The Royal Society

The structure of cynipid oak galls: patterns in the evolution of an extended phenotype

Graham N. Stone[1*] **and James M. Cook**[2]

[1]*Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK* (graham.stone@zoology.ox.ac.uk)
[2]*Department of Biology, Imperial College, Silwood Park, Ascot, Berkshire SL5 7PY, UK*

Galls are highly specialized plant tissues whose development is induced by another organism. The most complex and diverse galls are those induced on oak trees by gallwasps (Hymenoptera: Cynipidae: Cynipini), each species inducing a characteristic gall structure. Debate continues over the possible adaptive significance of gall structural traits; some protect the gall inducer from attack by natural enemies, although the adaptive significance of others remains undemonstrated. Several gall traits are shared by groups of oak gallwasp species. It remains unknown whether shared traits represent (i) limited divergence from a shared ancestral gall form, or (ii) multiple cases of independent evolution. Here we map gall character states onto a molecular phylogeny of the oak cynipid genus *Andricus*, and demonstrate three features of the evolution of gall structure: (i) closely related species generally induce galls of similar structure; (ii) despite this general pattern, closely related species can induce markedly different galls; and (iii) several gall traits (the presence of many larval chambers in a single gall structure, surface resins, surface spines and internal air spaces) of demonstrated or suggested adaptive value to the gallwasp have evolved repeatedly. We discuss these results in the light of existing hypotheses on the adaptive significance of gall structure.

Keywords: galls; Cynipidae; enemy-free space; extended phenotype; *Andricus*

1. INTRODUCTION

Galls are plant tissues, induced by another organism, which provide that organism with food and a measure of physical protection (Cornell 1983; Price *et al*. 1987). Oak gallwasps (Hymenoptera: Cynipidae, tribe Cynipini) induce the most structurally complex and diverse galls known of any gall-inducing group (Dreger-Jauffret & Shorthouse 1992; Rohfritsh 1992). Cynipid gall structures are characteristic of the gall inducer, rather than of the host-plant (Ambrus 1974; Rohfritsch 1992), and result from cynipid traits expressed at two stages in the wasp's life cycle. Morphogens (as yet uncharacterized), which are probably secreted by the larva, are thought to control the type and structure of plant tissues forming the gall (Rohfritsh 1992), whereas the ovipositional behaviour of the female determines how many larvae develop within a single gall. Gall structures, although constructed of plant tissues, thus represent the extended phenotypes of gallwasp genes (Stern 1995; Crespi *et al*. 1997).

Structurally, cynipid galls can be divided into two parts: the larval chamber and the outer gall. The larval chamber, which is structurally similar in all cynipid galls (Bronner 1992), is lined with nutritive plant tissues on which the larva feeds, and is surrounded by a thin wall of sclerenchyma. The cynipid larva completes its entire development within this chamber. The diversity of cynipid galls is the result of variation in gall tissues that develop outside the larval chamber. These include surrounding layers of woody or spongy tissue, complex air spaces within the gall, and surface coats of sticky resins, hairs or spines. Mature galls formed by members of the same genus may also differ enormously in size and colour. A long-standing challenge in understanding the evolution of gall structure has been to explain why such a diversity of morphologies may be found at the same time on the same part of the same host oak species (Askew 1984; Price *et al*. 1987).

Considerable debate surrounds the possible significance of gall structures, and both non-adaptive and adaptive hypotheses for the genesis of gall diversity have been proposed (Cornell 1983; Price *et al*. 1987; Crespi & Worobey 1998). If some gall structures evolve more often than others (perhaps as an emergent property of the developmental processes involved in gall formation), but go extinct at random, sets of gall morphologies could evolve without selection acting on gall shape. Several adaptive alternatives have been proposed. Gall structure may affect the ability of galls to protect the gall inducer from fluctuations in abiotic conditions, or affect the allocation of nutrients to gall tissues (and hence to growth of the gall inducer) by the host-plant (Cornell 1983; Price *et al*. 1987; Bagatto *et al*. 1996; Crespi & Worobey 1998). There is a general consensus, however, that the strongest selection pressure acting on gall form (with the exception of thrips galls (Crespi & Worobey 1998)) is probably associated with avoidance of mortality inflicted by natural enemies. The greatest cause of gallwasp mortality occurring after successful gall formation results from attack by two groups of wasps (chalcid parasitoids and inquiline cynipids), which reach the gallwasp by penetrating the gall with a drilling ovipositor (Askew 1965, 1984; Washburn & Cornell 1981;

*Author for correspondence.

Cornell 1983; Schönrogge *et al*. 1995, 1996). High mortality of gall inducers can also be caused by vertebrate predators, which also reach the gall inducer by removing the gall wall (e.g. Abrahamson *et al*. 1989; Csóka 1997). Gall structures therefore represent the interface through which the gall inducer and two principal causes of mortality interact. Gall traits reducing gallwasp mortality inflicted by natural enemies, and which are under the control of cynipid genes, should spread through natural selection, and defence is the commonest function attributed to gall traits (Cornell 1983; Askew 1984; Price *et al*. 1987). To date, however, the impact on survivorship of only a few gall characters (diameter, hardness and recruitment of ant guards through nectar secretion) has been demonstrated, and the role of the majority remains unknown (Washburn 1984; Weis *et al*. 1985; Price & Clancy 1986; Abe 1992; Stiling & Rossi 1996).

In addition to this structural diversity, a characteristic of cynipid oak galls is that all of the outer gall characteristics mentioned above are shared by sets of sympatric species (e.g. Weld 1960; Ambrus 1974; Askew 1984). This prompts two fundamental questions.

1. What are the phylogenetic relationships between gallwasps inducing structurally similar galls? Is the evolution of novel gall structures a rare event in oak cynipid evolution, such that phylogenetic proximity and similarity in gall structure are highly correlated, or have certain gall traits evolved independently many times?
2. What underlying processes have generated the observed patterns?

Here we address the first question by examining the distribution of gall traits across the phylogeny of a selected group of oak gallwasp species. We then consider the patterns we observe in the light of existing non-adaptive and adaptive hypotheses for the evolution of gall structure. The selected oak gallwasp taxon for study is the genus *Andricus*, which includes the most complex and diverse oak cynipid galls. We present a molecular sequence phylogeny for 28 members of the genus, which constitute clear sets of species sharing similar gall morphology (figure 1). We test the null hypothesis that gall traits are randomly distributed through the gallwasp phylogeny and ask (i) which traits, if any, are conserved within clades; (ii) which traits, if any, have evolved repeatedly in independent lineages; and (iii) how rapidly (in terms of sequence divergence and existing intermediate forms) have novel structures arisen within lineages?

A further characteristic of cynipine life cycles allows us to ask an additional question about the evolution of gall structure. Many oak gallwasp species have two generations each year, typically a sexual generation in the spring and a parthenogenetic generation in the summer/autumn. These generations often develop in structurally very different galls, and may develop on different host oak species, or plant organs (Askew 1984). By examining patterns in these two generations separately, we ask whether there is any evidence that evolution of gall structure in these two generations is correlated.

Within the Cynipini, phylogenetic relationships between genera are largely unknown (Ronquist 1995; Liljeblad & Ronquist 1997), and which of the diverse gall structures induced by *Andricus* species are primitive and which are derived remains unknown. Our last question is, therefore, whether phylogenetic patterns allow us to infer ancestral states for sexual and asexual generation galls in this genus.

2. MATERIALS AND METHODS

(a) *Study species*

Collection locations, type of material used in DNA extraction, and character values for each generation of the species studied are given in table 1. We selected 28 European *Andricus* species (38% of all European *Andricus*) representing the principal structural types present in the genus (described below). Of the selected species, 12 have both sexual and asexual generations in their life cycle, 14 have only a known asexual generation, and two species have only a known sexual generation. Gall structures are known for the generations given of each species except for the sexual generation of *A. viscosus*, for which the sexual adult female is known, but whose gall has yet to be identified. The asexual generation galls of the selected *Andricus* species develop on *Quercus petraea*, *Q. pubescens* or *Q. robur*, while sexual generation galls develop either on these species or on *Q. cerris* (Ambrus 1974). Many of the galls, particularly of the asexual generations, develop on more than one closely related oak species (Ambrus 1974). In all cases, the galls have the same structure (in terms of the character states used here) on all alternative hosts (Ambrus 1974).

To check the monophyletic status of *Andricus*, we have included in our analysis five species from other oak cynipid genera: *Aphelonyx cerricola* Gir., *Biorhiza pallida* Oliv., *Cynips cornifex* Htg., *Cynips divisa* Htg., and *Cynips quercus* Fourcr. To allow tree rooting we also included *Diplolepis rosae* Htg. in the tribe Rhoditini, the sister group to the Cynipini/Pediaspini clade (Ronquist 1995; Liljeblad & Ronquist 1997).

(b) *Scoring gall character states*

We map three binary characters and one multistate character across our oak cynipid phylogeny (table 1). Character states are illustrated for representative examples in figure 1. The binary traits are as follows.

1. Gall surface covered in sticky resin or not sticky.
2. Gall surface smooth or bearing spines (compare figure 1*a–d*, *m* with figure 1*n*,*q*).
3. Gall single chambered or multichambered (compare figure 1*a–d* with figure 1*e–s*).

Overall gall structure is a multistate character with seven states, and is scored separately for sexual and asexual generations where both are present.

S1. Gall consists of larval chamber only, without exterior structures (not illustrated).
S2. Larval chamber surrounded by modified bud scales, but not completely enclosed by gall tissue (figure 1*r*,*s*).
S3. Larval chamber completely surrounded by, and in direct contact with, woody outer gall tissue (figure 1*p*,*q*).
S4. Larval chamber completely enclosed, but separated from the outer gall by an air space (figure 1*e–k*).
S5. Gall woody, multichambered, and spiny (figure 1*a–d*).
S6. Gall single-celled, with larval chamber in a thin-walled structure at the end of a short stalk. Among the sampled species, this structure is shown only by the asexual generation of *A. solitarius*.
S7. Gall structure possessed by a single species within the genus (figure 1*l–o*). This includes the unique and distinct asexual gall structures of *A. conificus*, *A. gemmea* and *A. hartigi*, which are described individually below.

Table 1. *Gall character states, life cycle stages used in DNA extractions and collection locations for cynipid species in this study*

(Structure codes are explained in the § 2. For species with a known two-generation life cycle, only one generation was used in DNA extraction and sequencing. An asterisk (*) in the 'tissue used in DNA extraction' and 'collecting location' columns indicates that DNA from the alternate generation of the species was used.)

species	structure type	larval cells per gall	spines	sticky surface	tissue used in DNA extraction	collecting location
sexual generation galls						
genus *Andricus*						
A. burgundus Gir.	S1	one	no	no	adult	Madrid, Spain
A. corruptrix Schldl.	S1	one	no	no	*	*
A. curvator Htg.	S4	one	no	no	adult	Bükk Mountains, Hungary
A. fecundator Htg.	S1	one	no	no	*	*
A. gallaeurnaeformis Fonsc.	S7	one	no	no	*	*
A. gemmea Gir.	S1	one	no	no	*	*
A. grossulariae Gir.	S4	one	no	no	adult	Romhány, Hungary
A. inflator Htg.	S4	one	no	no	adult	Oxford, UK
A. kollari Htg.	S1	one	no	no	*	*
A. lignicola Htg.	S1	one	no	no	*	*
A. quercuscalicis Burgsdorf	S1	one	no	no	*	*
A. solitarius Fonsc.	S1	one	no	no	*	*
A. tinctoriusnostrus Stef.	S1	one	no	no	*	*
A. viscosus Nieves-Aldrey	a	a	a	a	*	*
outgroups						
Biorhiza pallida Oliv.	b	many	no	no	adult	Zliv, Slovakia
Cynips divisa Htg.	S1	one	no	no	*	*
Cynips quercus	S1	one	no	no	*	*
Diplolepis rosae Htg.	S5	many	yes	no	larva	Gödöllõ, Hungary
asexual generation galls						
genus *Andricus*						
A. caliciformis Gir.	S3	one	no	no	pupa	Gödöllõ, Hungary
A. caputmedusae Htg.	S5	many	yes	yes	adult	Valtice, Czech Republic
A. conglomeratus Gir.	S3	one	no	no	adult	Gödöllõ, Hungary
A. conificus Htg.	S7	one	no	no	pupa	Veszprem, Hungary
A. coriarius Htg.	S5	many	yes	no	larva	Mátrafüred, Hungary
A. coronatus Gir.	S4	one	no	yes	adult	Sopron, Hungary
A. corruptrix Schldl.	S3	one	no	no	adult	Oxford, UK
A. curvator Htg.	S2	one	no	no	*	*
A. fecundator Htg.	S2	one	no	no	larva	Gödöllõ, Hungary
A. gallaeurnaeformis Fonsc.	S7	one	no	no	larva	Szentkut, Hungary
A. gemmea Gir.	S7	one	no	no	larva	Gödöllõ, Hungary
A. hartigi Marschal	S7	one	yes	no	adult	Szentkut, Hungary
A. hungaricus Htg.	S4	one	no	no	adult	Gödöllõ, Hungary
A. hystrix Trotter	S7	one	yes	no	larva	Szentkut, Hungary
A. inflator Htg.	S2	one	no	no	*	*
A. kollari Htg.	S3	one	no	no	adult	Gödöllõ, Hungary
A. lignicola Htg.	S3	one	no	no	adult	Randalstown, Ireland
A. lucidus Htg.	S5	many	yes	yes	adult	Gödöllõ, Hungary
A. mayri Wachtl.	S5	many	yes	yes	adult	Bra, Italy
A. polycerus Gir.	S3	one	no	no	pupa	Ruffeno, Italy
A. quercuscalicis Burgsdorf	S4	one	no	yes	adult	Oxford, UK
A. quercustozae Bosc.	S4	one	no	yes	adult	Sopron, Hungary
A. seckendorffi Wachtl.	S5	many	yes	yes	adult	Bra, Italy
A. solitarius Fonsc.	S6	one	no	no	adult	Gödöllõ, Hungary
A. tinctoriusnostrus Stef.	S3	one	no	no	pupa	Gödöllõ, Hungary
A. viscosus Nieves-Aldrey	S4	one	no	yes	adult	Sopron, Hungary
outgroups						
Aphelonyx cerricola Gir.	S4	one	no	no	adult	Valtice, Czech Republic
Biorhiza pallida Oliv.	b	many	no	no	*	*
Cynips cornifex Htg.	b	one	no	no	pupa	Szentkut, Hungary
Cynips divisa Htg.	b	one	no	no	adult	Gödöllõ, Hungary
Cynips quercus Fourcr.	b	one	no	no	adult	Szentkut, Hungary

[a] Character states for the sexual gall of *A. viscosus* are unknown.

[b] The sexual gall of *Biorhiza pallida* is large, soft and spongy whereas the asexual gall is a woody, many-celled structure, lacking spines, which is subterranean on roots. The asexual galls of *A. solitarius* and *Cynips cornifex* are both club-shaped, with a single larval cell at the end of a short stalk. The asexual galls of *C. divisa* and *C. quercus* are spherical, non-woody, leaf galls.

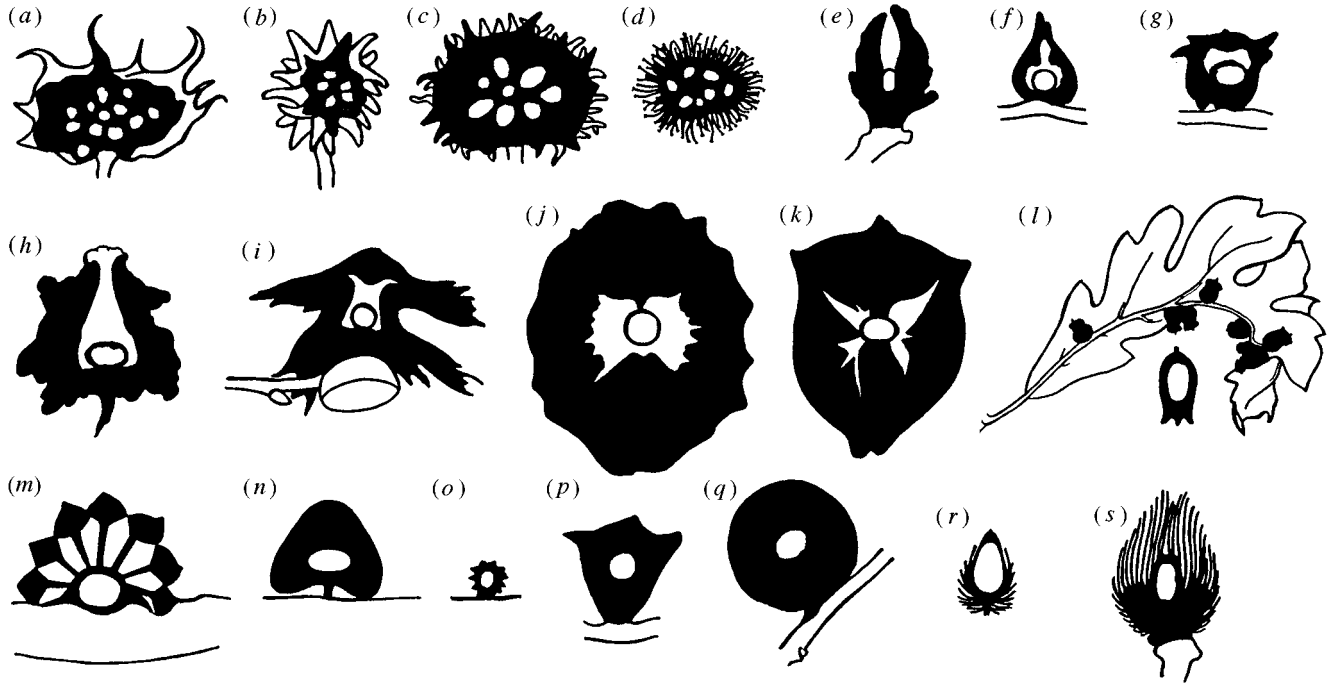


Figure 1. Gall structures induced by representative Andricus species discussed in this study. In each case, each unshaded central ovoid structure is a larval inner cell and dark shaded areas are cut gall tissue. (*a*) *A. coriarius* (asexual), (*b*) *A. mayri* (asexual), (*c*) *A. seckendorffi* (asexual), (*d*) *A. lucidus* (asexual), (*e*) *A. inflator* (sexual), (*f*) *A. grossulariae* (sexual), (*g*) *A. coronatus* (asexual), (*h*) *A. quercuscalicis* (asexual), (*i*) *A. viscosus* (asexual), (*j*) *A. hungaricus* (asexual), (*k*) *A. quercustozae* (asexual), (*l*) *A. gallaeurnaeformis* (asexual), showing position on leaf midrib and a single gall, (*m*) *A. hartigi* (asexual), (*n*) *A. conificus* (asexual), (*o*) *A. gemmea* (asexual), (*p*) *A. polycerus* (asexual), (*q*) *A. kollari* (asexual), (*r*) *A. curvator* (asexual) and (*s*) *A. fecundator* (asexual). All are shown life size except for (*f*, *l*), single cell gall and (*o*) and (*r*) which are 2 × life size.

(c) *Molecular methods*

DNA was extracted from single larvae, pupae or adults (table 1) using either a proteinase-K/SDS digestion followed by 'salting out', or a simple chelex procedure (Werren *et al*. 1995). A 433 base pair (b.p.) fragment of the mitochondrial cytochrome *b* gene was then amplified by PCR (35 cycles of denaturation at 92 °C for 60 s, annealing at 45–55 °C for 60 s and extension at 72 °C for 90 s) using the primers CB1 and CB2 (Jermiin & Crozier 1993) in a 50 µl reaction. To check the amplicon, 10 µl of each PCR product was then electrophoresed in a 1.5% agarose gel. Of the remaining 40 µl, 6 µl were used in standard ligation and transformation reactions using the TA-cloning kit (Invitrogen). Plasmid DNA was purified using Wizard miniprep kits (Promega) and sequenced using Taq-FS (Perkin-Elmer) chemistry and an ABI 373 sequencer.

(d) *Phylogenetic analyses*

Amplicons for each species were sequenced in both directions and the sequences (all 433 b.p. long, GenBank accession numbers AJ228448–AJ228481) aligned by eye. Maximum-parsimony (Farris 1970) and neighbour-joining methods (Saitou & Nei 1987) were used to generate phylogenies from the sequence data, using test version 4.0 d60–63 of PAUP*, written by D. L. Swofford, and with *Diplolepis rosae* as an outgroup. Eight shortest maximum-parsimony (MP) trees were found using 100 random additions in a heuristic search, with codons weighted equally. Although changes were more common at third positions (454) than first (148) or second (51) positions, downweighting or exclusion of third positions did not alter the deeper branches of the trees and severely reduced resolution within the main *Andricus* clade. Neighbour-joining (NJ) trees were generated using uncorrected *p*-distance and three corrected distance measures (Jukes–Cantor, Tamura–Nei and general time-reversible) (Swofford *et al*. 1996). All four algorithms returned the same topology, termed the NJ tree.

Parsimony reconstruction of character evolution was done using MacClade 3.04 (Maddison & Maddison 1992), and rather than assuming a single working phylogeny, we have mapped character state changes over each of the eight MP trees and the NJ tree in turn. All binary traits are mapped over the full phylogeny. The outgroup taxa possess diverse asexual gall structures absent from *Andricus*, and are thus unsuitable for inferring ancestral states for *Andricus* gall form. For simplicity, we have therefore used a pruned version of figure 2 (the main clade, including *A. fecundator*, see below) in mapping this trait.

To test whether gall-form states are conserved within clades or randomly distributed through the phylogeny, we compare the minimum number of character changes inferred for the actual character distribution with minimum numbers of changes required when the same character states are randomly reallocated to species on the same tree topology (using the shuffle command in MacClade). Two hundred and fifty replicates were used to generate frequency distributions using random reallocation for each of two tree topologies: that illustrated in figure 2, and a second corresponding to one of the MP trees in which *A. lucidus* is excluded from the *A. mayri* clade (see below).

3. RESULTS

(a) *Status of the genus* Andricus

There is considerable agreement between the MP and NJ trees, and most nodes relevant to the following analyses receive high bootstrap support in both the strict consensus of the eight MP trees and the NJ tree (figure 2). In all trees, all *Andricus* species bar four (*A. gallaeurnaeformis*,

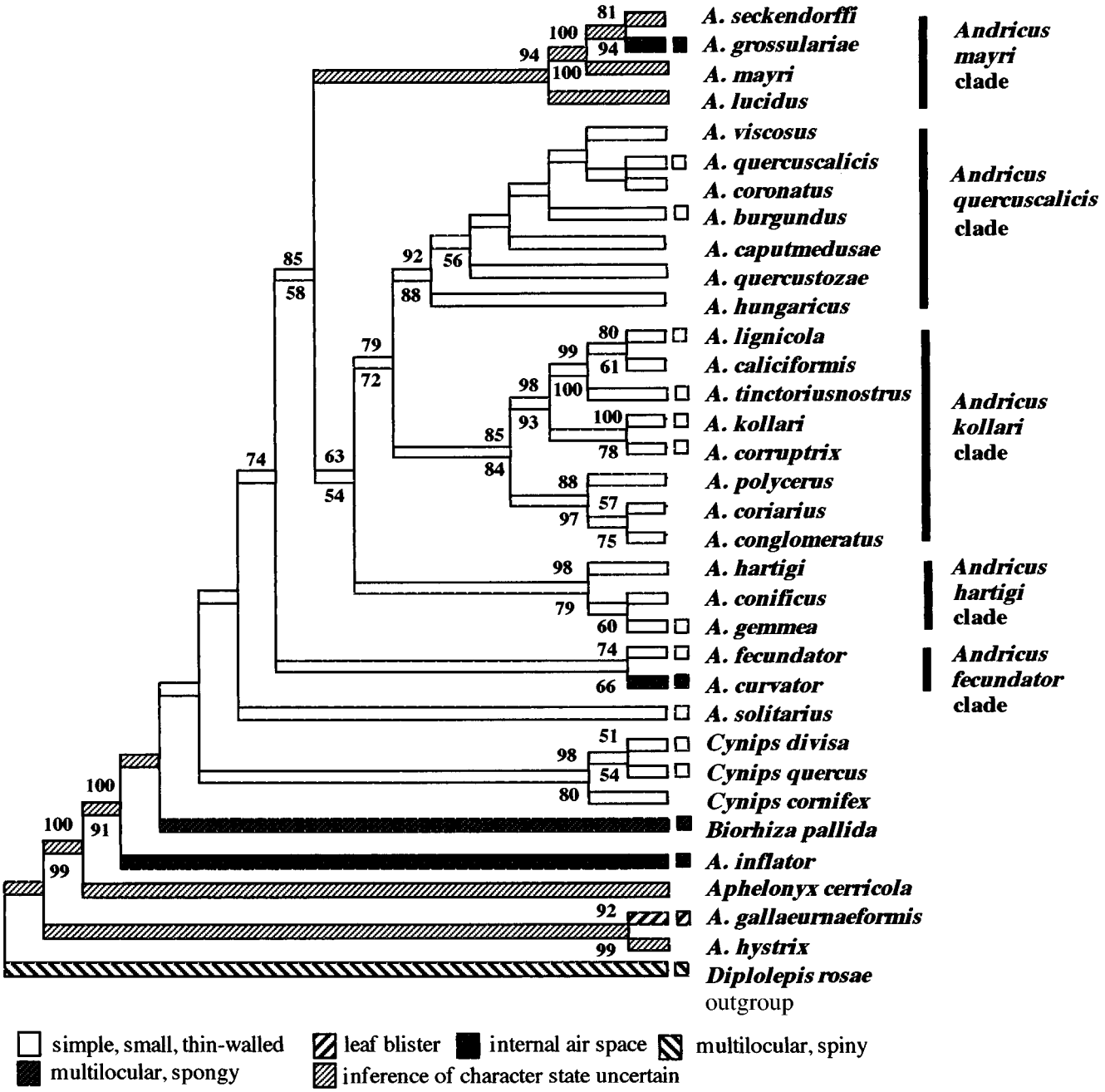


Figure 2. Phylogenetic relationships between species in this study. The topology shown is one of the eight shortest maximum parsimony trees. Only variation in the position of *A. lucidus* (discussed in the text) has any impact on inferred patterns of evolution of gall form. Numbers shown at nodes are bootstrap percentages; values above the node represent scores for the Tamura–Nei corrected NJ tree; values below the node are those for the strict consensus of the MP tree. Branches without values were supported by less than 50% of bootstrap replicates. The character mapped on the tree is sexual generation gall form, reconstructed by MacClade 3.04 (Maddison & Maddison 1992). The presence of a small square between a branch tip and a species name on the tree indicates that the species possesses a sexual generation in its life cycle for which the gall structure is known.

A. hystrix, *A. inflator* and *A. solitarius*) form a large monophyletic clade (termed the main clade). In the NJ tree and six of the eight MP trees, *A. solitarius* is also part of this clade (figure 2). *A. inflator* is always excluded from the main clade, but with low bootstrap support. Only six additional steps (an increase from 614 to 620 steps) are required to enforce monophyly on the main clade + *A. solitarius* + *A. inflator*. Among these species, the greatest sequence divergence (between *A. inflator* and *A. coriarius*) is 14.5%.

Within the main clade, there are five clear groups of species, named as follows for ease of reference (figure 2): (i) the *A. mayri* clade; (ii) the *A. quercuscalicis* clade; (iii) the *A. kollari* clade; (iv) the *A. hartigi* clade; and (v) the *A. fecundator* clade. All but the first are monophyletic in all nine tree topologies. The only variation in tree topology to have any impact in inferring patterns of gall evolution is the position of *A. lucidus*. In four of the MP trees and the NJ tree, this species is part of the *A. mayri* clade (figure 2). In the remaining four MP trees *A. lucidus* is a monospecific taxon diverging immediately basal to the *A. mayri* clade. The latter topology requires a single additional step in parsimony reconstruction of the three binary asexual gall

characters. In all other respects, however, conclusions from all nine trees are the same, and we do not consider the effects of variation in tree topology further.

All trees place the remaining two *Andricus* species (*A. gallaeurnaeformis* and *A. hystrix*) basal to all of the other oak cynipids sequenced, with high bootstrap support (figure 2). Although these two species appear as sister groups in this analysis, they show considerable sequence divergence from each other (16.4%) as well as from other *Andricus* (21.5–25.5% for *A. gallaeurnaeformis* and 22.9–26.3% for *A. hystrix*). Enforcing monophyly for all *Andricus* species results in a shortest MP tree of 642 steps, 28 steps more than the unconstrained MP tree. The position of these two divergent species suggests that *Andricus*, as currently defined, is at least diphyletic (triphyletic if *A. inflator* is genuinely separated from the main clade).

(b) *Phylogenetic patterns in sexual generation gall structure*

Only two structural types are induced by the 12 *Andricus* species that have a sexual generation; nine species form small, thin-walled galls, and three species (*A. grossulariae*, *A. curvator* and *A. inflator*) induce a more complex structure in which the inner cell is surrounded by an air space (figure 1*e*,*f*). The simpler sexual gall structure is found in *Cynips*, sister group to *Andricus*, resulting in the inference that this is the most probable ancestral state for the main *Andricus* clade. The more complex state is thus derived, and has evolved at least three times in *Andricus* (figure 2).

(c) *Phylogenetic patterns in asexual generation gall structure*

(i) *Overall gall form*

Four groups within the main *Andricus* clade each consist entirely or predominantly of species sharing a common asexual gall structure (figure 3*a*).

1. The *A. mayri* clade (see figure 1*b–d*) all have multichambered asexual galls in which the larval chambers are entirely surrounded with extensive woody tissue. The gall surface is covered in spines, and coated in sticky resin.
2. The *A. quercuscalicis* clade contains six species with asexual generation galls, all but one of which contain a single larval chamber and have an air space between this and the outer gall wall (figure 1*g–k*). Five species have outer surfaces covered in sticky resin.
3. The *A. kollari* clade all have solid asexual galls with extensive development of a hard, woody outer gall entirely surrounding the larval chamber (figure 1*p*,*q*). All but one species have a single larval chamber, and lack a sticky surface coating or spines.
4. The *A. fecundator* clade has an asexual gall in which the inner cell is surrounded by modified scale leaves (figure 1*r*,*s*).

For both of the tree topologies used in the test, reconstructions following random character reallocation required a minimum of 10–18 transitions between alternative gall structures, with a mean ±1 standard error of 13.5 ± 0.1. The actual number of transitions inferred in the main clade (figure 3*a*) is nine for all MP and NJ tree topologies, below the minimum value obtained by random allocation. This result confirms that similar gall forms are significantly aggregated within the phylogeny, and the null hypothesis of random gall form distribution through the phylogeny must thus be rejected. Transitions between alternate overall gall morphologies have been rare in the radiation of *Andricus*, and speciation in the genus is thus generally not associated with changes in gall structure. The diversity of asexual gall forms outside the *Andricus* clade, however, means that it is difficult to infer with any certainty which of the structures present in *Andricus* is ancestral, and which is derived.

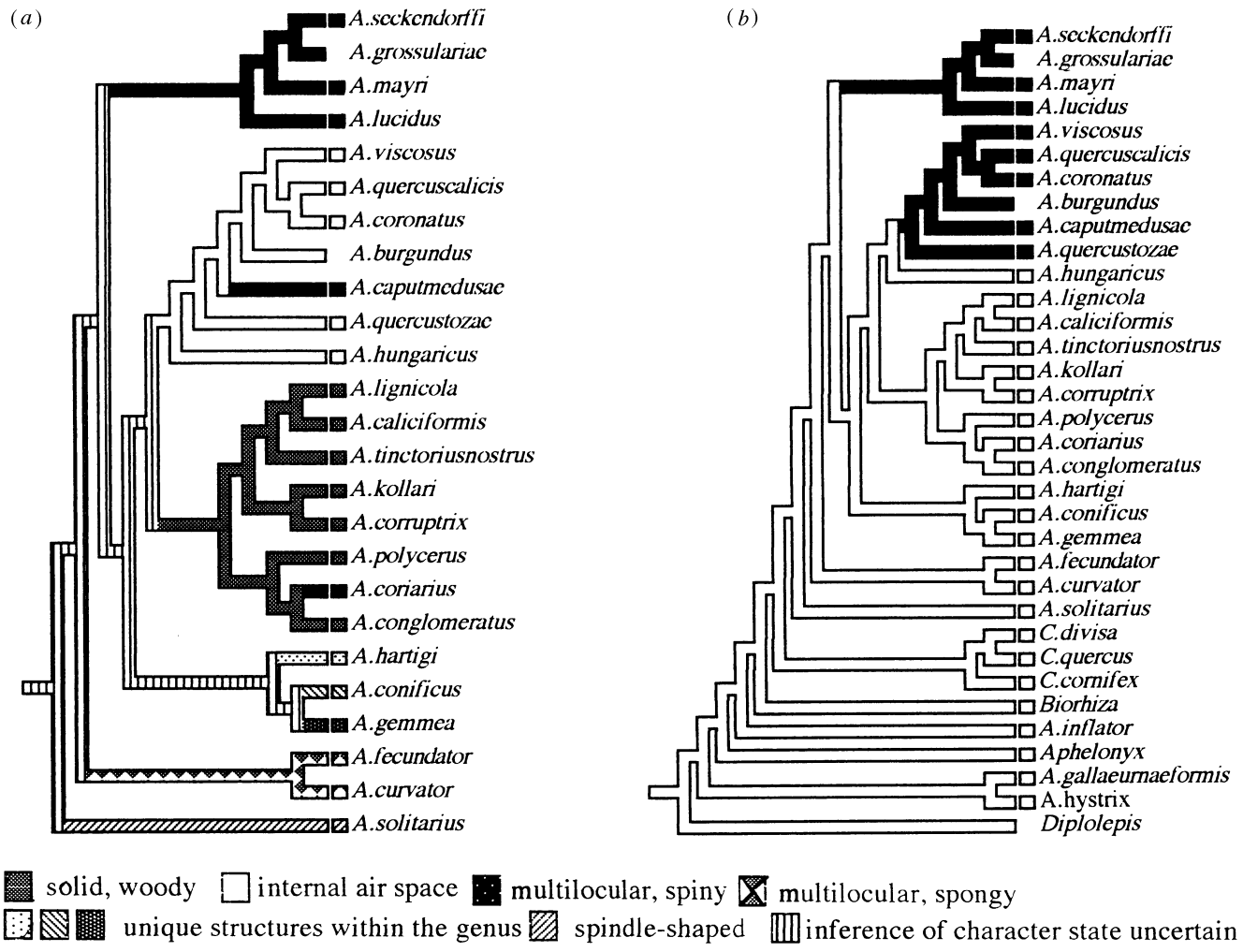
There are two types of exception to the rule that closely related species induce similar galls. First, spiny and multichambered asexual galls have evolved at least three times in *Andricus* (figure 3*a*): once in the *A. mayri* clade (figure 1*b–d*), once in *A. caputmedusae* (in the *A. quercuscalicis* clade) and once in *A. coriarius* (in the *A. kollari* clade; figure 1*a*). In the latter two cases, a multichambered spiny gall has been derived from quite different inferred ancestral states: one in which an air space separates the inner cell and outer wall (the *A. quercuscalicis* clade), and one in which the gall is solid (the *A. kollari* clade) (figure 3*a*). The combination of surface spines and the multichambered state represents the co-occurrence of changes in a trait under maternal control (number of larval chambers per gall) and the extended phenotype of the gallwasp larva (presence or absence of spines).

Second, the *A. hartigi* clade (figure 2) contains three species whose outer asexual gall structures are unique within the genus. In *A. hartigi* the larval chamber is surrounded by an air space formed from a roof of club-shaped spines (figure 1*m*). *A. conificus* has a well-developed outer gall which is spineless, fleshy and soft, without any internal air space (figure 1*n*). *A. gemmea* is a small, fleshy gall whose surface is covered with red tubercles (figure 1*o*). Divergence in gall structure between the members of the *A. hartigi* clade is not associated with high sequence divergence relative to clades whose members share similar gall structure: sequence divergence between *A. hartigi* and *A. conificus* is 2.8%, whereas divergence within each of the *A. kollari* and *A. quercuscalicis* clades reaches 3.5–4.5%. Phylogenetic proximity thus does not guarantee structural similarity in gall form.

(ii) *Sticky surface resins*

Stickiness represents a derived state that has evolved from a non-sticky ancestor at least twice within the genus (figure 3*b*): once in the common ancestor of the *A. mayri* clade, and once in the *A. quercuscalicis* clade.

Figure 3. (*opposite*) Parsimony reconstruction of the evolution of asexual gall characters using MacClade 3.04 (Maddison & Maddison 1992) over the tree topology shown in figure 2. The presence of a small square between a branch tip and a species name on the tree indicates that the species possesses an asexual generation in its life cycle. (*a*) Asexual gall form (a multistate character). Shading patterns as in figure 2. (*b*) Surface stickiness (a binary character): black = sticky, white = non-sticky. (*c*) Surface spines (a binary character): black = with spines, white = without spines. (*d*) Number of larval chambers per gall (a binary character): black = many, white = one.


(a)
A.seckendorffi
A.grossulariae
A.mayri
A.lucidus
A.viscosus
A.quercuscalicis
A.coronatus
A.burgundus
A.caputmedusae
A.quercustozae
A.hungaricus
A.lignicola
A.caliciformis
A.tinctoriusnostrus
A.kollari
A.corruptrix
A.polycerus
A.coriarius
A.conglomeratus
A.hartigi
A.conificus
A.gemmea
A.fecundator
A.curvator
A.solitarius
(b)
A.seckendorffi
A.grossulariae
A.mayri
A.lucidus
A.viscosus
A.quercuscalicis
A.coronatus
A.burgundus
A.caputmedusae
A.quercustozae
A.hungaricus
A.lignicola
A.caliciformis
A.tinctoriusnostrus
A.kollari
A.corruptrix
A.polycerus
A.coriarius
A.conglomeratus
A.hartigi
A.conificus
A.gemmea
A.fecundator
A.curvator
A.solitarius
C.divisa
C.quercus
C.cornifex
Biorhiza
A.inflator
Aphelonyx
A.gallaeurnaeformis
A.hystrix
Diplolepis
solid, woody
internal air space
multilocular, spiny
multilocular, spongy
unique structures within the genus
spindle-shaped
inference of character state uncertain
(c)
A.seckendorffi
A.grossulariae
A.mayri
A.lucidus
A.viscosus
A.quercuscalicis
A.coronatus
A.burgundus
A.caputmedusae
A.quercustozae
A.hungaricus
A.lignicola
A.caliciformis
A.tinctoriusnostrus
A.kollari
A.corruptrix
A.polycerus
A.coriarius
A.conglomeratus
A.hartigi
A.conificus
A.gemmea
A.fecundator
A.curvator
A.solitarius
C.divisa
C.quercus
C.cornifex
Biorhiza
A.inflator
Aphelonyx
A.gallaeurnaeformis
A.hystrix
Diplolepis
(d)
A.seckendorffi
A.grossulariae
A.mayri
A.lucidus
A.viscosus
A.quercuscalicis
A.coronatus
A.burgundus
A.caputmedusae
A.quercustozae
A.hungaricus
A.lignicola
A.caliciformis
A.tinctoriusnostrus
A.kollari
A.corruptrix
A.polycerus
A.coriarius
A.conglomeratus
A.hartigi
A.conificus
A.gemmea
A.fecundator
A.curvator
A.solitarius
C.divisa
C.quercus
C.cornifex
Biorhiza
A.inflator
Aphelonyx
A.gallaeurnaeformis
A.hystrix
Diplolepis

(iii) *Surface spines*

Surface spines represent a derived state that has evolved from a non-spiny ancestor at least three times in the main *Andricus* clade (figure 3*c*): once in the common ancestor of the *A. mayri* clade, and once within each of the *A. quercuscalicis* and *A. kollari* clades. *A. hystrix* represents a fourth, independent evolution of surface spines.

(iv) *Number of chambers per gall*

Multichambered asexual galls represent a derived state that has evolved from single chambered ancestors at least three times in the main *Andricus* clade (figure 3*d*): once in the common ancestor of the *A. mayri* clade, and once within each of the *A. quercuscalicis* and *A. kollari* clades. *Biorhiza pallida* represents a fourth, independent evolution of a multilocular asexual gall. Multichambered galls are also induced by two sexual generation galls (*Biorhiza pallida* and *Diplolepis rosae*; figure 2).

4. DISCUSSION

Our results reveal five characteristics of the evolution of gall traits in *Andricus*.

1. In general, closely related species induce galls of similar structure.
2. Closely related species can, however, induce extremely divergent gall structures.
3. Both traits under maternal control (single or many larval chambers) and traits under larval control (gall tissue types) have evolved repeatedly.
4. One trait (an air space separating the larval chamber from the outer wall) has evolved in sexual generation galls of one group of species, and in asexual generation galls of an entirely different group of species.
5. Derived states within *Andricus* are more structurally complex than inferred ancestral states.

We now discuss these patterns with reference to hypotheses on the adaptive significance of gall form.

(a) *Structural similarities within clades*

Our finding of a general correlation between gall morphology and gallwasp phylogeny parallels findings in other gall-forming insects (Stern 1995; Crespi *et al*. 1997; Plantard *et al*. 1998; Crespi & Worobey 1998). Conservation of gall form within clades does not necessarily imply any adaptive significance for gall shape, but could result simply through low rates of generation of structural novelty. Two patterns in *Andricus*, however, suggest that novel gall structures have arisen relatively rapidly and repeatedly during the radiation of the genus. First, two of the clades whose other members induce structurally similar galls (the *A. kollari* clade and the *A. quercuscalicis* clade) contain species with non-typical structures (*A. coriarius* and *A. caputmedusae*, respectively). Second, if a constant rate of sequence divergence over time is assumed within the genus, the three members of the *A. hartigi* clade have evolved radically divergent gall structures over a shorter time-scale than was required for divergence of structurally similar galls within either the *A. kollari* or *A. quercuscalicis* clades.

As an alternative to a non-adaptive hypothesis, shared characters may be maintained by strong stabilizing selection (Price *et al*. 1987). Alternative adaptive functions of gall structure include effects of structure on gall internal microclimate, and the impact of gall structure on its function as a sink for plant nutrients contributing to growth of the gall and gall inducer (Price *et al*. 1987; Shorthouse & Rohfritsch 1992). Causal links between variation in outer gall structures and these two impacts on the gall inducer remain little understood, however, and the generalist parasitoids that inflict high mortality on many cynipids are regarded as a more probable selective agent (Askew 1965, 1984). If the ancestor of a clade possessed a gall trait limiting mortality inflicted by a generalist parasitoid, and this parasitoid continued to attack the descendant cynipid species during radiation of the clade, then selection could act to retain that gall trait in all descendant species. Furthermore, were the generalist parasitoid to selectively attack less well-defended galls, it could mediate competition for enemy-free space between the members of the clade (Holt & Lawton 1994; Berdegue *et al*. 1996).

Evidence for such an adaptive explanation is currently limited. Although generalist parasitoids commonly inflict mortalities of 40–100% on oak gallwasps (Washburn & Cornell 1981; Askew 1984; Schönrogge *et al*. 1995; Stone *et al*. 1995; Plantard *et al*. 1996), it remains unclear to what extent particular gall traits affect parasitoid attack rates. Of the characters shared by different *Andricus* clades, two (high gall hardness and large gall diameter) have been shown to impede attack by certain parasitoid species in cynipid galls (Askew 1965; Washburn & Cornell 1979) and other insect gall-inducer systems (Weis *et al*. 1985; Price & Clancy 1986; Craig *et al*. 1990). Although defensive functions have been suggested for the other traits conserved within *Andricus* clades (sticky outer surfaces, an air space between the larval chamber and the outer wall, and surface spines (Askew 1984)), their adaptive significance has yet to be demonstrated. Testing the defensive effects of particular gall traits is difficult for two reasons. First, current gall morphologies may include traits (such as increased gall diameter) that, although once effective in defence, have now been circumvented by parasitoid coevolution (for example, evolution of longer ovipositors) (Price & Pschorn-Walcher 1988; Hawkins 1993). Such gall traits, although representing the 'ghost of parasitism past' (Price & Pschorn-Walcher 1988), remain important in understanding the evolution of the gall inducer's extended phenotype.

Second, the impact of gall structure on parasitoid behaviour must be integrated over the entire period of gall development. Structures present in the mature gall are often absent from earlier developmental stages, and some generalist parasitoids attack at this time (Askew 1984; Schönrogge *et al*. 1995; Plantard *et al*. 1996). Some generalist parasitoids (such as *Torymus* and *Megastigmus* species, family Torymidae) do attack mature galls. These species are generalists which can inflict high gallwasp mortality (Askew 1965; Schönrogge *et al*. 1995, 1996), and are thus potential agents of selection of gall traits appearing late in gall development. The long ovipositors of these parasitoids have been interpreted as coevolutionary responses by the parasitoids to large gall size (Askew 1965).

Whatever the adaptive significance of these gall traits, conservation within clades shows that speciation in *Andricus* is rarely associated with large-scale changes in gall morphology (but see §4*c* below). The patterns we

describe suggest that at least one of the possible evolutionary scenarios proposed for diversification of gall structure—disruptive selection within clades (Price *et al*. 1987)—has been rare in *Andricus*.

(b) *Convergent evolution of gall structures*

Four gall traits have evolved convergently in *Andricus*: (i) air spaces between the inner cell and the outer gall, (ii) surface coatings of resins, (iii) surface spines and (iv) production of a multichambered gall. Regardless of their adaptive significance, two interesting conclusions result from repeated evolution of traits. First, if we take the parsimonious view that ancestors of clades possessed the gall morphology now shared by most of the members of that clade, this pattern shows that similar galls can result from modification of quite different ancestral structures. Second, an air space between the larval chamber and the outer wall has evolved in the sexual generations of one set of species, and in the asexual generations of an entirely different set of species. This suggests that the evolution of gall form in these two generations is not tightly coupled.

A non-adaptive explanation for repeated evolution is that the traits concerned represent a set of most probable morphologies resulting from the underlying mechanism of gall formation. If gall formation involves the expression of suites of plant genes associated with the development of certain structures, then it is perhaps to be expected that certain patterns should be repeated (Jenkins & Mabberly 1994). This type of explanation may well be important in understanding the diversity of gall structures induced by eriophyid mites and pemphigine aphids (Price *et al*. 1987). Both of these groups of gall inducers have no known enemies that attack them through the gall wall, and the selective hypotheses presented here for cynipid gall structures thus cannot currently apply to them.

An alternative is that selective retention of advantageous gall traits has resulted in convergent evolution. The same traits conserved within clades show repeated evolution, and again mortality imposed by generalist parasitoids or predators is the most probable selective pressure. The extensive overlap in parasitoid communities associated with different oak gallwasp species shows that the shared selective pressures required for convergent evolution certainly exist (Askew 1965, 1984). The challenge is now to assess which of the traits showing repeated evolution actually have any impact on natural enemy attack rates (Berdegue *et al*. 1996).

An interesting pattern in *Andricus* galls is the repeated correlated evolution on three occasions (and again in *Diplolepis*) of the multichambered state and the presence of surface spines. One possibility is that spininess is an inevitable and non-adaptive consequence of the development of many larvae in the same structure. Not all multichambered *Andricus* galls are spiny, however (e.g. Ambrus 1974), and the two traits are therefore not inevitably linked. Furthermore, because the number of chambers in a gall is maternally controlled, whereas spininess is controlled by the larva, it seems unlikely that these two gall traits are genetically linked. An alternative is that some multichambered galls face particular selective pressures that have resulted in the evolution of additional defensive structures. Multichambered galls are typically larger than single-chambered structures, and while increased size may confer partial protection from insect parasitoids, larger galls are attacked preferentially by opportunist vertebrate predators (Abrahamson *et al*. 1989; Weis *et al*. 1985; Weis 1993). This may be because it is less costly for a predator to extract a given number of food items from a single multilocular gall than from many single-chambered galls. Most vertebrate predation on cynipid galls is opportunistic, and even a slight decrease in the reward obtained from a multichambered gall can result in a switch to alternate foods (Lima 1984). The spines present on multichambered galls such as *A. coriarius* are almost certainly too large to effectively exclude insect parasitoids, and we suggest that they may have evolved to extend the handling times required by vertebrate predators to open multichambered galls, and so reduce their profitability relative to other prey.

(c) *Rapid evolution of divergent gall forms*

The *A. hartigi* clade and the atypical members of the other clades both show that closely related gallwasps can produce very different gall structures. If gall traits are associated with defence against natural enemies, rapid changes in gall morphology may allow the gall inducer to attain a measure of enemy-free space (Jeffries & Lawton 1984; Price *et al*. 1987). Too little is currently known of the parasitoid assemblages associated with different gall morphologies for this possibility to be assessed. It is important to note that such an impact of novel gall structures could well be transitory, however, and may no longer be apparent in contemporary patterns of mortality (Price & Pschorn-Walcher 1988; Berdegue *et al*. 1996).

(d) *The wider significance of patterns in gall evolution*

Patterns of gall evolution shown here—both conservation within clades and convergence across clades—remain compatible with a number of adaptive and non-adaptive explanations which are not mutually exclusive. Further understanding of the evolution of gall form requires advances in two main areas. First, more work is needed on the implications of gall morphology for gallwasp mortality, whether mediated by natural enemies, abiotic factors, or variation in the allocation of plant resources to gall tissues. Second, to understand whether there are constraints on the potential set of gall structures offered to selection, we require deeper understanding of the developmental basis of interspecific differences in gall structure.

We thank Dr Alex Rowe, Dr Sue Kyes and Dr Tim Anderson for their generous help and advice in this work, and Dr Louis Miller and Dr Karen Day for permission to work in their laboratories. We thank Dr György Csóka, Dr George Mélika, Dr Juli Pujadei-Villar and Dr José-Luis Nieves-Aldrey for their hospitality, help during fieldwork and samples of rare species. We thank Dr Fredrik Ronquist, Dr Olivier Plantard, Professor Peter Price, Dr Jackie Brown and Dr Karsten Schönrogge for their many helpful comments on earlier versions of this paper. This work was funded by the Royal Society (G.N.S.; Standard Research Grant and a Collaborative Research with Eastern Europe Grant) and the NERC Taxonomy Initiative (J.M.C.).

REFERENCES

Abe, Y. 1992 The advantage of attending ants and gall aggregation for the gall wasp *Andricus symbioticus* (Hymenoptera: Cynipidae). *Oecologia* **89**, 166–167.

Abrahamson, W. G., Sattler, J. F., McCrea, K. D. & Weis A. E. 1989 Variation in selection pressure on the goldenrod gall fly and the competitive interactions of its natural enemies. *Oecologia* **79**, 15–22.

Ambrus, B. 1974 Cynipid galls. In *Fauna Hungariae* (ed. S. Gusztáv), vol. 12, part 1/a. Budapest, Hungary: Academic Press.

Askew, R. R. 1965 The biology of the British species of the genus Torymus Dalman (Hymenoptera: Torymidae) associated with galls of Cynipidae (Hymenoptera) on oak, with special reference to alternation of forms. *Trans. Soc. Brit. Ent.* **16**, 217–232.

Askew, R. R. 1984 The biology of gallwasps. In *The biology of galling insects* (ed. T. N. Ananthakrishnan), pp. 223–271. New Delhi: Oxford and IBH Publishing Co.

Bagatto, G., Paquette, L. C. & Shorthouse, J. D. 1996 Influence of galls of *Phanacis taraxaci* on carbon partitioning within common dandelion, *Taraxacum officinale*. *Ent. Exp. Appl.* **79**, 111–117.

Berdegue, M., Trumble, J. T., Hare, J. D. & Redak, R. A. 1996 Is it enemy-free space? The evidence from terrestrial insects and freshwater arthropods. *Ecol. Entomol.* **21**, 203–217.

Bronner, R. 1992 The role of nutritive cells in the nutrition of cynipids and cecidomyiids. In *Biology of insect-induced galls* (ed. J. D. Shorthouse & O. Rohfritsch), pp. 118–140. Oxford University Press.

Cornell, H. V. 1983 The secondary chemistry and complex morphology of galls formed by the Cynipinae (Hymenoptera): why and how? *Am. Midl. Nat.* **110**, 220–234.

Craig, T. P., Itami, J. K. & Price, P. W. 1990 The window of vulnerability of a shoot-galling sawfly to attack by a parasitoid. *Ecology* **59**, 297–308.

Crespi, B. & Worobey, M. 1998 The evolution and adaptive significance of gall morphology in australian gall thrips. *Evolution*. (In the press.)

Crespi, B. J., Carmean, D. A. & Chapman, T. W. 1997 Ecology and evolution of galling thrips and their allies. *A. Rev. Ent.* **42**, 51–71.

Csóka, G. 1997 *Plant galls*. Budapest: Forest Research Institute and Agroinform.

Dreger-Jauffret, F. & Shorthouse, J. D. 1992 Diversity of gall-inducing insects and their galls. In *Biology of insect-induced galls* (ed. J. D. Shorthouse & O. Rohfritsch), pp. 8–34. Oxford University Press.

Farris, J. S. 1970 Methods for computing Wagner trees. *Syst. Zool.* **18**, 374–385.

Hawkins, B. A. 1993 Refuges, host population dynamics, and the genesis of parasitoid diversity. In *Hymenoptera and biodiversity* (ed. J. La Salle & I. D. Gauld), pp. 235–256. Wallingford, UK: CAB International.

Holt, R. D. & Lawton, J. H. 1994 The ecological consequences of shared natural enemies. *A. Rev. Ecol. Syst.* **25**, 495–520.

Jeffries, M. J. & Lawton, J. H. 1984 Enemy-free space and the structure of ecological communities. *Biol. J. Linn. Soc.* **23**, 269–286.

Jenkins, R. M. & Mabberly, D. J. 1994 A breadfruit among the dipterocarps: galls and atavism. In *Plant galls: organisms, interactions, populations* (ed. M. A. Williams), pp. 183–200. Special Volume of the British Systematics Association, vol. 49. Oxford Science Publications.

Jermiin, L. S. & Crozier, R. H. 1994 The cytochrome-*b* region in the mitochondrial DNA of the ant *Tetraponera rufoniger*—sequence divergence in Hymenoptera may be associated with nucleotide content. *J. Molec. Evol.* **38**, 282–294.

Liljeblad, J. & Ronquist, F. 1997 A phylogenetic analysis of higher-level gall wasp relationships. *Syst. Ent.* (In the press.)

Lima, S. L. 1984 Downy woodpecker foraging behaviour: efficient sampling in simple stochastic environments. *Ecology* **65**, 166–174.

Maddison, W. P. & Maddison, D. R. 1992 *MacClade v. 3.04*. Sunderland, MA: Sinauer.

Plantard, O., Rasplus, J.-Y. & Hochberg, M. E. 1996 Resource partitioning in the parasitoid assemblage of the oak galler *Neuroterus quercusbaccarum* L. (Hymenoptera: Cynipidae). *Acta Oecologia* **171**, 1–15.

Plantard, O., Shorthouse, J. D. & Rasplus, J.-Y. 1998 Molecular phylogeny of the genus *Diplolepis* (Hymenoptera: Cynipidae). In *Biology of gall inducing arthropods* (ed. G. Csóka, W. Mattson, G. N. Stone & P. W. Price). Forest Service, United States Department of Agriculture. (In the press.)

Price, P. W. & Clancy, K. L. 1986 Interactions among three trophic levels: gall size and parasitoid attack. *Ecology* **67**, 1593–1600.

Price, P. W. & Pschorn-Walcher, H. 1988 Are gall insects better protected against parasites than exposed feeders? *Ecol. Ent.* **13**, 195–205.

Price, P. W., Fernandes, G. W. & Waring, G. L. 1987 Adaptive nature of insect galls. *Env. Ent.* **16**, 15–24.

Rohfritsch, O. 1992 Patterns in gall development. In *Biology of insect-induced galls* (ed. J. D. Shorthouse & O. Rohfritsch), pp. 60–87. Oxford University Press.

Ronquist, F. 1995 Phylogeny and early evolution of the Cynipoidea (Hymenoptera). *Syst. Ent.* **20**, 309–335.

Saitou, N. & Nei, M. 1987 The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molec. Biol. Evol.* **4**, 1406–1425.

Schönrogge, K., Stone, G. N. & Crawley, M. J. 1995 Spatial and temporal variation in guild structure: parasitoids and inquilines of *Andricus quercuscalicis* (Hymenoptera: Cynipidae) in its native and alien ranges. *Oikos* **72**, 51–60.

Schönrogge, K., Stone, G. N. & Crawley, M. J. 1996. Alien herbivores and native parasitoids: rapid development of guild structure in an invading gall wasp, *Andricus quercuscalicis* (Hymenoptera: Cynipidae). *Ecol. Ent.* **21**, 71–80.

Shorthouse, J. & Rohfritsch, O. (eds) 1992 *Biology of insect-induced galls*. Oxford University Press.

Stern, D. L. 1995 Phylogenetic evidence that aphids, rather than plants, determine gall morphology. *Proc. R. Soc. Lond.* B **260**, 85–89.

Stiling, P. & Rossi, A. M. 1996 Complex effects of genotype and environment on insect herbivores and their enemies. *Ecology* **77**, 2212–2218.

Stone, G. N., Schönrogge, K., Crawley, M. J. & Fraser, S. 1995 Geographic variation in the parasitoid community associated with an invading gallwasp, *Andricus quercuscalicis* (Hymenoptera: Cynipidae). *Oecologia* **104**, 207–217.

Swofford, D. L., Olsen, G. J., Waddell, P. J. & Hillis, D. M. 1996 Phylogenetic inference. In *Molecular systematics* (ed. D. M. Hillis, C. Moritz & B. K. Mable), pp. 407–514. Sunderland, MA: Sinauer.

Washburn, J. O. 1984 Mutualism between a cynipid wasp and ants. *Ecology* **65**, 654–656.

Washburn, J. O. & Cornell, H. V. 1979 Chalcid parasitoid attack on a gallwasp population (*Acraspis hirta* [Hymenoptera: Cynipidae]) on *Quercus prinus* (Fagaceae). *Can. Ent.* **111**, 391–400.

Washburn, J. O. & Cornell, H. V. 1981 Parasitoids, patches and phenology: their possible role in the local extinction of a cynipid wasp population. *Ecology* **62**, 1597–1607.

Weis, A. E. 1993 Host gall size predicts host quality for the parasitoid *Eurytoma gigantea*, but can the parasitoid tell? *J. Insect Behav.* **5**, 591–602.

Weis, A. E., Abrahamson, W. G. & McCrea, K. D. 1985 Host gall size and oviposition success by the parasitoid *Eurytoma gigantea*. *Ecol. Ent.* **10**, 341–348.

Weld, L. H. 1960 *Cynipid galls of the Southwest*. Ann Arbor, Michigan. Privately printed.

Werren, J. H., Windsor, D. & Guo, L. R. 1995 Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond.* B **262**, 197–204.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.