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Phylogeny and ecological evolution
of gall-inducing sawflies
(Hymenoptera: Tenthredinidae)

by
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The nematine sawflies (Hymenoptera: Tenthredinidae) that induce galls on willows (*Salix* spp.) form one of the most abundant and speciose herbivore groups in the Holarctic region. The purpose of this thesis was to study the evolutionary history of the nematine gallers. The three main questions addressed were: (1) what is the sequence in which different gall types have evolved in the nematines; (2) how has the utilization of host plants evolved in the group; and (3) why has gall induction evolved in the nematines?

The first question was studied by reconstructing the phylogeny of representative nematine species by using enzyme electrophoresis and DNA sequencing. According to the results, species that induce true closed galls evolved from species that induce leaf folds or rolls. Thereafter, several different gall types evolved gradually, and the evolution of each gall type was followed by a radiation to new host species. The phylogenies show that gall morphology is mainly determined by the insects, i.e., the gall represents an extended phenotype of the galler.

The two phylogenies were also used to study the second question. The results show that parallel cladogenesis between willows and the gallers is not a sufficient explanation for the current host associations, since many willow species have been colonized multiple times by nematine species representing different gall types. Likewise, it seems that the overall chemical similarity of hosts does not determine the direction of host shifts. Consequently, other explanations, such as host phenology, geographical distribution, or habitat, are probably needed for explaining the patterns of host use in the nematine sawflies. Similar results were obtained on a smaller scale in an enzyme electrophoretic study of the species that induce bud galls.

The third question was studied by comparing the chemical properties of sawfly galls to the chemical properties of the ungalloed tissues of the willow hosts. The galls were found to contain comparatively low levels of most phenolic defense compounds that are found in other willow tissues. Thus, it appears that the galls are nutritionally beneficial for the sawfly larvae. The changes in chemistry occur in a highly coordinated pattern in all studied galler-host pairs, which suggests that the sawflies are able to manipulate the phenolic biosynthesis in their hosts. The end result is a striking convergence of the chemical properties of the galls both within and between host species.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

- I Nyman, T., Roininen, H. & Vuorinen, J. A. 1998. Evolution of different gall types in willow-feeding sawflies (Hymenoptera: Tenthredinidae). *Evolution* 52: 465-474.
- II Nyman, T., Widmer, A. & Roininen, H. 2000. Evolution of gall morphology and host-plant relationships in willow-feeding sawflies (Hymenoptera: Tenthredinidae). *Evolution* 54: 526-533.
- III Nyman, T. The willow bud galler *Euura mucronata* Hartig (Hymenoptera: Tenthredinidae): one polyphage or many monophages? *Manuscript*.
- IV Nyman, T. & Julkunen-Tiitto, R. 2000. Manipulation of the phenolic chemistry of willows by gall-inducing sawflies. *Proc. Natl. Acad. Sci. USA* 97: 13184-13187.

Some unpublished results are also presented.

1. INTRODUCTION

1.1. Gall-inducing insects

Meyer (1987) defines galls as “all manifestations of growth, whether positive or negative, and of abnormal differentiation induced on a plant by animal and plant parasites”. Usually galls can be seen as abnormal growth caused by an increase in the number (hyperplasia) or size (hypertrophy) of plant cells at the site of the gall (Meyer 1987). Galls can be induced on virtually all parts of plants, and their complexity varies from simple pouches to highly structured organs with specifically differentiated tissues (Dreger-Jauffret & Shorthouse 1992).

The ability to induce galls has evolved convergently in many taxa, ranging from microbes and fungi to nematodes and arthropods (Meyer 1987). In insects alone, gall induction has evolved in at least the following seven orders: Thysanoptera, Hemiptera, Homoptera, Lepidoptera, Coleoptera, Diptera, and Hymenoptera (Meyer 1987, Dreger-Jauffret & Shorthouse 1992). The number of independent origins of gall induction in insects is, however, considerably higher, since within most of these orders there have been several separate lineages leading to the galling habit (Dreger-Jauffret & Shorthouse 1992, Roskam 1992).

The fossil record of plant galls shows that unknown gall-inducing insects were already present during the Late Carboniferous period, over 300 million years ago (Labandeira & Phillips 1996). Numerous galls have been found in younger plant fossils, but in most cases the identity of the gall inducer remains unknown (Larew 1992, Scott *et al.* 1994). However, it is evident that several gall-inducing insect groups were already in existence at the time of the initial radiation of angiosperms during the mid-Cretaceous; possible taxa include aphids, midges, and cynipid wasps (Scott *et al.* 1994).

Although galls are common, the mechanism by which insects induce galls remains largely unknown. Possible mecha-

nisms include mechanical damage, plant hormone analogs, and genetic manipulation (Hori 1992, Price 1992). Genetic manipulation is involved in the process of gall induction by the bacterium *Agrobacterium tumefaciens*. The bacterium inserts a portion of a Ti plasmid into the genome of some of the cells of its host, which eventually leads to abnormal growth (Davey *et al.* 1994). Although it has been suggested that such manipulation may also occur in the case of insect gallers (Cornell 1983), no evidence for such a mechanism has been found (Price 1992). It is likely that the mechanism of gall induction varies between galler taxa, but currently it would seem that the production of plant hormone analogs represents the most likely explanation (Hori 1992, Roskam 1992). For example, McCalla *et al.* (1962) found uric acid, glutamic acid, and adenine derivatives in the female accessory glands of the sawfly galler *Pontania pacifica* Marlatt. These compounds are closely related to cytokinins, which are natural growth-promoting hormones in plants (Hartley 1992). High concentrations of cytokinins have been found in galls induced by *Pontania proxima* Lepeletier, but apparently they are produced by the plant (Leitch 1994). Furthermore, studies on other galler/plant combinations have found that the relative concentrations of cytokinins are not necessarily higher in all galls (Mapes & Davies 1998). Mapes and Davies (1998) found cytokinins and IAA in larvae of the dipteran stem galler *Eurosta solidaginis* Fitch, but it is not clear whether the larvae produce these hormones *de novo*, or sequester them from their diets.

Different galler taxa tend to specialize in different plant taxa, but some groups of plants are attacked more than others (Roskam 1992). For example, relatively few insect species induce galls on ferns and gymnosperms (Roskam 1992). In contrast, over 90% of galls occur on dicots, and within dicots the vast majority of galls occur on Fagaceae, Asteraceae, and Rosaceae (Abrahamson & Weis 1987). In Britain,

Salix (Salicaceae) and *Quercus* (Fagaceae) species support the largest numbers of insect gallers (Kennedy & Southwood 1984).

A common observation is that gall-inducing insect species are strictly host-specific (Meyer 1987, Dreger-Jauffret & Shorthouse 1992). This is probably a result of the tight link between the gallers and their hosts, because gall induction may be possible only during a short period of time during the growing season, and the available plant species (or even individuals or clones within species) may differ in their reactions to the gall-inducing stimuli (Smith 1970, Price 1992, Whitham 1992, Craig *et al.* 1994, Peries 1994, Csóka 1994, Kokkonen 2000). It is important to note that the degree of host specificity may have been underestimated, since galler species are often extremely difficult to identify by morphological methods, and many taxa have not been studied in depth (Smith 1968, Harris 1994).

Obviously, the repeated evolution of the galling habit in highly divergent insect taxa presents an excellent opportunity for studying convergent evolution. Furthermore, the existence of various gall types makes it possible to study how gall morphology evolves, and whether the morphological and chemical features of the galls are determined by the host plants or by the gallers. Finally, the existence of groups of strictly monophagous gallers on various host species facilitates comparative studies of the speciation patterns in the host and galler taxa.

1.2. Purpose of this study

The purpose of this thesis was to study various questions relating to the ecology and evolution of galling insects by using the nematine sawflies (Hymenoptera: Tenthredinidae) that induce galls on willows as a model system. The main questions addressed in the thesis are: (1) what is the sequence in which different gall types have evolved in the group; (2) how has the utilization of host plants evolved in the group; and (3) why has gall induction evolved in the group? The first

two questions were studied by reconstructing the phylogeny of representative nematines using allozyme electrophoresis and DNA sequencing, and the last one was studied by analyzing the chemical properties of sawfly galls using high-performance liquid chromatography.

2. THE STUDY SYSTEM

2.1. Willows

Willows (*Salix* spp.) form one of the taxonomically and ecologically most diverse plant genera in the Northern Hemisphere (Argus 1973). The oldest willow fossils have been found from North American deposits dating from the early Eocene, ca. 55-65 million years ago (Collinson 1992). However, Skvortsov (1968, 1999) argued that the group originated in the warm temperate region or the subtropics, with the main radiation occurring later in the temperate region and the arctic (see also Dorn 1976). There are currently over 350 willow species, most of which occur in wet (riparian) habitats, or as colonizers in the early successional stages of vegetation (Skvortsov 1968, 1999; Argus 1973). Most species are medium-sized bushes, but the size range is from creeping species, less than one centimeter in height, to large trees.

The taxonomy of willows is still debated. This is partly due to the extreme morphological variation in many species (Skvortsov 1968, 1999). A further complication is caused by hybridization between species, although this is now considered to be less frequent than previously thought (Skvortsov 1968, 1999; Argus 1973, Rechinger 1992). It is, however, clear that hybridization occurs (Rechinger 1992, Brunsfeld *et al.* 1992, Hardig *et al.* 2000), and in some areas hybrid swarms can be found (Meikle 1992). Many species are polyploid (Dorn 1976, Argus 1973, Hämet-Ahti *et al.* 1998), but apparently the possibility and extent of allopolyploidy

ploid speciation in willows has not been given serious study.

Recently, the problems of willow taxonomy have been tackled using molecular methods, but they have not proved successful in reconstructing the phylogeny of the group. Surprisingly, this is explained by a lack of informative variation in molecular markers that are generally highly variable in other plant genera (Leskinen & Alström-Rapaport 1999, Azuma *et al.* 2000, C. K. Anttila, *personal communication*).

There is, however, agreement that morphologically, the genus *Salix* can be divided into three (Skvortsov 1968, 1999) or four (Argus 1997) subgenera, of which the subgenus *Salix* is the most primitive (Skvortsov 1968, 1999). The subgenus *Salix* is also the first one to appear in the fossil record (Collinson 1992). The subgenus *Chamaetia* includes low arctic species, and the subgenus *Vetrix* mostly bushes (Skvortsov 1968, 1999). The molecular studies indicate, however, that the subgenus *Salix* is paraphyletic, and also that the subgenera *Chamaetia* and *Vetrix* may not be monophyletic (Leskinen & Alström-Rapaport 1999, Azuma *et al.* 2000, see also Dorn 1976, Chong *et al.* 1995).

Chemically, willows are characterized by low-molecular weight phenolic glycosides that occur in various species-specific arrays (Palo 1984, Julkunen-Tiitto 1986, 1989; Shao 1991). Many of these phenolic compounds presumably have a defensive function in willows, since salicylates (Lindroth *et al.* 1988, Lindroth & Bloomer 1991, Lindroth & Hwang 1996), cinnamic acid derivatives (Matsuda & Senbo 1986, Summers & Felton 1994), and flavonoids (Shaver & Lukefahr 1969, Elliger *et al.* 1980, Dreyer & Jones 1981, Harborne & Grayer 1993) have been found to act as feeding or oviposition deterrents, growth inhibitors, and toxins against various herbivores. Willows also contain condensed tannins, a diverse class of high-molecular weight phenolics, that may have detrimental effects on herbivorous mammals (Hagerman & Butler 1992) and

insects (Ayres *et al.* 1997, Mutikainen *et al.* 2000).

It is probable that the presence of numerous phenolic compounds in varying concentrations makes willows a challenging food source. In spite of this, willows are utilized by a wide variety of mammalian and insect herbivores (Kontuniemi 1960, Seppänen 1970, Pasteels & Rowell-Rahier 1992). Some specialist insect herbivores that have overcome the chemical defense of willows use the phenolics as feeding or oviposition cues (Tahvanainen *et al.* 1985, Pasteels & Rowell-Rahier 1992, Kolehmainen *et al.* 1994, 1995; Roininen *et al.* 1999a), or even use the compounds for their own defense (Pasteels & Rowell-Rahier 1992, Köpf *et al.* 1998). There are hundreds of insect species adapted to use willows as food, and one of the most conspicuous and common groups is formed by the nematine gallers.

2.2. The nematine gallers

Within the large symphytan family Tenthredinidae, gall induction has evolved twice, in the subfamilies Blennocampinae and Nematinae (Price & Roininen 1993). Of these, the former includes only a couple of species that induce galls on Caprifoliaceae species (Price & Roininen 1993). By contrast, the subfamily Nematinae includes hundreds of galling and non-galling species (Smith 1979, Viitasaari & Vikberg 1985, Gauld & Bolton 1988). The non-galling species have various larval habits. Most of them have typical free-feeding (exophagous) larvae, whereas some have endophytous larvae feeding, for example, in fruits, leaf mines, berries, or willow catkins (Smith 1979, Gauld & Bolton 1988, Zinovjev & Vikberg 1998).

The gall-inducing nematines occur almost exclusively on *Salix* species; the few exceptions feed on the closely related *Populus* (Price & Roininen 1993). There are over 200 species of gall-inducing nematines, which, like their host plants, are common in arctic and subarctic regions (Smith 1979,

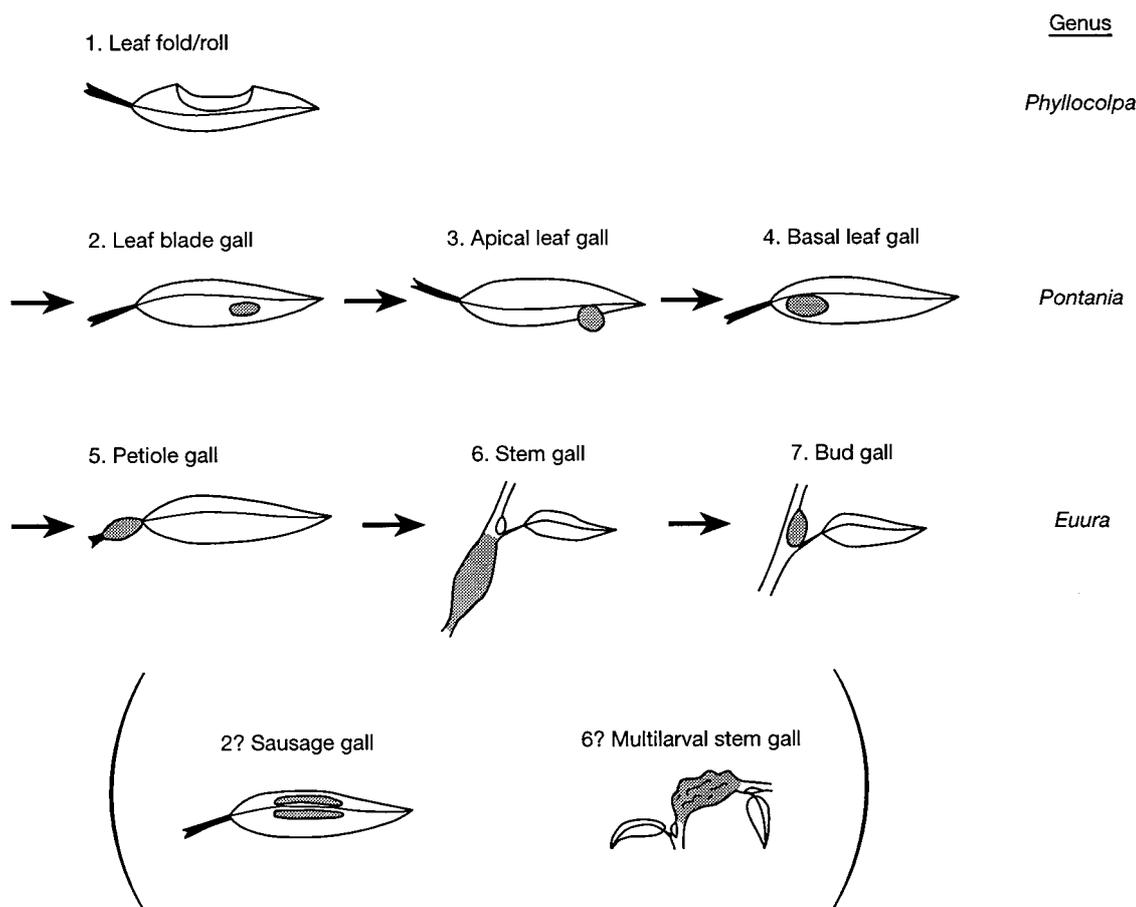


Figure 1. The different gall types induced by nematine sawflies on willows. Arrows indicate the Price-Roininen hypothesis of how the gall types have evolved (Price 1992; see also Roininen 1991a, Price & Roininen 1993). The three genera of nematines are indicated to the left of the gall type figures.

Kouki *et al.* 1994). These species have traditionally been classified in the subtribe *Euurina* (Vikberg 1982). *Euurina* is divided into three genera based on the type of gall that the species induce: *Phyllocolpa* species induce leaf folds or rolls, *Pontania* species induce bean- or pea-shaped galls on leaves, and *Euura* species induce galls on midribs, petioles, buds, or stems (Smith 1979, Price & Roininen 1993). In all, about eight different gall types can be recognized in the nematine gallers (Fig. 1).

Typically, the galling sawflies emerge in the spring or early summer, mate, and the females then oviposit into young growing tissues of the willow hosts (Price 1992). Gall

formation is initiated by a secretion that the females inject into the oviposition wound together with the egg (McCalla *et al.* 1962, Rey 1992, Leitch 1994, Higton & Maberley 1994). In some species, further gall development requires additional stimulation by the growing larva (McCalla *et al.* 1962, Smith 1970, Zinovjev & Vikberg 1998). The larvae grow during the summer and emerge in late fall to pupate, usually on the ground (Smith 1970, Price 1992, Kopelke 1998). Some species, especially leaf folders, can produce multiple generations per summer (Smith 1970, Price 1992).

Most nematine gallers are fairly host-specific, but the degree of specificity may

vary between the three genera. Smith (1970) argued that the *Phyllocolpa* leaf folders/rollers are the least host-specific (oligo- or polyphages), whereas the *Pontania* galls are more restricted in their host range, and *Euura* species are almost strictly restricted to one willow species (see also Zinovjev 1995). Such a generalization should, however, be treated with caution, since the species in *Euurina* are very difficult to identify by morphological methods, and in some cases oviposition and/or crossing experiments may provide the only possibility for distinguishing between the species (Smith 1970, Kopelke 1990a, 1990b, 1991; Zinovjev 1993). Molecular methods might also provide tools for species identification in the nematines, but so far they have been used only rarely (Roininen *et al.* 1993, III).

3. THE EVOLUTION OF DIFFERENT GALL TYPES

3.1. Hypotheses

The existence of numerous different gall types naturally raises the question of the order in which these types have evolved. Theoretically, multiple gall types could have evolved independently, but most authors have assumed that the galling species form a monophyletic group (Gauld & Bolton 1988), although this view has also been disputed (Zinovjev & Vikberg 1999). In addition, if the galling species are monophyletic as a whole, similar gall types could have evolved independently on different willow species, or each gall type could have arisen only once, with subsequent radiations to multiple willow species (Smith 1970, Price & Roininen 1993).

Price (1992; see also Roininen 1991a, Price & Roininen 1993) suggested that species inducing true closed galls evolved via leaf folders/rollers, and that leaf blade galls (*P. proxima*-type) would be the first true galls to appear. Thereafter, the sequence would have been apical leaf galls – basal

leaf galls – midrib/petiole galls – stem galls – bud galls (Fig. 1). The Price-Roininen hypothesis is based mainly on the location of the gall on the host, so that the oviposition site would have “wandered” towards the more central parts of the host plant (Price & Roininen 1993).

Similar views had been proposed earlier by Smith (1970). Smith claimed that the most primitive galler species were the *Phyllocolpa* leaf rollers, since the color of their larvae resembles the larval coloration of the nematine species that have free-feeding larvae. Smith also proposed that the *Pontania* species closest to the *Phyllocolpa* species belong to the *P. proxima*-group (leaf blade galls). The first *Euura* species would be the *Euura (Gemmura)* bud galls, as their ovipositor shape is intermediate between *Pontania* and other *Euura* species. The next ones to evolve would be the *Euura* petiole galls, and the last ones the *Euura* stem galls.

Although there are differences between the two hypotheses, they share the view that each gall type has arisen only once, followed by a subsequent radiation to new willow species. The hypotheses are also essentially gradualistic in the sense that gall type shifts would have occurred in small steps. The gradualistic view is, indeed, tempting, since gall formation is probably dependent on the exact positioning of the gall-forming substance (McCalla *et al.* 1962, Kopelke 1990a). Thus, a change in gall type would require multiple, co-occurring changes in the behavior of the ovipositing females and in the shape of the ovipositor. In the initial stages of a gall type shift the population must be polymorphic, which seems much more probable if the gall types are relatively similar. This constraint is, however, somewhat relaxed by the fact that oviposition occurs during the early development of the willow shoots when different parts of the leaves are in close proximity, which can enhance the possibility of oviposition jumps that appear more dramatic than they really are (Price & Roininen 1993).

The evolution of different gall types was studied by reconstructing the phylogeny of representative nematine species using allozyme electrophoresis (I) and DNA sequence data (II). The sequence in which the gall types evolved could then be inferred by plotting the gall types on the resulting phylogenies.

3.2. Allozyme results

The evolution of different gall types was first studied by using allozyme electrophoresis to reconstruct the phylogeny of 18 nematine species (I). The selected species included 1-3 species per gall type. Four non-galling nematine species were used as an outgroup. Eight informative enzyme loci were screened using standard starch gel electrophoresis according to the protocols outlined in Vuorinen (1984).

The data was analyzed by maximum parsimony methods in PAUP version 3.1.1 (Swofford 1993). The loci were treated as characters, and the allelic combinations as character states (Character = Locus coding; Buth 1984, Mabee & Humphries 1993, Mardulyn & Pasteels 1994). The numbers of steps between character states were defined using the step matrix option in PAUP. Gains and losses of alleles were weighted equally. Hypothetical ancestral states were created when needed, as described by Mardulyn and Pasteels (1994). Branch support was determined by bootstrapping 100 times over loci (Felsenstein 1985) and by determining the Decay index (Bremer 1988, 1994) for each branch. The evolution of gall types was inferred by plotting the (unordered) gall types on the trees using MacClade version 3.01 (Maddison & Maddison 1992). Restrictions in gall type evolution were tested by comparing the length of the original (ordered) gall type character to the character length distribution from 200 random characters, in which states had been shuffled between gall type groups.

The Character = Locus analysis produced two equally parsimonious trees, both of

which could be rooted so that the ingroup remains monophyletic (Fig. 2 in I). The two trees differ only in the structure of the *Euura* clade. In addition, there is a polytomy at the base of the *Euura* clade. If the polytomy is resolved in all possible ways, there are six equally parsimonious trees. The bootstrap supports of most branches were relatively low, and Decay indices ranged from one to three.

When the gall types are plotted on the phylogeny (Fig. 4 in I), it can be seen that: (1) leaf folders do indeed seem to be a basal group; (2) leaf blade gallers evolved independently of other true gallers (i.e., the species forming closed galls are polyphyletic); (3) apical and basal leaf gallers are not the ancestors of petiole and bud gallers, but they may share a common galling ancestor; (4) bud gallers evolved from midrib/petiole gallers; and (5) stem gallers are polyphyletic. The results also show that gall type changes occur less frequently than host shifts, and that there are restrictions affecting the evolution of different gall types: >95% of the randomized gall type characters are longer than the original character (Fig. 3 in I).

3.3. DNA results

In order to gain a more robust view of the evolution of gall types, a second study was made, using DNA sequence data and a larger number of sawfly species (II). In this study, 31 galler species representing seven different gall types were included (1-10 species per gall type), and five non-galling species were used as an outgroup. An approximately 750-bp portion of the mitochondrial Cytochrome *b* gene was amplified from each species using "universal" primers (Simon *et al.* 1994), and the amplified fragments were sequenced using an ABI 310 automated sequencer (Perkin Elmer, Foster City, CA). The sequences (678 bp) could be unambiguously aligned, since no insertions/deletions were observed.

Maximum-parsimony analyses of the data set were performed using PAUP* ver-

sion 4.0b1 (Swofford 1998). The analyses were first performed using 3:1 transversion:transition weights (216 informative characters), and then transversions only (123 informative characters). Unweighted maximum parsimony was not used, because a plot of transitions and transversions against sequence divergence indicated that transitions have become saturated (Fig. 2). Branch supports were again determined by bootstrapping (1000 replicates) and by calculating Bremer's (1988, 1994) support indices. In addition, the data was analyzed using quartet puzzling maximum likelihood analysis in PUZZLE version 4.0.1 (Strimmer & von Haeseler 1999), with parameters estimated from the data and a neighbor joining tree. Again, the evolution of different gall types was inferred by plotting the (unordered) gall types on the resulting phylogeny, and the possibility of restrictions in gall type evolution was inferred by comparing the length of the original (ordered) gall type character to the character length distribution of 1000 randomized characters, in which gall types had been shuffled between groups (Fig. 3).

The different phylogenetic analyses resulted in essentially identical results with

high support for important branches (Fig. 1 in II). The weakly supported branches are mainly within the outgroup and in the clade formed by the apical leaf galls (*P. viminalis*-group). The trees could not be rooted so that the ingroup remains monophyletic, because *Phyllocolpa anglica* Cameron is grouped within the outgroup. However, this grouping is not strongly supported, and if ingroup monophyly is enforced, the tree does not become significantly less parsimonious (Templeton's test, $P = 0.63$).

The phylogeny and the reconstruction of the evolution of different gall types (Fig. 2 in II) indicate that: (1) the nematine gallers probably form a monophyletic group; (2) true closed galls evolved only once, via leaf folders; (3) the genus *Euura* and subgenus *Eupontania* Zinovjev (apical + basal leaf galls) are monophyletic; (4) with the possible exception of leaf rollers, all gall type groups are mono- or paraphyletic; (5) similar gall types are closer on the phylogeny than would be expected by a random process; and (6) there is an apparent evolutionary trend in galling site from the leaf edge towards the more central parts of the host plant.

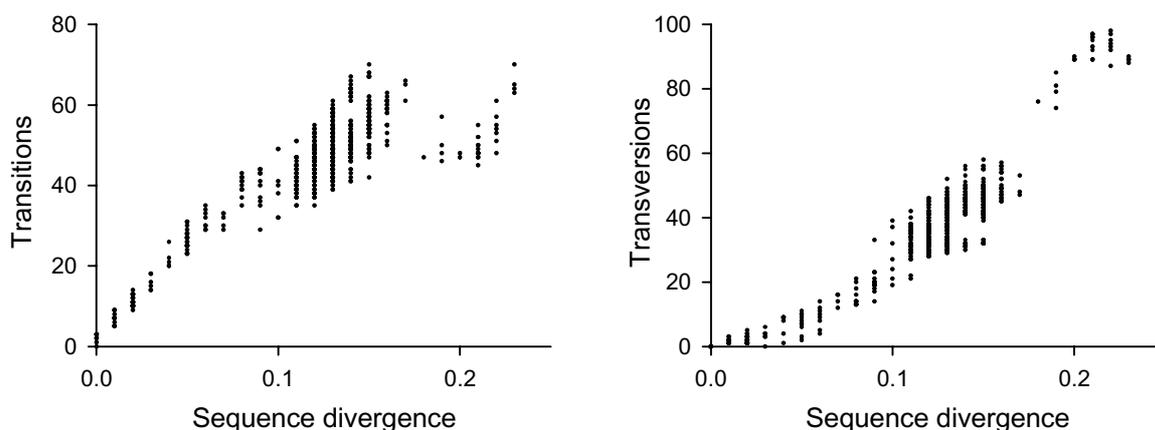


Figure 2. Numbers of transitions and transversions plotted against sequence divergence in pairwise comparisons of the DNA sequences used in (II). Note the saturation in transitions after ca. 10-15% sequence divergence.

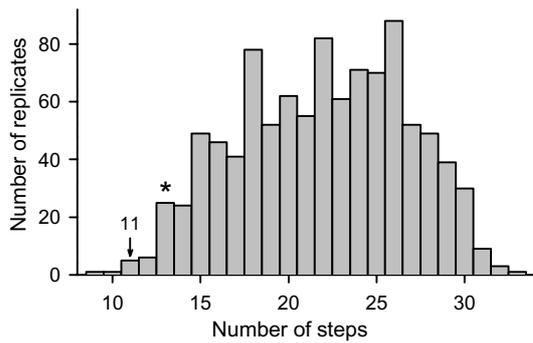


Figure 3. Frequency distribution of the number of steps in 1000 random (ordered) gall type characters when the tree is as shown in Fig. 1 in (II). Gall types were shuffled between gall type groups, but the outgroup state (= 0) was kept constant. Alpha = 0.05 level shown by star, number of steps in the original gall type character (= 11) shown by arrow (the *P. dolichura* samples and *E. americanae* were excluded from the analysis).

3.4. Combined evidence

Although there are slight differences between the allozyme tree and the DNA sequence tree, they also have important common features. For example, both trees agree that the species that induce true closed galls evolved via leaf folders, as had been suggested already by Smith (1970). As stated above, the DNA tree could not be rooted so that the ingroup remains monophyletic, because *Phyllocolpa anglica* is grouped within the outgroup. However, the exact location of *P. anglica* does not affect the inferences on the sequence in which the gall types evolved. It must be noted that Zinovjev and Vikberg (1999) claimed that the genus *Phyllocolpa* is rather heterogeneous, and that the leaf-rolling habit may be polyphyletic (see also Vikberg 1982).

Both phylogenies leave open the question of the next step in the evolutionary history. The inclusion of the *P. proxima*-group within the *Phyllocolpa* species in the allo-

zyme tree is probably erroneous, and if monophyly of the closed galls is enforced in the allozyme tree, the tree does not become significantly less parsimonious (Templeton's test, $P = 0.32$). Thus, it is likely that the species inducing true closed galls are monophyletic (as indicated by the DNA tree), and that the first species to appear were leaf blade gallers (*P. proxima*-group) or sausage gallers (*P. dolichura*-group). In both of these gall types, an inner green layer can be found, as in the rudimentary galls that form at the site of egg-laying in some leaf rollers (Zinovjev & Vikberg 1998). Furthermore, ovipositing females in the *P. dolichura*-group make multiple insertions with their ovipositor, and this habit is also present in the *Phyllocolpa* leaf folders/rollers (Kopelke 1985, 1998; Price & Roininen 1993). It is also interesting to note that late-instar larvae of some species in the *P. proxima*- and *P. dolichura*-groups occasionally come out of the gall to feed on the leaf edge (Roininen *et al.* 1999b).

The allozyme and DNA trees both support the monophyly of the *Euura* + *Eupontania* clade, but it is unclear what the ancestral gall type in the group was. Considering the similarities in the structure of the galls induced by *Pontania* s. str. gallers (leaf blade gallers + sausage gallers) and *Eupontania* basal leaf gallers, it seems very probable the ancestor induced galls on leaves, but a confirmation of this will require further study. It is possible that the ancestral gall type can be found by including more species in the analysis. The grouping of *E. atra* within the *Eupontania* in the allozyme tree is clearly erroneous, and *E. atra* can be moved to the *Euura* clade without the allozyme tree becoming less parsimonious (Templeton's test, $P = 0.18$). The DNA tree indicates that the apical leaf gallers evolved from basal leaf gallers, and this view is not contradicted by the allozyme evidence. Both the allozyme and DNA results contradict Smith's (1970) suggestion that *Euura* gallers evolved via the *Euura* (*Gemmura*) bud gallers.

The main result of both phylogenetic analyses is that similar gall types tend to be closer on the phylogenies than would be expected by a random process. This indicates that gall type changes have occurred in small steps, which is in accordance with the traditional gradualistic view of evolution, and seems reasonable, given the need for a polymorphic condition in the galler population during a gall type shift. Both studies also suggest that there has been a trend towards gall induction in the more basal parts of the host plants.

3.5. Discussion

The fact that gall morphology reflects the phylogeny of the gallers, not the phylogeny of the willow hosts, demonstrates that the location and shape of the gall is determined mainly by the galler, not by the host. Thus, gall morphology can be considered to represent an extended phenotype (*sensu* Dawkins 1982) of the galler. This interpretation is essentially identical to the results obtained from phylogenetic studies of gall-inducing psyllids (Yang & Mitter 1994), aphids (Stern 1995), cynipid wasps (Stone & Cook 1998), and thrips (Crespi & Worobey 1998), and the view has also been supported by some nonphylogenetic studies (Dodson 1991). To date, the only exception seems to be in the forinine aphids that induce galls on *Pistacia* species, since Wool (1997) suggested that in this case the host plants may play a more important role in the control of gall morphology than the insects. Furthermore, some studies indicate that the host plant can have an effect on the *size* of the galls (Price & Clancy 1986, Weis 1996, Crespi & Worobey 1998).

Many common features are shared by the independent radiations of diverse insect gallers on unrelated host plants. For example, there are apparently constraints on how gall morphology can change, since gall types seem to change gradually in sawflies (I, II), psyllids (Yang & Mitter 1994), cynipid wasps (Stone & Cook 1998) and thrips (Cre-

spi & Worobey 1998). Interestingly, the galling habit seems to be preceded by leaf folding also in the thrips that induce galls on *Acacia* species (Crespi & Worobey 1998). This suggests that leaf folding can act as a preadaptation that may lead to the evolution of true closed galls.

Assuming that the sawfly gallers control the location and morphology of the galls, then why do these features change? The reasons for changes in gall type could be adaptive. If parasitoids or predators search for galls according to stereotypical search patterns, changes in gall morphology could help the gallers to avoid these mortality factors (Cornell 1983). This may well be the case in the genus *Pontania*, where there are great differences between the parasitoid communities on different gall types (Kopelke 1985, 1994). For example, in sympatric populations of *P. samolad* Malaise (an apical leaf galler) and *P. lapponica* Malaise (a basal leaf galler) on *Salix lapponum* L., the parasitoid *Adelognathus cubiceps* Roman occurs only in *P. lapponica* galls (Roininen *et al.* 1999c).

It is also possible that shifts in gall type are nonadaptive. In the initial polymorphic stage, the galler could induce different galls on different hosts. After speciation, the species with the novel gall type could radiate to new hosts but still retain the newly acquired gall type. Indeed, in some cases nematine gallers have been found to induce abnormal galls on hosts that are not normally used by the galler (Zinovjev 1994, 1995). This could, for example, be a result of differences in size or phenology between willows: the length of the sawfly ovipositor might not be suitable for oviposition at the appropriate site on a novel host. This could explain, among other things, the transition from bud gallers to petiole gallers, since in both groups oviposition occurs through the petiole base (Smith 1968, Roininen 1988, Kopelke 1998).

However, the trend-like pattern observed in the evolution of the galling site suggests that adaptive explanations may be needed. There are several factors that might promote

oviposition in more basal parts of the hosts. For example, the reason could be linked to the control of resource allocation in the plant. Galls have been shown to behave as sinks for nutrients and photosynthetic assimilates in plants (Larson & Whitham 1991, 1997), and, thus, intraspecific competition could lead to unequal transition probabilities, if galls can intercept nutrient flow to other galls situated in more distal positions. This type of competition-driven evolution of galling site was also suggested by Yang and Mitter (1994) in the case of psyllid galls on American *Celtis* species. The suggestion is not entirely speculative: in pemphigine aphids, females fight for the basal parts of the leaves (Whitham 1979), where reproductive success is higher (Whitham 1978). In the oak leaf galler *Cynips divisa* Hartig, gall location in relation to other galls and the midrib affects gall size and growth (Sitch *et al.* 1988, Hartley 1998) and galler mortality (Gilbert *et al.* 1994). Fritz *et al.* (1986) demonstrated the possibility of asymmetric competition between the stem galler *E. lasiolepis* Smith and three other species representing different gall types on *Salix lasiolepis* Benth. Obviously, this explanation is dependent on the abundance of the gallers, and it is not known whether galler densities are normally high enough for serious competition to occur.

Competition, however, is not necessarily needed for selection favoring oviposition in more basal parts of the plant. Gall induction in basal positions could help in evading abscission reactions (Williams and Whitham 1986), and, as Larson and Whitham (1991) demonstrated, the sink strength of galls depends on their location. In their study of aphid galls, a difference of 12 mm caused a fourfold difference in the amount of resources drawn to the gall from adjacent leaves. This kind of positional effect may be unimportant under normal conditions, but it may, on the other hand, be important when resources are scarce.

4. THE EVOLUTION OF HOST PLANT USE

4.1. Hypotheses

Phytophagous insects have been used extensively to study various questions concerning the evolutionary relationships between animals and plants. In this respect, insects are a good model group, because they are abundant, speciose, and they reproduce rapidly. Furthermore, although some insect groups have been associated with the same host plants for millions of years (Farrell *et al.* 1992, Wilf *et al.* 2000), it is evident that host-associated evolutionary changes can occur very rapidly in some species (Bush 1975, Carroll & Boyd 1992, Radkey & Singer 1995, Carroll *et al.* 1997, 1998; Groman & Pellmyr 2000).

Insect herbivores are commonly more or less restricted in their use of potential host plants: most species feed on one or a few host species, and usually the hosts are closely related (Jermy 1984, Bernays & Graham 1988). This restricted diet is usually attributed to the “jack of all trades, master of none” hypothesis, which predicts that a given insect species cannot be adapted to the various chemical defenses of all potential host plants (Dethier 1954, Fraenkel 1959, Ehrlich & Raven 1964, Futuyma & Keese 1992). Restrictions in terms of host use can also be caused, for example, by phenological differences between hosts (Smith 1988, Craig *et al.* 1993, Groman & Pellmyr 2000), differences in predation/parasitism on hosts (Bernays & Graham 1988, Crespi & Sandoval 2000), and morphological or habitat differences between hosts (Futuyma & Keese 1992).

In fact, it appears that many phytophagous insects utilize only a small fraction of the host plants that are suitable as food for them (Wiklund 1975, Smiley 1978, Roininen & Tahvanainen 1989, Nylin & Janz 1999). This has been explained by constraints on the information processing capacities of the insects: if some of the potential host plants

can be confused with unsuitable hosts, the situation could lead to the evolution of an “unnecessarily” narrow host range (Levins & MacArthur 1969, Futuyma 1983, Courtney 1983, Janz & Nylin 1997).

The causes of host specificity are directly linked to the evolution of host shifts by monophagous insects. During a host shift, the insect species must be at least oligophagous and, thus, it is likely that the direction of host shifts is constrained by some of the aforementioned factors. For example, it has been suggested that a given insect species is more likely to colonize hosts that are chemically (Dethier 1954, Ehrlich & Raven 1964, Futuyma & McCafferty 1990, Becerra 1997, Wahlberg 2000) or ecologically (Dobler *et al.* 1996) similar to the original host. On the other hand, specialist insects could speciate simply by tracking the speciation events in their hosts (Mitter & Brooks 1983, Mitter *et al.* 1991). It has to be noted that speciation is not an automatic outcome of the colonization of a new host. Especially if the new host closely resembles the original host, the end result will only be an expansion of the insect’s feeding range (Thompson 1994, Groman & Pellmyr 2000).

Insect herbivores have also been studied extensively in order to find out whether sympatric speciation is possible in nature. Many phytophagous insect species exhibit features that are likely to enhance the possibility of sympatric speciation: mating commonly occurs on or near the host plant, the adults determine the larval host, and host preference (and performance on different hosts) may be subject to simple genetic control (Bush 1975). Indeed, some of the best putative cases of sympatric speciation are from insect herbivores (Bush 1969, 1994; Tauber & Tauber 1989, Barraclough & Vogler 2000). Sympatric speciation has been considered unlikely (Mayr 1963, Futuyma 1983), but mathematical models have shown that it may be possible under some circumstances (Diehl & Bush 1989, Dieckmann & Doebeli 1999).

The evolution of host use in the galling nematines was first studied by using the allozyme (I) and DNA phylogenies (II) to interpret the patterns of host associations in relation to the aforementioned hypotheses. The evolution of host associations was then further studied by analyzing host-associated enzyme electrophoretic variation in the presumed polyphagous bud galler *Euura mucronata* Hartig (III).

4.2. Host-plant relationships in the nematines

The allozyme study (I) indicated that host associations are rather labile in comparison to the evolution of gall types, and this result was confirmed by the results of the DNA study (II). It is clear that many willow species have been colonized several times. For example, *S. lapponum* L. has been colonized at least three times independently by species representing different gall types, and *S. phylicifolia* L. and *S. pentandra* L. at least two times each (Fig. 1 in II). Evidently, such a pattern of host associations excludes any coevolutionary hypotheses in the sense of gallers “tracking” speciation events in willows (Fig. 4). Considering the host plant records of Eurasian galler species not included in this study (Table 2 in II, Zinovjev 1998), it seems likely that the numbers of independent colonizations for many *Salix* species are actually considerably higher.

Furthermore, the results are not concordant with the escape-and-radiate model of coevolution (Ehrlich & Raven 1964), since there cannot be any concordant clades in the willow and galler phylogeny (Thompson 1999). Likewise, there is no clear connection between the phylogeny of the nematine gallers and the overall chemical similarity (Julkunen-Tiitto 1986, 1989; Shao 1991) of willows.

In the DNA study (II), the subgenera of the willow hosts were plotted on the galler phylogeny, but a randomization test did not indicate any statistically significant conservatism in host use even at the level of willow

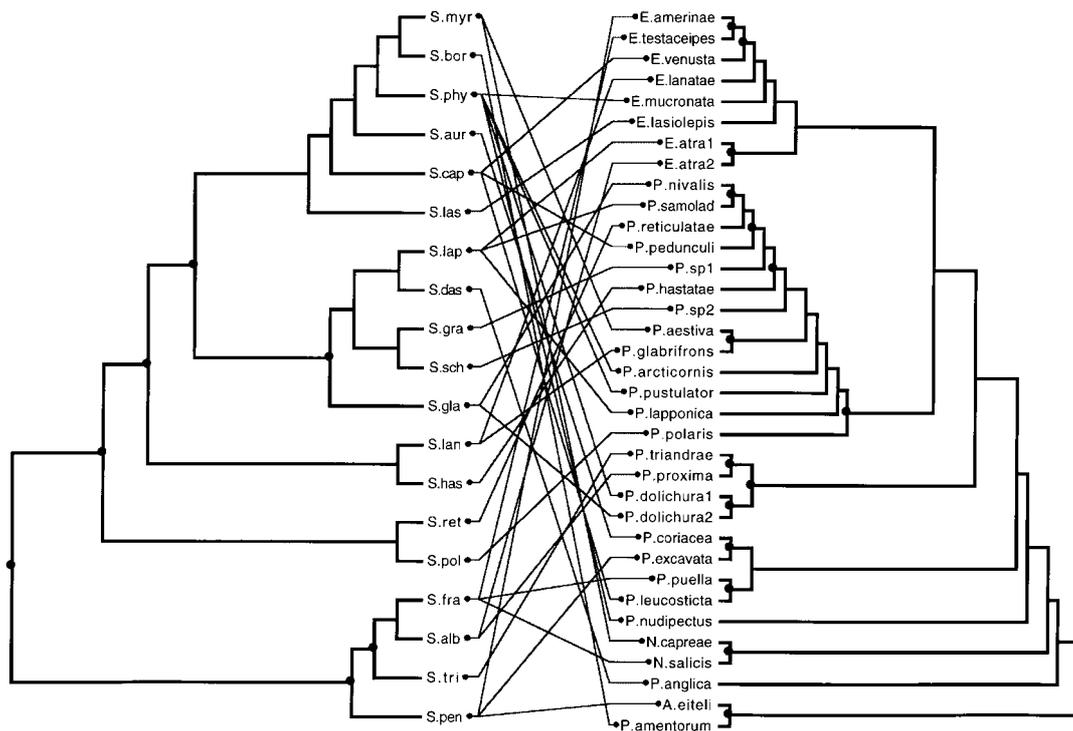


Figure 4. The evolution of host associations in nematine sawflies, plotted using TreeMap version 1.0b (Page 1995). The phylogeny of gallers is from (II) (*T. arquata* has been excluded), the willow phylogeny is mainly according to the phenetic tree of Argus (1997). Locations of host species not included in Argus (1997) are according to Skvortsov (1968, 1999). Potential cospeciation events are shown by black dots on the nodes of the sawfly tree. Hosts are abbreviated by using only the three first letters of the species name (e.g., *S. lan* = *Salix lanata*).

subgenera. This could, however, partly be a result of the limited sampling of species feeding on the subgenera *Salix* and *Chamaetia*. When all available host plant records are considered, there are signs of conservatism in host use (Table 2 in II).

4.3. *Euura mucronata*: a polyphage or a sibling species complex?

The results from the phylogenetic analyses (I, II) raise a problem: the monophyletic genus *Euura* contains several sibling species complexes comprised of strictly monophagous species (Smith 1968, 1970; Kopelke 1996), but the rather derived bud-galling *Euura mucronata* is considered to be polyphagous. According to the available host

records, *E. mucronata* induces galls on over 30 willow species in the Palearctic and Nearctic Regions (Smith 1970, Hartley 1992, Price & Roininen 1993, Kopelke 1998, H. Roininen & A. G. Zinovjev, *unpublished data*). Thus, the evolution of bud galling would have been accompanied by a switch from monophagy to extreme polyphagy.

Although switches from monophagy to polyphagy can and do occur (Thompson 1994, Kelley & Farrell 1998, Nylin & Janz 1999), the case of *E. mucronata* should be interpreted with caution. In many cases, insect species presumed to be polyphagous have been found to consist of numerous morphologically indistinguishable monophagous sibling species or host races (Mayr 1963, Diehl & Bush 1984) that may be iden-

tifiable only by using molecular markers (see, e.g., Menken 1981, Sturgeon & Mitton 1986, Feder *et al.* 1988, McPheron *et al.* 1988, Herbst & Heitland 1994, Emelianov *et al.* 1995, Condon & Steck 1997, Groman & Pellmyr 2000). Furthermore, even the stem-galling *Euura atra* complex was previously considered to be one polyphagous species until it was discovered that it is probably a sibling species complex (Roininen *et al.* 1993, Kopelke 1996). *E. mucronata* has been split into three species based on morphological features, but the feeding ranges of the species, and even their existence, is unclear (Viitasaari & Vikberg 1985, Kopelke 1998, Zinovjev & Vikberg 1998). This is partly a result of extreme size variation in the bud galls (Benson 1958).

To reveal the specific status of *E. mucronata*, enzyme electrophoretic variation was studied in bud galls collected from different willow species occurring in the surroundings of the Abisko research station in Sweden and the Kilpisjärvi research station in Finland. The distance between the two locations is approximately 120 km, which should be well above the dispersal capabilities of individual sawflies. Collections were made from randomly selected willow individuals in six host species (*S. lanata* L., *S. glauca* L., *S. lapponum* L., *S. phylicifolia* L., *S. myrsinifolia* Salisb., and *S. hastata* L.). In both locations bud galls can also be found on *S. myrsinites* L., but not enough larvae were found on this host for analyses. The larvae were reared to adults, which were sexed and then frozen until electrophoresis.

Seven variable enzyme systems were surveyed using standard starch gel electrophoresis, mainly according to the protocol outlined in Vuorinen (1984). The data was first analyzed by performing preliminary clustering analyses using the TFPGA version 1.3 program (Miller 1997). Population structure was then further studied by calculating hierarchical *F*-statistics (Wright 1978) within the subgroups formed by the clustering analyses. Fixation indices were calculated according to the method of Weir &

Cockerham (1984) using the Arlequin version 2.000 program (Schneider *et al.* 2000).

The results show that: (1) in northern Fennoscandia, "*E. mucronata*" comprises at least three mono- or oligophagous species or host races, but occasional hybridization between the species may occur; (2) the pattern of host use excludes the possibility of parallel cladogenesis between the bud galls and their hosts; (3) the overall chemical similarity of the hosts does not explain the pattern of host use; and (4) simple allopatric speciation does not seem to provide a sufficient explanation.

4.4. Discussion

Both the overall nematine phylogeny (II) and the pattern of host use in *E. mucronata* (III) exclude an explanation based on simple tracking of host speciation. This does not, however, imply that host phylogeny has no effect. For example, species in the monophyletic subgenus *Eupontania* Zinovjev (apical + basal leaf galls) are almost exclusively associated with willow species in the subgenera *Chamaetia* and *Vetrix* (Zinovjev 1993, 1995, 1998). Furthermore, there are differences in how separate groups of nematines use the three willow subgenera (Table 2 in II), which can be taken as an indication of constraints and phylogenetic inertia in host use. A similar combination of conservatism and promiscuity in host use has been observed in galling thrips (Crespi *et al.* 1997).

It must be noted that strictly concordant phylogenies between phytophagous insects and their hosts are probably rare (Mitter *et al.* 1991). In fact, the best demonstrated cases of parallel cladogenesis come from mammals and their ectoparasites (Hafner & Nadler 1988), and insects and their endosymbionts (Moran *et al.* 1993, Clark *et al.* 2000). The close association between the galling insects and their hosts would seem to predispose the system for parallel cladogenesis, but, on the other hand, it would be unlikely if the mechanism of gall induction is

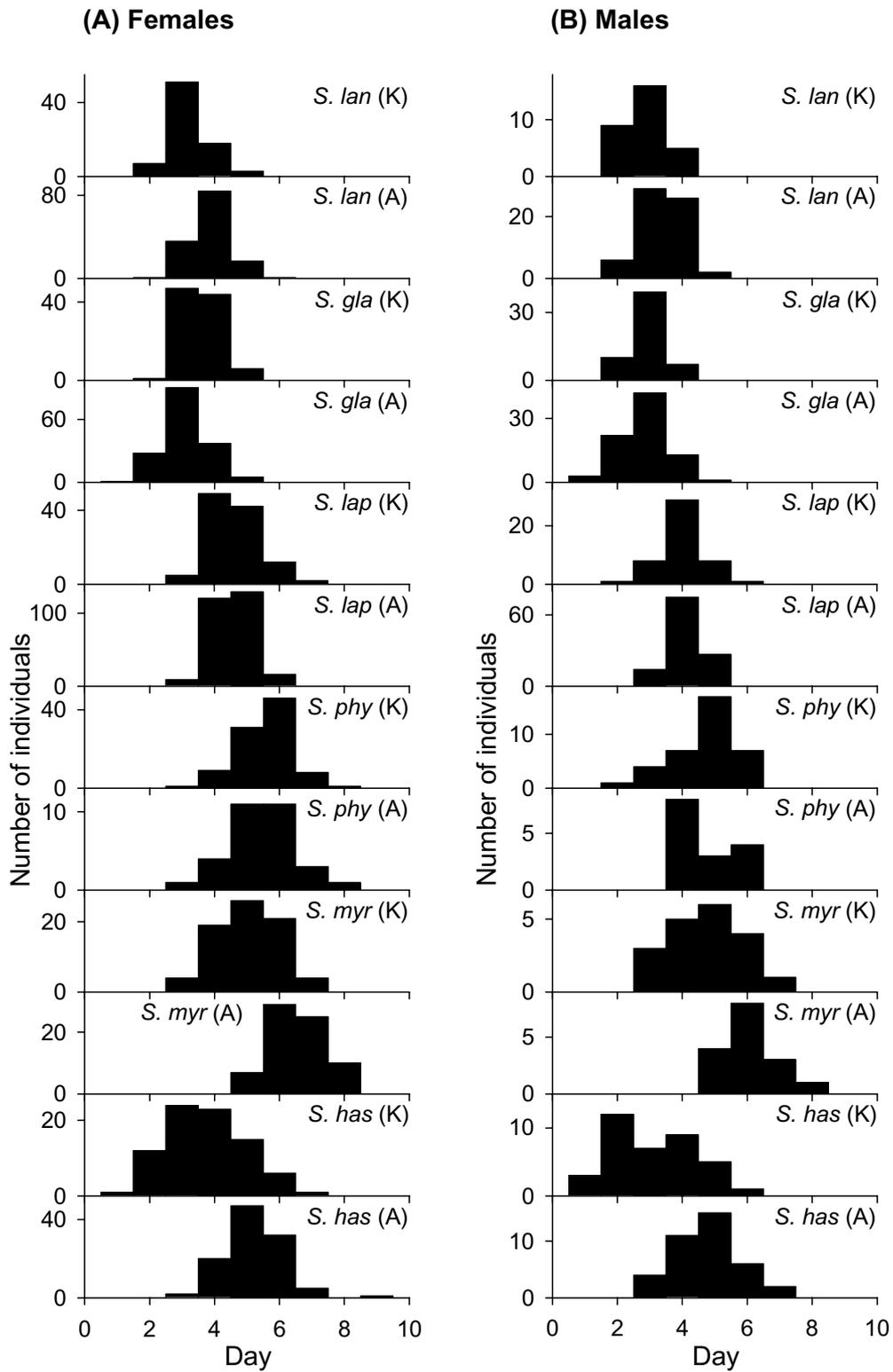


Figure 5. Emergence times of the *E. mucronata* adults used in (III). On the x-axis, 1 = the day when the first individual emerged. Hosts are abbreviated by using only the three first letters of the species name (e.g., *S. lan* = *Salix lanata*). Locations are shown in parentheses after the host name: K = Kilpisjärvi, A = Abisko.

general enough to produce galls on phylogenetically distant host species.

The studies also show that the evolution of host use in the nematines is not connected with the overall chemical similarity (Julkunen-Tiitto 1986, 1989; Shao 1991) of the willow hosts. Similar results were earlier found by Roininen *et al.* (1993) in the *Euura atra* complex. This is not surprising considering that the chemical properties of galls may differ radically from that of the host plants (Hartley 1992, 1998; IV). Furthermore, if diet chemistry is not important, then the oviposition behavior of females may be determined by only a few chemical compounds. Results indicating that this is the case have been found in the stem galls *Euura amerinae* L. (Kolehmainen *et al.* 1994) and *E. lasiolepis* Smith (Roininen *et al.* 1999a).

Recent studies have emphasized the importance of phenological adaptation to dif-

ferent host species, and the resulting possibility of allochronic speciation (Craig *et al.* 1993, Feder *et al.* 1997a, 1997b; Filchak *et al.* 2000, Groman & Pellmyr 2000, Tikkanen 2000). It is possible that phenological factors influence the pattern of host use in the nematines, but this is more difficult to study. The possibility should, however, be taken seriously, and at least in the case of *E. mucronata* there seem to be differences in the emergence times of adults collected from different willow species (Fig. 5). The oviposition period of *E. mucronata* lasts for weeks (Roininen 1991b), but this does not mean that timing is irrelevant. For example, the weight of the larvae may generally be lower in the more distal positions on the shoots (Fig. 6), which may have consequences if fitness correlates positively with adult weight, as has been demonstrated in many insect species (see, e.g., Charnov *et al.* 1981, Haukioja & Neuvonen 1985, Ueno 1999). If there are phenological differences between willows, it is possible that the most “profitable” host species changes during the summer.

Another possibility is that the sizes of the oviposition target tissues vary between willow species. Gall induction in the nematines require exact positioning of the egg in the host tissue (McCalla *et al.* 1962, Kopelke 1990a), which means that size differences between willow species could restrict the range of available hosts for an individual sawfly. The possibility is especially relevant in certain *Euura* species that are extremely variable in size (Benson 1958, Smith 1970). It should be noted that if the willow host affects the size of adult sawflies (via the size of galls), and if sawflies exhibit size-dependent assortative mating, then reproductive isolation between host races could evolve almost automatically. The result would essentially be analogous to the “conditioning to the larval host” effect, which can enhance the likelihood of sympatric speciation (Bush 1975). Indeed, the galling habit may even predispose insects to “accidental” sympatric speciation.

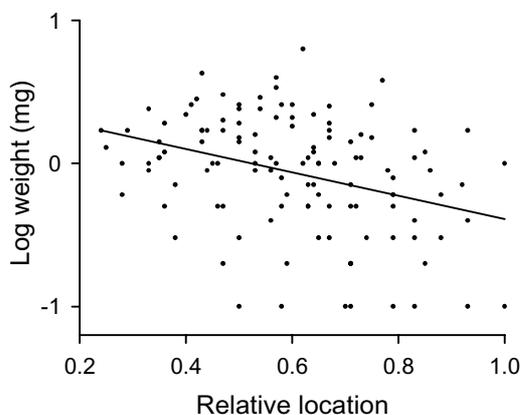


Figure 6. Log-transformed weight (mg) of *E. mucronata* larvae in different relative positions on *S. phlyicifolia* shoots collected on August 28, 1998, from the surroundings of the Kilpisjärvi Research Station. Relative position = number of the galled bud on the shoot (counting up from the base of the shoot) / the total number of buds on shoot. ($r = -0.35$, $n = 122$, $P < 0.001$).

5. THE CHEMICAL ECOLOGY OF NEMATINE SAWFLIES

5.1. The Nutrition Hypothesis and previous studies

The convergent evolution of the galling habit in highly diverse organisms suggests that galls are beneficial for the organisms that induce them. Price *et al.* (1986) summarized the possible adaptive reasons that have promoted gall induction, and concluded that the three most probable hypotheses are the Microenvironment Hypothesis (“galls create an optimal microenvironment”), the Enemy Hypothesis (“galls protect gallers from predators/parasitoids”), and the Nutrition Hypothesis (“galls are nutritionally of better quality than the host”).

Of these three hypotheses, the Nutrition Hypothesis has been studied most frequently, and it is evident that the chemical properties of galls may differ from the chemistry of the normal tissues of the host plant. For example, Brewer *et al.* (1987) found that the galls induced by five species of cecidomyiid midges on the needles of *Pinus edulis* Engelm. generally had lower concentrations of nitrogen than ungalled needles, but in other inorganic nutrients (P, S, Ca, Mg, Na, Zn, Mn, Cu, B) the pattern was not as clear. Low levels of nitrogen have also been found in galls induced by homopterans (Andersen & Mizell 1987), cynipid wasps (Hartley & Lawton 1992, Hartley 1998) and dipterans (Hartley 1998). On the other hand, in some galler-host pairs, nitrogen levels are higher in galls than in comparable plant tissues (Abrahamson & McCrea 1986, Hartley 1998). Several studies have found increased levels of starch (Shannon & Brewer 1980, Bronner 1992, De Bruyn *et al.* 1998) and proteins (Price *et al.* 1986, Bronner 1992, Gopichandran *et al.* 1992, De Bruyn 1994, De Bruyn *et al.* 1998) in galls, which seems to support the Nutrition Hypothesis.

The Nutrition Hypothesis also predicts that the levels of plant defense compounds

should be lowered in galls. The support for this prediction is currently vague and contradictory. Although Price *et al.* (1986) found low levels of phenolics in galls induced by the nematine stem galler *Euura lasiolepis* Smith on *Salix lasiolepis* Benthams, many galls have in fact been found to contain relatively high concentrations of phenolics (Abrahamson *et al.* 1991, Gopichandran *et al.* 1992, Hartley 1992, 1998, 1999), especially condensed tannins (Cornell 1983, Ananthakrishnan & Gopichandran 1993, Yang *et al.* 1998).

However, the interpretation of most studies is rather problematic. Many studies have analyzed whole galls (e.g., Shannon & Brewer 1980, Andersen & Mizell 1987, Brewer *et al.* 1987, Gopichandran *et al.* 1992, Hartley 1992, Hartley & Lawton 1992, Yang *et al.* 1998), although it is known that the chemical properties of the outer layers can differ considerably from the chemistry of the inner layers (Cornell 1983, Abrahamson & McCrea 1986, Bronner 1992). Since the larvae feed only on the inner layers of the gall, chemical analyses of whole galls can lead to severely misleading results. Many studies have also analyzed compounds that are of unknown importance to phytophagous insects (e.g., most inorganic nutrients). When defensive compounds have been analyzed, all studies have been performed with low chemical resolution, i.e., they have analyzed total concentrations of various chemical classes (e.g., phenolics) rather than the concentrations of individual compounds. Obviously, such analyses can obscure ecologically meaningful variation in the levels of individual compounds (Berenbaum 1995, Keinänen *et al.* 1999). Thus, for a chemical analysis to be meaningful, it should be carried out separately for different parts of the galls, it should measure compounds with known importance, and it should analyze individual compounds rather than totals of chemical classes.

The purpose of the chemical study (IV) was to gain insights into the chemical ecology of *Pontania* galls by following the

three aforementioned guidelines: (1) the analyses were performed separately for the outer and inner layers of the galls; (2) the analyzed phenolic compounds have demonstrated significant effects on insects; and (3) individual compounds were analyzed by High-performance liquid chromatography (HPLC), and totals were calculated by summing the concentrations of the individual phenolics.

5.2. The chemical properties of *Pontania* galls on willows

Apical leaf galls were collected from six willow species from the surroundings of the Kilpisjärvi research station. Samples were collected from ten individuals per host species. From each willow individual, a leaf with a gall, and the leaf immediately below the galled one, were collected. Each gall was cut open, and the larva was removed. Thereafter the tissue samples were dried at room temperature, and then frozen until analysis. Before the chemical analyses, the outer and inner layers of the galls were separated using a scalpel.

The concentrations of various phenolic compounds in the samples were analyzed using High-performance liquid chromatography (HPLC), essentially according to the protocol outlined in Julkunen-Tiitto *et al.* (1996). This allowed identification and quantification of 36 low-molecular weight phenolic compounds in the samples. Only compounds that could be reliably scored in all species were analyzed, but the phenolics included represent on average over 90% of the absorbance in the chromatograms at 270 nm. In addition, the concentration of condensed tannins was determined using a colorimetric test (Hagerman & Butler 1994).

The data was first analyzed by calculating the mean concentrations of different compounds in each of the four sample classes (gall cortex, gall interior, galled leaf, and ungalled leaf) in each galler-host pair. In addition, compounds were classified into different categories based on their structure

(Seigler 1998), and the total concentrations of the different chemical categories were calculated for each sample class. Differences in the totals were tested by a repeated-measures analysis of variance (RM-ANOVA) design in SPSS version 8.0 (SPSS, Inc., Chicago, IL), using the samples as within-subjects factors and species as between-subjects factors. The overall chemical similarities of samples and sample classes were analyzed using clustering and ordination methods in PC-ORD version 4.01 (MjM Software Design, Gleneden Beach, OR).

The results show that: (1) the concentrations of most low-molecular weight phenolics are clearly lower in gall interiors than in the leaves of the respective host willows, and the levels of virtually all analyzed compounds are lower in gall interiors than in gall cortices; (2) the levels of condensed tannins are elevated in gall interiors, but the highest concentrations are found in the gall cortices; (3) the pattern of the chemical changes indicates that the phenolic biosynthesis of willows is redirected in gall tissues; (4) within each host species, the overall variation in the chemical properties of gall interiors is markedly lower than in the leaf samples collected from the same host individuals; and (5) similarly, the between-species variation in willows is drastically reduced in gall interior samples. Indeed, the maximum level of variation in gall interiors is comparable to (or lower than) within-species variability in leaf samples.

5.3. Discussion

The results clearly suggest that the galls induced by *Pontania* gallers on willows are nutritionally beneficial for the sawfly larvae, because the concentrations of most phenolic compounds are clearly lower in gall interiors than in the leaves of the host plants. As stated above, there is a substantial amount of evidence indicating that the individual compounds that were measured have detrimental effects on insects. On the other hand, the benefits conferred by the reduction in the

levels of low-molecular weight phenolics could be counterbalanced by the increase in the concentration of condensed tannins in the galls. It must be noted, however, that condensed tannins have been found to have a rather low level of toxicity in some feeding tests (Ayres *et al.* 1997), but evidently further studies on the relative toxicity and other harmful effects of the various phenolics are needed.

Additional benefits for the larvae could be caused by the reduction in the *number* of different phenolics present in the gall tissues. It is probable that different defensive compounds can have synergistic effects (Lindroth *et al.* 1988, Harborne & Grayer 1993, Berenbaum 1995, Berenbaum & Zangerl 1999). Consequently, it may be easier for an insect to adapt to one or a few toxins. This is also supported by the fact that many commercial pesticides have lost their effectiveness in one or two decades (McKenzie & Batterham 1994). Conversely, the application of a mixture of compounds can delay the evolution of resistance, or prevent it altogether (Pimentel & Bellotti 1976, Berenbaum & Zangerl 1999).

It is interesting to note that the concentrations of virtually all analyzed compounds are higher in the outer layers of the galls than in the gall interiors, and especially the high concentrations of condensed tannins in the cortices are striking. The pattern is, however, not so surprising considering that the gall must be protected against attack by other phytophages (Schulz 1992), especially if the gall interiors are of a high nutritional quality. High concentrations of tannins have also been found in the outer layers of galls induced by cynipid wasps (Cornell 1983), where they may protect the gall from fungal invasion (Taper *et al.* 1986, Taper & Case 1987).

In addition to the sawfly (IV) and cynipid wasp galls (Cornell 1983), high concentrations of condensed tannins have also been found in galls induced by thrips (Ananthakrishnan & Gopichandran 1993), cecidomyiid midges (Yang *et al.* 1998), and adelgid

aphids (W. J. Mattson, *personal communication*). Although very little is known about the chemical properties of these other insect galls, the abundance of condensed tannins in galls induced by various gallers on highly diverse host plants is a pattern that should be further studied. It could reflect some common principle in the gall-induction process, such as, for example, a general response of plants to gall-inducing stimuli. On the other hand, the pattern could be caused by convergent selection pressures on the insects, which may be able to control the chemical properties of the galls.

In the sawfly galls, different phenolic categories react in a highly coordinated pattern in all the studied galler-host pairs. Since the analyzed compounds are all produced via the phenylpropanoid pathway in willows (Seigler 1998, Cooper-Driver & Bhattacharya 1998), this suggests that the biosynthesis of phenolic compounds is redirected in gall tissues. The observed pattern could result from a specific blocking of only a few branching points in the phenylpropanoid pathway (Fig. 7), which raises the possibility that the mechanism of manipulation is rather simple. For example, the specific enzymes acting at the branching points could be affected somehow in gall tissues. On the other hand, the mechanism could also operate by an overstimulation of the production of condensed tannins, which would lead to a depletion of intermediate substrates for the other phenolic classes. Theoretically, the observed pattern could arise as a consequence of the rapid growth of the gall tissues, since there may be a trade-off between growth and the production of defensive chemicals in plants (Herms & Mattson 1992). However, such a trade-off cannot by itself explain the observed change in allocation to different compounds.

The changes in chemistry are so great that they probably have far-reaching consequences for the evolutionary relationships between the nematine gallers and their willow hosts. The most interesting possibility concerns the causes of host specificity and

the evolution of host shifts by the gallers: since the galls induced on chemically divergent host species are chemically almost identical, then why are the gallers monophagous? It is unlikely that the constraints in host use are caused by an inability to adapt to variable diet chemistry. Instead, specialization may be caused by the other possible causes mentioned above (phenological differences between hosts, differential predation/parasitism, sensory constraints, plant responses to gall-inducing stimuli, etc.). Evidently, if the causes of host specificity change, then there should also be consequences for the evolution of host use on a larger scale. Thus, studies on gall-inducing insects can provide valuable insights into the (co)evolutionary relationships between insects and plants.

Although it seems that the results support the Nutrition Hypothesis, one should be cautious about stating that the evolution of gall induction in the nematines was originally *caused* by nutritional benefits. The *Pontania* species used in the study represent a rather derived group within the gall-inducing nematines (I, II). Thus, more information is needed on the chemical composition of the galls induced by the *P. proxima*-type leaf blade galls, and on the chemistry of the leaf folds induced by *Phyllocolpa* species. Leaf folding probably does not markedly alter the quality of the leaf as food (but see Sagers 1992), but it may help in avoidance of predation or parasitism (Damman 1987, Sipura 1999, but see Murakami 1999). This is also supported by the fact that many lepidopteran larvae make leaf rolls mechanically (Seppänen 1970). Thus, it is possible that the original cause for the evolution of gall induction was a reduction in parasitism and/or predation, and more sophisticated host manipulation evolved only subsequently in the *Pontania* galls.

The results also reinforce the importance of analyzing individual compounds rather than total concentrations of chemical classes. If only total phenolics had been measured, the result would have been that the total con-

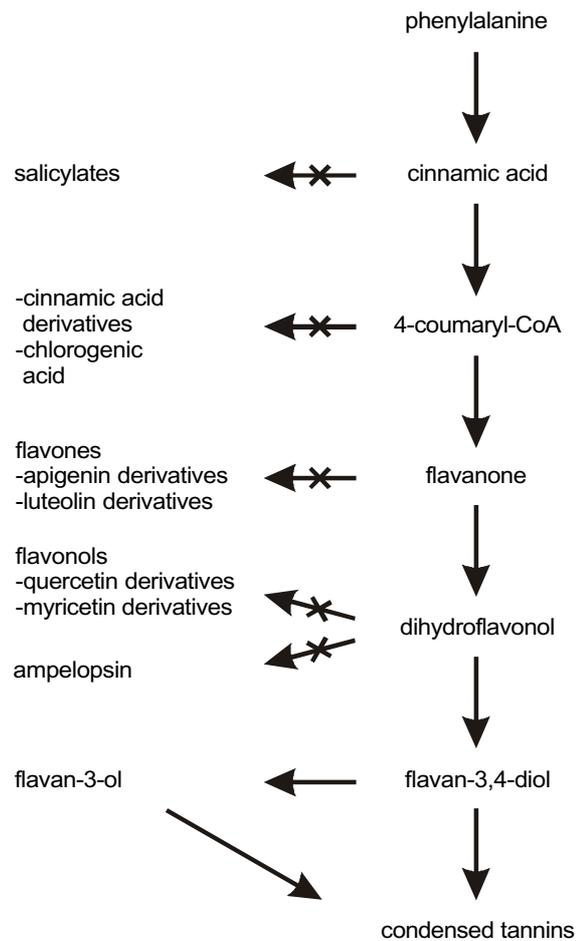


Figure 7. A simplified scheme of the phenylpropanoid pathway, along which different phenolic compounds are produced in willows (modified from Seigler (1998) and Keinänen *et al.* (1999)). Crossed arrows indicate branching points that seem to be partially blocked in *Pontania* galls (IV).

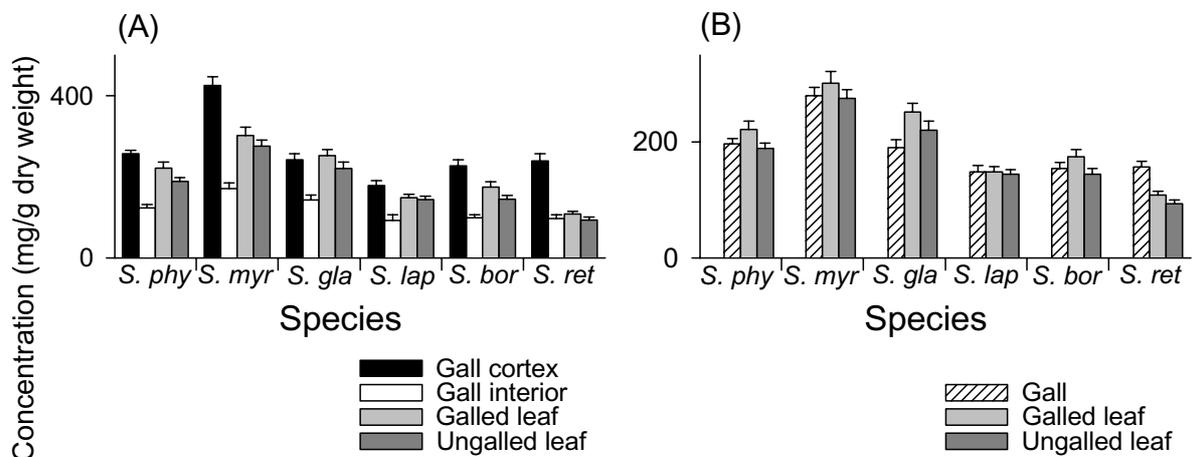


Figure 8. (A) Total phenolics (condensed tannins + other phenolics) in different sample classes in (IV); (B) Total phenolics concentrations when galls are treated as one sample. The mean concentration in galls (B) was calculated by using the weighted average of the concentrations in the cortex and interior samples for each individual gall.

centration is only slightly reduced in galls (Fig. 8A). More importantly, if whole galls had been used in the analyses, the conclusion would have been that phenolic levels do not really change at all in galled tissues (Fig. 8B). Since all possible compounds were not measured in the study, these results must be treated with caution, but they illustrate the point that measuring compounds in bulk can severely affect the conclusions that are drawn.

6. CONCLUDING REMARKS

It is evident that the nematine sawflies and their willow hosts offer an excellent model system for testing various evolutionary hypotheses, since both the hosts and gallers are abundant and speciose in northern ecosystems. However, it is clear that many interesting questions remained outside the scope of this thesis, and that further studies are needed. There are four main topics that should be studied:

(1) The phylogenetic relationships within the Nematinae. One direction would be to go “up” on the phylogenies (I, II) by making separate phylogenetic studies of the main groups. The phylogeny in (II) demonstrates the existence of three to five independent radiations on the genus *Salix*, at least in the *Pontania* s. str., the *Eupontania*, and in the *Euura*. Separate phylogenetic analyses of these groups would facilitate a comparative analysis of the radiation patterns in the groups. Combining the separate studies would also provide additional insights into the evolution of different gall types. The current phylogenies provide a rough view of the evolution of gall morphology and location in the nematine sawflies, but the study should be continued. There are still some Nearctic gall types that are relatively unknown. Molecular methods should also be used more often to study the specific composition of problematic nematine groups. Another promising direction would be to go “down” on the phylogeny by adding species to the base. There are numerous different

larval habits (and very divergent host associations) in other groups within the Nematinae, and a broader sampling of species could provide interesting insights into the ecological evolution in these sawflies.

(2) The phylogenetic relationships within the genus *Salix*. Currently, the interpretation of possible macroevolutionary patterns in the evolution of host use in the nematines is limited by the lack of knowledge about the relationships between the willow hosts. Since morphological studies have not succeeded in elucidating the phylogenetic relationships within the genus *Salix*, one can only hope that molecular methods will be more successful. Thus far, the results have not been promising, but hopefully something will emerge from the intensive research that is currently being carried out on molecular *Salix* taxonomy. It should be noted that knowing the phylogeny of the *Salix* would help in many other research areas as well.

(3) The chemical properties of *Salix* species. With improved methods of chemical analyses, the identification and quantification of compounds has become easier. Currently it is possible to reliably measure many more compounds than in the pioneering studies of Julkunen-Tiitto (1986, 1989). A thorough chemical analysis of northern willow species would be extremely valuable for interpreting the results from the phylogenetic

analyses of the nematine sawflies, and knowing the chemical properties of *Salix* species could also help in reconstructing the phylogeny of willows.

(4) The chemical composition of the galls induced by the nematine gallers. Detailed knowledge about the chemistry of the willow hosts would also be valuable when the chemistry of galls is studied. It is likely that the galls induced by, for example, leaf blade gallers are chemically quite different from those induced by the apical leaf gallers studied in (IV). This can be suspected since the coloration of the leaf blade galls is usually greenish, in contrast to the pale galls induced by the apical leaf gallers. Likewise, the color of *Euura* galls is also usually greenish. The interesting question is whether different galler groups produce chemically similar galls, or whether there are differences between the galler groups.

Evidently, the end goal must be a situation in which detailed information on the phylogeny of the nematines can be combined with information on the phylogeny of the willow hosts, and with information on the chemical properties of both the hosts and the galls. This would make it possible to study how the speciation patterns evolve in relation to host chemistry and phylogeny, and how the chemistry of the galls changes during the evolutionary history of the sawflies.

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