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Research

Molecular Phylogenetic Analysis and Species Delimitation in the Pine Needle-feeding Aphid Genus *Essigella* (Hemiptera, Sternorrhyncha, Aphididae)

Thomas Théry,[1,3] Mariusz Kanturski,[2] and Colin Favret[1]

[1]Department of Biological Sciences, Biodiversity Centre, University of Montreal, 4101 E. Sherbrooke Street, Montreal, QC H1X 2B2, Canada, [2]Department of Zoology, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Bankowa 9, 40-007 Katowice, Poland, and [3]Corresponding author, e-mail: thomasjcthery@gmail.com

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Abstract

Species of the genus *Essigella* Del Guercio, 1909 (Hemiptera: Aphididae) are known to be specific to one or a few related species of Pinaceae hosts. The current *Essigella* classification includes 15 species-group taxa, inferred with morphological and ecological data. We present a phylogeny of *Essigella* using maximum likelihood and Bayesian inference using DNA sequences from three genomes: mitochondrial (*COI*, *ATP6*), nuclear (*EF-1α*), and endosymbiont (*Gnd*). We also challenged the taxonomy of *Essigella* species using four species delimitation methods: the 2% *COI* barcode threshold, the ABGD (Automatic Barcode Gap Discovery), the GMYC (General Mixed Yule Coalescent), and the bPTP (Bayesian Poisson Tree Process) methods. Fifty-three populations of *Essigella* were studied, with the eulachnine genera *Cinara* Curtis, 1835, *Eulachnus* Del Guercio, 1909 and *Pseudessigella* Hille Ris Lambers, 1966 as outgroups. Phylogenetic analyses support *Pseudessigella* as sister-group of *Essigella*. They confirm that all the known species are valid and mostly linked to a specific host plant, but also that *E. pini* Wilson, 1919 encompasses two species, the second probably being *E. patchae* Hottes, 1957, currently considered a synonym. ABGD and *COI* barcoding species delimitation analyses were partially congruent, although the barcoding threshold was less than 2% for the latter. They suggest the existence of several cryptic species also supported by ecological data. Methods of bPTP and GMYC gave conflicting results, possibly due to inadequate sampling. Our results highlight that substantial data are often required to delimit species with confidence.

Key words: Lachninae, Eulachnini, host plant, speciation, barcoding

Aphids (Hemiptera Aphididae) are sap-feeding insects. More than 5,000 species are known (Favret 2017), with some considered important economic pests (Eastop 1977, Foottit *et al.* 2006, Blackman and Eastop 2017). Aphid taxonomy is mainly based on morphology and host plant identity, given their high host plant specificity (Blackman and Eastop 2017). Intraspecific polymorphism can make species identification difficult and inaccurate (Hille Ris Lambers 1966a). Aphids can have different life cycles, patterns of host alternation, and different morphs depending on seasonal or climatic parameters (Hille Ris Lambers 1966a, von Dohlen and Moran 2000). Their morphology and general appearance can be also modified by their relationships with other organisms (Weisser *et al.* 1999, Johnson *et al.* 2003, Tsuchida *et al.* 2010, Yao 2012), most notably with their host plant (Wool and Hales 1997, Margaritopoulos *et al.* 2000, Favret and Voegtlin 2004). In some cases, the paucity or complete lack of diagnostic morphological characters remains the main issue (Sorensen 1983, 1994; Favret 2009). Host plant specificity is not always a reliable indicator, because several aphid species are oligophagous or polyphagous.

DNA barcoding is a method used in recognition of animal species using a 658 base-pair fragment of the 5′ end of the mitochondrial gene cytochrome *c* oxidase I (*COI*) (Hebert *et al.* 2003a,b; Hajibabaei *et al.* 2006). It is a powerful tool providing a rapid and accurate identification of animal species (Armstrong and Ball 2005, Hajibabaei *et al.* 2006) and can be used regardless of the condition of a specimen or of its life stage. Thus, it is commonly used in pathogen and pest control (Zhang *et al.* 2016, Cock *et al.* 2017, Sulaiman *et al.* 2017) and notably in aphid identifications (Lee *et al.* 2011, Cœur d'acier et al. 2014). The method is also employed in integrated insect taxonomy and systematics, for example, in Coleoptera (Beeren *et al.* 2016, Magoga *et al.* 2016), Diptera (Montagna *et al.* 2016, Chroni *et al.* 2017), Hymenoptera (Packer and Ruz 2016, Schmidt *et al.* 2017), Lepidoptera (Hajibabaei *et al.* 2006, Buchner *et al.* 2017), and Plecoptera (Avelino-Capistrano *et al.* 2016). *COI* DNA barcoding can be used to delimit species assuming a threshold of sequence divergence between animal species in general (Hebert *et al.* 2003b, Ratnasingham and Hebert 2013) and notably in aphids

(Foottit *et al.* 2009). Thus, it permits recognition of cryptic species, and several species of aphids have been discovered using this method (Miller *et al.* 2009, Lee *et al.* 2017). However, species delimitation using the *COI* barcode is sometimes not precise enough in some groups of Sternorrhyncha, notably for those already known to be problematic (Zurovcová *et al.* 2010, Cœur d'acier et al. 2014, Lee *et al.* 2014). Consequently, other barcoding genes with similar properties as *COI* have been considered in aphids: *Gnd*, a gene of the obligate bacterial endosymbiont *Buchnera aphidicola*, and *ATP6*, a mitochondrial gene, were successfully tested (Chen *et al.* 2013, Lee *et al.* 2014). Some nuclear genes can also be used for these same purposes; notably *EF-1α*, with its exon–intron structure, can provide relevant phylogenetic or population information (Simon *et al.* 2010, Savory and Ramakrishnan 2015, Théry *et al.* 2017).

Several other methods have been developed for molecular species delimitation. For example, the Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2012) is a method that delimits groups by comparing the gap existing between the range of intra- and interspecific sequence distances. After having detected a first gap value and delimited initial partitions, the program recursively applies the method to the new entities in order to refine the partition. Two input priors are proposed to the user in order to tighten the location of the barcode gap and the sensitivity of the method (minimum and maximum intraspecific P-distances and an X relative gap width) (Puillandre *et al.* 2012). Other methods based on phylogenetic analyses use the theory of coalescence (Fujita *et al.* 2012), such as the General Mixed Yule Coalescent (GMYC) (Pons *et al.* 2006, Fujisawa and Barraclough 2013) or the Bayesian Poisson Tree Process (bPTP) (Zhang *et al.* 2013). GMYC is based on an ultrametric gene tree. This method seeks to differentiate the shift between a speciation event (Yule process) and intraspecific diversification (coalescent process) by comparing the lengths of branches (Pons *et al.* 2006, Fujisawa and Barraclough 2013). The bPTP method is similar to GMYC but does not require an ultrametric tree. Contrary to GMYC, which compares long interspecific branches and short, polytomous intraspecific branches, bPTP uses rates of nucleotide substitution following a Poisson distribution to distinguish both events (Zhang *et al.* 2013).

Essigella Del Guercio, 1909 (Aphididae, Lachninae) (Fig. 1) is a small genus of narrow-bodied aphids encompassing 13 species with two species having two subspecies (Sorensen 1994). All species feed on the needles of *Pinus* Linnaeus, with the exception of *E. wilsoni* Hottes, 1957, which feeds only on the needles of *Pseudotsuga* Carrière, and *E. alyeska* Sorensen, 1988, which has been known to feed on *Picea* A. Dietrich as well as *Pinus*. According to Sorensen (1994), all species of *Essigella* are restricted to one or a few closely related host species, the oligophagous *E. californica* (Essig, 1909) and *E. pini* Wilson, 1919 being the only exceptions. All species are originally Nearctic, but one species, *E. californica*, was inadvertently introduced in several parts of the world (Théry *et al.* 2017). *Essigella* exhibits high intra- and interspecific variation in external morphology, making species identification difficult (Sorensen 1994). Sorensen (1994) fully revised the genus with 'discriminant function and principal component analyses, using morphometric data, and with principal coordinate analysis, multidimensional scaling and various UPGMA and single linkage clustering algorithms, using coded quantitative and qualitative data'. He split *Essigella* into three subgenera: *Archeoessigella*, *Essigella,* and *Lambersella* (Sorensen 1994) (Table 1). He further sorted them into two species series (Series A and B) and three species complexes (*E. californica*, *E. fusca,* and *E. knowltoni* complexes) (Sorensen 1994) (Table 1). *Essigella* belongs to the tribe Eulachnini along with *Cinara* Curtis, 1835 (including subgenus *Schizolachnus* Mordvilko, 1909), *Eulachnus* Del Guercio, 1909 and *Pseudessigella* Hille Ris Lambers, 1966 (Chen *et al.* 2016). Eulachnini feed on conifers either on bark (*Cinara* excluding *Schizolachnus*) or on needles (*Cinara* (*Schizolachnus*), *Eulachnus*, *Essigella,* and *Pseudessigella*) (Chen *et al.* 2016). Relationships between these genera are not yet fully resolved. *Schizolachnus* was considered a separate genus until its unification as a subgenus of *Cinara* by a recent molecular study (Chen *et al.* 2016). Furthermore, the relationship of *Pseudessigella*, a Himalayan genus, with *Essigella* and *Eulachnus* remains to be clarified (Sorensen 1991, 1994; Kanturski et al. 2017a,b).

Several *Essigella* species, notably those within each species complex, are morphologically very close, some also living on closely related species of host plant. Because populations of a same species can exhibit host-induced morphological variation (Favret and Voegtlin 2004, Jorge *et al.* 2011, Paris *et al.* 2016), Sorensen's taxonomy should be confirmed with molecular data. The present study tests Sorensen's (1994) taxonomy using four genes, *COI* and *ATP6* (mitochondrial), *EF-1α* (nuclear), and *Gnd* (of the bacterial primary nutritional symbiont *Buchnera aphidicola*). Phylogenetic analyses are used to study *Essigella* systematics within the genus itself and regarding its position within the Eulachnini. Notably, we sought to clarify the relationship of *Pseudessigella*, a Himalayan genus, with *Essigella* and *Eulachnus* (Sorensen 1991, 1994; Kanturski et al. 2017a,b). *Essigella* species validity was tested using both phylogenetic analyses and species delimitation methods.

Materials and Methods

Taxon Sampling

Fifty-three populations representing 13 species of *Essigella* were studied (Table 2). North American specimens were collected in Canada, Mexico, and the United States, overseas ones in Argentina, Australia, France, and New Zealand (Théry *et al.* 2017). Specimens of genera used as outgroups were collected in the USA for *Cinara* and *Eulachnus* and in India for *Pseudessigella* (Table 2). All specimens

Fig. 1. *Essigella hoerneri* on *Pinus monophylla* (left) and *Essigella sp.* on *Pinus ponderosa* (right) (pictures from C. Favret).

Table 1. Current classification of the genus *Essigella* (Sorensen, 1994)

		Genus *Essigella* Del Guercio, 1909**: 329** Type species: *Lachnus californicus* Essig, 1909: 1
		Subgenus *Archeoessigella* Sorensen, 1994**: 21** Type species: *Essigella kathleenae* Sorensen, 1988: 115
		Essigella (*Archeoessigella*) *kathleenae* Sorensen, 1988: 115; Sorensen, 1994: 26
		Essigella (*Archeoessigella*) *kirki* Sorensen, 1988: 121; Sorensen, 1994: 22
		Subgenus *Lambersella* Sorensen, 1994**: 29** Type species: *Essigella fusca* Gillette & Palmer, 1924: 6
	E. fusca complex	*Essigella* (*Lambersella*) *eastopi* Sorensen, 1994: 30
	E. fusca complex	*Essigella* (*Lambersella*) *fusca fusca* Gillette & Palmer, 1924: 6; Sorensen, 1994: 34 = *Essigella fusca* Gillette & Palmer, 1924: 6 = *Essigella agilis* Hottes, 1957: 71 (Synonymy by Sorensen, 1994: 34) = *Essigella palmerae* Hottes, 1957: 96 (Synonymy by Sorensen, 1994: 34)
	E. fusca complex	*Essigella* (*Lambersella*) *fusca voegtlini* Sorensen, 1994: 39
	E. fusca complex	*Essigella* (*Lambersella*) *hillerislambersi* Sorensen, 1994: 41
		Subgenus *Essigella* Del Guercio, 1909**: 329** Type Species: *Lachnus californicus* Essig, 1909: 1
Series A		*Essigella* (*Essigella*) *essigi* Hottes, 1957: 84; Sorensen, 1994: 45
Series A		*Essigella* (*Essigella*) *pini* Wilson, 1919: 2; Sorensen, 1994: 49 = *Essigella patchae* Hottes, 1957: 98 (Synonymy by Sorensen, 1994: 49)
Series A	*E. californica* complex	*Essigella* (*Essigella*) *californica* (Essig) 1909: 1; Sorensen, 1994: 53 = *Lachnus californicus* Essig, 1909: 1 = *Essigella claremontiana* Hottes, 1957: 79 (Synonymy by Sorensen, 1994: 53) = *Essigella cocheta* Hottes, 1957: 82 (Synonymy by Sorensen, 1994: 53) = *Essigella monelli* Hottes, 1957: 95 (Synonymy by Sorensen, 1994: 53) = *Essigella pineti* Hottes, 1957: 101 (Synonymy by Sorensen, 1994: 53) = *Essigella swaini* Hottes, 1957: 105 (Synonymy by Sorensen, 1994: 53)
Series A	*E. californica* complex	*Essigella* (*Essigella*) *hoerneri* Gillette & Palmer, 1924: 5; Sorensen, 1994: 62 = *Essigella gillettei* Hottes, 1957: 88 (Synonymy by Sorensen, 1994: 62) = *Essigella maculata* Hottes, 1957: 93 (Synonymy by Sorensen, 1994: 62)
Series A		*Essigella* (*Essigella*) *wilsoni* Hottes, 1957: 106; Sorensen, 1994: 67 = *Essigella pergandei* Hottes, 1957: 100 (Synonymy by Sorensen, 1994: 67) = *Essigella oregonensis* Hottes, 1958: 155 (Synonymy by Sorensen, 1994: 67)
Series B		*Essigella* (*Essigella*) *alyeska* Sorensen, 1988: 118; Sorensen, 1994: 72
Series B	*E. knowltoni* complex	*Essigella* (*Essigella*) *critchfieldi* Sorensen, 1994: 75
Series B	*E. knowltoni* complex	*Essigella* (*Essigella*) *knowltoni knowltoni* Hottes, 1957: 92; Sorensen, 1994: 78 = *Essigella knowltoni* Hottes, 1957: 92 (New status by Sorensen, 1994: 78)
Series B	*E. knowltoni* complex	*Essigella* (*Essigella*) *knowltoni braggi* Hottes, 1957: 73; Sorensen, 1994: 84 = *Essigella braggi* Hottes, 1957: 73 (New status by Sorensen, 1994: 84) = *Essigella robusta* Hottes, 1957: 103 (Synonymy by Sorensen, 1994: 84)

used for analysis were viviparous apterae. They were preserved in 95% ethanol after collecting and thereafter kept at −20°C or −80°C until DNA extraction. Specimens were slide-mounted in Canada Balsam and kept as voucher specimens (Favret 2005). Specimens were identified with the published key to the species of the genus *Essigella* by Sorensen (1994) and host-based keys by Blackman and Eastop (2017). Specimens were also compared with authoritatively identified reference material, including type specimens and material in the Sorensen Collection (Essig Museum of Entomology, Berkeley, CA). We followed the pinaceous host classification of the Gymnosperm database (Earle 2015). Voucher specimens are deposited in the Ouellet-Robert Collection of the University of Montreal (QMOR).

DNA Extraction, Amplification, and Sequencing

DNA extraction was nondestructive (Favret 2005), performed using the DNeasy Blood and Tissue kit (QIAGEN, Düsseldorf, Germany). PCR amplifications were carried out using Thermocycler Eppendorf Mastercycler ProS, with Phire Green Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Our protocols and primers were those of Théry *et al.* 2017 (Table 3). Amplicons were sequenced in both directions with their respective PCR primers at the McGill University and Génome Québec Innovation Centre (Montreal, Canada).

Phylogenetic Analyses

Chromatograms of each gene were edited using Geneious 9 software (Biomatters Ltd, Auckland, New Zealand) (Kearse *et al.* 2012). A GenBank BLAST search confirmed the aphid's generic identity. The sequences were aligned and compared with Bioedit Version 7.2.5 (Hall 1999) using the ClustalW multiple alignment program (Thompson *et al.* 1994). Alignments of *COI*, *ATP6*, and *Gnd* were straightforward due to a lack of length variation. Sequences of *EF-1α* were aligned with AphidBase transcript sequences of *Acyrthosiphon pisum* (Harris 1776) (Legeai *et al.* 2010) providing us the locations of introns in the *EF-1α* sequences. Phylogenetic analyses were performed with concatenated sequences, partitioned by gene. Following MrModeltest 2.3 to determine the best evolution model (Nylander 2004), we used GTR + Γ as models for *COI*, *ATP6*, and *EF-1α* and GTR + I + Γ for *Gnd*. Phylogenetic trees were estimated using maximum likelihood (ML) and Bayesian inference (BI) methods using RAxML-HP BlackBox 8.2.10 (Stamatakis 2014) and MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003, Ronquist *et al.* 2012), respectively. For ML analyses, we used the bootstrapping parameter proposed by the program ('let RAxML halt bootstrapping automatically'), other parameters were those by default. For BI analyses, we performed a run of 100 million generations including four chains (one cold chain and three heated) using Metropolis-coupled Markov Chain Monte Carlo (MCMC) with a burn-in of 25%. RAxML and MrBayes analyses were run via the CIPRES Science Gateway 3.3 (http://www.phylo.org/) (Miller *et al.* 2010). Each gene was first analyzed alone, then all four were analyzed together. Because of the low number of genes and populations, the concatenations were made manually. Data were partitioned into four parts: *ATP6*, *COI*, *EF-1α*, and *Gnd*. For ML analyses, we considered strong bootstrap support to be more than 95% and low bootstrap support to be less than 70%. For BI analysis, we considered strong support to be a posterior probability of more than 95% and low support to be a posterior probability of less than 90%.

Species Delimitation

We compared species identified with morphological characters, Operational Taxonomic Units or OTUs (Doyen and Slobodchikoff 1974), with those discriminated with molecular data, Molecular Operational Taxonomic Units or MOTUs (Floyd *et al.* 2002, Vogler and Monaghan 2007).

Table 2. Collecting data of specimens and Genbank accession numbers of DNA sequences

Species	Country	Locality	Host plant	Collection Number	Genbank accession numbers			
					ATP6	*COI*	*EF-1α*	*Gnd*
Essigella alyeska	Canada	Lac-Édouard (QC)	*Pinus banksiana*	QMOR50670	MG579864	MG579774	MG579909	MG579819
E. californica	Argentina	Malargüe (Mendoza)	*Pinus* sp.	QMOR50043	KY288967	KY288911	KY288929	KY288948
E. californica	Australia	Mt Mitchell (NSW)	*Pinus radiata*	QMOR50052	KY288976	KY288920	KY288938	KY288957
E. californica	France	Le Rheu (Ille et Vilaine)	*Pinus radiata*	QMOR50054	KY288978	KY288922	KY288940	KY288959
E. californica	New Zealand	Christchurch (Canterbury)	*Pinus resinosa/wallichiana*	QMOR50046	KY288970	KY288914	KY288932	KY288951
E. californica	United States	San Bernardino Co. (CA)	*Pinus coulteri*	QMOR50049	KY288973	KY288917	KY288935	KY288954
E. californica	United States	Ventura Co. (CA)	*Pinus* sp.	QMOR50051	KY288975	KY288919	KY288937	KY288956
E. californica	United States	Ventura Co. (CA)	*Pinus attenuata*	QMOR50047	KY288971	KY288915	KY288933	KY288952
E. californica	United States	Placer Co. (CA)	*Pinus ponderosa*	QMOR50048	KY288972	KY288916	KY288934	KY288953
E. californica	United States	Los Angeles Co. (CA	*Pinus coulteri*	QMOR50671	MG579865	MG579775	MG579910	MG579820
E. californica	United States	Monterey Co. (CA)	*Pinus sabiniana*	QMOR50672	MG579866	MG579776	MG579911	MG579821
E. californica	United States	Placer Co. (CA)	*Pinus ponderosa*	QMOR50673	MG579867	MG579777	MG579912	MG579822
E. californica	United States	Sonoma Co. (CA)	*Pinus muricata*	QMOR50674	MG579868	MG579778	MG579913	MG579823
E. californica	United States	Mendocino Co. (CA)	*Pinus muricata*	QMOR50675	MG579869	MG579779	MG579914	MG579824
E. californica	United States	El Dorado Co. (CA)	*Pinus monticola*	QMOR50676	MG579870	MG579780	MG579915	MG579825
E. californica	United States	Douglas Co. (NV)	*Pinus monticola*	QMOR50677	MG579871	MG579781	MG579916	MG579826
E. californica	United States	Alpine Co. (CA)	*Pinus albicaulis*	QMOR50678	MG579872	MG579782	MG579917	MG579827
E. californica	United States	Squamish (BC)	*Pinus contorta ssp. latifolia*	QMOR50679	MG579873	MG579783	MG579918	MG579828
E. critchfieldi	United States	Curry Co. (OR)	*Pinus contorta ssp. contorta*	QMOR50680	MG579874	MG579784	MG579919	MG579829
E. eastopi	United States	Los Angeles Co. (CA)	*Pinus coulteri*	QMOR50044	KY288968	KY288912	KY288930	KY288949
E. eastopi	United States	Los Angeles Co. (CA)	*Pinus coulteri*	QMOR50681	MG579875	MG579785	MG579920	MG579830
E. eastopi	United States	San Bernardino Co. (CA)	*Pinus coulteri*	QMOR50682	MG579876	MG579786	MG579921	MG579831
E. eastopi	United States	San Bernardino Co. (CA)	*Pinus coulteri*	QMOR50683	MG579877	MG579787	MG579922	MG579832
E. eastopi	United States	San Bernardino Co. (CA)	*Pinus coulteri*	QMOR50684	MG579878	MG579788	MG579923	MG579833
E. eastopi	United States	San Bernardino Co. (CA)	*Pinus coulteri*	QMOR50685	MG579879	MG579789	MG579924	MG579834
E. eastopi	United States	Monterey Co. (CA)	*Pinus coulteri*	QMOR50686	MG579880	MG579790	MG579925	MG579835
E. essigi	United States	Ventura Co. (CA)	*Pinus attenuata*	QMOR50687	MG579881	MG579791	MG579926	MG579836
E. essigi	United States	Monterey Co. (CA)	*Pinus radiata*	QMOR50688	MG579882	MG579792	MG579927	MG579837
E. fusca voegtlini	United States	Monterey Co. (CA)	*Pinus ponderosa*	QMOR50689	MG579883	MG579793	MG579928	MG579838
E. fusca voegtlini	United States	Placer Co. (CA)	*Pinus ponderosa*	QMOR50690	MG579884	MG579794	MG579929	MG579839
E. fusca voegtlini	United States	Placer Co. (CA)	*Pinus ponderosa*	QMOR50691	MG579885	MG579795	MG579930	MG579840
E. hillerislambersi	United States	Los Angeles Co. (CA)	*Pinus jeffreyi*	QMOR50692	MG579886	MG579796	MG579931	MG579841
E. hillerislambersi	United States	San Bernardino Co. (CA)	*Pinus jeffreyi*	QMOR50693	MG579887	MG579797	MG579932	MG579842
E. hillerislambersi	United States	San Bernardino Co. (CA)	*Pinus jeffreyi*	QMOR50694	MG579888	MG579798	MG579933	MG579843
E. hoerneri	United States	San Bernardino Co. (CA)	*Pinus monophylla*	QMOR50050	KY288974	KY288918	KY288936	KY288955
E. hoerneri	United States	San Bernardino Co. (CA)	*Pinus monophylla*	QMOR50695	MG579889	MG579799	MG579934	MG579844
E. hoerneri	United States	San Bernardino Co. (CA)	*Pinus monophylla*	QMOR50696	MG579890	MG579800	MG579935	MG579845
E. hoerneri	United States	Ventura Co. (CA)	*Pinus monophylla*	QMOR50697	MG579891	MG579801	MG579936	MG579846
E. hoerneri	United States	El Dorado Co. (CA)	*Pinus monophylla*	QMOR50698	MG579892	MG579802	MG579937	MG579847
E. kathleenae	United States	Los Angeles Co. (CA)	*Pinus lambertiana*	QMOR50699	MG579893	MG579803	MG579938	MG579848
E. kathleenae	United States	San Bernardino Co. (CA)	*Pinus lambertiana*	QMOR50700	MG579894	MG579804	MG579939	MG579849
E. kirki	Mexico	Sierra Norte (OAX)	*Pinus* sp.	QMOR50701	MG579895	MG579805	MG579940	MG579850
E. knowltoni braggi	United States	El Dorado Co. (CA)	*Pinus contorta ssp. murrayana*	QMOR50702	MG579896	MG579806	MG579941	MG579851

Table 2. *Continued*

Species	Country	Locality	Host plant	Collection Number	Genbank accession numbers			
					ATP6	*COI*	*EF-1α*	*Gnd*
E. knowltoni braggi	United States	El Dorado Co. (CA)	*Pinus contorta ssp. murrayana*	QMOR50703	MG579897	MG579807	MG579942	MG579852
E. knowltoni braggi	United States	Alpine Co. (CA)	*Pinus contorta ssp. murrayana*	QMOR50704	MG579898	MG579808	MG579943	MG579853
E. knowltoni knowltoni	Canada	Whistler (BC)	*Pinus contorta ssp. latifolia*	QMOR50705	MG579899	MG579809	MG579944	MG579854
E. knowltoni knowltoni	Canada	Squamish (BC)	*Pinus contorta ssp. latifolia*	QMOR50706	MG579900	MG579810	MG579945	MG579855
E. pini	United States	Swain Co. (NC)	*Pinus rigida*	QMOR50045	KY288969	KY288913	KY288931	KY288950
E. pini	Canada	St-Jérôme (QC)	*Pinus strobus*	QMOR50707	MG579901	MG579811	MG579946	MG579856
E. wilsoni	United States	Los Angeles Co. (CA)	*Pseudotsuga macrocarpa*	QMOR50708	MG579902	MG579812	MG579947	MG579857
E. wilsoni	United States	Ventura Co. (CA)	*Pseudotsuga macrocarpa*	QMOR50709	MG579903	MG579813	MG579948	MG579858
E. wilsoni	United States	San Mateo Co. (CA)	*Pseudotsuga menziesii*	QMOR50710	MG579904	MG579814	MG579949	MG579859
E. wilsoni	Canada	Vancouver (BC)	*Pseudotsuga menziesii*	QMOR50711	MG579905	MG579815	MG579950	MG579860
Cinara sp.	United States	El Dorado Co. (CA)	*Pinus contorta ssp. murrayana*	QMOR50712	MG579906	MG579816	MG579951	MG579861
Eulachnus sp.	United States	Monterey Co. (CA)	*Pinus radiata*	QMOR50713	MG579907	MG579817	MG579952	MG579862
Pseudessigella brachychaeta	India	Yousmarg (Jammu and Kashmir)	*Pinus wallichiana*	QMOR50714	MG579908	MG579818	MG579953	MG579863

We used four molecular species delimitation methods: a simple 2% *COI* DNA barcode threshold (Hebert *et al.* 2003b; Ratnasingham and Hebert 2013), the Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2012), the GMYC (Pons *et al.* 2006, Fujisawa and Barraclough 2013), and the bPTP (Zhang *et al.* 2013).

Assuming properties of a 658 base pairs fragment of *COI* as a standard DNA barcode (Hebert *et al.* 2003a,b), we compared Kimura 2 Parameter (K2P) distances of the *COI* sequences of all our *Essigella* populations. We chose a threshold of 2% because it was shown that *COI* divergence is usually more than 2% in animal species in general (Ratnasingham and Hebert 2013) and notably in aphids (Foottit *et al.* 2009). Those distances were obtained and compared using MEGA 6.0 (Tamura *et al.* 2013).

Our *Essigella* sequences were analyzed using the graphic web version of the ABGD method (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). Because of their prior use in species delimitation studies and their lack of indels, we analyzed *COI*, *ATP6*, and *Gnd* sequences separately and compared their results. For each gene, we used MEGA 6.0 (Tamura *et al.* 2013) to calculate distance values using a K2P model. Because a small number of populations (<3) in some species can distort ABGD resolution (Puillandre *et al.* 2012), we compared results obtained for all species with those for which we had ≥ 3 populations. We used a value of X = 1.25 (relative gap width), and values given by default with P_{min} = 0.001 and P_{max} = 0.1.

GMYC method is a tree-based likelihood method using the coalescent theory. It requires ultrametric trees based on single-sequence data (Pons *et al.* 2006; Fujisawa and Barraclough 2013). In consequence, we analyzed our four genes separately and built our trees using BEAST 1.8 (Drummond *et al.* 2012). We partially followed the protocol of Dumas *et al.* (2015) by using a Birth-Death model as tree prior and an uncorrelated lognormal relaxed clock as clock prior. To avoid biases of outgroups on our species delimitation results, we removed them from our analyses. We ran two independent analyses of 60 million generations for each gene, with trees sampled every 1,000 generations. Substitution models were those used in phylogenetic analyses. LogCombiner 1.8 (Drummond *et al.* 2012) was used to separately combine log and tree files obtained from our BEAST analyses. We used Tracer 1.6 (Rambaut *et al.* 2014) to check the convergence of parameters. Finally, TreeAnnotator 1.8 (Drummond *et al.* 2012) was used to summarize all obtained trees. Our output trees were converted to Newick files using FigTree 1.4.2 (Rambaut 2012) and analyzed via the GMYC web server (http://species.h-its.org/gmyc/).

bPTP is also a tree-based method based on a coalescent model, but contrary to GMYC it does not require ultrametric trees as input (Zhang *et al.* 2013). As with GMYC, we analyzed the four genes separately. For the same reasons explained above, we ran our analyses without outgroups. We built our trees using MrBayes 3.2.6, following same protocol described above. Our outcome trees were converted into NEXUS files using FigTree 1.4.2 (Rambaut 2012) and analyzed via the bPTP web server (http://species.h-its.org/ptp/). We used 300,000 generations and a burn-in of 25%. Other parameters were those given by default.

Results

DNA Extraction, Amplification, and Sequencing

All genes were sequenced and analyzed for all populations. The amplicon lengths were 657–663 base pairs (bp) for *ATP6*, 658 bp for *COI*, 661–778 bp for *EF-1α* (including introns), and 749 bp for *Gnd* (see Table 1 for GenBank accession numbers).

Table 3. Primers and PCR protocols

Genes	Primers	Primers sequences	Initial denaturation: time and T°C	Number of cycles	Denaturation: time and T°C	Annealing: time and T°C	Elongation: time and T°C	Final Elongation: time and T°C
ATP6	tRNALysAf2	GACTGAAAAGCAAAGTAATGATCTCT	94°C for 3 min	35	94°C for 30 s	55°C for 30 s	65°C for 1 min	None
	CO3WWRD	TCWCGAATWACATCWCGTCATCA						
COI	Lep-F1	ATTCAACCAATCATAAAGATATTGG	98°C for 30 s	35	98°C for 10 s	50°C for 20 s	72°C for 20 s	72°C for 2 min
	Lep-R1	TAAACTTCTGGATGTCCAAAAAATCA						
EF-1α	EF-1-F	GAACGTGAACGTGGTATCAC	98°C for 30 s	35	98°C for 20 s	51°C for 20 s	72°C for 20 s	72°C for 2 min
	EF-1-R	TGACCAGGGTGGTTTCAATAC						
Gnd	BamHI	CGCGGATCCGGWCCWWSWATWATGCCWGGWGG	98°C for 1 min	35	98°C for 20 s	51°C for 40 s	72°C for 40 s	72°C for 3 min
	ApaI	CGCGGGCCCCGTATGWGCWCCAAAATAATCWCKTTGWGCTTG						

Phylogenetic Analyses

Trees obtained for each gene separately showed few important incongruences for both ML and BI analyses. Observed incongruences coincided with low branch support. ML and BI trees of concatenated sequences were identical and are presented in a single dendrogram (Fig. 2). The main branches were strongly supported except for Clade A, grouping all *Essigella* species except *E. kirki* Sorensen, 1988 (posterior probabilities [*PP*] = 64%), Clade D (*pp* = 89%), and Clade E (*pp* = 53%). *Pseudessigella* appeared as the sister-group of *Essigella* (*pp* = 100%) and *Eulachnus* as the sister-group of *Pseudessigella* + *Essigella* (*pp* = 100%). Species of the subgenus *Lambersella* were clustered with strong support (*pp* = 100%) (Clade F, Fig. 2). They were included in the same clade (Clade C, Fig. 2) (*pp* = 100%) as *E. essigi* Hottes, 1957 (subgenus *Essigella*) and *E. kathleenae* Sorensen, 1988 (subgenus *Archeoessigella*). The two species of *Archeoessigella* (*E. kathleenae* and *E. kirki*) were not found together, *E. kirki* branching basally as the sister-group to all other *Essigella*, and *E. kathleenae* being found in Clade C. Species of the subgenus *Essigella* were split into three different groups (Fig. 2). The first one corresponded to the Clade B (*pp* = 100%); the second was represented by *E. essigi* of the Clade C; the third corresponded to Clade D (*pp* = 89%). Sorensen's (1994) Species Series A was split into Clade B for *E. californica* and *E. hoerneri* Gillette & Palmer, 1924, Clade C for *E. essigi*, and Clade D for *E. wilsoni*. Populations of *E. pini* were divided between Canadian and US populations in Clades B and D, respectively. Sorensen's (1994) Species Series B were recovered within the Clade D (*pp* = 100%). The *Essigella californica, E. fusca,* and *E. knowltoni* complexes (Sorensen 1994) were also recovered. Among species with several populations, some showed little genetic variability, such as *E. hillerislambersi* Sorensen, 1994 and *E. hoerneri*. In contrast, others showed high variability and formed several clear sub-specific groups: notably *E. californica*, *E. eastopi* Sorensen, 1994, *E. essigi, E. fusca voegtlini* Sorensen, 1994, *E. knowltoni* Hottes, 1957, and *E. wilsoni*. *E. californica* was divided into two main clades, Clades G and H, with a *pp* = 100% for the first and a *pp* = 81% (with a bootstrap value = 91%) for the second. Clade G was divided into two groups: the first (G1) gathered 11 populations collected on diverse pine species, the second (G2) was represented by a population collected on *P. contorta* Douglas ex Loudon. Clade H was split into three groups: populations collected on *P. muricata* D. Don (H1), those collected on *P. monticola* Douglas ex D. Don (H2), and those collected on *P. albicaulis* Engelm. (H3).

Essigella knowltoni was divided into two clades corresponding to the subspecies *E. knowltoni braggi* Hottes, 1957 (*pp* = 87%) and *E. knowltoni knowltoni* Hottes, 1957 (*pp* = 100%). We did not observe a correlation between individual populations and host plant identity with *E. eastopi, E. fusca voegtlini*, and *E. wilsoni* but we did with *E. essigi*. However, in this last case, both populations were collected on pine species of the subsection *Attenuatae*.

EF-1α Sequences

Our *EF-1α* sequences included two introns of variable size (Introns 3 and 4), the second showing the more informative structure (Fig. 3). We observed a similar pattern of intron insertions and deletions (indels) for *E. californica*, *E. hoerneri* and the Canadian population of *E. pini*. *Essigella kirki* exhibited a similar intron indel pattern as that of the previous species, but it was completely different from that of its supposed sister species, *E. kathleenae*. Populations of the *E. knowltoni* ssp. and *E. critchfieldi* Sorensen, 1994 showed similar patterns except a deletion of 12 nucleotides in Intron 4 (5′ TTAAATATACTA 3′) in *E. knowltoni knowltoni*. The complete

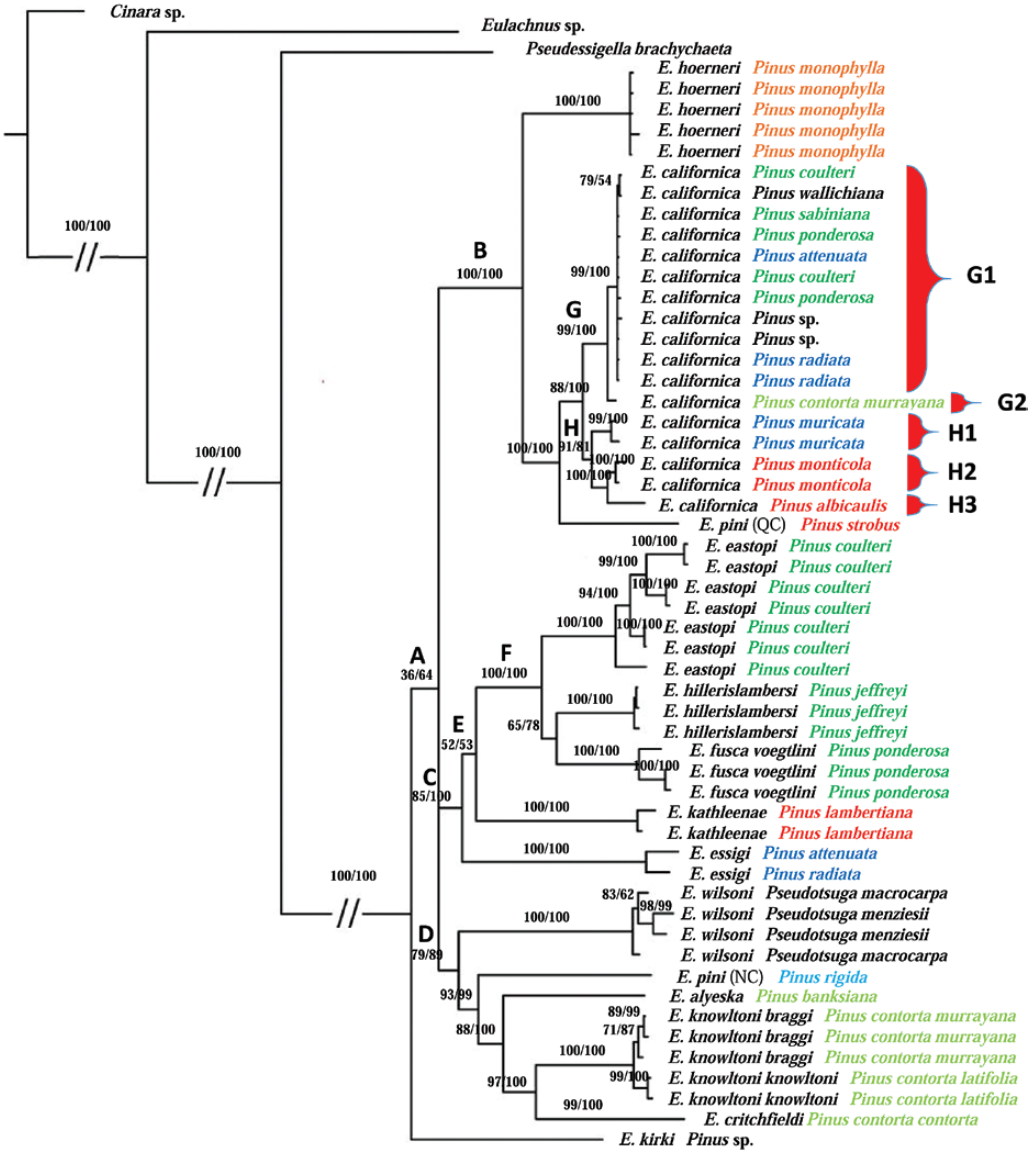

Fig. 2. Phylogenetic tree (ML and BI) of *Essigella* species using concatenate *ATP6, COI, EF-1α* and *Gnd*. *Pinus* species of the subgenus *Strobus* appear either in orange or in red, those of the subgenus *Pinus* either in green or in blue. Color nuances represent infragroup within each subgenus. Nonidentified *Pinus* and other Pinaceae are in black. Values indicate ML bootstrap % values followed by Bayesian posterior probabilities % values.

sequence was present in our populations of *E. knowltoni braggi* and *E. critchfieldi*. *Essigella alyeska* showed a similar pattern (with one nucleotide added to Intron 4) as that of *E. knowltoni braggi* and *E. critchfieldi*. All other species each had their own unique intron indel pattern.

Species Delimitation

COI barcoding

According to the 2% DNA barcode threshold, 16 MOTUs were revealed, including 13 that had been identified morphologically as species-group taxa (Supplementary Table 1; Table 4). Two MOTUs were found within *E. pini* with a divergence value of $P = 4.1\%$ between them. Three MOTUs appeared within *E. californica*: sequence divergences between populations of Clades G and H were 1.1–3.1%, specifically, $P = 1.1–1.5\%$ between Clades G and H1, $P = 1.9–2.3\%$ between Clades G and H2 and $P = 2.8–3.1\%$ between Clades G and H3. Divergences between Clades H1 and H2 were $P = 1.2–1.5\%$, those between Clades H1 and H3 were $P = 2.2–2.3\%$, and those between Clades H2 and H3 were $P = 2.0\%$.

Automatic Barcode Gap Discovery (ABGD)

We obtained the same number of MOTUs for taxa for which we had three or more populations (i.e., *E. californica*, *E. eastopi*, *E. fusca voegtlini*, *E. hillerislambersi*, *E. knowltoni*, and *E. wilsoni*), whether or not we included the taxa with fewer than three. *Essigella pini* consisted of two MOTUs for all three genes. In all, we obtained 18, 17, and 16 MOTUs for *COI*, *ATP6*, and *Gnd*, respectively (Table 4); the variability was due to *E. californica* for which we obtained five, four, and three different MOTUs for *COI*, *ATP6*, and *Gnd*, respectively.

GMYC and bPTP

We obtained 15, 29, 16, and 14 MOTUs with GMYC for *ATP6*, *COI*, *EF-1α*, and *Gnd*, respectively (Table 5). In each case, *Essigella pini* was always divided into two distinct MOTUs. The bPTP web

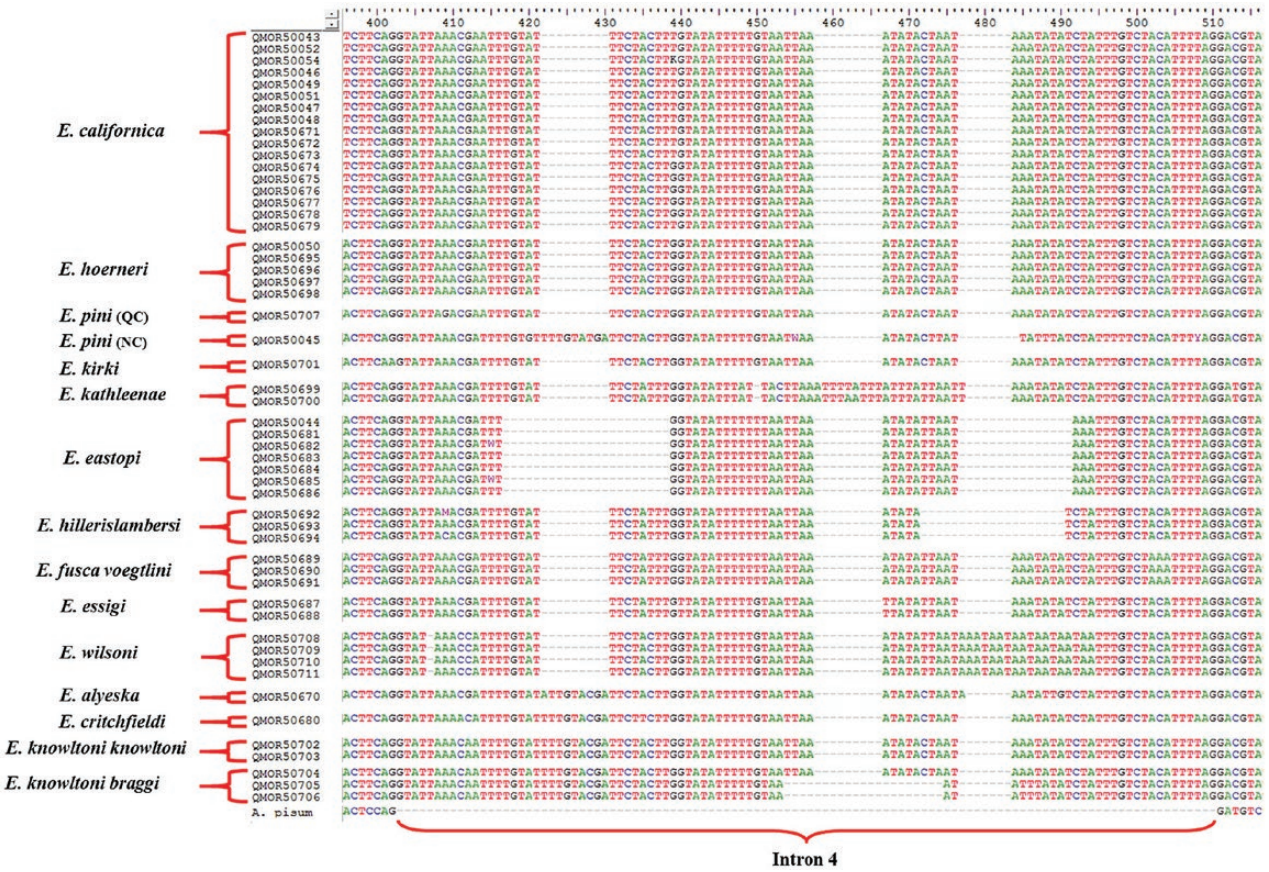

Fig. 3. *Essigella* specific differences in intron region 4 of *EF-1α*.

server proposes both a maximum likelihood and a Bayesian solution for each analysis. We obtained 25/23, 26/26, 14/22, and 21/21 (ML/BI) MOTUs for *ATP6*, *COI*, *EF-1α,* and *Gnd*, respectively. As with GMYC, *E. pini* consistently included two MOTUs (Table 6).

Discussion

Our results provide relevant information that clarifies *Essigella* and Eulachnini systematics. They also bring up concerns regarding molecular species delimitation methods.

Species Delimitation Methods

The four species delimitation methods we used in this study presented variable and incongruent results and therefore must be considered carefully. Usually, bPTP and GMYC provide similar results (Dumas *et al.* 2015, Jasso-Martinez et al. 2016, Zhu *et al.* 2017). It was shown that GMYC may be less reliable than bPTP, overestimating the number of MOTUs (as in our case with *COI*), due to the ultrametricization of the input trees (Zhang *et al.* 2013, Ahrens *et al.* 2016). In contrast, for *ATP6* and *Gnd*, we observed more MOTUs with bPTP than with GMYC. The origins of these incongruities between GMYC and bPTP may be diverse, but are likely due to the small size of our population sampling. Indeed, our sampling was reduced in comparison with those of other studies that employed both GMYC and bPTP methods (Dumas *et al.* 2015, Ahrens *et al.* 2016, Jasso-Martinez et al. 2016, Zhu *et al.* 2017). The problem of inadequate sampling per species was mentioned for ABGD as well: ABGD works best when there are more than to three to five populations per species (Puillandre *et al.* 2012). However, we observed no differences in our ABGD results by calculating with species containing three or more populations alone, or with other species showing fewer populations. Because ABGD and *COI* barcoding are distance-based methods, they may be less sensitive to sample size than bPTP and GMYC. In consequence, below we will only discuss results obtained with ABGD and *COI* barcoding.

Within Eulachnini

Since its description (Hille Ris Lambers 1966b), *Pseudessigella* has always been classified as a genus intermediate between *Eulachnus* and *Essigella*. Indeed, *Pseudessigella* shares characters with *Eulachnus*, such as simple tarsal claws (not incised), and with *Essigella*, such as 5-segmented antennae (Hille Ris Lambers 1966b; Sorensen 1991, 1994). *Pseudessigella* also shares with *Essigella* a head fused with the pronotum (Kanturski *et al.* 2017a) and with *Eulachnus* a membranous abdominal dorsum (Kanturski *et al.* 2017a; Sorensen 1991, 1994). Sorensen, in his revision of the genus *Essigella* (1994), highlighted morphological proximities between *Pseudessigella* and *Essigella*, notably in the close patterns of their abdominal dorsal chaetotaxy. In contrast, a more recent morphological study pointed out that, except for its 5-segmented antennae, the general morphology of *Pseudessigella* was closer to that of *Eulachnus* (Kanturski *et al.* 2017a). Our analyses places *Pseudessigella* as sister-group to *Essigella* (Fig. 2, *pp* = 100%), as predicted by Sorensen (1991, 1994). The 5-segmented antennae and the head fused with the pronotum are therefore synapomorphies of *Essigella* and *Pseudessigella*, and the incised tarsal claws are an autapomorphy of *Essigella*. In contrast, the membranous abdominal dorsum present in *Eulachnus* and *Pseudessigella* is plesiomorphic.

Table 4. OTUs and MOTUs according to morphology, 2% threshold *COI* and ABGD

Morphology (Sorensen 1994)	Barcoding (Hebert *et al.* 2003a, b)	ABGD (Puillandre *et al.* 2012)		
		ATP6	*COI*	*Gnd*
OTU 1 *E. alyeska* (QMOR50670) OTU 2 *E. californica* (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50674, QMOR50675, QMOR50676, QMOR50677, QMOR50678, QMOR50679) OTU 3 *E. critchfieldi* (QMOR50680) OTU 4 *E. eastopi* (QMOR50044, QMOR50681, QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686) OTU 5 *E. essigi* (QMOR50687, QMOR50688) OTU 6 *E. fusca voegtlini* (QMOR50689, QMOR50690, QMOR50691) OTU 7 *E. hillerislambersi* (QMOR50692, QMOR50693, QMOR50694) OTU 8 *E. hoerneri* (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698) OTU 9 *E. kathleenae* (QMOR50699, QMOR50700) OTU 10 *E. kirki* (QMOR50701) OTU 11 *E. knowltoni braggi* (QMOR50702, QMOR50703, QMOR50704) *E. knowltoni knowltoni* (QMOR50705, QMOR50706) OTU 12 *E. pini* (QMOR50045, QMOR50707) OTU 13 *E. wilsoni* (QMOR50708, QMOR50709, QMOR50710, QMOR50711)	MOTU 1 *E. alyeska* (QMOR50670) MOTU 2 *E. californica* (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50674), (QMOR50675, QMOR50679) MOTU 3 *E. californica* (QMOR50676, QMOR50677) MOTU 4 *E. californica* (QMOR50678) MOTU 5 *E. critchfieldi* (QMOR50680) MOTU 6 *E. eastopi* (QMOR50044, QMOR50681, QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686) MOTU 7 *E. essigi* (QMOR50687, QMOR50688) MOTU 8 *E. fusca voegtlini* (QMOR50689, QMOR50690, QMOR50691) MOTU 9 *E. hillerislambersi* (QMOR50692, QMOR50693, QMOR50694) MOTU 10 *E. hoerneri* (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698) MOTU 11 *E. kathleenae* (QMOR50699, QMOR50700) MOTU 12 *E. kirki* (QMOR50701) MOTU 13 *E. knowltoni braggi* (QMOR50702, QMOR50703, QMOR50704) *E. knowltoni knowltoni* (QMOR50705, QMOR50706) MOTU 14 *E. pini* (QMOR50045) MOTU 15 *E. pini* (QMOR50707) MOTU 16 *E. wilsoni* (QMOR50708), QMOR50709, QMOR50710, QMOR50711)	MOTU 1 *E. alyeska* (QMOR50670) MOTU 2 *E. californica* (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50679) MOTU 3 *E. californica* (QMOR50674, QMOR50675) MOTU 4 *E. californica* (QMOR50676, QMOR50677) MOTU 5 *E. californica* (QMOR50678) MOTU 6 *E. critchfieldi* (QMOR50680) MOTU 7 *E. eastopi* (QMOR50044, QMOR50681, QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686) MOTU 8 *E. essigi* (QMOR50687, QMOR50688) MOTU 9 *E. fusca voegtlini* (QMOR50689, QMOR50690, QMOR50691) MOTU 10 *E. hillerislambersi* (QMOR50692, QMOR50693, QMOR50694) MOTU 11 *E. hoerneri* (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698) MOTU 12 *E. kathleenae* (QMOR50699, QMOR50700) MOTU 13 *E. kirki* (QMOR50701) MOTU 14 *E. knowltoni braggi* (QMOR50702, QMOR50703, QMOR50704) *E. knowltoni knowltoni* (QMOR50705, QMOR50706) MOTU 15 *E. pini* (QMOR50045) MOTU 16 *E. pini* (QMOR50707) MOTU 17 *E. wilsoni* (QMOR50708, QMOR50709, QMOR50710, QMOR50711)	MOTU 1 *E. alyeska* (QMOR50670) MOTU 2 *E. californica* (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673) MOTU 3 *E. californica* (QMOR50679) MOTU 4 *E. californica* (QMOR50674, QMOR50675) MOTU 5 *E. californica* (QMOR50676, QMOR50677) MOTU 6 *E. californica* (QMOR50678) MOTU 7 *E. critchfieldi* (QMOR50680) MOTU 8 *E. eastopi* (QMOR50044, QMOR50681, QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686) MOTU 9 *E. essigi* (QMOR50687, QMOR50688) MOTU 10 *E. fusca voegtlini* (QMOR50689, QMOR50690, QMOR50691) MOTU 11 *E. hillerislambersi* (QMOR50692, QMOR50693, QMOR50694) MOTU 12 *E. hoerneri* (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698) MOTU 13 *E. kathleenae* (QMOR50699, QMOR50700) MOTU 14 *E. kirki* (QMOR50701) MOTU 15 *E. knowltoni braggi* (QMOR50702, QMOR50703, QMOR50704) *E. knowltoni knowltoni* (QMOR50705, QMOR50706) MOTU 16 *E. pini* (QMOR50045) MOTU 17 *E. pini* (QMOR50707) MOTU 18 *E. wilsoni* (QMOR50708, QMOR50709, QMOR50710, QMOR50711)	MOTU 1 *E. alyeska* (QMOR50670) MOTU 2 *E. californica* (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50679) MOTU 3 *E. californica* (QMOR50674, QMOR50675) MOTU 4 *E. californica* (QMOR50676, QMOR50677, QMOR50678) MOTU 5 *E. critchfieldi* (QMOR50680) MOTU 6 *E. eastopi* (QMOR50044, QMOR50681, QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686) MOTU 7 *E. essigi* (QMOR50687, QMOR50688) MOTU 8 *E. fusca voegtlini* (QMOR50689, QMOR50690, QMOR50691) MOTU 9 *E. hillerislambersi* (QMOR50692, QMOR50693, QMOR50694) MOTU 10 *E. hoerneri* (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698) MOTU 11 *E. kathleenae* (QMOR50699, QMOR50700) MOTU 12 *E. kirki* (QMOR50701) MOTU 13 *E. knowltoni braggi* (QMOR50702, QMOR50703, QMOR50704) *E. knowltoni knowltoni* (QMOR50705, QMOR50706) MOTU 14 *E. pini* (QMOR50045) MOTU 15 *E. pini* (QMOR50707) MOTU 16 *E. wilsoni* (QMOR50708, QMOR50709, QMOR50710, QMOR50711)

Table 5. MOTUs according to GMYC

GMYC (Pons *et al.* 2006)			
ATP6	*COI*	*EF-1α*	*Gnd*

MOTU 1
E. alyeska (QMOR50670)
MOTU 2
E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50674, QMOR50675, QMOR50676, QMOR50677, QMOR50678, QMOR50679)
MOTU 3
E. critchfieldi (QMOR50680)
MOTU 4
E. eastopi (QMOR50044, QMOR50681, (QMOR50682, QMOR50683, QMOR50684, QMOR50685)
MOTU 5
E. eastopi (QMOR50686)
MOTU 6
E. essigi (QMOR50687, QMOR50688)
MOTU 7
E. fusca voegtlini (QMOR50689, QMOR50690, QMOR50691)
MOTU 8
E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
MOTU 9
E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
MOTU 10
E. kathleenae (QMOR50699, QMOR50700)
MOTU 11
E. kirki (QMOR50701)
MOTU 12
E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
E. knowltoni knowltoni (QMOR50705, QMOR50706)
MOTU 13
E. pini (QMOR50045)
MOTU 14
E. pini (QMOR50707)
MOTU 15
E. wilsoni (QMOR50708, QMOR50709, QMOR50710, QMOR50711)
	MOTU 1
	E. alyeska (QMOR50670)
	MOTU 2-MOTU 7
	E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672) // *E. californica* (QMOR50673) // *E. californica* (QMOR50674, QMOR50675) // *E. californica* (QMOR50676, QMOR50677) // *E. californica* (QMOR50678) // *E. californica* (QMOR50679)
	MOTU 8
	E. critchfieldi (QMOR50680)
	MOTU 9 - MOTU 12
	E. eastopi (QMOR50044, QMOR50681) // *E. eastopi* (QMOR50682, QMOR50684) // *E. eastopi* (QMOR50683, QMOR50685) // *E. eastopi* (QMOR50686)
	MOTU 13 - MOTU 14
	E. essigi (QMOR50687) // *E. essigi* (QMOR50688)
	MOTU 15
	E. fusca voegtlini (QMOR50689)
	MOTU 16
	E. fusca voegtlini (QMOR50690, QMOR50691)
	MOTU 17
	E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
	MOTU 18
	E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
	MOTU 19 - MOTU 20
	E. kathleenae (QMOR50699) // *E. kathleenae* (QMOR50700)
	MOTU 21
	E. kirki (QMOR50701)
	MOTU 22 - MOTU 23
	E. knowltoni braggi (QMOR50702, QMOR50703) // *E. knowltoni braggi* (QMOR50704)
	MOTU 24
	E. knowltoni knowltoni (QMOR50705, QMOR50706)
	MOTU 25
	E. pini (QMOR50045)
	MOTU 26
	E. pini (QMOR50707)
	MOTU 27 - MOTU 29
	E. wilsoni (QMOR50708, QMOR50709) // *E. wilsoni* (QMOR50710) // *E. wilsoni* (QMOR50711)
		MOTU 1
		E. alyeska (QMOR50670)
		MOTU 2
		E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50679)
		MOTU 3
		E. californica (QMOR50674, QMOR50675, QMOR50676, QMOR50677, QMOR50678)
		MOTU 4
		E. critchfieldi (QMOR50680)
		MOTU 5
		E. eastopi (QMOR50044, QMOR50681, (QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686)
		MOTU 6
		E. essigi (QMOR50687, QMOR50688)
		MOTU 7
		E. fusca voegtlini (QMOR50689, QMOR50690, QMOR50691)
		MOTU 8
		E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
		MOTU 9
		E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
		MOTU 10
		E. kathleenae (QMOR50699, QMOR50700)
		MOTU 11
		E. kirki (QMOR50701)
		MOTU 12
		E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
		MOTU 13
		E. knowltoni knowltoni (QMOR50705, QMOR50706)
		MOTU 14
		E. pini (QMOR50045)
		MOTU 15
		E. pini (QMOR50707)
		MOTU 16
		E. wilsoni (QMOR50708, QMOR50709, QMOR50710, QMOR50711)
			MOTU 1
			E. alyeska (QMOR50670)
			MOTU 2
			E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50674, QMOR50675, QMOR50679, QMOR50676, QMOR50677, QMOR50678)
			MOTU 3
			E. critchfieldi (QMOR50680)
			MOTU 4
			E. eastopi (QMOR50044, QMOR50681, (QMOR50682, QMOR50683, QMOR50684, QMOR50685, QMOR50686)
			MOTU 5
			E. essigi (QMOR50687, QMOR50688)
			MOTU 6
			E. fusca voegtlini (QMOR50689, QMOR50690, **QMOR50691**)
			MOTU 7
			E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
			MOTU 8
			E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
			MOTU 9
			E. kathleenae (QMOR50699, QMOR50700)
			MOTU 10
			E. kirki (QMOR50701)
			MOTU 11
			E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
			E. knowltoni knowltoni (QMOR50705, QMOR50706)
			MOTU 12
			E. pini (QMOR50045)
			MOTU 13
			E. pini (QMOR50707)
			MOTU 14
			E. wilsoni (QMOR50708, QMOR50709, QMOR50710, QMOR50711)

Table 6. MOTUs according to bPTP

bPTP (Zhang *et al.* 2013)			
ATP6[a]	*COI*	*EF-1α*[b]	*Gnd*

MOTU 1
E. alyeska (QMOR50670)
MOTU 2-MOTU 5
E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50679) // *E. californica* (QMOR50674, QMOR50675) // *E. californica* (QMOR50676, QMOR50677) // *E. californica* (QMOR50678)
MOTU 6
E. critchfieldi (QMOR50680)
MOTU 7 - MOTU 10
E. eastopi (QMOR50044, QMOR50681) //
E. eastopi (QMOR50682, QMOR50684) //
E. eastopi (QMOR50683, QMOR50685) //
E. eastopi (QMOR50686)
MOTU 11 - MOTU 12
E. essigi (QMOR50687) // *E. essigi* (QMOR50688)
MOTU 13 - MOTU 14
E. fusca voegtlini (QMOR50689) // *E. fusca voegtlini* (QMOR50690, QMOR50691)
MOTU 15
E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
MOTU 16
E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
MOTU 17
E. kathleenae (QMOR50699, QMOR50700)
MOTU 18
E. kirki (QMOR50701)
MOTU 19
E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
E. knowltoni knowltoni (QMOR50705, QMOR50706)
MOTU 20
E. pini (QMOR50045)
MOTU 21
E. pini (QMOR50707)
MOTU 22 - MOTU 25
E. wilsoni (QMOR50708) //
E. wilsoni (QMOR50709) //
E. wilsoni (QMOR50710) //
E. wilsoni (QMOR50711) [a]
	MOTU 1
	E. alyeska (QMOR50670)
	MOTU 2-MOTU 6
	E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673) // *E. californica* (QMOR50679) // *E. californica* (QMOR50674, QMOR50675) // *E. californica* (QMOR50676, QMOR50677) // *E. californica* (QMOR50678)
	MOTU 7
	E. critchfieldi (QMOR50680)
	MOTU 8 - MOTU 11
	E. eastopi (QMOR50044, QMOR50681) //
	E. eastopi (QMOR50682, QMOR50684) //
	E. eastopi (QMOR50683, QMOR50685) //
	E. eastopi (QMOR50686)
	MOTU 12 - MOTU 13
	E. essigi (QMOR50687) // *E. essigi* (QMOR50688)
	MOTU 14 - MOTU 15
	E. fusca voegtlini (QMOR50689) // *E. fusca voegtlini* (QMOR50690, QMOR50691)
	MOTU 16
	E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
	MOTU 17
	E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
	MOTU 18 - MOTU 19
	E. kathleenae (QMOR50699) // *E. kathleenae* (QMOR50700)
	MOTU 20
	E. kirki (QMOR50701)
	MOTU 21
	E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
	E. knowltoni knowltoni (QMOR50705, QMOR50706)
	MOTU 22
	E. pini (QMOR50045)
	MOTU 23
	E. pini (QMOR50707)
	MOTU 24 - MOTU 26
	E. wilsoni (QMOR50708, QMOR50709) // *E. wilsoni* (QMOR50710) // *E. wilsoni* (QMOR50711)
		MOTU 1
		E. alyeska (QMOR50670)
		MOTU 2-MOTU 5
		E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50679) // *E. californica* (QMOR50674, QMOR50675) // *E. californica* (QMOR50676, QMOR50677) // *E. californica* (QMOR50678)
		MOTU 6
		E. critchfieldi (QMOR50680)
		MOTU 7
		E. eastopi (QMOR50044, QMOR50681, QMOR50682, QMOR50684, QMOR50683, QMOR50685, QMOR50686)
		MOTU 8
		E. essigi (QMOR50687, QMOR50688)
		MOTU 9 - MOTU 11
		E. fusca voegtlini (QMOR50689) // *E. fusca voegtlini* (QMOR50690) // *E. fusca voegtlini* (QMOR50691)
		MOTU 12- MOTU 14
		E. hillerislambersi (QMOR50692) // *E. hillerislambersi* (QMOR50693) // *E. hillerislambersi* (QMOR50694)
		MOTU 15
		E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
		MOTU 16
		E. kathleenae (QMOR50699, QMOR50700)
		MOTU 17
		E. kirki (QMOR50701)
		MOTU 18
		E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
		MOTU 19
		E. knowltoni knowltoni (QMOR50705, QMOR50706)
		MOTU 20
		E. pini (QMOR50045)
		MOTU 21
		E. pini (QMOR50707)
		MOTU 22
		E. wilsoni (QMOR50708, QMOR5070, QMOR50710, QMOR50711)
			MOTU 1
			E. alyeska (QMOR50670)
			MOTU 2-MOTU 4
			E. californica (QMOR50043, QMOR50046, QMOR50047, QMOR50048, QMOR50051, QMOR50052, QMOR50054, QMOR50671, QMOR50672, QMOR50673, QMOR50679) // *E. californica* (QMOR50674, QMOR50675) // *E. californica* (QMOR50676, QMOR50677, QMOR50678)
			MOTU 5
			E. critchfieldi (QMOR50680)
			MOTU 6 - MOTU 9
			E. eastopi (QMOR50044, QMOR50681) //
			E. eastopi (QMOR50682, QMOR50684) //
			E. eastopi (QMOR50683, QMOR50685) //
			E. eastopi (QMOR50686)
			MOTU 10 - MOTU 11
			E. essigi (QMOR50687) // *E. essigi* (QMOR50688)
			MOTU 12 - MOTU 13
			E. fusca voegtlini (QMOR50689) // *E. fusca voegtlini* (QMOR50690, QMOR50691)
			MOTU 14
			E. hillerislambersi (QMOR50692, QMOR50693, QMOR50694)
			MOTU 15
			E. hoerneri (QMOR50050, QMOR50695, QMOR50696, QMOR50697, QMOR50698)
			MOTU 16
			E. kathleenae (QMOR50699, QMOR50700)
			MOTU 17
			E. kirki (QMOR50701)
			MOTU 18
			E. knowltoni braggi (QMOR50702, QMOR50703, QMOR50704)
			E. knowltoni knowltoni (QMOR50705, QMOR50706)
			MOTU 19
			E. pini (QMOR50045)
			MOTU 20
			E. pini (QMOR50707)
			MOTU 21
			E. wilsoni (QMOR50708, QMOR50709, QMOR50710, QMOR50711)

[a]Populations in red indicate difference in BI results. They represent one unique MOTU.
[b]Only the BI results are indicated. ML results are similar to those with *Gnd* using GMYC.

Within *Essigella*

Our phylogenetic results supported neither the division of *Essigella* into three subgenera *Archeoessigella*, *Essigella*, and *Lambersella*, nor the validity of one of the two species series created by Sorensen (1994) (i.e., Series A). However, our molecular results largely corroborate Sorensen *Essigella* species concepts. Indeed, we recovered all species complexes and all species that Sorensen delimited with his multivariate analyses, the one significant exception being the two populations of *E. pini* (Clades B and D, Fig. 2). Several species showed clear internal cladistic structure, however, and merit discussion.

A clear division of *E. californica* into several clades appeared in our results. The species appeared as two lineages represented by Clades G and H (Fig. 2). With a *COI* DNA barcode using a 2% threshold, Clades G1, G2, and H1 may represent one unique species. However, the *COI* barcode results were not congruent with our ABGD results which consistently separated the Clade H1 from the other *E. californica* populations (Table 4). The gaps discovered by the ABGD method can vary from group to group. This flexibility increases the reliability of this method over classical static barcode methods. In aphids, as in animals in general, the genetic distance between two species is considered to be predominantly greater than 2%. However, this useful value is arbitrary and several aphid species have been found showing sequence divergence less than 2%, and even less than 1% (Rakauskas *et al.* 2011). In consequence, and because of their placement in our tree (Fig. 2), we conclude that populations of Clades G and H correspond to at least two distinct species. Moreover, if Clades G and H1 are to be considered two distinct species with a sequence divergence between them of 1.1–1.5%, we would have to consider that the interspecific *COI* threshold is lower than 2% in *Essigella*, and possibly near 1.1%. Thus, with a *COI* sequence divergence $P = 2.0\%$ between them (Supplementary Table 1), we consider populations of the Clades H2 and H3, i.e., those found on *P. monticola* and those on *P. albicaulis*, to be distinct species as well.

The populations of G1 and the population collected on *P. contorta* (G2) showed a sequence divergence of $P = 0.9–1.2\%$. These weak and transitional values and the phylogenetic position of G2 do not allow us to decide on the presence of two distinct taxa; additional data are needed to evaluate their species identity. It is possible that these two different groups represent subspecies or that population G2 represents an incipient species.

Morphological comparison with the type series and ecological data suggest that Clade G corresponds to the true *E. californica*. Sorensen's analyses (1983), revealed several groups within the *E. californica* species complex. He divided the complex into two groups, the one with populations developing on pinyon pines (i.e., on pines of the subsection *Cembroides* like *P. monophylla*) which he discriminated as *E. hoerneri*, and populations developing on nonpinyon pines, which he discriminated as *E. californica*. Beyond this division, Sorensen (1983) mentioned several other groups within *E. californica* that were slightly distinct in comparison with the other populations and that could be linked to specific pine species. More specifically, he singled out populations living on *Pinus flexilis* and *P. lambertiana*. Despite these observations and following the results of his analyses, Sorensen decided that all these populations living on non-pinyon pines belong to *E. californica* and that observed variance between them in his analyses could be considered intraspecific variation. No populations of our discriminated MOTUs were collected on *P. flexilis* or *P. lambertiana*. Thus, we suspect that *E. californica* may include two additional cryptic species beyond those revealed in our study.

Species that compose the *Essigella knowtoni* complex, i.e., *E. knowltoni* and *E. critchfieldi* are morphologically similar. Identity of host plant and geographic data are required for species identification (Sorensen 1994). Despite the strong proximity between these species and the *E. knowltoni* subspecies, our phylogenetic and molecular delimitation results support their validity. The subspecies appear in different clades and their *COI* barcode sequence divergence did not exceed 0.8%, whereas the divergence between *E. knowltoni* and *E. critchfieldi* was more than 4% (Supplementary Table 1).

Two genetically distinct species were revealed within *E. pini* in the phylogenetic results, regardless of the genetic locus examined. In our dendrogram, the Canadian population of *E. pini* clearly belonged to the *E. californica* complex, whereas the American population appeared related to species of Series B and the *E. knowltoni* complex (Fig. 2). Those two species were also revealed as different MOTUs with all species delimitation methods and exhibited a high *COI* DNA barcode sequence divergence ($P = 4.1\%$). According to these results, one of those species may correspond to *E. patchae* Hottes, 1957. Indeed, before Sorensen's revision, two species were known to occur in the Eastern part of North America. Because Sorensen's (1994) multivariate analyses did not find any differences between them, he made *E. patchae* a synonym of *E. pini*. The issue of multiple species within *E. pini* will be explored in a future publication.

Species belonging to subgenus *Lambersella* (also corresponding to the *Essigella fusca* complex (Sorensen 1994)) are difficult to distinguish, showing high morphological variability, notably in the length of dorsal metatibial setae (Sorensen 1994). More particularly, *E. eastopi* is itself a highly variable species, being easily confused with *E. fusca voegtlini*. According to Sorensen (1994), several populations of both taxa occurring together in southern California are not distinguishable, suggesting to Sorensen that *E. eastopi* might be a diminutive form of *E. fusca voegtlini*. Indeed, during the initial stages of this study, we misidentified all but one population of *E. eastopi* as *E. fusca voegtlini* (both species occurring on *P. coulteri*). We also misidentified our populations of *E. fusca voegtlini* as *E. fusca fusca* Gillette & Palmer, 1924 (both subspecies occurring on *P. ponderosa*). We had initially concluded that *E. eastopi* and *E. fusca voegtlini* may represent the same species. In the light of our molecular results and following a reappraisal of our slide-mounted specimens, our populations misidentified as *E. fusca fusca* appear to be closer to *E. hillerislambersi* than to our *E. fusca voegtlini*. Moreover, the two subspecies of *E. fusca* are allopatric (Sorensen 1994), and according to our collecting data, we collected both subspecies in relative proximity and in a region where *E. fusca fusca* does not occur (Sorensen 1994). Either our identifications of *Lambersella* species were inaccurate, or both subspecies occur in sympatry. Because it is more likely we made wrong identifications, we concluded that we had only collected *E. eastopi*, *E. fusca voegtlini,* and *E. hillerislambersi*. Our mistake underlines the high morphological variability of *E. eastopi*.

The number of MOTUs from ABGD using *COI* appeared more important than those obtained using *ATP6* and *Gnd*. However, those obtained with *ATP6* appeared more coherent with groups observed with the phylogenetic analyses. This corroborates observations of Lee *et al.* (2014), who considered that *ATP6* would be a better molecular marker than *COI* in the discrimination of aphid species.

EF-1α Sequences

Indel regions of *EF-1α* introns provided pertinent phylogenetic information in *Essigella* systematics. *E. californica*, *E. hoerneri,* and the Canadian population of *E. pini* which form the *E. californica* complex all showed the same indel pattern (Fig. 3). Surprisingly, the same pattern was also found for *E. kirki*, which was totally different

from that of its supposed sister species, *E. kathleenae*. Our first suspicion is that this pattern might correspond to the ancestral pattern, conserved in the *E. californica* complex, but which was progressively modified in other species by the addition or deletion of nucleotides. In contrast, the general pattern found in species of the *E. knowltoni* complex and in *E. alyeska* was also found in the American population of *E. pini*, with a loss of one nucleotide in the latter, showing a close relationship between all those species (Fig. 3). The *EF-1α* intron indel patterns also indicated a divergence between *E. knowltoni* subspecies. This difference corresponded to 12 missing nucleotides in *E. knowtoni knowltoni* as compared with of *E. knowtoni braggi*, but also with all species of Series B (Fig. 3). Because that loss was only found in our *E. knowtoni knowltoni* sequences, it may be considered an autapomorphy of that subspecies. The indel patterns of *E. eastopi*, *E. fusca voegtlini,* and *E. hillerislambersi* appeared different in Intron 4 despite their close relationship, that of *E. fusca voegtlini* being similar to *E. essigi.* However, the indel patterns of the three first species were similar in Intron 3, that of *E. essigi* being different by the insertion of one nucleotide.

The Host Plant Issue

Although several well-known species of aphids are oligophagous or polyphagous, most aphids are associated with one or a few closely related host plants (Heie 1986, Lee *et al.* 2015). Some related species or populations of *Essigella* do inhabit closely related pines. For example, species of Series B (*E. knowltoni* complex + *E. alyeska*) are known to develop on pine species of the *Contortae* subsection (Sorensen 1994), as shown in our phylogenetic results (Fig. 2). We made similar observations with the closely related *E. eastopi*, *E. fusca voegtlini,* and *E. hillerislambersi*, all feeding on pines of the subsection *Ponderosae* (Sorensen 1994) (Fig. 2). These observations may be explained by the fact that host-shift speciation is more common on phylogenetically-related host plants (Ehrlich and Raven 1964, Roskam 1985, Farrell and Mitter 1990, Percy *et al.* 2004, Ouvrard *et al.* 2015). This pattern was not observed for all *Essigella* species, however, thus our results do not adequately support the host-shift with phylogenetic tracking model (Mitter and Brook 1983, Tilmon 2008, Peccoud *et al.* 2010; Althoff *et al.* 2014) suspected by Sorensen ('tracking resource model') (1983, 1994).

We previously saw that a specific threshold of *COI* sequence divergence of 2% was overly conservative in the genus *Essigella* and that a threshold of around 1.1% may be more credible. In our analyses, several populations showed *COI* sequence divergences nearly equal to or greater than this threshold, but no MOTUs were revealed within them using ABGD. This applied to populations of *E. eastopi* (P = 0.8–1.5%), *E. essigi* (P = 1.5%), *E. fusca voegtlini* (P = 1.4%), and *E. wilsoni* (P = 0.2–1.5%) (Supplementary Table 1). *Essigella essigi* develops on *Pinus attenuata* and *P. radiata*, both pine species belonging to the subsection *Attenuatae*. Considering the *COI* value and ecological data, it is possible that these two populations represent sub- or incipient species. Respective populations of the other species were collected on the same host plants. Because it is less likely to have several cryptic species on the same host plant than on different ones, we cannot conclude that *E. eastopi* and *E. fusca voegtlini* include cryptic species. The same can be said for *E. wilsoni*. We found a P = 1.1–1.5% between populations collected on *Pseudotsuga macrocarpa* and *Ps. menziesii*. But we also had a P = 1.1% between populations on *Ps. menziesii* alone.

We revealed that several cryptic species occurred within *E. californica* and that a *COI* barcode threshold of 2% was overly conservative in *Essigella*. However, the reassessment of that threshold challenged our ABGD results and our ecological observations regarding the presence or absence of cryptic species within *E. eastopi*, *E. essigi*, and *E. wilsoni*. Despite that we found no differences in our ABGD results by testing our analyses with or without species with fewer than three populations, our sparse sampling may nevertheless have had a negative effect on the ABGD resolution.

We recognized several MOTUs in *E. californica* because it was the species for which we had the most populations. Perhaps we would have uncovered cryptic species among other complexes had they been more fully sampled.

In addition to the population size issue, ABGD can also be affected by recent speciation events (Puillandre *et al.* 2012). If the speciation event is not old enough, not all species will be delimited (Puillandre *et al.* 2012). In consequence, ABGD may not have detected speciation events in *E. eastopi*, *E. essigi*, and *E. wilsoni*. Thus, a comparison and a combination of several species delimitation methods appear to be required to better understand the complexity of the species reality. The use of as much data as possible appears to be important: an additional study using substantial material and taxa would be required to resolve the issue of cryptic species within *E. californica*, and other species of the genus.

According to Sorensen, *E. fusca* encompasses two subspecies: *E. fusca fusca* and *E. fusca voegtlini*. Because we did not have populations of *E. fusca fusca* in our study, we were unable to confirm the validity of both subspecies. Neither were we able to conclude on the relationship between the two subspecies of *E. fusca* and the other species of the *E. fusca* complex, *E. eastopi* and *E. hillerislambersi*. Likewise, populations of *E. californica* collected on *Pinus flexilis* and *P. lambertiana* would have provided more complete understanding of the cryptic species belonging within that complex.

Supplementary Data

Supplementary data are available at *Insect Systematics and Diversity* online.

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