Was species diversification in Tenthredinoidea (Hymenoptera: Symphyta) related to the origin and diversification of angiosperms?

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Abstract—The paraphyletic grouping "Symphyta" (8353 described species) represents the basal lineages of the insect order Hymenoptera. The most species-rich superfamily in Symphyta is Tenthredinoidea (7390 species), with six extant families. Most of tenthredinoids species are phytophagous at the larval stage, and the species using angiosperms as a host are more numerous (6265 species) than those using gymnosperms (140 species) or pteridophytes (985 species). In this study, we investigated whether diversification of Tenthredinoidea could be attributed to their use of angiosperms as hosts by examining host plant usage by lineage. We performed molecular phylogenetic and divergence time estimation analyses using molecular data (~2 kilobase sequence in five DNA regions) and conducted a diversification analysis. Our results suggest that Tenthredinoidea (excluding Blasticotomidae) had used angiosperms since its origin; the phylogeny of Tenthredinoidea showed a significant shift in diversification at two nodes, and those nodes overlap with the periods of origin and diversification of angiosperms.

Introduction

Insects are the most diverse class of organisms (Grimaldi and Engel 2005). Within Insecta, Hymenoptera are one of the most successful orders, comprising ~120 000 described species. This high diversity places it just behind Coleoptera and Lepidoptera, but some hymenopterists argue that if undescribed species were included, Hymenoptera would be a more species-rich taxon than any other insect order. Hymenoptera are presently divided into two suborders: Symphyta, a paraphyletic grouping that comprises the basal lineages branching off from the branch leading to the second suborder, the monophyletic Apocrita (Grimaldi and Engel 2005; Sharkey 2007). Symphyta includes 8353 described species (Taeger *et al.* 2010; Taeger and Blank 2011), all of which, except those in Orussidae, are phytophagous at the larval stage (Grimaldi and Engel 2005; Sharkey 2007).

Tenthredinoidea, the most species-rich superfamily in Symphyta, comprises six families with ~7400 species (Taeger *et al.* 2010; Taeger and Blank 2011). Each family or subfamily is associated with a main host plant group. Argidae, Cimbicidae, Pergidae, and Tenthredinidae (except for Selandriinae) include ~6300 species (85% in Tenthredinoidea) and mainly use angiosperms as a host. The families not using angiosperms as host plants are less species-rich, *e.g.*, Diprionidae (2%; 140 species) use gymnosperms, and Blasticotomidae and Selandriinae (Tenthredinidae) (13%; ~1000 species) mainly use pteridophytes (Table 1; *e.g.*, Goulet and Huber 1993; Costa and Louque 2001; Naito 2004; Grimaldi and Engel 2005; Schmidt and Smith 2006; Yoshida 2006; Badenes-Perez and Johnson 2007; Blank *et al.* 2012). The species richness of Tenthredinoidea may have resulted from its use of angiosperms as host plants because insect groups that feed on angiosperms are generally species-rich (Futuyma and Agrawal 2009). Several studies have reported the relationship between a host plant group and lineage diversity. For example, Farrell (1998) reported that diversity in Coleoptera was caused by a host shift to angiosperms from

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Table 1. The number of species, main food resource and remark for Tenthredinoidea.

Family, subfamily, or tribe	Number of species**	Main food resource	Remarks
Blasticotomidae	13	Pteridophytes	
Pergidae	442	Angiosperms	Acordulecerinae (105) + Perreyiinae (90) + 12 others (247)
Argidae	913	Angiosperms	Arginae (443) + Sterictiphorinae (330) + 5 others (140)
Diprionidae	140	Gymnosperms	Diprioninae (121) + 19
Cimbicidae	205		
Cimbicidae Part 1	177	Angiosperms	Cimbicinae (105) + others (72)
Cimbicidae Part 2	28	Angiosperms	Coryninae (28)
Tenthredinidae	5677		
Athaliini	104	Angiosperms	*Athalia* + *Hennedyia* + *Hennedyella* + *Hypsathalia* (Benson 1963)
Tenthredinidae*	5573		Tenthredinidae excluding Athaliini
Nematinae	1251	Angiosperms	
Selandriinae	972	Pteridophytes	Several species use monocots
Allantinae*	740	Angiosperms	Allantinae excluding Athaliini
Heterarthrinae	250	Angiosperms	
Blennocampinae	636	Angiosperms	
Tenthredininae	1724	Angiosperms	Few species use pteridophytes or bryophytes

Note: *According to result from molecular phylogeny in this study (see Fig. 2).
Note: **According to Taeger and Blank (2011).

gymnosperms, and Wiegmann *et al.* (2002) reported that in Lepidoptera, the number of species in the basal lineage that feeds on gymnosperms is much smaller than that in a sister lineage that feeds on angiosperms. Janz *et al.* (2006) showed that species diversity in Nymphalinae (Lepidoptera: Nymphalidae) was driven by host plant diversity.

Thus, heterogeneity in species richness among tenthredinoid species could be related to host plant usage, and the shift in diversification could have occurred during the evolution of Tenthredinoidea. To clarify heterogeneity in species richness among families and subfamilies belonging to Tenthredinoidea, we performed a comparative analysis of species richness among sister taxa similar to the analysis of Mayhew (2002) and also used in several other studies of species richness in insects and plants (Davies *et al.* 2004; Hunt *et al.* 2007; Davis *et al.* 2009, 2010). Among these studies, Davis *et al.* (2010) demonstrated the supertree method and the comparison of species richness in sister taxon analysis to identify the origin of species richness in Hymenoptera. Their results indicated that the possible drivers of specific adaptive radiations included key anatomical innovations, the exploitation of species-rich host groups, and an association with angiosperms. In contrast, low species richness may have resulted from geographical isolation, specialisation for a narrow ecological niche, a habitat loss, and competition. In Tenthredinoidea, Davis *et al.* (2010) attributed the low species richness of Blasticotomidae to the specialised ecological niche occupied by this family.

To compare species richness among sister taxa, a phylogenetic tree must first be constructed. Recent phylogenetic analyses of Tenthredinoidea have been based on molecular data (Heraty *et al.* 2011); molecular and morphological data (Schulmeister *et al.* 2002; Schulmeister 2003b; Sharkey *et al.* 2012); or molecular, morphological, and fossil data (Ronquist *et al.* 2012). The resulting phylogenetic trees have some features in common, namely the monophyly of Tenthredinoidea, Argidae, Pergidae, and Argidae + Pergidae; the ancestral state of Tenthredinoidea represented by Blasticotomidae; and construction of a single lineage from Diprionidae, Cimbicidae, and Tenthredinidae. They disagree on the phylogenetic relationships among Diprionidae, Cimbicidae, and Tenthredinidae and on the monophyly of Tenthredinidae (including *Athalia*). Thus, several phylogenetic relationships within Tenthredinoidea require clarification. Vilhelmsen (2006) suggested that to resolve these relationships, a large sample of Tenthredinidae, in particular, would be needed because Tenthredinidae is the most diversified family in Tenthredinoidea. Of the six subfamilies in Tenthredinidae, these

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previous phylogenetic analyses mainly used the three most species rich (Tenthredininae, Allantinae, and Nematinae). Although Selandriinae are relatively species rich, few species belonging to this subfamily were included. As a result of this omission, these phylogenies may not have been reconstructed correctly. Recently, two more relevant molecular phylogenetic studies have appeared. Malm and Nyman (2014) present a new, extremely interesting phylogeny of Symphyta with emphasis on Tenthredinoidea using ~6.8 kilobases (kb) of molecular data obtained 164 Symphyta specimens. Boevé *et al.* (2013) examined phylogenetic correlations among ecological and defensive traits in Tenthredinoidea, and suggested that the evolution and radiation of several tenthredinid subgroups have been driven by invertebrate rather than vertebrate predators. We expected that the findings of these studies will improve the reliability of our phylogenetic reconstruction and aid in our analysis of diversification of Tenthredinoidea.

The shift in diversification should be uncovered by analysing a combination of independent data sets (*e.g.*, phylogenetic relationships among families and subfamilies and the number of species in each family or subfamily along with their divergence date and the divergence date of their host plant group). In this study, we obtained specimens from as many tenthredinoids species distributed in Japan as possible and reconstructed molecular phylogeny of Tenthredinoidea by using not only previously reported DNA sequences (*e.g.*, Schulmeister *et al.* 2002; Schulmeister 2003b; Ronquist *et al.* 2012) but also sequences from new specimens belonging to 14 genera (31 species). We used ~2 kb of the DNA sequence from five gene regions to reconstruct the molecular phylogeny. Then, based on the resulting our molecular phylogeny and focussing on the host plant group used by each lineage, we tested the hypothesis that diversification of Tenthredinoidea was related to their use of angiosperms as a host plant. Specifically, we tested the hypothesis by estimating divergence times, assessing ancestral host usage, and comparing species richness among sister taxa.

Materials and methods

Taxon sampling and DNA extraction

In total (*i.e.*, including both new and old samples), we analysed 56 species and 40 genera belonging to six extant families of Tenthredinoidea, plus four species of Xyeloidea and Pamphilioidea as outgroups. In addition to the Tenthredinoidea taxa used in previous phylogenetic reconstructions (Schulmeister *et al.* 2002; Schulmeister 2003b; Ronquist *et al.* 2012), we included 31 new specimens recorded mainly in eastern Asia from 14 genera not used before: *Trichiosoma* Leach (Cimbicinae: Cimbicidae); *Allantus* Panzer and *Empria* Lepeletier and Serville (Allantinae: Tenthredinidae); *Paracharactus* MacGillivray (Blennocampinae: Tenthredinidae); *Aneugmenus* Hartig, *Stromboceros* Konow, *Nipponorhynchus* Takeuchi, *Rocalia* Takeuchi, and *Thrinax* Konow (Selandriinae: Tenthredinidae); and *Lagidina* Malaise, *Macrophya* Dahlbom, *Pachyprotasis* Hartig, *Rhogogaster* Konow, and *Siobla* Cameron (Tenthredininae: Tenthredinidae) and from seven genera included previously: *Blasticotoma* Klug (Blasticotomidae), *Athalia* Leach (Allantinae: Tenthredinidae), *Nematinus* Rohwer (Nematinae: Tenthredinidae), *Dolerus* Panzer and *Strongylogaster* Dahlbom (Selandriinae: Tenthredinidae), and *Aglaostigma* Kirby and *Tenthredo* Linnaeus (Tenthredininae: Tenthredinidae).

Of the 56 species, 31 were collected in the field and fixed and preserved in 99.5% ethanol until dissection. They were then identified by their morphological characteristics, and the target genes sequenced for them. For the remaining 25 species belonging to Tenthredinoidea, we used GenBank data. We also used the GenBank data of four species of superfamilies closely related Tenthredinoidea as outgroups (Xyeloidea and Pamphilioidea).

Total genomic DNA was extracted from the samples of ethanol-preserved insects using a salting-out protocol (Sunnucks and Hales 1996). We targeted five gene regions, ribosomal 12S, 16S, 18S, and 28S sequences, and the mitochondrial cytochrome oxidase I (COI), using ~2 kb of the DNA sequence. We amplified five gene regions by polymerase chain reaction (PCR) using primer sets and protocols described by Schulmeister *et al.* (2002), Schulmeister (2003b), and Nyman *et al.* (2006). After amplification, the PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio, United States of America). Cycle sequencing reactions for both strands were performed using a BigDye Terminator v.3.1 Cycle Sequencing Kit (ABI; Applied Biosystems,

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Forster City, California, United States of America) on an Applied Biosystems 3130 Genetic Analyzer (ABI). The taxa analysed in this study are listed with their GenBank accession numbers in Table 2, and the PCR primer sets are listed in Table S1.

Molecular phylogeny

The partial DNA sequences were aligned with ClustalX (Larkin *et al*. 2007) using default parameter settings, and ambiguously aligned regions were removed before further analysis. A total of 2356 sites from the 12S (426 sites), 16S (243 sites), 18S (748 sites), 28S (538 sites), and COI (401 sites) sequences were used for the phylogenetic analysis. Bayesian molecular phylogenies were reconstructed with MrBayes v.3.1.7 (Ronquist and Huelsenbeck 2003) using a substitution model for each ribosomal gene and each codon position belonging to COI calculated by Kakusan4 (Tanabe 2007). Information on the sequences used to reconstruct the molecular phylogeny is given in Table S2.

In MrBayes, the molecular phylogeny was reconstructed with default priors, and each of the seven partitions (12S, 16S, 18S, 28S, and COI first, second, and third position) was allowed to have its own unlinked substitution model (Table S2). Two parallel Markov Chain Monte Carlo (MCMC) runs having six incrementally heated chains were computed for five million generations, while sampling trees from the current cold chain every 100 generations. Log files of the runs were inspected in Tracer v.1.5 (Rambaut and Drummond 2009) to confirm reaching chain stationarity and adequate effective sample sizes (ESS; > 200) for the estimated parameters. After ESS were identified, the first 12 501 of the sampled trees were discarded as burn-in from both runs, and the last 75 000 trees were used to calculate 51 percentage Bayesian majority rule consensus tree.

Optimisation of host plant usage

The host plant use in tenthredinoid species was evaluated in relation to mainly three large plant groups, angiosperms, gymnosperms, and pteridophytes. To optimise the host plant usage in Tenthredinoidea, ancestral associations were reconstructed with parsimony optimisation across the in-group part of the MrBayes phylogeny using Mesquite v.2.75 (Maddison and Maddison 2011). Host plants (angiosperms, gymnosperms, or pteridophytes) for each species were selected according to the Electronic World Catalog of Symphyta (ECatSym) v.4.0 beta (Blank *et al*. 2012), but host plant groups of the unidentified species were given host characteristics inferred from the data on what host plants were used by other members of the genus listed in ECatSym v.4.0 beta (Blank *et al*. 2012), or they were described in general.

Divergence time estimation

Divergence times were estimated using BEAST v.1.8.0 (Drummond and Rambaut 2007) and the substitution model for an unpartitioned matrix (12S + 16S + 18S + 28S + COI) calculated by Kakusan4 (Tanabe 2007, Table S2). When seven partitions, a matrix similar to the reconstructed phylogeny, or five partitions (12S, 16S, 18S, 28S, and COI) were used, there were too many parameters to perform the analysis on BEAST. An uncorrelated lognormal relaxed molecular clock model (Drummond *et al*. 2006) was implemented as a tree before using the Birth–Death process (Gernhard 2008). This analysis provided two credible internal node points for calibration with fossil records (Ronquist *et al.* 2012) with lognormal priors: Pamphilioidea (161 million years ago (Ma); Rasnitsyn and Zhang 2004) with applied Log(Mean) = 4.3, Log(SD) = 0.5, and offset = 161 and Tenthredinoidea excluding Blasticotomidae (140 Ma; Zhang 1985) with applied Log(Mean) = 4.1, Log(SD) = 0.5, and offset = 140. In addition, Xyelidae, all specimen excluding Xyelidae, Pamphilioidea (Megalodontesidae and Pamphiliidae), Tenthredinoidea excluding Blasticotomidae, Argidae + Pergidae, Cimbicidae + Diprionidae, Athaliini (*Athalia*), Tenthredinidae* (Tenthredinidae excluding Athaliini), and Selandriinae were constrained as monophyletic based on the MrBayes phylogeny (see Results, Fig. 1), because an unconstrained analysis of an unpartitioned matrix would lead to a different phylogeny than the one using the seven partitions matrix (see Fig. S1). Two independent MCMC runs were performed for 50 million generations while sampling trees and parameter estimates once every 1000 generations. Log files of the runs were inspected in Tracer v.1.5 (Rambaut and Drummond 2009) to confirm that chain stationarity was reached and that the ESS was adequate (> 200)

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Table 2. Taxa analysed in this study, with information on host plant groups and GenBank accession numbers.

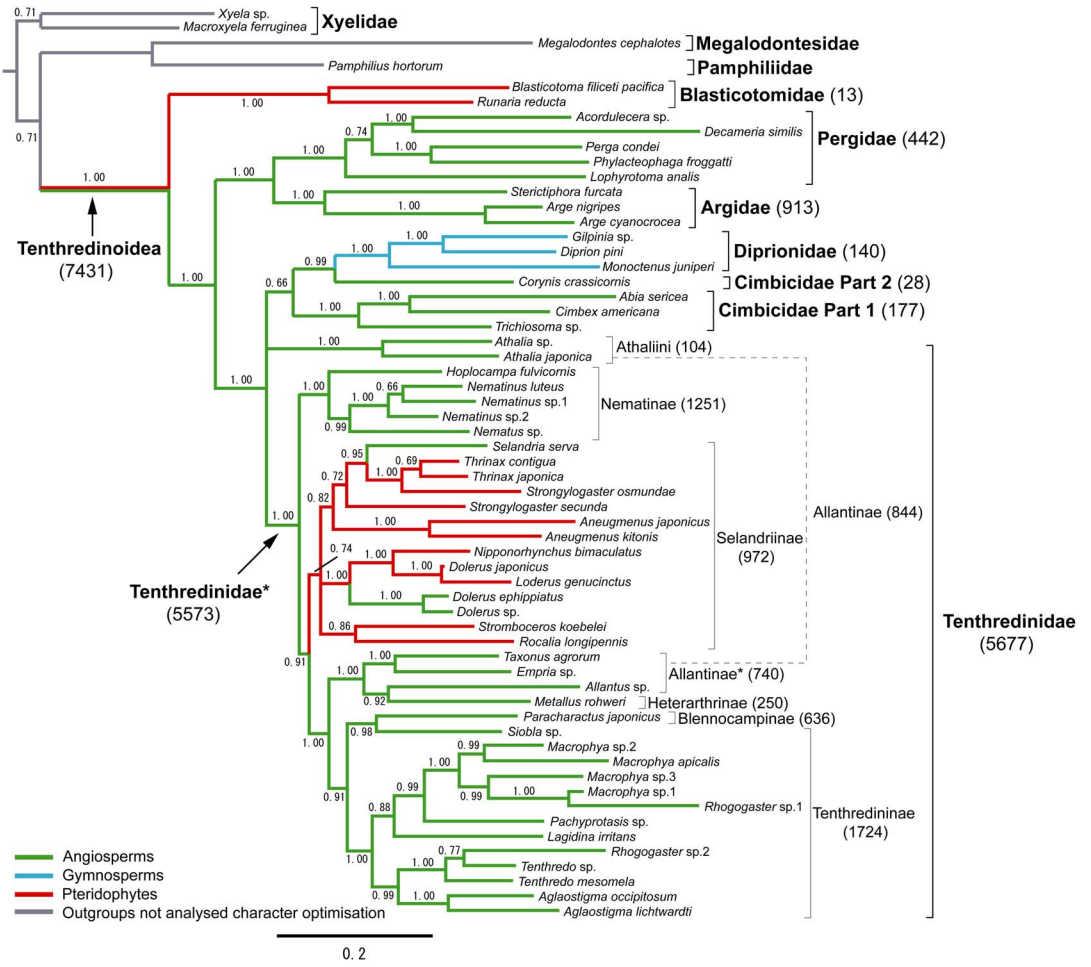
				GenBank accession numbers				
Family	Subfamily	Species	Host plant groups	12S	16S	18S	28S	COI
Argidae	Arginae	*Arge cyanocrocea*	Angiosperms	EF032193	AY206783	AY621130	EF032254	EF032221
		Arge nigripes	Angiosperms	EF032209	EF032175	EF032320	AF146659	EF032285
	Sterictiphorinae	*Sterictiphora furcata*	Angiosperms	EF032194	AY206784	AY621131	EF032255	EF032222
Blasticotomidae		***Blasticotoma filiceti pacifica***	Pteridophytes	AB859174	AB859197	AB859795	AB859818	AB858494
		Runaria reducta	Pteridophytes	EF032186	AY206774	AY621121	EF032245	EF032212
Cimbicidae	Abiinae	*Abia sericea*	Angiosperms	EF032207	EF032170	EF032314	EF032297	EF032280
	Cimbicinae	*Cimbex americanus*	Angiosperms	EF032191	AY206780	AY621127	EF032251	EF032218
		***Trichiosoma* species**	Angiosperms	AB859175	AB859198	AB859796	AB859819	AB858495
	Coryninae	*Corynis crassicornis*	Angiosperms	EF032192	AY206782	AY621129	EF032253	EF032220
Diprionidae	Monocteninae	*Monoctenus juniperi*	Gymnosperms	EF032205	EF032168	EF032312	EF032295	EF032278
	Diprioninae	*Diprion pini*	Gymnosperms	EF032206	EF032169	EF032313	EF032296	EF032279
		Gilpinia species	Gymnosperms	JF505471	AY206779	AY621126	EF032250	EF032217
Pergidae	Acordulecerinae	*Acordulecera* species	Angiosperms	JF505472	JF505487	EF032316	EF032299	JF505400
	Perginae	*Pergagrapta condei*	Angiosperms	EF032200	U06953	AY621132	EF032271	EF032238
	Perreyiinae	*Decameria similis*	Angiosperms	JF505473	JF505488	JF505496	JF505461	JF505401
	Phylactophaginae	*Phylacteophaga froggatti*	Angiosperms	EF032201	U06954	AY621133	EF032272	EF032239
	Pterygophorinae	*Lophyrotoma analis*	Angiosperms	EF032208	EF032171	EF032315	EF032298	EF032281
Tenthredinidae	Allantinae	***Allantus* species**	Angiosperms	AB863648	AB863662	AB863676	AB863690	AB863634
		Athalia japonica	Angiosperms	AB859158	AB859181	AB859779	AB859802	AB858478
		Athalia species	Angiosperms	EF032189	AY206777	AY621124	EF032248	EF032215
		***Empria* species**	Angiosperms	AB863647	AB863661	AB863675	AB863689	AB863633
		Taxonus agrorum	Angiosperms	JF505468	JF505484	JF505492	JF505459	JF505397
	Blennocampinae	***Paracharactus japonicus***	Angiosperms	AB863649	AB863663	AB863677	AB863691	AB863635
	Heterarthrinae	*Metallus rohweri*	Angiosperms	JF505467	JF505483	JF505491	JF505458	JF505396
	Nematinae	*Hoplocampa fulvicornis*	Angiosperms	JF505469	JF505485	JF505493	JF505462	JF505398
		Nematinus luteus	Angiosperms	JF505470	JF505486	JF505494	JF505460	JF505399
		***Nematinus* species 1**	Angiosperms	AB859176	AB859199	AB859797	AB859820	AB858496
		***Nematinus* species 2**	Angiosperms	AB859177	AB859200	AB859798	AB859821	AB858497
		Nematus species	Angiosperms	EF032190	AY206778	AY621125	EF032249	EF032216
	Selandriinae	***Aneugmenus japonicus***	Pteridophytes	AB859156	AB859179	AB859777	AB859800	AB858476
		Aneugmenus kiotonis	Pteridophytes	AB859155	AB859178	AB859776	AB859799	AB858475

Table 2. *Continued*

Family	Subfamily	Species	Host plant groups	GenBank accession numbers				
				12S	16S	18S	28S	COI
		Dolerus ephippiatus	Angiosperms	AB859159	AB859182	AB859780	AB859803	AB858479
		Dolerus japonicus	Pteridophytes	AB859160	AB859183	AB859781	AB859804	AB858480
		Dolerus genucinctus	Pteridophytes	AB859161	AB859184	AB859782	AB859805	AB858481
		Dolerus species	Angiosperms (?)	EF032188	AY206776	AY621123	EF032247	EF032214
		Nipponorhynchus bimaculatus	Pteridophytes (?)	AB859162	AB859185	AB859783	AB859806	AB858482
		Rocalia longipennis	Pteridophytes	AB859173	AB859196	AB859794	AB859817	AB858493
		Selandria serva	Pteridophytes	EF032203	EF032166	EF032310	EF032293	EF032276
		Stromboceros koebelei	Pteridophytes	AB859157	AB859180	AB859778	AB859801	AB858477
		Strongylogaster osmundae	Pteridophytes	AB859168	AB859191	AB859789	AB859812	AB858488
		Strongylogaster secunda	Pteridophytes	AB859169	AB859192	AB859790	AB859813	AB858489
		Thrinax contigua	Pteridophytes	AB859170	AB859193	AB859791	AB859814	AB858490
		Thrinax japonica	Pteridophytes	AB859171	AB859194	AB859792	AB859815	AB858491
	Tenthredininae	*Aglaostigma lichtwardti*	Angiosperms	EF032202	EF032165	EF032309	EF032292	EF032275
		Aglaostigma occipitosum	Angiosperms	AB863658	AB863672	AB863686	AB863700	AB863644
		Lagidina irritans	Angiosperms	AB863650	AB863664	AB863678	AB863692	AB863636
		Macrophya apicalis	Angiosperms	AB863652	AB863666	AB863680	AB863694	AB863638
		***Macrophya* species 1**	Angiosperms	AB863653	AB863667	AB863681	AB863695	AB863639
		***Macrophya* species 2**	Angiosperms	AB863654	AB863668	AB863682	AB863696	AB863640
		***Macrophya* species 3**	Angiosperms	AB863655	AB863669	AB863683	AB863697	AB863641
		***Pachyprotasis* species**	Angiosperms	AB863651	AB863665	AB863679	AB863693	AB863637
		***Rhogogaster* species 1**	Angiosperms	AB863656	AB863670	AB863684	AB863698	AB863642
		***Rhogogaster* species 2**	Angiosperms	AB863657	AB863671	AB863685	AB863699	AB863643
		Tenthredo mesomela	Angiosperms	EF032187	AY206775	AY621122	EF032246	EF032213
		***Tenthredo* species**	Angiosperms	AB863659	AB863673	AB863687	AB863701	AB863645
		***Siobla* species**	Angiosperms	AB863660	AB863674	AB863688	AB863702	AB863646
Outgroups								
Megalodontesidae		*Megalodontes cephalotes*		EF032197	AY206789	AY621138	EF032260	EF032227
Pamphiliidae	Pamphiliinae	*Pamphilius hortorum*		JF505474	EF032172	EF032317	EF032300	EF032282
Xyelidae	Xyelinae	*Xyela* species		EF032184	AY206772	AY621119	EF032243	EF032210
	Macroxyelinae	*Macroxyela ferruginea*		EF032185	AY206773	AY621120	EF032244	EF032211

Note: New specimen not included in previous molecular phylogenetic study (Schulmeister *et al.* 2002; Schulmeister 2003b; Ronquist *et al.* 2012) shown with bold character.

Fig. 1. The reconstructed molecular phylogeny calculated a 51 percentages Bayesian majority rule. Numbers above or below the branches are posterior probabilities. Each number in parentheses on the taxon name indicates the number of species. Branch colours show host plant groups (angiosperms, gymnosperms, or pteridophytes inferred from character optimisation analysis).



for each estimated parameter. The tree files were then combined using LogCombiner v.1.8.0 (part of the BEAST package), with the burn-in set to 12 501 trees and resampling trees every 10 000 generations. The remaining 7500 trees were thereafter used to generate maximum clade credibility trees with mean node heights using TreeAnnotator v.1.8.0 (part of the BEAST package).

Diversification analyses

Based on simple null models of cladogenesis, the comparison of species richness in the sister taxon analysis (Mayhew 2002) was performed using BEAST phylogeny. If two sister taxa radiated at equal (but not necessarily constant) rates through time (Nee *et al*. 1994), the all possible partitions of N species into two clades were equally probable (Farris 1976). The (two-tailed) probability of an equal or greater magnitude of split under the null model is given by the following formula: $2[N_{small}/(N_{small} + N_{large} - 1)]$. Caution is advised before attributing a significant result to the shift in diversification at that node; a shift occurring only among derived taxa will automatically raise the species richness of higher clades to which they belong. It is therefore

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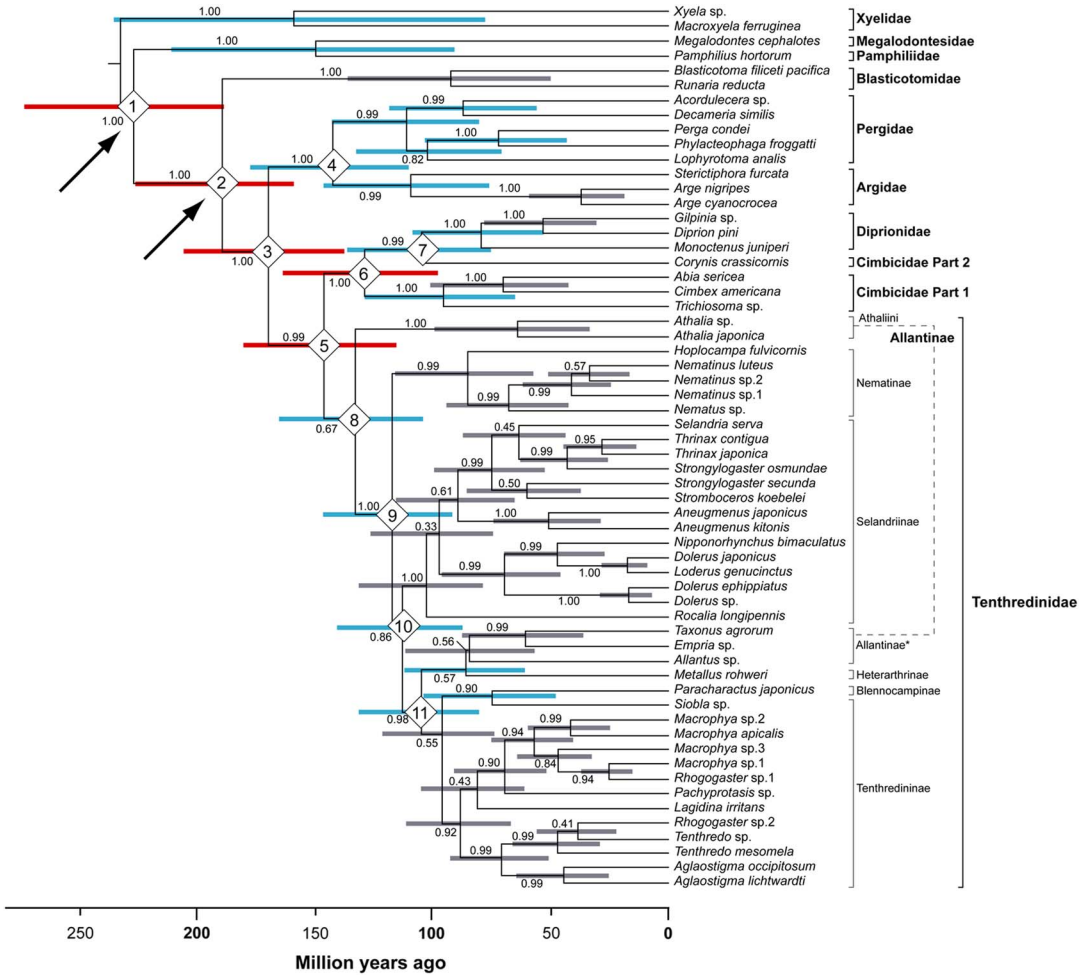
Fig. 2. Relaxed molecular clock phylogeny of Tenthredinoidea and representative outgroup taxa of Megalodontesidae, Pamphiliidae, and Xyelidae. Numbers above or below the branches are posterior probabilities. The divergence time of each crown group above the subfamily level in Tenthredinoidea is based on the 95% higher posterior density intervals of the node ages. Red bars represent node ages of groups above family level, blue bars represent node ages of families and nodes below family level, and grey bars represent node ages at the other levels. The numbers enclosed in rhombuses at the nodes correspond to the node numbers in Table 3. Arrows indicate points of calibration against fossil records.



essential to examine the components of species-rich taxa to determine if the most primitive members of the group are also species-rich. If not, it is probable that the actual shift occurred at some more derived node (Sanderson and Donaghue 1994). This analysis was performed by observing if any further significant results occur within the most species-rich taxon and, if so, the test was repeated excluding those taxa. In addition, the diversification rate was estimated from the clade's current age (*t*) and current species richness (*n*; Mayhew 2002).

For this analysis, the number of species in each tenthredinoid family or subfamily was taken from the ECatSym v.3.10 (Taeger and Blank 2011). Then, because the molecular phylogenetic analysis showed that most of the lineages were monophyletic (see Results, Fig. 2), we were able to use the number of species directly from those data. Nonetheless, five lineages (Cimbicidae Part1

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and Part 2, Athaliini, Tenthredinidae*, and Allantinae*; see Table 1) resulted in a complex number of species as follows. Cimbicidae Part 1 (177 species); this lineage consists of Cimbicidae (205 species) but excludes Coryninae. Cimbicidae Part 2 (28 species); this lineage consists of Coryninae (28 species) contributed by *Corynis* Thunberg. Athaliini (104 species); this lineage consists of all species of *Hennedyia* Cameron, *Hennedyella* Forsius, *Hypsathalia* Benson, and *Athalia*. These species were included in Athaliini by Benson (1963). Tenthredinidae* (5573 species); this lineage consists of Tenthredinidae (5677 species) excluding Athaliini (104 species). Allantinae* (740 species); this lineage consists of Allantinae (844 species) excluding Athaliini (104 species).

Results

Molecular phylogeny

The molecular phylogeny reconstructed using MrBayes (Fig. 1) supports the monophyletic status of Tenthredinoidea and of four of the six families in Tenthredinoidea (Argidae, Blasticotomidae, Diprionidae, and Pergidae) with high posterior probabilities ($PP = 1.00$). Cimbicidae was not supported as monophyletic because *Corynis* was found to be more closely related to the diprionids than to other genera in Cimbicidae (*Trichiosoma*, *Cimbex* Olivier, and *Abia* Leach). Tenthredinidae also was not supported as monophyletic because it was divided into Athaliini and Tenthredinidae*, and their basal node which consisted of them and the Cimbicidae + Diprionidae clade, was unresolved in the majority-rule consensus.

In the reconstructed phylogeny, three of the six subfamilies in Tenthredinidae (Nematinae, Tenthredininae, and Selandriinae) analysed in this study were monophyletic. Allantinae, however, was non-monophyletic because *Athalia* was found not to be closely related to the other Allantinae genera (*Allantus*, *Empria*, and *Taxonus*). No conclusions could be made about the monophyly of Blennocampinae or Heterarthrinae because they were each represented by only one species.

Optimisation of host plant usage in Tenthredinoidea

Host plant usage and ancestral states inferred by the parsimony method in Tenthredinoidea analysed in this study are shown with coloured branches in Fig. 1 (and Fig. S2). The results show that the host used at the origin of Tenthredinoidea was angiosperms or pteridophytes. Nonetheless, two major shifts in host usage were observed at the branching of Diprionidae or Selandriinae. These results suggested that Tenthredinoidea used angiosperms or pteridophytes ancestrally; however, when Blasticotomidae originated, the ancestral Tenthredinoidea excluding Blasticotomidae would change the main host to angiosperms. Thus, angiosperm usage would be the basal plan in Tenthredinoidea excluding Blasticotomidae. Therefore, when Diprionidae or Selandriinae originated, they changed the host to gymnosperms or pteridophytes from angiosperms, respectively.

Divergence time estimation

To estimate divergence time, we first confirmed that the molecular phylogeny reconstructed with BEAST using the no partitions strategy (Fig. 2) was generally congruent with that reconstructed by the MrBayes phylogeny using seven partitions (Fig. 1). The difference was that in the BEAST phylogeny, Tenthredinidae was monophyletic, albeit with low support ($PP = 0.67$). The BEAST analysis estimated the split between Blasticotimidae and the rest of the superfamily at 190 Ma (95% high posterior density (HPD), 159–223), Argidae and Pergidae 143 Ma (95% HPD, 110–178), Diprionidae 104 Ma (95% HPD: 75–137), Cimbicidae Part 1 129 Ma (95% HPD: 98–164), and Tenthredinidae 146 Ma (95% HPD: 115–181; Table 3 and Fig. 2). Thus, the mean estimated time of origin of families in Tenthredinidae ranged from 190 to 104 Ma, meaning the early Jurassic to the early Cretaceous period. In addition, Athaliini and Tenthredinidae* originated at 133 Ma (95% HPD, 104–165).

Diversification analysis

Results of sister taxon comparison based on the BEAST phylogeny are shown in Table 4. In five tests, significant differences were found, and in the others, no significant difference was observed.

There was no evidence that the origin of Cimbicidae + Diprionidae and Tenthredinidae represents a shift in diversification. Neither the sister taxon comparison nor the estimation of the divergence rate yielded unexpected results (Table 3, node 5; Table 4, comparison 6). The comparison

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Table 3. Estimated node ages and 95% highest posterior density intervals for the divergence time of each crown-group in this study and the diversification rate.

Number of node	Divergence time of each crown-group in this study (number of species = *n*)	Estimated node ages (Ma = *t*)			Diversification rate ln (*n*)/*t*
		Mean	Min	Max	
1	Tenthredinoidea (7390)	227	189	274	0.039
2	Blasticotomidae (13)	190	159	223	0.014
	Tenthredinoidea – Blasticotomidae (7377)				0.047
3	Pergidae + Argidae (1355)	170	138	206	0.042
	Cimbicidae + Diprionidae + Tenthredinidae (6022)				0.051
4	Pergidae (442)	143	110	178	0.043
	Argidae (913)				0.048
5	Cimbicidae + Diprionidae (345)	146	115	181	0.040
	Tenthredinidae (5677)				0.059
6	Cimbicidae Part 1 (177)	129	98	164	0.040
	Cimbicidae Part 2 + Diprionidae (168)				0.040
7	Diprionidae (140)	104	75	137	0.047
	Cimbicidae Part 2 (28)				0.032
8	Athaliini (104)	133	104	165	0.035
	Tenthredinidae* (5573)				0.065
9	Nematinae (1251)	117	92	147	0.061
	Tenthredinidae* – Nematinae (4322)				0.071
10	Selandriinae (972)	113	89	142	0.061
	Allantinae* + Heterarthrinae + Blennocampinae + Tenthredininae (3350)				0.072
11	Allantinae* + Heterarthrinae (990)	105	80	131	0.066
	Blennocampinae + Tenthredininae (2360)				0.074

Note: Each number of node corresponds to it in Fig. 2.

between Pergidae + Argidae and Cimbicidae + Diprionidae + Tenthredinidae also did not yield significant differences (Table 3, node 3; Table 4, comparison 4). This result suggested that the high species richness of Tenthredinoidea excluding Blasticotomidae is a general property of that group and is not a feature of just a few derived clades (Table 3, node 2; Table 4, comparison 1); thus, the species richness of Tenthredinoidea excluding Blasticotomidae represents a shift in diversification. On the other hand, although this result indicates a relative shift in diversification, it cannot tell us whether we were dealing with a shift to a slower speciation rate (be species-poor) in Blasticotomidae or to a faster rate (be species-rich) in Tenthredinoidea excluding Blasticotomidae.

In the same way, one suggestion was applied to comparative analyses related to the split between Athaliini and Tenthredinidae* (Table 3, node 8; Table 4, comparison 9, 10, and 11). In particular, with respect to the split between Athaliini and Tenthredinidae*, if there were defined putative sister taxa Athaliini and Nematinae or Athaliini and Tenthredinidae* excluding Nematinae, the former set did not show significant results, but the latter one did (Table 4, comparison 10 and 11). These results suggested that the shift in diversification found among Tenthredinidae* was caused by a shift to a faster speciation rate among Tenthredinidae* excluding Nematinae.

Discussion

Molecular phylogeny

The molecular phylogenies reconstructed in this study using MrBayes (Fig. 1) and BEAST (Fig. 2) are supported fairly well by the morphology-based classification of Tenthredinoidea (Goulet and Huber 1993). Our data confirm some results (namely, that Tenthredinoidea, Argidae, Pergidae, and Argidae + Pergidae are monophyletic; Blasticotomidae is sister to the rest of Tenthredinoidea; and Diprionidae, Cimbicidae, and Tenthredinidae belong to the same

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Table 4. Sister taxon comparisons between tenthredinoid lineages and associated probabilities under the null model of equal (but not necessarily constant) rate of speciation and extinction in the two lineages (smaller and larger taxon).

Comparison	Smaller taxon	Species	Larger taxon	species	*P* (two-tailed)
1	Blasticotomidae	13	Tenthredinoidea excluding Blasticotomidae	7418	**0.003**
2	Blasticotomidae	13	Argidae + Pergidae	1355	**0.019**
3	Blasticotomidae	13	Cimbicidae + Diprionidae + Tenthredinidae	6063	**0.004**
4	Argidae + Pergidae	1355	Cimbicidae + Diprionidae + Tenthredinidae	6063	0.365
5	Pergidae	442	Argidae	913	0.653
6	Cimbicidae + Diprionidae	345	Tenthredinidae	5718	0.114
7	Diprionidae + Coryninae	168	Cimbicidae*	177	0.977
8	Coryninae	28	Diprionidae	140	0.335
9	Athallini	104	Tenthredinidae*	5614	**0.036**
10	Athallini	104	Nematinae	1251	0.154
11	Athallini	104	Tenthredinidae* excluding Nematinae	4322	**0.047**
12	Nematinae	1251	Tenthredinidae* excluding Nematinae	4322	0.449
13	Selandriinae	972	Allantinae* + Heterarthrinae + Blennocampinae + Tenthredininae	3350	0.450
14	Allantinae* + Heterarthrinae	990	Blennocampinae + Tenthredininae	2360	0.591

Note: *P*-value with bold character indicates significant shift in diversification.

lineage group) reported by other recent molecular phylogenetic studies (Schulmeister *et al.* 2002; Schulmeister 2003b; Heraty *et al.* 2011; Ronquist *et al.* 2012; Sharkey *et al.* 2012; Malm and Nyman 2014). This observation indicated that the molecular phylogenies reconstructed in this study were reliable.

An important difference between phylogenies reconstructed using MrBayes and BEAST is the monophyletic status of Tenthredinidae. The MrBayes phylogeny indicates that Tenthredinidae is non-monophyletic (Fig. 1) whereas the BEAST phylogeny shows it as monophyletic (Fig. 2). This discrepancy is due to the unpartitioned matrix and constraint setting used in BEAST, because the MrBayes phylogeny that was reconstructed using an unpartitioned matrix without constraints, showed that Tenthredinidae was non-monophyletic (Fig. S2).

On the other hand, our BEAST phylogeny showed that Tenthredinidae is likely to be monophyletic (Fig. 2). Previous studies (Schulmeister *et al.* 2002; Schulmeister 2003b; Heraty *et al.* 2011; Ronquist *et al.* 2012; Sharkey *et al.* 2012), however, reported that Tenthredinidae*, and accordingly, Tenthredinidae was non-monophyletic. In morphological phylogenetic studies (Vilhelmsen 2001; Schulmeister 2003a), Tenthredinidae is also considered non-monophyletic because morphological information on *Athalia* (Allantinae) is complex; *Athalia* has morphological characteristics that correspond to those found in Blasticotomidae. Benson (1963), however, in his description of Athaliini (comprising four genera, *Athalia*, *Hennedyia*, *Hennedyella*, and *Hypsathalia*), considered *Athalia* to be near the base of the Tenthredinidae stem because it shows some primitive features. Thus, although our results differ from those of previous work – in that we found Tenthredinidae to be possibly monophyletic – they are consistent with the above comment by Benson (1963) because our phylogeny has *Athalia* in a basal position in the Tenthredinidae topology. This status of *Athalia* is also supported by Malm and Nyman (2014). The inclusion of additional rarely analysed species, such as additional Selandriinae species, in future molecular phylogenetic studies may help to resolve this discrepancy.

At the family level, our results for Cimbicidae were not congruent with the morphological classification because we found *Corynis* to be closer

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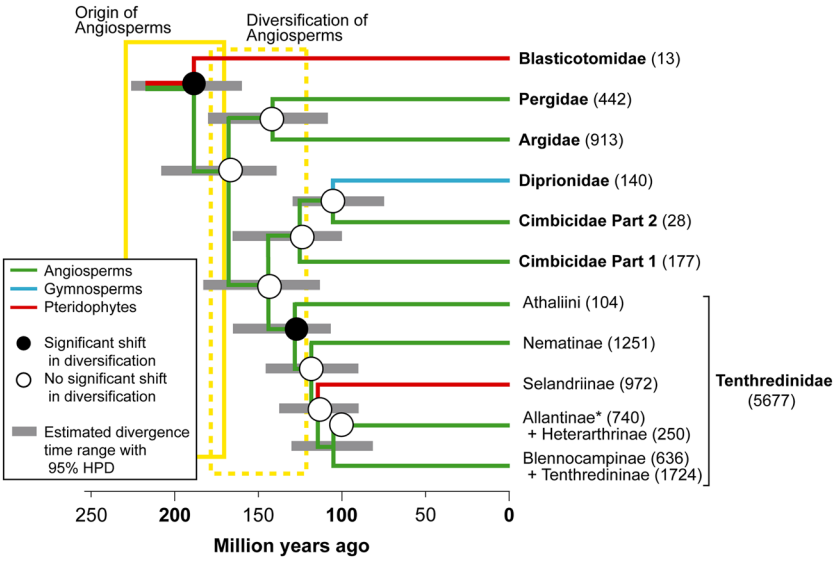
Fig. 3. Summarised results of this study. Phylogeny shows relationships within the Tenthredinoidea and tenthredinid subfamilies. Circles on the phylogeny indicate the mean values of estimated divergence time with a significant (black) or not a significant (white) shift in diversification. Grey bars show 95% highest posterior density intervals for a node age. Each number in parentheses on the taxon name indicates the number of species. The branch colour shows a host plant group (angiosperms, gymnosperms, or pteridophytes inferred from character optimisation analysis). Each yellow square indicates intervals for the origin and diversification of angiosperms.



to the diprionid genera than to other genera in Cimbicidae (*Trichiosoma*, *Cimbex*, and *Abia*; Fig. 1). This result shows that Cimbicidae may be related to Diprionidae. The nature of the relationships among Cimbicidae, Diprionidae, and Tenthredinidae is controversial (Schulmeister *et al*. 2002; Schulmeister 2003b; Heraty *et al*. 2011; Ronquist *et al*. 2012; Sharkey *et al*. 2012). In particular, Schulmeister (2003b) suggested that the topological relationships among these three families are not stable because the difference in the number of steps between the Tenthredinidae* + Diprionidae clade and the Cimbicidae + Diprionidae clade was only one in the most parsimonious reconstruction of the molecular phylogeny. Malm and Nyman (2014) have proposed a resolution for these problems. In their study, these three families and clade comprised of Cimbicidae and Diprionidae are monophyletic. The results of our phylogenetic analyses are mostly consistent with this proposal. Therefore, the findings of Malm and Nyman (2014) indicate that the results we obtained by our analysis of DNA sequences available to us are generally reliable.

Diversification of Tenthredinoidea

The summarised result of this study is shown in Figure 3. It suggests that Tenthredinoidea have used angiosperms since their origin, two nodes showed a shift in diversification on the BEAST phylogeny of Tenthredinoidea and an overlap with the periods of origin and diversification of angiosperms. Therefore, diversification of Tenthredinoidea seems to have been related to the origin and diversification of angiosperms and by adaptation to them.

The character optimisation analysis indicated that angiosperms probably have been used as a host from the origin of Tenthredinoidea excluding Blasticotomidae to the present. At the same time, in basal nodes of Diprionidae or Selandriinae we found a host shift from angiosperms to gymnosperms or pteridophytes, respectively. The result of the optimisation seems to be approximate but it is plausibly correct, although not all tenthredinoid species were included. Although most of the tenthredinoid species excluding gymnosperm or pteridophyte feeders (~6300 species) could have used angiosperms, some of them have no reliable

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host information. Their hosts are possibly angiosperms according to the host information of the species related to them in ECatSym v.4.0 beta (Blank *et al*. 2012). Even if all tenthredinoid species that use angiosperms, gymnosperms, or pteridophytes as a host could be included in this analysis, the species using angiosperms would be dominated and lead to the same result in the parsimony optimisation analysis.

We consider the estimated ages to be mainly reliable even though our divergence time estimation analysis was excluded derived lineage Unicalcarida (Cephoidea + Siricoidea + Xiphydrioidea + Vespina; Schulmeister *et al.* 2002). Since the estimated ages of some of the major tenthredinoid subclades (Table 3, node 2, 3, and 4) were in the range estimated based on the fossil record (Rasnitsyn 2010) and similar to those estimated by the most recent divergence time estimation for symphytan clades (Ronquist *et al.* 2012), we believe that our results are relatively reliable even though our divergence time estimation analysis excluded the derived lineage Unicalcarida (Schulmeister *et al.* 2002).

The divergence time of the nodes Blasticotomidae–Tenthredinoidea excluding Blasticotomidae and Athaliini–Tenthredinoidea* (which shows a significant shift in diversification) was estimated at 190 Ma (95% HPD, 159–223) and 133 Ma (95% HPD, 104–165) respectively. The 95% HPD interval of the former overlaps with the periods of origin of angiosperms and the latter with the periods of diversification of angiosperms. Recent studies show widely varying estimates of the origin of the crown clade Angiospermae, ranging from the late Triassic (Bell *et al*. 2010; Smith *et al*. 2010) to the early Jurassic period (Clarke *et al*. 2011), with some signs that Bell *et al*. (2010) is converging on the range 229–170 Ma. In addition, estimates of the time of diversification of angiosperms converged on the range 179–120 Ma, according to a suggestion that crown angiosperms were in existence for some 50 Ma (or longer) before their radiation (Smith *et al*. 2010). We therefore inferred that the shift in the diversification of Tenthredinoidea was related to the origin and diversification of angiosperms. Alternatively, there is some evidence that angiosperms originated 145 Ma estimated with fossil records (Crane *et al*. 1994; Friis *et al*. 2006). If the origin of angiosperms had been later, then the diversification of Tenthredinoidea, especially of the most species-rich Tenthredinidae, would have occurred contemporaneously, coinciding with the evolutionary event of angiosperms.

The sister taxon comparison results showed a significant shift in diversification between Blasticotomidae and Tenthredinoidea excluding Blasticotomidae and between Athaliini and Tenthredinidae*. The first significant shift in the diversification was probably due to a shift to a slower speciation rate in Blasticotomidae. Though we suggested slower shift related to not using angiosperms (see above), Davis *et al*. (2010) reported that the shift to a slower speciation rate in Blasticotomidae was caused by its stem minor strategy of their larvae, which is unique among Tenthredinoidea (Shcherbakov 2006). The significant shift in diversification between Athaliini and Tenthredinoidea* (Table 4, comparison 9) was caused by a shift to a faster speciation rate in Tenthredinidae* excluding Nematinae (Table 4, comparison 10 and 11). These data indicate that the whole Tenthredinidae* has a stronger propensity for diversification, especially in later periods. We suggested that this shift to a faster speciation was related to diversification of angiosperms (see above), whereas Boevé *et al.* (2013) suggested diversification of especially Nematinae, Selandriinae, and Tenthredininae had been related to predatation. We also performed sister taxon comparison based on the latest molecular phylogeny (Malm and Nyman 2014), and found shifts to faster speciation rates (Table S3 and 4). These results suggest that diversification of Tehthredinoidea was related to multiple, interrelated factors: not only origin and diversification of angiosperms but also predation pressure and ecological niches of tenthredinoids species.

The insect group that feeds on angiosperms is generally species-rich (Farrell 1998; Wiegmann *et al*. 2002; Futuyma and Agrawal 2009). In fact, in Tenthredinoidea, the number of species of angiosperm eaters was 85%, gymnosperm eaters 2%, and pteridophyte eaters (some Selandriinae feed on angiosperms) 13%. Futuyma and Agrawal (2009) pointed out that increasing host diversity might contribute to speciation by enabling geographic expansion and therefore increasing opportunities for spatial isolation and genetic divergence. It is known that angiosperms are the most species-rich plant group compared with

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gymnosperms or pteridophytes (angiosperms: 250 000–300 000 species, gymnosperms: ~1000 species, and pteridophytes ~10 000 species; Crane *et al*. 1994; Schneider *et al*. 2004; Christopher 2013). Therefore, it is plausible that species diversification in Tenthredinoidea may be related to the species richness or general abundance of angiosperms.

Leppänen *et al*. (2012) reported that the diversification of Heterarthrinae (Tenthredinidae) is related to their host plant groups, and some of them have diversified through a host shift, while others did so in tandem with the diversification of host plants (codiversification; Quek *et al*. 2004). In addition Isaka and Sato (2014) suggested that the diversification of Selandriinae (Tenthredinidae) was also related to their host plant groups. Similarly, some tenthredinoid species may have diversified in lock step with their host plant group, mainly angiosperms.

Conclusion

Our phylogenetic analysis indicates that diversification in Tenthredinoidea may have been related to the origin and diversification of angiosperms. Because Tenthredinoidea excluding Blasticotomidae has used angiosperms since its origin, phylogeny of Tenthredinoidea has a significant shift in diversification at two nodes, and the 95% HPD interval of those nodes overlaps with the periods of origin and diversification of angiosperms. Molecular phylogeny in this study showed relationships among tenthredinoid families that are generally congruent with recent molecular phylogenetic studies (Schulmeister *et al*. 2002; Schulmeister 2003b; Heraty *et al*. 2011; Ronquist *et al*. 2012; Sharkey *et al*. 2012; Malm and Nyman 2014). Phylogenetic analyses in this study assessed the diversification process in Tenthredinoidea by means of character optimisation, sister taxon comparison, and estimation of diversification time. In future, to elucidate diversification in Symphyta, phylogenetic analyses involving various taxon levels would be warranted.

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Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.4039/tce.2014.60.

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