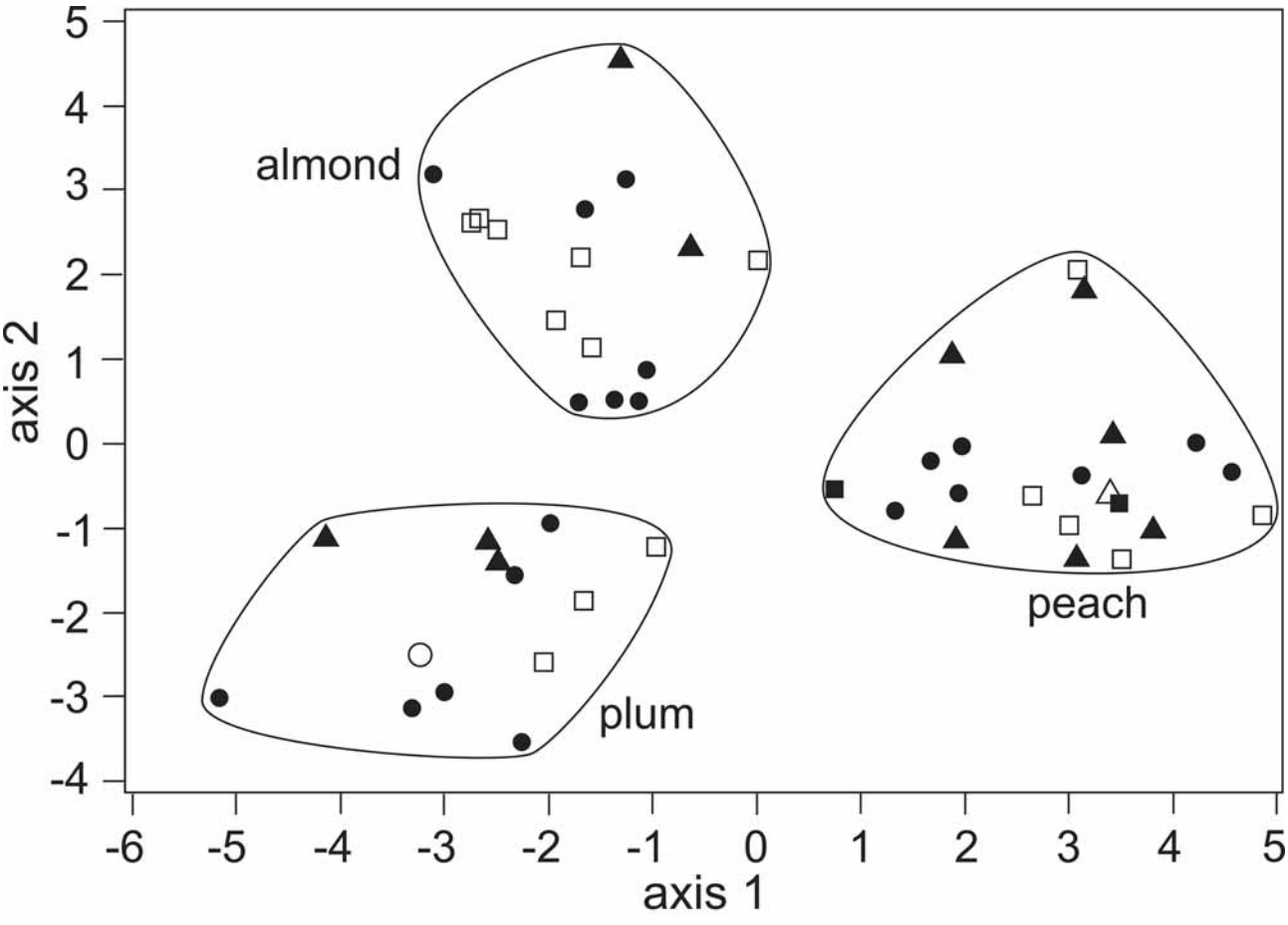

FIGURE 5. Canonical discriminate analysis of 50 specimens of *Hyalopterus* based on the analysis of 16 morphological variables; specimens projected on to the first and second principal axes (Table 5). Country of origin for the specimens are indicated by the following symbols: Greece (●), Italy (□), Spain (▲), Republic of Georgia (○).

Other specimens examined. **REPUBLIC OF GEORGIA**: Karsp District, Doesi, 5-IX-2005, on *P. persica*, S. Barjadze coll. (12 apterous viviparae and 24 immatures on 12 slides) CNCI, USNM; **IRAN**: Tabriz, 8-VIII-1958, on peach, Hambleton coll. (3 apterous viviparae on 1 slide) USNM; **AFGANISTAN**: Kabul, 29-V-1961, on apricot, E. R. Millet and E. J. Hambelton coll. (2 apterous viviparae, 2 alate viviparae, 2 immatures on 1 slide) USNM; **IRAQ**: Baghdad, 23-III-1965, on apricot, on nectarine, A. K. Daoud coll. (8 apterous viviparae and 5 immatures on 5 slides) USNM.

Comments. *Hyalopterus persikonus* is very similar to *H. amygdali* and *H. pruni*, morphologically. Characters useful for species separation are included within the following key. In addition, host plant association of the specimen is critical. However, the use of the key for field identification remains difficult as evidenced by the overlap of the various ranges. Single individual specimens may be problematic and it is advisable to use multiple specimens from the same collection series. However, molecular data and morphometic analyses can be applied for separating *Hyalopterus* species.

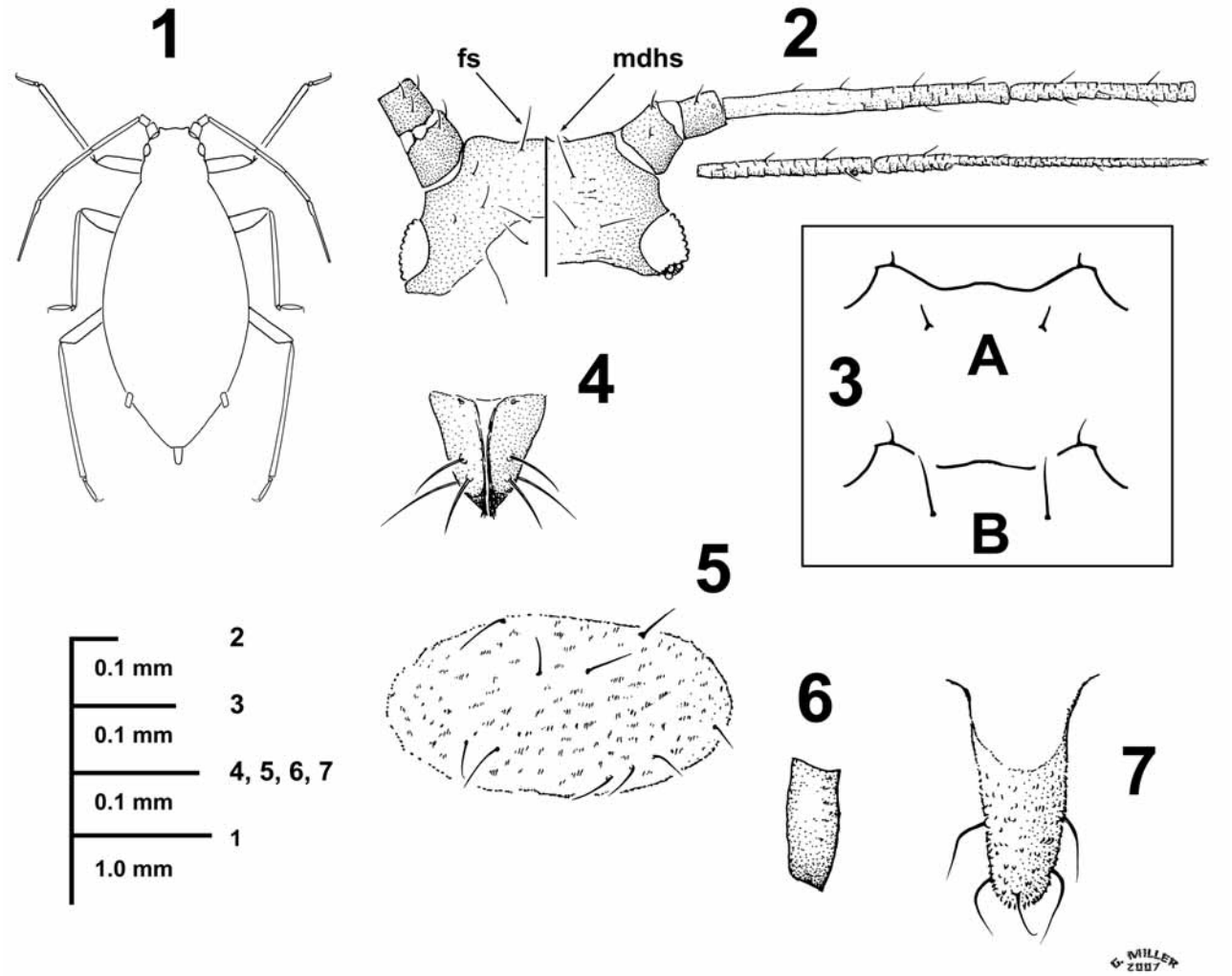

FIGURE 6. Morphology of apterous vivipara of *Hyalopterus persikonus* n. sp. 6.1. Dorsal habitus of slide-mounted specimen; 6.2. Head dorsum (left) and venter (right), median dorsal head setae (mdhs), frons seta (fs); 6.3 Head dorsum, A, *Hyalopterus amygdali* (Blanchard), B, *Hyalopterus persikonus* n. sp.; 6.4. Ulltimate rostral article; 6.5. Genital plate; 6.6 Siphunculus; 6.7. Cauda.

Key to *Hyalopterus* apterous viviparae
on primary hosts of *Prunus armeniaca, P. domestica, P.dulcis,* or *P.persica*

1. Setae on frons stout, approximately half the width of antennal segment II; distance of the bases median dorsal head setae much greater than their lengths (Fig. 6.3A); most tips of median dorsal head setae, meso- and metathoracic seatae, and spinal and pleural setae on abdominal segments I–III apically blunt, almost capitate. Colonizing *P. dulcis* and *P. armeniaca* ..... *Hyalopterus amygdali* (Blanchard)
- Setae on frons filiform, nearly as long as width of antennal segment II; distance of the bases median dorsal head setae nearly equal to or slightly less than their lengths; thoracic and most dorsal body setae acuminate. Colonizing *P. domestica, P. persica,* or *P. armeniaca* 2
2 . Ratio of length of antennal segment III/ length of cauda 1.8–3.5 (2.5); ratio of length of antennal III/ length antennal segment IV 1.3–1.9 (1.5); ratio of length of hind tibia/ length of cauda 4.6–8.3 (6.1); ratio of length of hind tibia/ length of antennal segment III 2.1– 4.2 (3.5)[1] Colonizing *P. domestica* .... *Hyalopterus pruni* (L.)
- Ratio of length of antennal segment III/ length of cauda 1.3–2.7 (2.0); ratio of length of length of antennal

III/ length antennal segment IV 1.0–1.8 (1.4); ratio of length of hind tibia/ length of cauda 3.7–7.6 (5.1); ratio of length of hind tibia/ length of antennal segment III 2.2–3.0 (2.6)[1]. Colonizing *P. persica* and *P. armeniaca* ..... *Hyalopterus persikonus* n.sp.

Discussion

The present study has expanded upon previous research on the aphid genus *Hyalopterus* through the use of additional DNA regions, a greater number of morphological characters, and greater geographic representation. The morphological and molecular data sets presented here reveal three distinct clades in *Hyalopterus* and correspond with previous morphological and molecular findings (Mosco *et al*. 1997; Lozier *et al.* 2007; Poulios *et al.* 2007). Together these studies provide compelling evidence for the presence of three host plant associated *Hyalopterus* species: one species associated largely with plum (our Clade A, *H. pruni*), one with almond (our Clade B, *H. amygdali*, previously *H. amygdali* A; Mosco *et al.* 1997; Poulios *et al.* 2007), and one with peach (our Clade C, *H. persikonus* n. sp., previously *H. amygdali* B; Mosco *et al*. 1997; Poulios *et al.* 2007). No one study has found these associations to be perfect, however, with *H. pruni*, for example, feeding on several other species of *Prunus*, such as *P. spinosa*, *P. cerasifera*, and *P. pissardi*. The molecular phylogeny presented here also shows that both *H. amygdali* (Clade B) and *H. persikonus* n. sp. (Clade C) are present on apricot, and though not shown, *H. pruni* (Clade A) is also common on apricot (Lozier *et al.* 2007; Poulis *et al*. 2007), suggesting that this host plant acts as a shared resource among *Hyalopterus* species.

Our results demonstrate the utility of examining multiple types of data when attempting to identify and classify problematic species so that potential discrepancies can be revealed and considered when evaluating phylogenetic hypotheses. Overall, the use of any single DNA region would have resulted in moderate support for the relationships among *Hyalopterus* clades. The addition of 12S and EF-1α to the data set increased the total support for relationships among *Hyalopterus* species compared to previous analyses (Lozier *et al.* 2007) and also provided increased intraspecific resolution, though detailed analysis of this structure will be presented in a companion study. There was general agreement across loci for individual membership in each of the major clades (e.g., no individual had a plum-type sequence at one locus an almond-type at another), though there were some topological discrepancies worth noting that highlight the importance of examining each gene region individually as well as in combination. First, the relationships among lineages was somewhat rearranged for the 12S data set, with the plum associated clade (A) placed ancestrally to the peach and almond clades (B+C). While topological incongruence is not uncommon among mitochondrial genes (Caterino *et al.* 2000), the B+C clade was poorly supported in the 12S data set ($PP = 0.64$) and, excluding the presence of gaps, this gene had low overall levels of differentiation which may have obscured the true clade relationships. Furthermore, the B+C node was not recovered at all in the parsimony analysis (not shown). Examination of the raw 12S sequences reveals that *H. pruni* and *H. amygdali* share a unique and seemingly homologous gap not present in *H. persikonus* n. sp., so it was somewhat surprising that these aphids were not resolved as sister taxa in the Bayesian analysis, as indels are considered to be informative for phylogenetic inference (Simmons & Ochoterena 2000; Kawakita *et al.* 2003). Incorporating more realistic modeling and weighting of indel regions in analyses may help resolve such discrepancies and improve resolution of topology and branch lengths, though currently no such models are supported by MrBAYES.

Secondly, the EF-1α tree showed no differentiation between *H. pruni* and *H. amygdali* in our analysis. While nuclear introns are thought to be highly variable, in some cases more so than mitochondrial markers (Villablanca *et al.* 1998), this is not always true (Simon *et al.* 2006). Given the recent divergence times likely

1. Comparison for *H. amygdali* is as follows: ratio of length of antennal segment III/ length of cauda 1.6–3.0 (2.3); ratio of length of length of antennal III/ length antennal segment IV 1.4–2.0 (1.6); ratio of length of hind tibia/ length of cauda 4.2–6.8 (5.4); ratio of length of hind tibia/ length of antennal segment III 2.1–2.8 (2.4).

involved between these species, it may simply be the case that insufficient time has passed for informative mutations to occur in the EF-1α region analyzed. The use of a longer sequence fragment in the future could help overcome this lack of phylogenetic signal and reveal a greater number of diagnostic polymorphisms. Another distinct possibility is that ongoing gene flow between aphids in the plum and almond-associated clades has limited divergence at EF-1α. Hybridization between recently divergent species is relatively common, and can contribute to topological incongruence in phylogenetic trees due to introgression (Funk & Omland 2003). Indeed, microsatellite analyses have revealed a substantial level of introgression between plum and almond associated *Hyalopterus* lineages, though results suggested that hybridization was restricted to apricot trees, and introgression of plum aphid type alleles into aphids on almond or almond aphid type alleles into aphids on plum was not observed (Lozier *et al* 2007).

Lastly, our morphometric data also clearly separated the three *Hyalopterus* species. While no geographic separation was possible in the PCA or CDA, the separation by host plant was impressive (Figure 3, 4, 5). Along CDA axis 1, the almond and plum groups (both negative) were strongly separated from the peach group (positive), though they could not be separated along this axis. Along CDA axis 2, however, plum (negative) and almond (positive) groups were well defined, and the peach group was intermediate (overlapping zero). We purposely chose to exclude apricot aphids from our morphometric analyses because of the possible presence of almond-type/plum-type hybrid aphids that could have obscured results for aphids collected from the other primary hosts, though we expect that they would be placed in all three groups (Lozier *et al*. 2007; Poulios *et al*. 2007). Certainly, there was some degree of within group variation in morphometric data, suggesting the possibility of geographic or environmental contributions to character differences within each of the host associated species. Climatic effects on character measurements has been noted for *Hyalopterus* (Eastop 1966), and future investigation of this phenomenon would be worthwhile.

Our phylogenetic analysis does conflict somewhat with other studies, however. Our phylogeny demonstrates that *H. pruni* and *H. amygdali* are more closely related to each other than to *H. persikonus* n. sp. Yet, Poulios *et al*. (2007) suggest that the almond and peach associated species are more closely related, based on UPGMA dendrograms constructed from their morphometric data. The morphometric data we present cannot resolve these relationships, but the findings from 43 DNA and protein markers all suggest that the plum and almond associated species are the more recently diverged sister taxa (Mosco *et al*. 1997; Lozier *et al*. 2007; present study).

An applied perspective

An understanding of higher level genetic structure and taxonomy of pest insects, as presented here, is a crucial step for any management program. Rapid species identification is critical for the detection and assessment of new pest threats and the implementation of a successful control strategy. Currently *Hyalopterus* is found in California only on varieties of plum, apricot, and pluot (not sampled for this study). Primary host plant use for other North American populations is uncertain, though they are readily abundant on their secondary *Phragmites* or *Typhus* hosts (pers. obs.). If mealy aphids were to be detected on peach or almond in California, distinguishing the minor threat posed by individuals of *H. pruni* that had landed on the wrong host plant from the potentially major threat of the invasion of a different *Hyalopterus* species that could threaten the economically-important peach or almond industries would be a priority. Similarly, regular screening of secondary hosts (on which all three species are known to co-occur, pers. obs.) for the presence of *H. amygdali* or *H. persikonus* n. sp. could also be useful for revealing a potential new threat. The accurate identification of adventive pests is also of particular importance in biological control, where introduction of natural enemies for biological control may not be effective unless there is a suitable genetic and ecological match to the pest (Gordh & Beardsley 1999; Mills 2000; Hufbauer & Roderick 2005). The suite of natural enemies that prey upon *Hyalopterus* may, for example, vary across host plant associated lineages or may themselves be cryptically structured in association with host plants. In addition, association of previously recorded natural enemies

(Golfari 1946; Basky 1982) may be in question due to prior species concepts of *Hyalopterus*. The results presented here for the genetic structure of *Hyalopterus* provide the framework to investigate the biological differences among these lineages and their associated natural enemies.

Unfortunately, the use of some morphological characters to distinguish between *Hyalopterus* species has proved unreliable (Basky & Szalay-Marszó 1987; Blackman & Eastop 1994, 2000, 2006), making traditional identification problematic. This is still reflected within the key provided herein, though we have shown that genetic data and multiple morphological characters can be used to clearly distinguish species within *Hyalopterus*. While morphology is often the easiest method of identification, discrimination in these insects is difficult, particularly if only sub-adult or damaged material is available. Because of its wide use in genetic studies and its straightforward amplification with generic primers (Simon *et al.* 2006), COI has become the marker of choice for diagnostics for animal groups where morphological data is inadequate, and has potential for identifying intercepted or quarantine species (Armstrong & Ball 2005). Sequences of the COI gene should be sufficient for distinguishing between different *Hyalopterus* aphids given the low observed intraspecific diversity. By comparison with the GENBANK sequences supplied here, and in conjunction with the morphological key provided above, researchers will be able to identify *Hyalopterus* species.

Acknowledgements

We gratefully acknowledge the technical and analytical support of Eric Maw (Agriculture and Agri-Food Canada, Ottawa), Andrew Carmichael and Ashley Kaippallil (USDA, Systematic Entomology Laboratory, Beltsville, MD). We are grateful to Shalva Batzagan (Tbilisi, Republic of Georgia) for collection of specimens, and Mike Pogue (USDA, Systematic Entomology Laboratory, Washington, D.C.), Ron Ochoa (USDA, Systematic Entomology Laboratory, Beltsville, MD), Colin Favret (Illinois Natural History Survey), and Hugh Loxdale (Friedrich Schiller University & Max Planck Institute for Chemical Ecology, Jena, Germany) for their manuscript reviews. We thank the University of California Exotic/Invasive Pests and Diseases Research Program, the U. S. Department of Agriculture, and the U. S. Environmental Protection Agency STAR fellowship program for funding this research.

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