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The scale and parasitoid community on native hemlocks in Japan

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ABSTRACT

Elongate hemlock scale (EHS), *Fiorinia externa* (Hemiptera: Diaspididae), is an invasive species in the United States of Japanese origin that was first detected in New York in 1908. In the United States, EHS attacks eastern hemlock (*Tsuga canadensis*) and Carolina hemlock (*Tsuga caroliniana*), and has established in most states where these trees occur. Elongate hemlock scale density can be several orders of magnitude higher in the United States than in its native Japan despite the presence of the parasitoid *Encarsia citrina* (Hymenoptera: Aphelinidae) in both locations. We postulated three possible reasons for this: (1) cryptic diversity of *E. citrina* attacking EHS exists in Japan, but not in the United States, (2) additional parasitoid species attack EHS in Japan at significant rates, or (3) alternate armored scale hosts on hemlock in Japan enhance rates of parasitism of EHS by *E. citrina* or other parasitoid species. We surveyed 88 sites (or subsites) throughout Japan for all armored scales on hemlock to explore the scale community and the genetic diversity of the associated parasitoids, as well as the relationship between scale community species richness and percent parasitism of EHS. Six armored scale species were found on hemlock in Japan. Using two gene loci (28S - D2 and COI), 18 identified or putative species of parasitoids were reared from these six armored scale species. Only five of the 18 parasitoid species were found in more than one scale host species. Eleven of the 18 parasitoid species were found in EHS, and six of these were exclusive to EHS. *Encarsia citrina* was recovered from all six armored scale species, and there was no evidence for cryptic species of *E. citrina* or that it differed genetically from populations of this species found in the eastern United States. There was

no relationship between the number of armored scale species present in a given location and percent parasitism of EHS by all parasitoid species. Our phylogenetic analysis corroborated the morphological identification of the parasitoid species, including those newly described, published in an earlier study. The results from this study suggest that the high densities and low parasitism rates of EHS in the United States may be due to a depauperate scale-parasitoid community compared to Japan. In particular, this study identified ten new parasitoid species attacking EHS in Japan, which suggests strong parasitoid pressure on EHS populations there. Despite the diverse parasitoid community associated with EHS, parasitism rates were often low. This may be indicative of natural variation in spatial/temporal host-parasitoid population dynamics, scale suppression from plant-mediated factors, or a combination of both. The hemlock scale parasitoids found in this study provide many potential biological control candidates should management of EHS or other hemlock scales become necessary in the future.

Keywords: *Encarsia citrina*, *Fiorinia externa*, cryptic species, parasitoid phylogeny, invasive species

1. Introduction

Elongate hemlock scale (*Fiorinia externa* Ferris) (EHS) (Hemiptera: Diaspididae) is a non-indigenous pest of hemlocks (*Tsuga* spp.) in the eastern United States. Originating from Japan (Takagi, 1963; Watanabe and Uchida, 1967), EHS was first detected in the United States in 1908 on Long Island, New York (Ferris, 1942). Since that time, EHS has spread throughout most of the range of eastern and Carolina hemlocks. Between

1998 and 2005, EHS spread further north in New England and increased in density (Orwig et al., 2002; Preisser et al., 2008a). This spread, together with experimental evidence (Preisser et al., 2008b), suggests that EHS is evolving greater cold tolerance and may continue to move north. This evolution poses a threat to eastern hemlock forests in areas previously protected from infestation by cold winter temperatures.

EHS feeds on the fluid within hemlock needles. Several scales feeding on the same needle can cause needle death. Heavy infestations result in the loss of a large proportion of a tree's foliage, and sustained defoliation can cause tree mortality. In North America, EHS density varies from 21 to 420 scales per 100 needles in Connecticut (McClure and Fergione, 1977; McClure, 1978) and as high as 400 scales per 100 needles in North Carolina and Tennessee (Lambdin et al., 2005). In Japan, density of elongate hemlock scale is low, ranging from 0.0 to 0.15 scales per 100 needles (McClure, 1986). These low densities are associated with attack by the parasitoid *Encarsia citrina* Crawford (Hymenoptera: Aphelinidae) (Watanabe and Uchida, 1967; McClure, 1986). Parasitism rates by this wasp of over 90% that prevent EHS from reaching injurious levels were reported by McClure (1986) in Japan (Kyoto, 34°55'-35°05' latitude, 80-150 m elevation). One additional parasitoid of EHS in Japan, *Arrhenophagus albitibiae* Gerault (Hymenoptera: Encyrtidae), was reported, but only accounted for 11.6% of all parasitized scales (McClure, 1986). *Encarsia citrina* is a generalist parasitoid that also occurs in the United States, attacking various armored scales (Diaspididae) (Krombein and Hurd., 1979), including 2nd instar female EHS. Parasitism of EHS by *E. citrina* in the United States varies widely from near zero to greater than 90% (McClure, 1981; Lambdin et al., 2005; Abell and Van Driesche, 2012).

In this study we use data from surveys of the community of hemlock scales and parasitoids found in Japan to examine three possible reasons why *E. citrina* fails to suppress the density of EHS in the United States to levels comparable to those in Japan. First, we considered the possibility that, due to the presence of cryptic species, the entities described as *E. citrina* in Japan and the United States might not be identical. Many studies have found cases where what was once thought to be a generalist parasitoid species was in fact a complex of cryptic species each with more specific host preferences (e.g., Heraty et al., 2007; Smith et al., 2008; Desneux et al., 2009; Zhu and Fang, 2009). Second, there might be species of parasitoids attacking EHS in Japan that are not present in the United States; this is plausible since previous surveys in Japan to find EHS parasitoids were confined to the Kyoto area (McClure, 1986). Finally, there may be other species of armored scales on hemlock in Japan that act as alternate hosts for *E. citrina*, collectively supporting a larger *E. citrina* population better able to suppress EHS. To address these hypotheses, the objectives of this study were to (1) conduct surveys throughout Japan to discover new parasitoid species that attack EHS, (2) document other species of armored scales that occur on hemlocks in Japan, namely the two native species *Tsuga diversifolia* [M.] Masters and *Tsuga sieboldii* Carrière and two introduced species, the North American *Tsuga canadensis* [L.] Carrière and the Chinese species *Tsuga chinensis* [Franch.] Pritzel ex Diels, (3) explore the parasitoid community attacking these armored scales using phylogenetic analysis, and (4) determine if the presence of other scale species on hemlock affected parasitism of elongate hemlock scale.

2. Materials and Methods

2.1. Specimen collection

A survey of diaspidid scales on *T. diversifolia*, *T. sieboldii*, *T. chinensis*, and *T. canadensis* throughout the main island of Japan (Honshu) was conducted in May of 2009. Surveys of *T. diversifolia* and *T. sieboldii* were primarily done in natural areas, while *T. chinensis* and *T. canadensis* were confined to botanical gardens. Eighty-eight geographically distinct sites (or subsites, see Table 1) from N 34° 03.540' to N 40° 38.167' latitude, and E 135° 10.386' to E 140° 52.527' longitude, and ranging in altitude from 30 to 2324 m were surveyed. Forty-six sites had *T. sieboldii*, 37 sites had *T. diversifolia*, four sites had *T. canadensis*, and one had *T. chinensis* (Fig. 1). Sites were chosen based on the knowledge of local guides and foresters. The number of scales collected and the time spent collecting was variable depending on the number of hemlock trees present at a site and limitations set by foresters and land managers on the amount of material that could be taken. Trees were searched by visually scanning the undersides of needles for the presence of scales around the entire circumference of each tree when possible for all or most branches within arm's reach, or using a pole pruner when needed. Small branches and sometimes individual needles with scales were cut off and placed inside a sealable plastic bag (Zip-Loc). Once a scale species was collected, subsequent sampling effort was focused on discovery of other scale species so as to maximize the number of scale species collected. Therefore, the number of each scale species collected was not representative of the population at each site and density could not be estimated.

Within 48 hours, individual hemlock needles bearing scales of any species were placed in gelatin capsules, and checked every two days during the survey for newly emerged parasitoids. Upon emergence of a parasitoid, the scales on the needle were checked for an emergence hole to confirm the species of scale that had been parasitized, and the parasitoid was placed in 100% ethanol. At the conclusion of the survey, all parasitoids reared were shipped to the University of Massachusetts for later molecular analysis. Scales that did not produce parasitoids were shipped live to the USDA-FS quarantine facility in Ansonia, Connecticut, where they were held for further observation. Scales were monitored for five more weeks in quarantine for additional parasitoids. After five weeks, all scales were placed in 100% ethanol and taken to the University of Massachusetts where they were counted, identified, and dissected to look for evidence of parasitoid pupae or larvae in hosts. Armored scales collected in Japan were identified to species by one of us (BBN). The total number of scales collected and the total percent parasitism (all parasitoid species combined) was calculated for each scale species by collection site and *Tsuga* species (Table 1). Twenty-four additional parasitoid specimens collected from scales on hemlock in Japan by Suzanne Lyon in 2006 were also included in the phylogenetic analyses of this study.

Encarsia citrina from EHS on *T. canadensis* in the eastern United States included in the analyses in this study were collected in separate surveys, whose methods are described in Abell and Van Driesche (2012).

2.2. DNA extraction, amplification, sequencing, and alignment

To obtain sequences from collected parasitoids for phylogenetic analyses, DNA was extracted from whole adults or pupae dissected from scales using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). For all specimens, we amplified a ~685 bp section of the D2-D3 portion of the 28S large ribosomal subunit rRNA using the polymerase chain reaction (PCR). Primers and protocols used for 28S were those of Schmidt et al. (2006). For a subset of specimens in the genus *Encarsia*, we also amplified an 1148 bp section of the Cytochrome Oxidase 1 (COI) mtDNA gene using primers we developed: s1699-ka, CGTTATACCAGTTATAATAGGA and a2961-ka, CATAAAAAGAATGAGATCTAGAAGGA. Successful amplification was determined by visualizing PCR products on 1.5% agarose gel in 0.5X TBE stained with SYBRsafe™ (Invitrogen, Grand Island, NY). PCR products were cleaned with Exo SAP-IT enzymatic digestion (USB Corporation, Santa Clara, CA), and sequenced on a 3730 DNA Analyzer (Applied Biosystems, Grand Island, New York) at the Yale University DNA Analysis Facility, New Haven, Connecticut. All sequences were assembled and edited using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan). The 28S sequences were aligned using a profile alignment by the “add” algorithm of the online MAFFT server (Kato and Frith, 2012; Kato and Standley, 2013). For the profile, we used the secondary-structure based alignment from the Chalcidoidea phylogeny of Munro et al. (2011) containing specimens from 19 families, 72 subfamilies, 343 genera, as represented by 649 species. COI sequences were aligned using ClustalW 2.1 (Larkin et al., 2007) as implemented in Mesquite v2.73 (Maddison and Maddison, 2009). Two alignments were produced for separate analyses: (1) the MAFFT aligned 28S sequences, including the Munro et al. (2011) sequences and (2) the aligned COI dataset.

2.3. *Phylogenetic analysis*

Maximum Likelihood (ML) bootstrap analyses were run on both alignments using the Extreme Science and Engineering Discovery Environment (XSEDE) via the CIPRES computer portal (Miller et al., 2010). The 28S analyses followed those of Munro et al. (2011) using RAxML 7.3.2, with an unpartitioned GTRGAMMA model. This alignment and analysis is directly comparable to the “secondary structure-derived matrix with MAFFT-aligned RAA regions (SSME)” alignment by Munro et al. (2011) used for their Fig. 1.

Five thousand rapid bootstrap replicates were performed on each dataset, and bootstrap percentages (BP) were drawn on the tree with the best known likelihood score. Sequences from Munro et al. (2011) retain their original names.

2.4. *Preliminary identifications*

All sequence data for this study have corresponding vouchers of adult parasitoids at either the University of Massachusetts or the Agricultural University of Georgia. Many of these vouchers have been morphologically identified to species (or if new species, have been described -- see names in Figs. 2, 3, 4, and 5) by George Japoshvili at the Agricultural University of Georgia (Japoshvili et al., 2013). Tentative phylogenetic identifications are based on sister-group relationships and relative branch lengths. A

singleton or clade that is nested within a monophyletic genus or higher taxon is hypothesized to be a member of that taxon. We consider relative branch lengths as a measure of heterospecific sequence divergence. Two individuals whose 28S sequences are identical are hypothesized to be conspecific. Branch lengths between species within a clade in the 2011 Chalcidoidea dataset (Munro et al., 2011) are interpreted as characteristic of heterospecific divergence within that clade. Thus, a singleton or clade that we have sampled is hypothesized to belong to a species absent from the 2011 Chalcidoidea dataset if the branch length separating it from individuals in that dataset is similar to, or greater than, the branch lengths that separate different species in that dataset.

2.5. Statistical analysis

To determine if the presence of additional scale species benefitted parasitoids by acting as alternate hosts when susceptible EHS were not available, we assessed the possible relationship, across all sample sites, between the number of scale species present and percent parasitism of EHS by all parasitoid species using logistic regression. The rarity of each scale species on each *Tsuga* species was calculated by dividing the number of sites in which each scale species was found (site x s_i occurrences) by the sum of this number across all scale species ($\sum_i^n \text{species} \times \text{all sites}$). All analyses were performed on data from the 2009 survey only using SAS Version 9.1 (SAS Institute, Cary, NC).

3. Results

3.1. Food web of trees, scales and parasitoids

In total, 88 sites or subsites of varying elevation were surveyed throughout Honshu (Table 1, Fig. 1). Six scale species were collected: *Aspidiotus cryptomeriae* Kuwana, *Fiorinia externa* Ferris, *Lepidosaphes japonica* Kuwana, *Lepidosaphes pseudotsugae* Takahashi, *Dynaspidiotus tsugae* Marlatt, and *Pseudaulacaspis momi* Kuwana. All scale species were found on both species of native *Tsuga*. In no case were all six scale species found in the same site.

On *T. diversifolia*, *L. pseudotsugae* was the most common species followed closely by *F. externa*, *L. japonica*, and *D. tsugae*; *A. cryptomeria* was rare and *P. momi* was absent (Fig. 6A). On *T. sieboldii*, *L. japonica*, *F. externa*, and *D. tsugae* were relatively common followed by *A. cryptomeria* and *P. momi*, while *L. pseudotsugae* was rare (Fig.

6B). On *T. canadensis*, *F. externa* was most common, followed by *A. cryptomeria*, *L. pseudotsugae*, and *D. tsugae*; *L. japonica* was rare and *P. momi* was absent (Fig. 6C). Too few *T. chinensis* were found to describe its scale community.

Parasitism of scales varied from zero to 100% (Table 1; Fig. 7). There was no significant relationship between the number of armored scale species present in a given location and percent parasitism of EHS by all parasitoid species ($\chi^2 = 0.0248$, $df = 1$, $P = 0.8750$) (Fig. 7).

3.2. Phylogenetic analyses

3.2.1. Phylogenetic analysis of clades

PCR and sequencing resulted in 218 28S sequences (55 were *E. citrina* from the USA), and 122 COI sequences (32 were *E. citrina* from the USA), which have been deposited in GenBank under numbers KF778517 through KF778734 for 28S, and numbers KF778395 through KF778516 for COI. Fewer COI sequences were obtained due to poor amplification. The topology, branch lengths and bootstrap support values of the 28S Chalcidoidea genealogy is consistent with the topology presented by Munro et al. (2011), and all genera relevant to specimens in this study are well supported (Figs. 2 and 3). Fifteen clades in the 28S genealogy, containing specimens in this study, are presented in Figs. 2 and 3, and these have been numbered sequentially. Sequences from this study are grouped in 12 clades of morphologically identified species and three clades of unidentified specimens (clades 4, 6, and 11). All sampled specimens fall into either of two families: Encyrtidae (five clades) or Aphelinidae (ten clades). Of the ten Aphelinidae

clades, six are in the genus *Encarsia* (Figs. 2 and 3). Almost all clades are monophyletic with respect to species, but this is not the case with clade 13, which contains both *Encarsia brimblecombei* Girault and *E. normarki* Japoshvili.

The topology of the *Encarsia* COI genealogy (Fig. 4) is generally consistent with that of 28S, where support for the placement of clades 11 and 14 is also low. Notably, species within clade 13 that are indistinguishable in the 28S genealogy are resolved as distinct monophyletic groups in the COI genealogy (clades 13-B, and 13-C). Clade 13-A consists of a single specimen from 28S clade 13 and another specimen not present in the 28S genealogy.

3.2.2. Species recognized from analyses

Based on the above molecular analyses and a morphological study (Japoshvili et al., 2013), we now recognize a total of 18 distinct parasitoids from six scale species on hemlock in Japan.

We found eleven genetically distinct parasitoids associated with EHS in Japan, and six of these were exclusive to EHS. Two of these EHS-specific parasitoids were in the family Encyrtidae: *Arrenophagus chionaspidis* Aurivillius (Fig. 2 clade 1) and a newly described species from this work (described in Japoshvili et al., 2013), *Thomsonisca noyesi* Japoshvili (Fig. 2 clade 3).

A third species, *Aphytis moldavicus* Yasnosh, was also exclusively recovered from EHS (Fig. 2 clade 7). This *Aphytis* species should be evaluated for EHS control since many *Aphytis* species have been successfully used as biological control agents against scales (Clausen, 1978; Rosen and DeBach, 1979). A fourth species represented by a

single specimen that we were unable to identify, appears likely to be another *Aphytis* species (Fig. 2, clade 6).

The remaining seven EHS-specific parasitoids are all *Encarsia* species: *E. citrina*, *E. guerrierii*, *E. normarki* Japoshvili, *E. schmidtii* Japoshvili, *E. brimblecombei* (Figs. 3 and 4, clades 15, 14, 13B, 12, and 13C respectively), and two other specimens we were unable to identify to species due to their poor condition, *E. sp. A*, and *E. sp. B* (Figs. 3 and 4, clades 11 and 13A). These seven *Encarsia* species represent new, possibly exclusive, host associations with elongate hemlock scale and are likely candidates for biological control in the United States.

Encarsia citrina was the most common parasitoid found in our survey and was the only parasitoid to attack all six scale species. Despite this fact, we found no evidence for cryptic or host specific races in Japan, nor did we find any genetic differences between specimens collected in Japan and the USA, suggesting that unknown cryptic diversity is probably not responsible for the low parasitism and lack of control of elongate hemlock scale in the United States.

4. Discussion

Using genetic data similar to those in this study, many studies have found that what was thought to be a single generalist parasitoid species was actually a complex of cryptic species, each being uniquely suited to a particular host and its environment (e.g., Heraty et al., 2007; Smith et al., 2008; Desneux et al., 2009; Zhu and Fang, 2009). In the scale-parasitoid community on hemlock in Japan examined in this study *E. citrina*

appears to be an extreme generalist on the Diaspididae, being the only parasitoid found to attack all six scale species present. However, we found no genetic differences between *E. citrina* collected in Japan or compared to those collected in the USA. Therefore, we reject our first hypothesis that cryptic diversity of *E. citrina* attacking EHS exists in Japan. Our data support *E. citrina* being a generalist scale parasitoid; however, it should be noted that the 28S gene sequence for *E. citrina* from Munro et al (2011) was different from our samples, suggesting that cryptic diversity may exist outside of the hemlock scale system in Japan or the USA.

We found strong evidence to support our second hypothesis that additional parasitoid species attack EHS in Japan other than the two reported in previous studies (Watanabe and Uchida, 1967; McClure, 1986). This result is consistent with diverse parasitoid communities described from other armored scales (Gwiazdowski & Normark 2014). In this study, ten new parasitoid species were found to attack EHS in Japan, six of which were exclusive to EHS. Should EHS require control efforts in the USA in the future, these ten parasitoid species could be valuable tools for classical biological control. In addition, we documented several new host-parasitoid associations for the other hemlock scales in Japan which could be valuable if these scales ever become pests elsewhere. Despite the apparent rarity of some parasitoids, one or more may play an important role in the host-parasitoid dynamics of hemlock scales.

The number of parasitoid species found in this study suggests the potential for top-down control of EHS in Japan, however, it is important to consider that parasitism rates were often low and variable across sites. This contradicts previous work that consistently found greater than 90% parasitism of EHS in Japan (McClure, 1986). Our

results suggest that parasitism by *E. citrina* may not control EHS in Japan as previously thought (McClure, 1986). What's more, even with the new parasitoid species found in this study, EHS parasitism remained low. This may be indicative of natural spatial or temporal fluctuation in host-parasitoid population dynamics, or that parasitoid pressure in the system is relatively weak. If that is the case, then biological control efforts utilizing other parasitoid species may be ineffective. With this in mind, the effects of plant-mediated factors on EHS in Japan should be considered. McClure (1980) found that foliar nitrogen was the primary factor influencing EHS mortality, fecundity, and developmental rate; however, foliar nitrogen does not always determine EHS' reproductive success (McClure, 1983), and exactly how foliar nitrogen varies among hemlock species, and the factors affecting that variation, remain unclear. Furthermore, little is known about how defensive chemistry varies among hemlock species or about its effect on hemlock scales. McClure and Hare (1984) found that EHS was negatively associated with the concentration of acyclic terpenes and had greater fecundity on *T. sieboldii* compared to on *T. canadensis* where it is associated with greater levels of terpene alcohols. Despite these findings, the effects of plant nutritional quality and defensive chemistry of Japanese and North American hemlock species on EHS is poorly understood. Therefore, it is difficult to speculate on the relative contribution of bottom-up and top-down control on EHS in Japan and North America. Further work is needed to assess the relative impacts and interaction of parasitoid pressure and plant-mediated factors on suppression of EHS and other hemlock scales in Japan.

Lastly, we found no evidence in support of our third hypothesis, that alternate armored scale hosts on hemlock in Japan enhance parasitism of EHS by *E. citrina* or

other parasitoid species. However, because of the unexpected complexity of the scale and parasitoid community on hemlock in Japan, our sampling methods may not have been adequate to definitively reject this hypothesis. Because *E. citrina* attacks all six scale species, it seems plausible that its population may be enhanced by the presence of other scale species, but more intensive and repeated sampling is needed to elucidate this. Much work remains to be done to fully describe the natural history and ecology of the hemlock scale-parasitoid community in Japan.

In summary, the parasitoid community associated with armored scales on hemlock in Japan, particularly EHS, is much more diverse than previously thought. This diversity provides great potential for possible future biological control efforts of EHS and other invasive hemlock scales with the caveat that the low parasitism rates found in this study may indicate that host-parasitoid dynamics are spatially/temporally variable, or that plant-mediated factors may also contribute to suppressing EHS and other scales.

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Table 1

Sites, GPS coordinates, elevation, *Tsuga* host species, number of specimens, and percent parasitism of scale species collected throughout Japan in 2009.

Site ID	Latitude	Longitude	Elevation (m)	<i>Tsuga</i> spp.	<i>A. cryptomeriae</i>		<i>F. externa</i>		<i>L. japonica</i>		<i>L. pseudotsugae</i>		<i>D. tsugae</i>		<i>P. momi</i>	
					N	%Par	N	%Par	N	%Par	N	%Par	N	%Par	N	%Par
1	34 03.685	135 31.532	698	<i>sieboldii</i>	12	0.0	113	9.7	5	40.0			9	33.3	9	33.3
2	34 03.540	135 31.439	710	<i>sieboldii</i>			27	3.7	26	0.0						
3	34 04.023	135 32.014	677	<i>sieboldii</i>									2	100	1	0.0
4	34 12.767	135 35.028	824	<i>sieboldii</i>			252	33.3	16	12.5	7	0.0	1	100	15	26.7
5	34 12.805	135 34.386	861	<i>sieboldii</i>			60	1.7	5	40.0			1	0.0	4	0.0
6	34 22.777	135 33.383	585	<i>sieboldii</i>	2	0.0	6	33.3	33	12.1			1	0.0	38	5.3
7	34 27.554	135 22.220	30	<i>sieboldii</i>	78	3.8			2	50.0						
8	34 57.564	135 36.682	424	<i>sieboldii</i>	1	0.0							2	0.0		
9	34 57.682	135 36.332	389	<i>sieboldii</i>	33	0.0							14	21.4	38	5.3
10	34 57.605	135 35.781	369	<i>sieboldii</i>	11	9.1			22	27.3			73	27.4		
11	34 57.609	135 35.697	369	<i>sieboldii</i>					54	1.9						
12	34 57.683	135 35.525	369	<i>sieboldii</i>			1	0.0					6	0.0	2	0.0
13	34 44.426	135 10.615	435	<i>diversifolia</i>	3	33.3	309	17.2	14	35.7			142	15.5		
14	34 44.426	135 10.615	435	<i>sieboldii</i>	12	25.0	3	0.0					52	23.1		
15	34 44.425	135 10.604	434	<i>diversifolia</i>			26	11.5								
16	34 44.449	135 10.571	438	<i>sieboldii</i>	1	0.0			10	0.0			105	13.3		
17	34 44.367	135 10.386	431	<i>diversifolia</i>	19	0.0	5	40.0					22	4.5		
18	34 44.443	135 10.617	452	<i>sieboldii</i>	2	0.0			8	12.5	1	100				
19	34 44.222	135 10.826	433	<i>canadensis</i>												
20	35 04.251	135 45.824	132	<i>chinensis</i>			2	0.0					12	8.3		
21	35 04.251	135 45.824	132	<i>sieboldii</i>			59	20.3					10	0.0		
22	35 04.222	135 45.818	149	<i>canadensis</i>			30	50.0			1	0.0	2	50.0		
23	35 04.221	135 45.811	146	<i>sieboldii</i>			58	22.4					1	0.0		

24	35	04.212	135	45.714	176	<i>canadensis</i>	3	0.0	96	21.9	8	0.0	1	100.0	8	0.0		
25	37	43.022	140	15.150	1605	<i>diversifolia</i>							105	8.6	8	25.0		
26	39	42.813	141	08.412	140	<i>diversifolia</i>			90	13.3	8	25.0			1	0.0		
27	40	38.167	140	52.527	1041	<i>diversifolia</i>					296	1.4	26	3.8				
27.5	40	35.406	140	28.418	56	<i>diversifolia</i>												
28	40	36.287	140	27.955	51	<i>canadensis</i>	7	0.0	7	0.0								
29	40	36.259	140	27.892	47	<i>diversifolia</i>												
30	35	55.546	138	49.842	752	<i>sieboldii</i>			240	11.3	39	12.8					4	0.0
31	35	55.596	138	49.807	767	<i>sieboldii</i>					32	0.0						
32	35	55.564	138	49.804	776	<i>sieboldii</i>			494	20.4	7	14.3						
33	35	55.501	138	49.774	833	<i>sieboldii</i>			8	25.0	14	14.3						
34	35	55.506	138	49.759	852	<i>sieboldii</i>					144	40.3						
35	35	55.268	138	48.425	1671	<i>diversifolia</i>												
36	35	55.179	138	48.256	1692	<i>diversifolia</i>					18	11.1	3	0.0				
37	35	55.110	138	48.199	1719	<i>diversifolia</i>							16	6.3				
38	35	55.214	138	48.983	1475	<i>diversifolia</i>					36	0.0						
39	35	54.913	138	49.143	1252	<i>sieboldii</i>			3	0.0	34	44.1					8	0.0
40	35	56.330	138	49.265	817	<i>sieboldii</i>			14	0.0	43	25.6						
41	35	56.459	138	47.910	1240	<i>sieboldii</i>					38	36.8			68	1.5		
42	35	56.391	138	48.014	1229	<i>sieboldii</i>			5	20.0	29	13.8			137	4.4	2	0.0
43	35	56.238	138	48.201	1200	<i>sieboldii</i>			58	10.3	124	42.7			34	26.5		
44	35	56.385	138	48.811	1061	<i>sieboldii</i>			16	12.5	124	29.0	3	100	8	25.0	2	0.0
45	35	56.380	138	49.211	834	<i>sieboldii</i>					26	19.2					4	0.0
46	35	54.244	138	59.610	930	<i>sieboldii</i>			14	42.9	100	49.0	2	100	11	9.1		
47	35	54.245	138	59.586	914	<i>sieboldii</i>					20	55.0			13	15.4	15	0.0
48	35	54.256	138	59.564	896	<i>sieboldii</i>			1	100	7	0.0			1	0.0	7	28.6
49	35	54.271	138	59.540	849	<i>sieboldii</i>					5	0.0						
50	35	54.278	138	59.544	841	<i>sieboldii</i>			13	7.7								
51	36	47.626	139	28.241	1511	<i>diversifolia</i>			4	0.0	8	12.5	48	35.4	26	23.1		
52	36	47.748	139	28.448	1503	<i>diversifolia</i>					2	100	675	16.9	8	0.0		
53	36	48.272	139	29.050	1680	<i>diversifolia</i>					1	100	41	14.6	1	0.0		
54	36	46.989	139	49.169	410	<i>sieboldii</i>	11	18.2									16	6.3
55	36	47.025	139	49.110	414	<i>sieboldii</i>											2	0.0

56	36 47.162	139 49.136	458	<i>sieboldii</i>	2	50.0													
57	36 47.177	139 49.104	465	<i>sieboldii</i>	4	25.0											41	0.0	
58	36 47.237	139 49.049	476	<i>sieboldii</i>													34	26.5	
59	36 46.766	139 49.400	398	<i>sieboldii</i>	8	25.0													
60	36 46.694	139 49.464	416	<i>sieboldii</i>	3	0.0					1	100					30	0.0	
61	36 46.680	139 49.453	1457	<i>sieboldii</i>	57	0.0	1	0.0	92	7.6	7	0.0	16	31.3					
62	35 34.925	138 21.595	1779	<i>diversifolia</i>			1	100	181	0.0	16	6.3	157	7.6					
63	35 55.676	138 25.447	1431	<i>sieboldii</i>	6	33.3	10	20.0	246	37.0	15	40.0	35	0.0					
64	35 55.381	138 25.187	1397	<i>sieboldii</i>					34	26.5	5	0.0	1	100					
65	35 23.625	138 43.915	2324	<i>diversifolia</i>															
65.1	35 23.137	138 41.868	2135	<i>diversifolia</i>															
65.2	35 23.099	138 41.832	2073	<i>diversifolia</i>							3	33.3							
65.3	35 22.502	138 41.597	2034	<i>diversifolia</i>							52	21.2							
65.4	35 23.994	138 41.519	1856	<i>diversifolia</i>					1	0.0	21	61.9							
65.5	35 24.916	138 42.099	1661	<i>diversifolia</i>					64	0.0	18	44.4							
65.6	35 25.795	138 44.322	1288	<i>diversifolia</i>			20	10.0			345	0.3							
65.7	35 24.866	138 44.303	1422	<i>diversifolia</i>					13	7.7	7	14.3							
65.8	35 26.841	138 45.341	1104	<i>diversifolia</i>			2	0.0			21	14.3	4	0.0					
65.9	35 27.352	138 45.308	1055	<i>diversifolia</i>			65	13.8	153	50.3									
66	35 52.372	137 30.219	2202	<i>diversifolia</i>															
67	35 52.302	137 30.253	2100	<i>diversifolia</i>															
68	35 52.062	137 30.820	1979	<i>diversifolia</i>							564	8.7							
69	35 52.071	137 31.067	1889	<i>diversifolia</i>							33	33.3							
70	35 51.932	137 31.251	1773	<i>diversifolia</i>							37	13.5	9	0.0					
71	35 51.790	137 31.556	1715	<i>diversifolia</i>			70	14.3			138	5.1	17	0.0					
72	35 51.530	137 31.610	1697	<i>diversifolia</i>			3	0.0	1	100	110	18.2	1	0.0					
72.1	35 51.423	137 31.683	1635	<i>diversifolia</i>															
73	36 07.691	137 39.489	1254	<i>diversifolia</i>			76	2.6	28	32.1	8	12.5	20	45.0					
74	36 08.614	137 59.734	843	<i>diversifolia</i>			395	25.6											
75	36 08.567	137 59.974	912	<i>diversifolia</i>			664	42.0	13	69.2			17	11.8					
76	34 41.464	135 51.155	323	<i>sieboldii</i>			18	33.3	10	10.0									
77	34 41.418	135 51.153	313	<i>sieboldii</i>															

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Parasitoid-Scale Host Associations on *T. diversifolia* in Japan

Parasitoid-Scale Host Associations on *T. sieboldii* in Japan

Parasitoid-Scale Host Associations on *T. canadensis* in Japan

Figure 6

Tsuga diversifolia

A

Tsuga sieboldii

B

Tsuga canadensis

C

Figure 7

Figure Captions

Fig. 1. Collection locations in Japan by *Tsuga* species.

Fig. 2. Clades containing specimens in this study, taken from the 28S (D2-D3 regions) maximum likelihood genealogy of the Chalcidoidea. Sequences in this study are named with accession number, country/locality, abbreviation of the scale host species, *Tsuga* species, and parasitoid species if identified. Taxon names for specimens in this study **appear in bold**. Clades containing specimens in this study are indicated by a vertical bar, and labeled with adjacent numbers 1 through 9. Family and subfamily assignments appear at right. Only bootstrap support values above 45 are displayed. The scale bar represents estimated substitutions per site, taken from the entire chalcid topology.

Fig. 3. Clades of *Encarsia* spp. containing specimens in this study, taken from the 28S (D2-D3 regions) maximum likelihood genealogy of the Chalcidoidea. Sequences in this study are named with accession number, country/locality, abbreviation of the scale host species, *Tsuga* species, and parasitoid species if identified. Taxon names for specimens in this study **appear in bold**. Clades containing specimens in this study indicated by a vertical bar, and labeled with adjacent numbers 10 through 15. To conserve space, a shallow polytomy of specimens identified as *Encarsia citrina* has been removed. The scale bar represents estimated substitutions per site, taken from the entire chalcid topology.

Fig. 4. Maximum likelihood genealogy based on COI mtDNA, from a subset of specimens appearing in Figs. 2 and 3. Sequences in this study are named with accession number, country/locality, abbreviation of the scale host species, *Tsuga* species, and

parasitoid species if identified. Clades containing specimens in this study are indicated by a vertical bar, and labeled with numbers corresponding to their presence in a clade based on the 28S data (Figs. 2 and 3). Here, independent clades present within a single one using 28S are additionally labeled with a letter (e.g. 13-A). Specimens 1382A and 1384A do not appear in the 28S genealogy and are labeled with an X. To conserve space, a shallow polytomy of specimens identified as *Encarsia citrina* has been removed. Only bootstrap support values above 45 are displayed. The scale bar represents estimated substitutions per site, set at the same scale as the 28S bars in Figs. 2 and 3.

Fig. 5. Connectance web of diaspidid scale host and parasitoid linkages found on *Tsugae diversifolia* (A), *T. sieboldii* (B), and *T. canadensis* (C) in Japan. Dashed lines represent parasitoid species identifications supported by morphological characters only, dotted lines represent parasitoid species identification supported by genetic data only, and solid lines represent parasitoid species identifications supported by both morphological and genetic data. Linkages supported by morphology only are from Japoshvili et al., (2013). The full names for species shown are *Aphytis chrysomphali*, *Aphytis moldavicus*, *Aphytis proclia*, *Encarsia brimblecombei*, *Encarsia citrina*, *Encarsia guerrierii*, *Encarsia vandrieschei*, *Encarsia normarki*, *Encarsia lambei*, *Encarsia schmidtii*, *Pteroptrix chinensis*, *Pteroptrix kamatai*, *Arrenophagus chionaspidis*, *Zaomma lambinus*, *Coccidencyrus shiyakei*, and *Thomsonisca noyesi*.

Fig. 6. Rarity of each scale species on (A) *Tsuga diversifolia*, (B) *T. sieboldii*, and (C) *T. canadensis* in Japan

Fig. 7. Relationship between percent parasitism of elongate hemlock scale and number of additional scale species present at sites in Japan.