Molecular evidence for sympatric taxa within *Pemphigus betae* (Hemiptera: Aphididae: Eriosomatinae)

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Abstract—Mitochondrial DNA cytochrome *c* oxidase subunit 1 (DNA barcode) and nuclear microsatellite flanking region sequences were used to analyse populations of putative "sugarbeet root aphid", *Pemphigus betae* Doane, from sites in Alberta, Canada. Three sympatric genotypes were revealed, identified as *P. betae*, *P. populivenae* Fitch, and an undetermined third species. All three genotypes formed morphologically indistinguishable galls on the same set of cottonwood (*Populus* L., Salicaceae) host species, often on the same tree. Gall morphology is frequently used to identify *Pemphigus* species. Our results indicate that this practice may be unreliable for these three taxa at least.

Résumé—Les séquences de la sous-unité 1 de la cytochrome *c* oxydase de l'ADN mitochondrial (code à barres de l'ADN) et de la région flanquante des microsatellites nucléaires nous ont servi à analyser les populations de l'espèce putative du « puceron de la betterave à sucre », *Pemphigus betae* Doane, de sites de l'Alberta (Canada). Il existe trois génotypes sympatriques, identifiés comme *P. betae*, *P. populivenae* Fitch et une troisième espèce non identifiée. Les trois génotypes forment tous des galles qui sont impossibles à distinguer sur le même ensemble d'espèces-hôtes de peupliers (*Populus* L., Salicaceae), souvent sur le même arbre. La morphologie des galles sert souvent à identifier les espèces de *Pemphigus*; nos résultats indiquent que cette procédure n'est pas fiable au moins pour ces trois taxons.

[Traduction par la Rédaction]

Introduction

Aphids of the genus *Pemphigus* Hartig (Hemiptera: Aphididae: Eriosomatinae) form galls on the leaf blades, petioles, and twigs of cottonwood trees (*Populus* L., Salicaceae). In the typical life cycle, galls are initiated in the spring on cottonwood (the "primary" host) by an aphid stem mother that survives the winter as an egg protected beneath the bark or in a crevice of the tree. The stem mother is both viviparous and parthenogenetic. This form produces one or more generations of wingless offspring ("apterae") that remain in the gall to feed on the content of phloem cells. In June and July the apterae give rise to winged individuals ("alatae") that exit the gall to colonize the roots of secondary herbaceous hosts. The alatae produce one or more generations of apterae on the secondary host that give rise in autumn to winged female "sexuparae". The sexuparae migrate back to the cottonwood host, where they produce wingless males and females that have no mouthparts. These wingless individuals are the only sexual generation of the life cycle. Females mate and then deposit a single egg that gives rise in the spring to the aphid stem mother. Some species have an abbreviated life cycle that eliminates the secondary-host phase. In these species there may be more than one apterous generation within the gall, and

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Fig. 1. Typical galls of *Pemphigus betae*. Galls usually form along the midrib, extend below the abaxial surface of the leaf, and open to the upper side.

the alates that leave the gall are sexuparae. The form and location (*e.g.*, leaf petiole *versus* leaf blade) of the gall are often characteristic of the species. Thus, galls are commonly used in field studies to distinguish among *Pemphigus* species (Palmer 1952; Harper 1959).

The sugarbeet root aphid, *Pemphigus betae* Doane, is of interest as both an economic pest and a "model" species for ecological studies of cottonwood. Among its secondary hosts, the aphid is occasionally a serious pest on common beet (sugarbeet), *Beta vulgaris* L. (Chenopodiaceae) (Harper 1963; Summers and Newton 1989; Hutchison and Campbell 1994). The aphid forms pouch-shaped galls on the leaf blades of its host; among its primary hosts are the balsam poplars, *Populus* section *Tacamahaca* (*e.g.*, *P. angustifolia* James, *P. balsamifera* L.), and hybrids involving these species, and cottonwoods in *Populus* section *Aigeiros* (*e.g.*, *P. deltoides* Marsh, *P. fremontii* S. Watson) (Fig. 1) (Palmer 1952; Harper 1959; Floate *et al.* 1997). Whether or not gall formation is successful depends upon the degree and nature of hybridization and this has provided insights into the role of the genetics of *Populus* species in structuring their associated arthropod assemblages (*e.g.*, Whitham 1989; Floate and Whitham 1993; Floate *et al.* 1997; Whitham *et al.* 1999).

Despite this interest, the taxonomic status of *P. betae* remains unclear. *Pemphigus betae* was originally described from specimens on sugarbeets, the gall form being unknown. *Pemphigus balsamiferae* Williams, described from the gall form, is currently considered a synonym of *P. betae* (Maxson 1916; Remaudière and Remaudière 1997). Grigarick and Lange (1962) considered *P. betae* to be synonymous with *Pemphigus populivenae* Fitch, which also

forms pouch-shaped galls on the leaf blades of species in *Populus* section *Tacamahaca*. Summers and Newton (1989) used the name *P. populivenae* for aphids on sugarbeets in California. Harper (1959) designated aphids that formed galls projecting below the plane of the leaf blade on *Populus* hosts as *P. betae* (Fig. 1) and used the name *P. populivenae* for aphids forming galls that projected above the plane of the leaf blade. However, according to Maxson and Knowlton (1929), in heavy infestations the latter can occur in the position typical of *P. betae.* In addition, *Pemphigus knowltoni* Stroyan has been reported from Utah, where it forms galls similar to those of *P. betae* on *P. angustifolia* (Stroyan 1970). In Europe the species on beets and other Chenopodiaceae is named *Pemphigus fuscicornis* (Koch) and has no known associated primary host form (Blackman and Eastop 2000). Secondary hosts for both *P. betae* and *P. populivenae* are reported to include species of *Rumex* L. (Polygonaceae) as well as Chenopodiaceae. In South America, where there are no indigenous *Populus* species, *Pemphigus solanophila* (Blanchard) feeds on *Rumex* roots, and there are specimens in the Natural History Museum, London, collected on *Rumex* in California that are attributed to this species.

During a preliminary survey of molecular variation within *Pemphigus*, we noted that putative "*Pemphigus betae*" samples were partitioned into three discrete groups by several different loci. Here we report the results of studies on samples of *Pemphigus* from galls of the form ascribed to *P. betae* and discuss the taxonomic implications.

Methods

Aphids were collected from "closed" galls in pure zones, and in zones of overlap and hybridization, of *Populus* species in Alberta, and from one site in New Brunswick. Aphids within a closed gall were assumed to be genetically identical. All galls exhibited the characteristic shape and location reported for *P. betae* (Fig. 1; see Harper 1959). Secondary-host samples were also collected from roots of sugarbeet and lambsquarters, *Chenopodium album* L. (Chenopodiaceae), at sites in southern Alberta. An additional secondary-host sample collected in Washington State from tubers of potato, *Solanum tuberosum* L. (Solanaceae) was obtained from Keith Pike (Washington State University, Prosser). Collection sites for all samples are provided in Table 1.

Aphids were preserved in 95% ethanol and held at −20 °C until processed. Standard protocols (deWaard *et al.* 2008) were used for DNA extraction, PCR (polymerase chain reaction) amplification, and sequencing of the barcode region (a 658 base-pair segment at the 5′-end of mitochondrial cytochrome *c* oxidase subunit 1 (COI)) at the Biodiversity Institute of Ontario (University of Guelph, Guelph, Ontario) using the primers given in Table 2. Sequence data and associated specimen details are available on the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007; http://www.barcodinglife.org, "Published Projects"/"Pemphigus–sugarbeet root aphid" links) and GenBank (accession Nos. GU66270−GU667401, HM64506−HM64544, HM64550−HM64513, HM64535−HM64609). Sequences for several other *Pemphigus* species are included for comparison (Foottit *et al.* 2008; available on BOLD, "Published Projects"/"Barcoding the Aphididae" link; GenBank accession Nos. EU701834, EU701836, EU701838, EU701841, EU701843, EU701844, EU701846, EU701849).

For a subset of samples, sequences were also obtained for three anonymous nuclear regions adjacent to microsatellites (microsatellite flanking region sequences (MFRS)): PEM60F and PEM83B (34 samples) and PEM19R (25 samples). Linkage status among these loci is unknown. See Table 2 for primer names, primer sequences, and annealing temperature for each locus, and for characteristics of the products. Each was amplified for 35 cycles of denaturation at 95 °C for 1 min, annealing at the temperature indicated in Table 2 for 1 min, and extension at 72 °C for 2 min (PEM19R and Pem60F) or 2.5 min (PEM83B). PCR products were sequenced directly, using the DYEnamic Sequencing Kit (GE Health Sciences, Birmingham, UK) and dye-labelled custom primers for both forward and reverse strands, for 30 cycles at an annealing temperature of 54 °C, with other parameters as specified by the manufacturer. Sequencing products were visualized by means

Table 1. Collection localities of specimens in the *Pemphigus betae* group in Alberta and New Brunswick, and Washington State, associated GenBank accession numbers for microsatellite flanking regions (MFRS), and the number of samples in each genotype group collected at each site.

			GenBank accession Nos. for MFRSs			No. of samples in genotype		
Code*	Location†	Host or host zone‡	PEM19R	PEM60	PEM83	A	B	C
IB	Alberta, Lethbridge, Indian Battle Park	*Populus angustifolia* × *balsamifera* × *deltoides* zone	GU991887 GU991907	GU991949 GU991953 GU991980 GU991982 GU991985	GU991910 GU991914 GU991941 GU991943 GU991946	18	0	11
PAV	Alberta, Lethbridge, Pavan Park	*P. angustifolia* × *balsamifera* × *deltoides* zone	GU991888 GU991889 GU991890 GU991892	GU991954 GU991955 GU991956 GU991958 GU991960 GU991981	GU991915 GU991916 GU991917 GU991919 GU991921 GU991942	30	0	12
POP	Alberta, Lethbridge, Popson Park	*P. angustifolia* × *balsamifera* × *deltoides* zone	GU991884 GU991893 GU991909	GU991950 GU991961 GU991977 GU991987	GU991911 GU991922 GU991938 GU991948	6	0	11
FMC	Alberta, Fort McLeod Wilderness Park	*P. angustifolia* × *balsamifera* zone	GU991894 GU991885	GU991962 GU991951	GU991912 GU991923	38	0	0
CRC	Alberta, Castle R. Recreation Area	*P. angustifolia* × *balsamifera* zone	GU991908	GU991986	GU991947	13	8	5
WP	Alberta, Woolford Provincial Park	*P. angustifolia* × *balsamifera* zone	GU991899 GU991902	GU991957 GU991968 GU991971 GU991979 GU991984	GU991918 GU991929 GU991932 GU991940 GU991945	4	12	4
KIM	Alberta, Aetna, Kimball Park	*P. angustifolia* × *balsamifera* zone	GU991895 GU991896	GU991963 GU991964 GU991965 GU991972 GU991973	GU991924 GU991925 GU991926 GU991933 GU991934	2	2	4
CHP	Alberta, Cypress Hills Provincial Park	*P. balsamifera*	GU991897 GU991898 GU991900 GU991901	GU991966 GU991967 GU991969 GU991970 GU991975	GU991927 GU991928 GU991930 GU991931 GU991936	0	4	2
FB	Alberta, Fabyan	*P. balsamifera*	GU991903	GU991974	GU991935	0	0	1
	Alberta, Lethbridge, Botterill Bottom Park	*P. angustifolia* × *balsamifera* × *deltoides* zone				1	0	0
	Alberta, Lundbreck Falls	*P. balsamifera*				0	3	2

Table 1 (*concluded*).

Code*	Location†	Host or host zone‡	GenBank accession Nos. for MFRSs			No. of samples in genotype		
			PEM19R	PEM60	PEM83	A	B	C
	Alberta, Spring Glen Park	*P. angustifolia* × *balsamifera* zone				0	3	4
	Alberta, Police Pt Provincial Park	*P. balsamifera*				0	2	0
	New Brunswick, Loggieville	*P. balsamifera*				0	0	1
	Alberta, Lethbridge	*Beta vulgaris* and *Chenopodium album*				7	3	0
	Washington, Puyallup	*Solanum tuberosum*				0	1	0

Note: Detailed georeferenced collection data for individual samples and associated COI GenBank accession numbers are available on BOLD (Ratnasingham and Hebert 2007; http://www.barcodinglife.org, "Published Projects", "Pemphigus–sugarbeet root aphid" links).

*Site identifier used in Figure 2.

†Additional data for Alberta localities are provided in Floate (2004).

‡Zones include morphologically pure zones of *Populus balsamifera* or overlap/hybrid zones of two or more *Populus* species.

of simultaneous bidirectional electrophoresis on a LI-COR/NEN Global IR2® automated sequencing system (LI-COR Biosciences, Lincoln, Nebraska, USA). Sequences are available on GenBank; accession numbers associated with each site are given in Table 1.

Pairwise distances among COI sequences were calculated using Kimura's two-parameter model (Kimura 1980), regarded as the best model for species-level analysis with low distances and the standard for barcode data analysis (Hebert *et al.* 2003). Distance matrices for MFRS data were calculated using several models: uncorrected distance and HKY85 models (determined to be the most appropriate model by ModelTest (Posada and Crandall 1998)) for data sets with gaps excluded; or mean distance, with gaps treated as additional characters, multibase gaps being recoded as single characters. The distance matrices are visualized through construction of neighbour-joining trees (Saitou and Nei 1987) as implemented in PAUP* version 4.0b10 (Swofford 2003). Note that the trees presented are data summaries only, and are not to be construed as phylogenetic hypotheses.

Results

Sequence data for COI define three distinct haplotype clusters (designated groups A, B, and C; Fig. 2). Mean pairwise within-group sequence distance (percent divergence, where 1 base change corresponds to about 0.15%) is 0.09% (range 0%−0.77%). Mean pairwise distance is 2.66% (range 2.36%−2.98%) between specimens in groups A and B, 3.08% (range 2.65%−3.79%) between those in groups A and C, and 3.59% (range 3.00%−3.96%) between those in groups B and C.

Segregation of the groups based on the ratio of within- to among-group distances for the MFRSs is not as well defined, *i.e.*, although the distribution of pairwise distances is bimodal, there is no sharp discontinuity. However, for each locus there are three sets of shared fixed differences, and these sets are entirely concordant across all MFRS loci and with the COI groups. Furthermore, most of the within-group variation is due to base changes that are shared by only a few samples or are unique. Tree topologies for each MFRS locus and for the combined data set were identical under all

Table 2. COI barcode and microsatellite flanking region loci, primers, annealing temperatures, and characteristics.

Locus	Primer name	Primer sequence	Annealing temp. (°C)	Product size (base pairs)*	No. of variable sites
COI	LepF1 (PCR/seq.)	ATTCAACCAATCA-TAAAGATATTGG		658	48
	LepR1 (PCR/seq.)	TAAACTTCTGGATG-TCCAAAAAATCA			
	LCO1490_t1 (PCR) (seq. with M13)	TGTAAAACGACGG-CCAGTGGTCAACA-AATCATAAA-GATATTGG			
	HCO2198_t1 (PCR) (seq. with M13)	CAGGAAACAGCTAT-GACTAAACTTCA-GGGTGACCAA-AAAATCA			
PEM19R	Pem19R-4F (PCR/seq.)	TGTGTCAAGAG-GCCCAGTAGT	61	302	8
	Pem19R-2R (PCR/seq.)	CTTCTCCGATT-CCACCGTTAT			
PEM60F	Pem60F-1F (PCR/seq.)	GCTATTGCGC-CGAGGTTGC	53	522	17
	Pem60F-3R (PCR)	GAGTGCAATTAT-TCGTACC-TACAGTCA			
	Pem60F-5R (seq.)	CTGACAGCAGTC-CAAGGTGAGC			
PEM83B	Pem83F-3F (PCR)	AACCCTTGCACAA-ATATTAGTTTC	55	880	26
	Pem83R-10R (PCR/seq.)	CGGTTGCTG-GGGTGCTG			
	Pem83F-5F (seq.)	GATACTTCAGACAC-TGGGTCGTTC			
	Pem83B-11R (seq.)	TTTTGTATTGGCGG-TGTATCATTAT			
	Pem83B-12F (seq.)	CGAGRAAACTGTC-ATATAACTATT			

*Size of the sequence fragment examined, after noisy data were trimmed, not the size of the PCR product (''PCR'' and ''seq.'' indicate whether the primer was used for PCR, sequencing, or both.).

models (uncorrected and HKY85 models for gaps excluded, mean distance with gaps included), with only minor differences in branch lengths. The neighbour-joining tree based on mean distances for the combined data set is shown at the right-hand side of Figure 2.

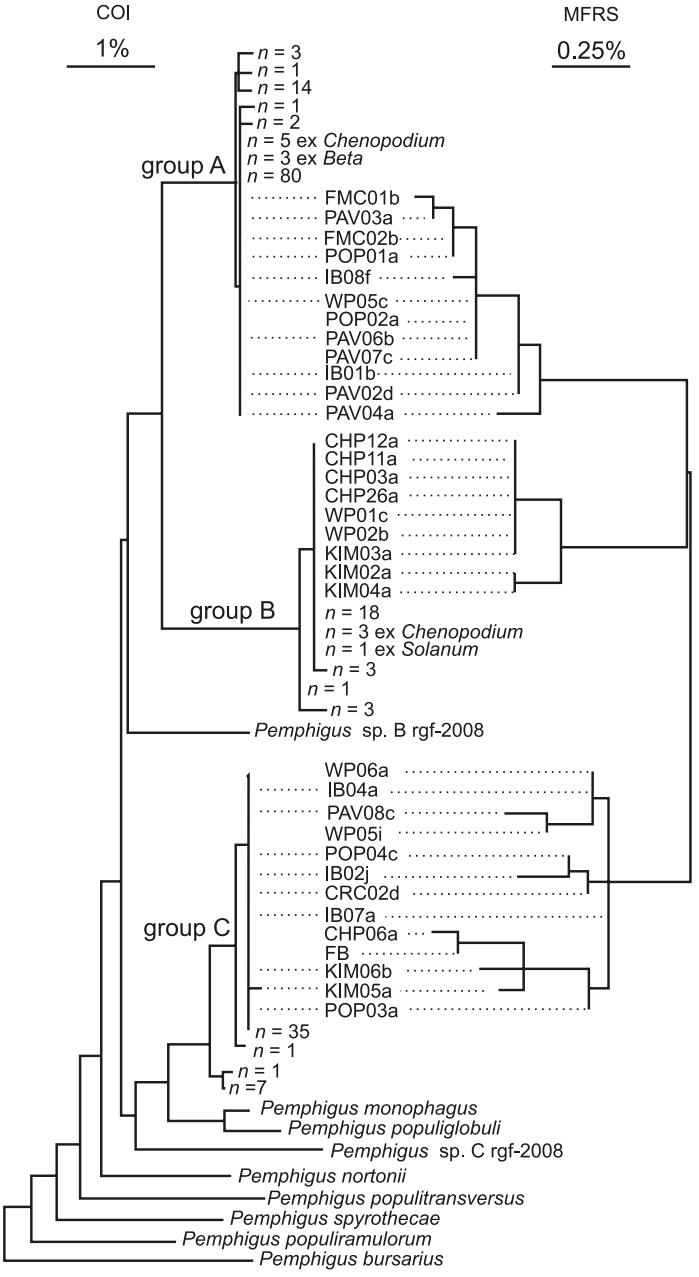
The clusters defined by COI were consistent with those defined by the set of MFRS loci for samples from throughout southern Alberta. The sample from New Brunswick belongs to group C, based on the COI sequence.

Three samples from sugarbeets belong to group A (Fig. 2). Seven samples from lambsquarters cluster with group A or group B. The sample from potato has a COI barcode sequence characteristic of group B.

Discussion

Collectively, these results identify at least three co-occurring populations forming similar galls on the same host trees. The concordance

Fig. 2. Neighbour-joining tree for mitochondrial COI and microsatellite flanking region sequences (MFRS) for samples of the "*P. betae*" complex, with COI sequences for representatives of other *Pemphigus* species. Sample identifiers, consisting of the site code (from Table 1) with a number and letter suffix indicating the tree and gall number, respectively, are given for 34 samples for which MFRS data were available. Other samples are not uniquely identified, but the number of specimens exhibiting an identical observed COI haplotype is indicated. The host genus for samples from secondary hosts is given; all other samples are from galls on *Populus* species.



of the mitochondrial and nuclear data suggests that the three groups are reproductively isolated.

Using the identification key provided by Smith (1984), based on the distribution and form of antennal rhinaria, specimens from group A (Fig. 2) key to *P. betae* (primary rhinarium of last antennal segment simple, and last segment without, rarely with 1 or 2 small, secondary rhinaria). They are also very similar to type material of *P. balsamiferae*, currently regarded as a synonym of *P. betae*, deposited in the National Museum of Natural History (Systematic Entomology Laboratory, Beltsville, Maryland, USA). Specimens from group C key to *P. populivenae* (last antennal segment with several well-developed secondary rhinaria and fusion of primary rhinarium with distal secondary rhinarium, the aggregate forming a large plate containing "islands"). Specimens from group B are intermediate, possessing several small secondary rhinaria, and the distal secondary rhinarium usually separate from the primary rhinarium, but occasionally partially fused with it. No specimens matched the state reported for *P. knowltoni* (last antennal segment with a large aggregate rhinarium, without additional secondary rhinaria).

The available evidence suggests that group B is an undescribed species. The COI distance between groups A and B (about 2.5%) relative to within-group values (less than 0.8%) is comparable to that observed among different *Pemphigus* species (lower part of Fig. 2) and among congeneric species in other aphid genera (Foottit *et al.* 2008, 2009). Other than *P. betae*, *P. populivenae*, and *P. knowltoni*, no North American *Pemphigus* species are known to regularly form elongate galls along the leaf midrib of species in *Populus* section *Tacamahaca*.

Although group C was identified as *P. populivenae*, there are several inconsistencies. Galls of this species are reported to project above the plane of the leaf blade (Harper 1959), but galls sampled in the present study resembled those of *P. betae*, *i.e.*, they project below the leaf blade. Galls of *P. populivenae* can apparently resemble those of *P. betae* when populations of the former are high (Maxson and Knowlton 1929). However, galls in the position normally ascribed to *P. populivenae* are uncommon in southern Alberta (Harper 1959; K.D.F., personal observations), whereas group C samples were common in our collections. These discrepancies are resolved if *P. populivenae* forms galls that can project either above or below the leaf blade regardless of population density. Alternatively, group C may represent an undescribed species morphologically similar to *P. populivenae*.

At least two groups were detected at most sites for which more than one sample was analysed, and all three groups were present at three sites (Table 1). Groups B and C co-occur in morphologically pure zones of *P. balsamifera* (samples from Cypress Hills and Lundbreck Falls, Alberta). Different groups can occur on the same tree (COI data; compare specimen identifiers in the "Isolate/Field No." field in data available on BOLD, in which the numeric part identifies the tree). Group B was not detected at sites where *P. deltoides* (section *Aigeiros*) or hybrids involving it occur. The significance of this finding is unclear. It may reflect an effect of the primary host on the occurrence of members of the *P. betae* complex, or could be an artefact of a relatively small sample size.

Secondary-host associations are currently unclear. Groups A and B were collected on both primary and secondary (*e.g.*, lambsquarters) hosts. However, we did not recover group C individuals from secondary hosts, so we cannot exclude the possibility that they rely on secondary-host species different from those used by groups A and B. If group C is indeed *P. populivenae*, as indicated by its morphology, secondary hosts in southern Alberta likely include species of *Chenopodium* L. For example, *P. populivenae* is reported to be a significant pest of quinoa, *Chenopodium quinoa* Willd., in Colorado (Cranshaw *et al.* 1990). *Pemphigus populivenae* has been reported to occur on sugarbeets in California (Summers and Newton 1989). If the interpretation of the California species is accurate, it is possible that groups A, B, and C all use sugarbeet as a secondary host in Alberta.

The reproductive mechanisms isolating these groups are unknown. Potential mechanisms include differences in host preference, evolution of differing recognition cues in geographic iso-

lation with subsequent sympatry, and differences in the timing of migration for sexuparae returning to the primary host to deposit the sexual forms. A variant of the last scenario is the omission of the secondary-host phase of the life cycle, so that alates emerging from galls are sexuparae, as in *Pemphigus monophagus* Maxson (Palmer 1952). However, secondary-host forms matching both group A and group B were found, and all alates from galls observed in this study contain embryos with mouthparts, and are thus clearly not sexuparae.

Analyses of samples from secondary hosts may identify additional genetic groups within the *P. betae* complex. Some colonies of *P. betae* persist year-round on the roots of secondary hosts, and so remain reproductively isolated from conspecific clones that alternate annually between secondary and primary hosts (Moran and Whitham 1988).

It is apparent that several taxa form similar galls on the leaf blades of species and hybrids of *Populus* section *Tacamahaca*. The form of a gall in a given position (petiole, petiole−blade junction, along midvein), may depend as much on host physiology or morphology as on the inducing aphid species. Anecdotal observations that we made during recent collections indicate that midvein galls containing morphologically similar aphids tend to be more globular when close the leaf base than those located more distally, especially on thicker leaves, sometimes approaching the state characteristic of *Pemphigus populiglobuli* Fitch. Maxson and Kowlton (1929) noted galls similar to those of *P. populivenae* produced by aphids they identified as *Pemphigus populicaulis* Fitch and *P. populiglobuli*. Reliance on gall position and form as indicators of species identity is clearly not a viable approach for use with this group of *Pemphigus* species, given the current state of knowledge.

Further study is currently being undertaken to establish the correlation between the observed genotypic groups and aphid morphology, gall location and morphology, primary-host genotypes, secondary-host utilization, and the broader geographic context. This information will be used to more firmly establish the taxonomic identity of each of the groups involved and to provide a full description.

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