Speciation by host-switching in pinyon Cinara (Insecta: Hemiptera: Aphididae)

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Abstract

Parasite–host cospeciation has received much attention as an important mechanism in the diversification of phytophagous insects. However, studies have shown that for certain taxa, it is not host fidelity but host-switching that plays the critical role in speciation. Cinara are aphids (Insecta: Hemiptera: Aphididae: Lachninae) that feed exclusively on the woody parts of conifers of the Cupressaceae and Pinaceae. They are unusual aphids because most Pinaceae play host to several species of Cinara. The aphids show relatively strong host fidelity, and as a consequence historically have been treated based on the taxonomy of their hosts. The historical paradigm of aphid evolution implies that Cinara species have radiated to different parts of the same host species and/or speciated with their host. Using mitochondrial cytochrome oxidase I and nuclear elongation factor 1-α DNA sequences, we performed molecular phylogenetic analysis of Cinara species, concentrating on those associated with pinyon pines in the southwestern USA. We determined that switching hosts has played a key role in the speciation of the genus, reflected in the polyphyly of pinyon-feeding Cinara. Furthermore, species sharing a common feeding site on different hosts were more closely related to each other than to those sharing the same host but at different feeding sites, suggesting that feeding site fidelity plays a more important role in speciation than does host fidelity in general. This study also elucidated the primary taxonomy of various species: it suggested that Cinara rustica Hottes is a junior synonym of C. edulis (Wilson) and that C. wahtolca Hottes represents two species on the two different pinyon pine species, Pinus edulis Englem. and P. monophylla Torr. & Frem.

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1. Introduction

Aphids of the genus Cinara (Insecta: Hemiptera: Aphididae: Lachninae) rarely achieve pest status (but see Kfir et al., 1985; Penteado et al., 2000; Watson et al., 1999) and hence are generally overlooked. They are monophyletic within the Aphididae (Heie, 1988; Normark, 2000), have simple life cycles compared to other aphids (Hottes and Frison, 1931), are diverse with 154 North American species (Voegtlin and Bridges, 1988), and have a narrow host range, feeding exclusively on the woody parts of conifers in the Pinaceae and Cupressaceae (Eastop, 1972).

Cinara are unique among aphids in that many species of the genus have been described from the same host. Other genera such as Aphis and Macrosiphum (Aphidinae) may be more speciose (Remaudière and Remaudière, 1997), but species of those genera are scattered over many more hosts and are not found in great diversity on any one. There are 14 species of Cinara recorded from Pinus edulis Engelm. and a subset of 5 recorded from P. monophylla Torr. & Frem. (Voegtlin and Bridges, 1988).

These hosts and their concomitant Cinara occur only in the mountains of the Desert Southwest, USA. The range of these two pinyon species extends from southwest Texas north to north-central Colorado, and west to southern and east-central California. They are allopatric, with P. edulis occurring in the eastern part, and P. monophylla occupying the Great Basin and westward. Some parapatric populations, hybrids, and unusual
populations exist, such as hybrid zones in the New York Mountains of southeastern California (Trombulak and Cody, 1980) and Zion National Park (Gafney and Lanner, 1987). Restricted to elevations between approximately 1300 and 2300 m, their distributions are scattered and island-like, especially in the Great Basin. The disjunct distribution of the hosts implies allopatric populations of *Cinara*, which may have consequences for island biogeography and speciation in *Cinara*. The isolation of populations on mountain ranges is made greater by the relative low vagility of some species of *Cinara*: some species are not recorded as producing winged dispersal forms, or alatae (Voegtltn and Bridges, 1988).

Phytophagous insects show a great range of host specificity, from monophagous to polyphagous species. Aphids tend toward the more host-specific end of the range of affinities, most species known to feed on only one or a few species of host plant (Eastop, 1979; Hille Ris Lambers, 1979). The tight affinity between many aphid species and their hosts has led to a proliferation of host-based taxonomic treatments (Bissell, 1978; Cook, 1984; Richards, 1972) which implicitly suggest that the phylogeny of Aphididae follows that of their host plants. Eastop (1986) explicitly suggested coevolution to be a common phenomenon between aphids and plants.

Meanwhile, morphological adaptation to host plants is evident, even within the same species (Margaritopoulos et al., 2000; Moran, 1986), and, under the assumption that aphid evolution largely parallels that of aphid hosts, has caused some concern that convergent morphological evolution may confound the study of evolutionary relatedness (Moran, 1986).

Like other aphids, *Cinara* have traditionally been treated on the basis of host association (Hottes, 1960a,b; 1961; Pepper and Tissot, 1973). In fact, Hottes (1960a) advanced the notion of separating the genus into more manageable groups based on host taxonomy: “Because species of the genus *Cinara* have either specific or closely allied species of *Coniferae* as hosts, it has seemed logical to use the host species as media to divide the species of the genus into more workable groups, hence this section of species which have *Pinus edulis* and *Pinus monophylla* for their host plants.” Whether host-associated *Cinara* form monophyletic groups has never been tested empirically.

No morphological phylogenetics has been done on *Cinara* because, as in all aphids, their morphology is simplified and difficult to interpret (Footitt, 1992; Footitt and Mackauer, 1990; Watson et al., 1999). The difficulty partially lies in the apparent convergent morphological evolution towards niche specialization. Bradley (1961) found that the rostra of *Cinara* feeding at the same site on different hosts were more similar in length than the rostra of *Cinara* feeding at different sites on the same host. For example, root- and trunk-feeding species had longer rostra, irrespective of host affiliation, than did shoot- and twig-feeding species. Given the tendency to treat groups of *Cinara* based on host affiliation, this feature of the aphid rostrum may seem to be an instance of convergence, although this hypothesis has never been tested.

The purpose of the present study was to disentangle the apparent discord between host association and feeding site and to determine whether treating *Cinara* based on host taxonomy reflects their phylogenetic relatedness. Which is more important in the process of *Cinara* speciation, host identity, or feeding site (or neither or both)? We addressed these questions using cytochrome oxidase 1 (CO-1) mitochondrial DNA sequence data to test the hypothesis of monophyly of a putatively host-associated group of aphids, the same pinyon pine-feeding *Cinara* as Hottes (1960a). Our hypotheses can be stated as follows: pinyon-feeding species of *Cinara* are most closely related to other pinyon-feeding species, or closely related species of *Cinara* do not share the same host species. If the latter is true: closely related species of *Cinara* share the same feeding site or closely related species of *Cinara* do not share the same feeding site.

2. Materials and methods

2.1. Collection and determination

Beating sheet and hand-collecting were used to collect *Cinara* during the summers of 1996–2003 throughout the range of pinyon pines in the United States, as well as on other hosts in other scattered localities. Photographs and notes on patterning and coloration were taken in vivo and the feeding sites recorded. *Cinara* were collected on *Pinus edulis*, *P. monophylla*, *P. discolor* D. K. Bailey & Hawksw., and *P. edulisimonophylla* hybrids. Hybrid pines were identified by the presence of both two- and one-needle fascicles in sufficient proportions to be seen by quick, casual observation (Gafney and Lanner, 1987; Lanner and Hutchinson, 1972; Trombulak and Cody, 1980). No attempt was made to distinguish *P. californiarum* D. K. Bailey or *P. californiarum* subsp. *fallax* Little (= *P. edulis* var. *fallax* Little) as these taxa are in dispute and difficult to identify (Bailey, 1987; Langer, 1996; Lanner, 1997). In order to compare pinyon *Cinara* to other species, we also collected on *P. banksiana* Lamb., *P. contorta* Doug., *P. flexilis* James, *P. jeffreyi* Grev. & Balf., *P. monticola* Doug., *P. nigra* Arnold, *P. ponderosa* Doug., *P. rigida* Mill., *P. strobiformis* Engelm., *P. strobus* L., *P. taeda* L., *P. virginiana* Mill., *Picea engelmannii* Parry, *Abies lasiocarpa* (Hook.) Nutt., *A. magnifica* A. Murr., *Pseudotsuga menziesii* (Mirb.) Franco, and *Juniperus virginiana* L. Aphids were preserved in 95% ethanol and kept in a cooler while in the field and in −20°C or −80°C freezers in the lab for immediate use or long-term storage.
Aphids reproduce parthenogenetically during the summer and often form colonies of genetically similar individuals (Loxdale and Lushai, 2003). Occasionally, multiple species aggregate in the same colony. Such mixed colonies were sorted and different species segregated into separate vials. Several individuals from each collection were cleared and mounted on microscope slides in Canada balsam for identification, description, and morphological analysis. Vouchers are deposited in the insect collection at the Illinois Natural History Survey, Champaign, IL, voucher numbers 16,401–16,763 and 18,297–18,298.

We collected 147 colonies of Cinara and 1 colony each of Longistigma caryae (Harris), Eulachnus rileyi (Williams), and Mindarus abietinus Koch (Table 1). Seven species of pinyon-feeding Cinara were identified from the collected material: C. atra (Gillette and Palmer), C. caliente Hottes, C. edulis (Wilson), C. puerca Hottes, C. rustica Hottes, C. terminalis (Gillette and Palmer), and C. watolka Hottes. Also collected were: C. arizonica (Wilson), C. atlantica (Wilson), C. coloradensis (Gillette), C. contortae Hottes, C. cronartii Tissot and Pepper, C. curveips (Patch), C. hottesi (Gillette and Palmer), C. juniperovora (Wilson), C. murrayanae (Gillette and Palmer), C. nigra (Wilson), C. pergandeii (Wilson), C. pinivora (Wilson), C. ponderosae (Williams), C. pseudotaxifoliae Palmer, C. schwarzi (Wilson), C. strobi (Fitch), C. villosa (Gillette and Palmer), and C. watsoni Tissot. There were two species that were not identifiable. One is either C. hirsuta Hottes and Essig or C. kuchea Hottes. The reference specimen to the other was accidentally lost after being sequenced, but its CO-1 DNA sequence is most similar to C. pinivora (differing by 2 bp). We called it C. near pinivora.

2.2. DNA extraction, amplification, and sequencing

Early in the project, a single individual from each colony was destroyed for the DNA studies. Aphid DNA was extracted using a standard phenol procedure (Hillis et al., 1996, pp. 342–343) with the following modifications: whole aphids were ground in buffer; extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform were each done once; and all centrifugation was done in a microcentrifuge at 14,000 rpm. Large (up to 3 mm) pellets were obtained and these were dissolved in 50 l of water. A new non-destructive DNA extraction protocol was developed later in the project. The procedure was the same as above, except that rather than destroying the aphid, a single longitudinal incision was made along the ventral aspect of the abdomen. The aphid was left in the extraction buffer for 2–3 days and the cleared cuticle dehydrated and mounted directly to a microscope slide.

We obtained universal PCR primer sequences from Simon et al. (1994) and had them synthesized by Operon Technologies (Alameda, CA). We tried using these primers to amplify a ~1300 base region of the CO-1 gene from the mitochondrial genome of individuals from six colonies chosen at random. PCR mixtures of 25 l consisted of 1 l of unquantified aphid DNA solution, 1.875 l (0.375 l) of Gibco-BRL Taq Polymerase (Invitrogen, Carlsbad, CA), 2.5 l of 10× PCR buffer, 1 l of 25 mM MgCl₂, 1 l of each primer at 10 mM, and 18.5 l of PCR-grade water. Forty reaction cycles were run: denature at 94°C for 30 s, anneal at 50°C for 45 s, and extend at 72°C for 100 s. The reaction mixtures were held for 5 min at 94°C prior to the first cycle and for 10 min at 72°C at the end of the last. Only two of the six individuals produced single clean bands on a 1% agarose gel (they were both C. edulis: haplotype 39 from White Pine, NV and haplotype 41 from Sevier, UT; Table 1). We cleaned the PCR product of these two individuals using a Bio 101 (Vista, CA) Gene Clean II kit. Cleaned PCR product was sent to the University of Illinois's (Urbana-Champaign) Biotechnology Center and sequenced in both directions using the same two primers, a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and a Perkin-Elmer (Boston, MA) 377 automated DNA sequencer.

Because DNA from only two of six individuals was successfully amplified, we proceeded to design new, aphid-specific primers. The two edited sequences were aligned by eye with four other aphid CO-1 sequences downloaded from GenBank: Acyrthosiphon pisum (Harris) AF068480, Eriaphis fimbriata (Richards) AF077768, Macrosiphum creelii Davis AF077770, and Schizaphis rotundiventris (Signoret) AF220511. The new 24-mer aphid-specific PCR primers were synthesized by Operon Technologies: sense, 5'-ACC AGT TTT AGC AGG TGC TAT TAC-3' and antisense, 5'-GTA TAT CGA TGT CAT TAC-3'. The primers border a ~700 base region in the center of the CO-1 gene. The aphid-specific primers were used in 25 l PCRs as above, with the following cycle protocol: 35 cycles, denature at 94°C for 45 s, anneal at 55°C for 90 s, and extend at 72°C for 120 s. This protocol and the new primers worked well for all aphids that we analyzed.

PCR products were cleaned as above and sequenced directly in both directions using a BigDye Terminator Cycle Sequencing Kit with the same primers. The sequencing products were cleaned on G-50 Sephadex columns and read by the University of Illinois’s Biotechnology Center’s sequencing gel reader. We visually compared chromatographs and text sequences for errors.

Rather than trust our results to a single mitochondrial locus that may be confounded by the presence of pseudogenes (Sunnucks and Hales, 1996; although there was no evidence of heteroplasm in the CO-1 sequencing chromatographs), we sought to validate our results by sequencing a portion of the nuclear elongation factor
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*a* # corresponds to a haplotype and terminal number in Figs. 1 and 2.

*b* Twig = 0.5–2 cm diameter and branch = 2–8 cm diameter.

*c* Mo/Dy/Yr.

*d* Degrees North, ±0.02°.

*e* Degrees West, ±0.02°.
1-2 (EF-1) gene from a subset of the CO-1 samples. PCR and sequencing protocols were exactly as above, with 20-mer primers EF3 and EF6 (von Dohlen et al., 2002).

2.3. Phylogenetic analysis

We used seven outgroup CO-1 sequences obtained from GenBank, members of the subfamily Aphidinae, believed to be the sister-group of the Lachninae (Heie, 1988; Normark, 2000). These included the four species used to construct the aphid-specific primers as well as: *Acyrthosiphon kondoi* Shinji AF077777, *A. macrosiphum* (Wilson) AF077769, and *Schizaphis graminum* (Rondani) AF220515. We included the three non-*Cinara* aphid sequences we obtained (Table 1) from our collections to ensure they would fall outside the *Cinara* ingroup: *Eulachmus rileyi* and *Longistigma caryae* are both Lachninae, and *Mindarus abietinus* is a mindarin. We aligned ingroup and outgroup sequences using Sequencher 4.1 (GeneCodes, Ann Arbor, MI) and checked the alignment visually for errors; there were no gaps in the CO-1 sequences. The EF-1 alignment included two regions rich in indels. We removed these introns from the alignment, spanning from the first indel to the last, leaving the analyzed alignment free of gaps. We included two other available aphid EF-1 sequences from GenBank: *Eulachmus rileyi* (AF163867) as outgroup and *C. glabra* (Gillette and Palmer) (AF163870) in the ingroup.

We used a PAUP* 4.0b10 (Swofford, 2002) heuristic parsimony search using 10 random addition sequence replicates and tree bisection–reconnection (TBR) branch swapping. A finite number of most parsimonious trees could not be found within the limits of the computer memory, so the maxtrees limit was set to 60,000. The same settings were used in a heuristic parsimony analysis in which the tree topology was constrained such that the pinyon-feeding *Cinara* would be monophyletic. To see if phylogenetic signal was being obscured by substitution saturation, we removed the third position from all codons and calculated the number of parsimony-informative characters. We ran 100 heuristic parsimony bootstrap replicates; each replicate used only a single random addition sequence and was permitted to swap down from the 60,000 maxtrees limit. Bremer support indices were calculated using TreeRot (Sorenson, 1999) with the same PAUP* settings.

Modeltest 3.06 (Posada and Krandall, 1998) was used on the entire data matrix to determine the best DNA substitution model for use in a single heuristic maximum likelihood analysis using PAUP*: neighbor-joining starting tree, TBR branch swapping, and no maxtrees limit. One hundred “fast step-wise addition” maximum likelihood bootstrap replicates were run. We also used PAUP* to perform neighbor-joining analyses using various distance models, including uncorrected P, Jukes–Cantor, Tajima–Nei, Tamura–Nei, Kimura 2-parameter, and Kimura 3-parameter. The EF-1 maximum parsimony and maximum likelihood analyses were as above, minus the distance analyses and with no maxtrees limit.

3. Results

As with most insect mitochondrial DNA (Hoy, 1994), the CO-1 sequences, ≤678 bp in length, were AT-rich, with mean base frequencies as follows: A: 0.311, T: 0.475, G: 0.124, and C: 0.090. Of the 145 *Cinara* individuals sequenced, we identified 66 haplotypes, 35 of which were of the species on pinyon pines. EF-1 primers amplified and sequenced a single ≤965 bp product for 18 individuals: A: 0.291, T: 0.316, G: 0.211, and C: 0.182.

3.1. Analysis of the entire matrix

 Parsimony analysis of the 76 CO-1 sequences (66 *Cinara*, 2 non-*Cinara* Lachninae, 1 mindarin, and 7 Aphidinae) contained 216 variable characters, 36 of which were autapomorphic and therefore not parsimony informative. It produced 60,000 most parsimonious trees, each of 873 steps with consistency and retention indices of 0.328 and 0.714, respectively (excluding uninformative characters). The strict consensus maximum parsimony tree is shown in Fig. 1. The maximum likelihood analysis produced a single tree which conformed precisely to the strict consensus parsimony tree (Fig. 1) when branches incongruent with it were collapsed. Also, all six distance models yielded similar trees. Combining all distance trees yielded a strict consensus cladogram that, with incongruent branches collapsed, matched the parsimony tree perfectly.

Branching resolution was greatest towards the terminals and weakest at deeper levels of the tree, but dropping the third codon position left only 22 parsimony-informative characters. Three large clades, six small ones, and five solitary terminal branches all shared a large basal polytomy in the strict consensus tree (Fig. 1). Five of the smaller clades contained paired species (haplotypes 51–53 and 57–64). The three major clades were all supported with moderate to high parsimony bootstrap values (A, 69; B, 100; and C, 72; Fig. 1), but their relationships to each other were lost in the basal polytomy. Likewise, the analyses with EF-1 recovered the three principal clades (Fig. 2), albeit with much lower parsimony bootstrap support (A, <50; B, 99; and C, 52). Although the results with EF-1 were not particularly strong (38 parsimony-informative characters, 543 equally parsimonious 146-step trees, Cl: 719, RI: 730), they were a validation of the CO-1 analyses.

Resolution of the unconstrained tree was insufficient to know if *Pinus*-feeding *Cinara* form a clade, although...
Fig. 1. CO-I strict consensus cladogram of all 60,000 most parsimonious trees, the maximum likelihood tree, and trees for all six distance models. Aphid species are listed with host and haplotype number (see Table 1). The maximum likelihood general time reversible model included six substitution types (A–C: 32, A–G: 55, A–T: 48, C–G: 22, C–T: 11, G–T: 1), unequal base frequencies (A: 0.311, C: 0.090, G: 0.124, T: 0.475), and a heterogeneous gamma distribution rate (0.7296). Left-hand numbers above internal branches are bootstrap values using 100 heuristic parsimony replicates. Right-hand numbers above internal branches are bootstrap values using 100 fast step-wise addition maximum likelihood replicates. Numbers below internal branches are Bremer support values for the parsimony tree.
Fig. 2. EF-1 strict consensus cladogram of 543 most parsimonious trees. The likelihood tree differed from the parsimony tree only as follows: the maximum likelihood tree joined C. caliente and C. strobi as sister species; the maximum parsimony tree placed C. curvipes basal to the rest of the ingroup. Aphid species are listed with host and haplotype number (see Table 1). The maximum likelihood general time reversible model included six substitution types (A–C: 2.52, A–G: 10.79, A–T: 0.284, C–G: 0.195, C–T: 0.234, T–G: 0.287), and a homogeneous variation rate. Left-hand numbers above internal branches are bootstrap values using 100 heuristic parsimony replicates. Right-hand numbers above internal branches are bootstrap values using 100 fast step-wise addition replicates. Numbers below internal branches are Bremer support values for the parsimony tree.

Abies- and Picea-feeding species, C. coloradensis and C. curvipes, grouped together to the exclusion of another Picea-feeding, C. hirta. The analysis of the entire data set constrained to render the pinyon-feeding Cinara monophyletic resulted in 10,094 most parsimonious trees, each 909 steps long, or 36 steps longer than the unconstrained analysis.

Four of the pinyon-feeding Cinara, C. caliente, C. puerca, C. terminalis (Clade A), and C. wahtolca (Clade B), segregated from each other, sharing only the large, basal, polytomy (Fig. 1). Only one haplotype was recovered for C. caliente and C. puerca, but the numerous haplotypes found for C. terminalis and C. wahtolca revealed internal structure within the species.

3.2. Smaller analyses of clades A, B, and C

All three large clades, labeled A, B, and C in Figs. 1 and 2, each contained one or more pinyon-feeding species and one or more non-pinyon-feeding species. These relationships render the pinyon Cinara polyphyletic. Within Clade A, the C. terminalis clade (haplotypes 1–10) had high parsimony bootstrap support (83), but the species separated into two smaller, well-supported clades (parsimony bootstrap support of 84 and 100), between which was a sequence divergence of 2.0–3.2%. Divergence within each of these two smaller clades was 0.15–0.6%. Cinara terminalis grouped in Clade A with five other species from different hosts: C. atlantica and C. pinivora on Pinus taeda, C. contortae and C. murrayanae on P. contorta, and C. ponderosae on P. ponderosa. Sequence divergence between C. terminalis and the other species ranged from 2.8 to 6.5%.

Two smaller clades were also evident for C. wahtolca (haplotypes 23–27) in Clade B, each associated with a different pinyon species (parsimony bootstrap supports of 100 and 97). However, the relationship of these two clades to each other was lost in a polytomy with C. caliente and C. coloradensis—feeding species, and C. puerca, C. terminalis, and C. wahtolca was 2.1–2.4%, whereas sequence divergence between any two species was 0.7–2.3%.

The last clade that included pinyon-feeders, Clade C, was less resolved. Cinara schwazii on Pinus ponderosa and P. jeffreyi (haplotypes 49–50) was clearly distinct from the clade of pinyon-feeding aphids (haplotypes 31–48) with parsimony bootstrap support of 100 and 96, respectively, and sequence divergence of 3.9–5.4%. However, relationships within the pinyon-feeding Cinara were ambiguous, C. atra and C. rustica falling within a larger C. edulis clade. Sequence divergence within the pinyon-feeding clade was 0.15–2.4%. Cinara rustica had the same haplotype as C. edulis (haplotype 42) and fell within a clade that included three haplotypes, within which sequence divergence was 0.16–0.46%, and for which parsimony bootstrap support was 72. These three haplotypes had a sequence divergence of 1.2–2.4% from their neighbors (haplotypes 31–39 and 43–48). Six haplotypes of C. atra (numbered 43–48), all on P. edulis, grouped with the larger C. edulis clade in a large polytomy.

4. Discussion

4.1. Host-switching

The pinyon pine-feeding Cinara are polyphyletic and we showed three instances where pinyon-feeding species have non-pinyon aphids as close relatives. Pinyon-feeder C. terminalis is more closely related to C. atlantica, C. contortae, C. murrayanae, C. pinivora, and C. ponderosae, than it is to other pinyon-feeding species.
(Fig. 1, Clade A). Four of these aphids do share similar biologies, however, in that they feed preferentially on the growing shoots of young trees, 3 m tall or less (Voegtlin and Dahlsten, 1982; Hottes, 1960a; pers. obs.). The exceptions are C. murrayanae and C. pinivora, which are sometimes found among needles but normally feed on small branches (Bradley, 1961). Cinara wahtolca is more closely related to other twig- and branch-feeding aphids, C. hirsuta (or C. kuchea), C. villosa, and C. near apini, than it is to other pinyon pine aphids (Clade B, Fig. 1).

Finally, the twig- and branch-feeding aphids of Clade C on pinyons group with the twig- and branch-feeding C. schwarzi on P. ponderosa and P. jeffreyi (Fig. 1).

Cinara evidently do not cospeciate with their hosts, but species of the genus are under certain host-based constraints: all Cinara feed on conifers, for instance, Eastop (1972). Although Cinara do not form clades based on host genus, as we have shown with C. curvipes, C. coloradensis, and C. hottesi on Abies and Picea, we have not shown whether the Cinara of the host genus Pinus form a natural group. Almost all of the phylogenetic information is in the third codon position; removing it would leave only 22 informative characters. There is not enough phylogenetic signal from these data to resolve the relationships of the Pinus-feeders. A larger sample size or a more highly conserved gene may resolve the deeper nodes of the cladogram. Further studies may expand the EF-1 analysis and add another mitochondrial gene.

Our results suggest that Cinara speciation is caused by host shifts and not by coevolving with the host (similarly to tephritid flies: Rhagoletis; Berlocher, 2000; Berlocher and Bush, 1982). The phylogenetic groupings of the Cinara we examined have no similarity to Pinus relationships (Liston et al., 1999; Price et al., 1998). Also, the aphids appear better able to switch to a similar microhabitat on another host than to partition the environment of the host (Condon and Steck, 1997). We did find occasional instances of species on atypical hosts (eg., C. wahtolca, haplotype 24, on P. jeffreyi) and suggest that such occurrences in ancestral species may have led to bifurcations in phylogeny. It appears that Cinara can speciate more easily by switching hosts than by moving to another part of the tree.

Rather than representing an instance of convergence, morphological correlation with feeding site (Bradley, 1961; Condon and Steck, 1997) appears to reflect phylogenetically based host-use patterns (although there are exceptions in Clade A). Morphological adaptation to feeding on shoots of one host species presumably pre-adapts the aphid to feeding on shoots of another host. Perhaps such morphological correlation is also the case for other aphids such as Moran’s (1986) Uroleucon. Certainly, our results support the use of supposed ecologically correlated morphological characters in phylogenetic inference.

Speciation through host-switch is often used as an example of sympatric speciation. Berlocher and Bush (1982), McPheron et al. (1988), and Berlocher (2000) found that host-switching was common in the Rhagoletis pomonella (Walsh) (Diptera: Tephritidae) species group, and was a key component in speciation. Aphids have also been found to diversify along host plant lines, although all of the work has been done with economically important species. Shufan et al. (2000) and Anstead et al. (2002) found different genetic lineages of Schizaphis graminum feeding on grain and non-crops. De Barro et al. (1995) and Lushai et al. (2002) found host-based genetic differentiation of Sitobion avenae (F.). Vanlerberghe-Masutti and Chavigny (1998) found differentiation of Aphis gossypii Glover on cucurbits and other hosts, and Via (1999) and Via et al. (2000) found differentiation between Acrystosiphon pisum on clover and alfalfa.

Our results match most closely those of Condon and Steck (1997), who not only found host-based differentiation in Blepharoneura (Diptera: Tephritidae), but also found morphological congruence based on host feeding site. Most instances of diversification were due to host shift, although they did find examples of speciation based on feeding-site shifts on the same host. Most importantly, we have shown that the current practice of defining groups of Cinara based on host taxonomy misinforms Cinara phylogenetics; the same has been recently found for lice (Johnson et al., 2002).

4.2. Defining a species

A ubiquitous problem in aphid taxonomy is also of concern to all biological systematics, that is, the species problem. At what point can two entities be considered different species? Morphologically, C. contortae and C. ponderosae are indistinguishable, and determinations for this study were made based on the identity of their occasionally sympatric hosts. However, the CO-1 DNA sequence divergence between the two was 3.7–4.5%, greater than between other morphologically distinguishable species (eg., C. hirsuta [or C. kuchea] and C. villosa, 1.0–1.3%), suggesting that C. contortae and C. ponderosae are indeed different species.

Sequence divergence within either of the two C. terminalis clades (haplotypes 1–6 and 7–10) was 0.15–0.6%, but between them was 2.0–3.2%. This level of divergence is lower than that between the other species of the clade (2.8–5.2%), and lower than what was found between pinyon-feeding sister species Ips confusus (LeConte) and I. hoppini Lanier (Coleoptera: Scolytidae) (3.3–4.0%; Cognato and Sperling, 2000). Stern et al. (1997) used 2.0% mtDNA sequence divergence as their cut-off point for re-evaluating aphid species, and synonymized several based on divergence below that threshold. However, mitochondrial sequence divergence much higher than
2.0% has been found in a single valid species (12.9%; Thomaz et al., 1996), so sequence divergence alone is an insufficient indicator of taxonomic cut-off. The two *C. terminalis* clades occupy non-overlapping, east and west ranges, suggesting they may have speciated allopatrically, but further analysis will be necessary to establish the validity of two species. More surprising is the sequence divergence between individuals of a single species, *C. ponderosa* (haplotypes 16–18), which reaches 3.0%. This species is widespread and may have split allopatrically into one or more cryptic species. A greater sample size and better understanding of the variation within the species will be necessary to evaluate it fully.

*Cinara wahtolca* is a better example of speciation through host shift. The two, well-supported clades (haplotypes 23–25 and 26–27) are restricted to different pinyon hosts, *P. edulis* and *P. monophylla*. Within the clades, CO-1 DNA sequence divergence is 0.1–0.5%, but between them it is 2.1–2.4%. This level of divergence between two clades of *C. wahtolca* is greater than that between it and the other named species, *C. near apini*, *C. hirsuta* (or *C. kuchea*), and *C. villosa*, 0.7–2.2%. Considering this level of divergence and their restriction to two different hosts, the two clades of *C. wahtolca* are likely different species. The distinction between the two *C. wahtolca* clades is also supported by the fact that the ranges of haplotypes 24 and 27 (Table 1) extend over very large portions of the host ranges. Haplotype 24, on *P. edulis*, extends from the far northeast corner of New Mexico, south to Texas, and west to the isolated and farthest west population of *P. edulis* in the New York Mountains of California (Critchfield and Little, 1966; Trombulak and Cody, 1980). Haplotype 27, on *P. monophylla*, ranges from southern California to northern Nevada. The broad ranges of these haplotypes suggest that the species is fairly vagile and able to maintain relative genetic homogeneity over great distances. That two clear clades exist despite such vagility amplifies their distinction.

The larger *C. edulis* plus *C. atra* clade (haplotypes 31–48) is well supported and shows a lower level of sequence divergence within itself (0.15–2.4%) than it does with a non-pinyon aphid, *C. schwarzii* (3.9–5.4%). All aphids in the *C. edulis* clade are clearly closely related. Whereas *C. terminalis* and *C. wahtolca* may contain undescribed species, the *C. edulis* plus *C. atra* clade (haplotypes 31–48) appears to contain synonyms. In particular, *C. rustica*, sharing the same haplotype as *C. edulis*, is likely a junior synonym of that species. *Cinara atra* is distinct from *C. edulis* in life, and the relationships between it and *C. edulis* are insufficienly resolved to come to a firm conclusion. Both of these supposed species allied with *C. edulis* have discrete geographic ranges. *Cinara rustica* was collected only in southeast New Mexico and Texas, and *C. atra* only in central and northern New Mexico and northeast Arizona. Contrasted with the very broad range of *C. edulis*, these isolated distributions suggest that these two species may be morphological variants of *C. edulis* or newly derived or incipient species arising from within *C. edulis*. Morphological study of these groups will hopefully clarify their relationships. Perhaps a contributing factor to the complexity of the *C. edulis* clade is the distribution pattern of the species’ hosts. *Pinus edulis* and *P. monophylla* grow only at montane elevations and as a consequence have disjunct, island-like, distributions across the mountain ranges of the Southwest (Critchfield and Little, 1966). The patchy distribution of the concomitant *Cinara* may lead to multiple instances of incipient speciation and reticulate phylogeny that would obscure phylogenetic relationships in the terminal nodes.

Shufran et al. (2000) found high and variable levels of mtDNA sequence divergence (0.08–6.2%) between various strains of the same aphid species, *Schizaphis graminum*. However, they also found that their biotypes segregated into three clades with divergences between members of a clade consistently less than 1%. The authors concluded that their three clades probably represented host-adapted races. Whether the various groups within the three *Cinara* clades represent new species, new synonyms, or host-adapted races remains to be assessed.

All three major clades in the present study both clarify and complicate *Cinara* taxonomy. We have identified host-switching as a major contributor to speciation and have begun to circumscribe taxonomic affinities in addressing the species problem in *Cinara*. Our next immediate step will be to evaluate the putative new species and synonyms with morphological and morphometric data.

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