

Evidence for widespread cryptic sexual generations in apparently purely asexual *Andricus* gallwasps

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Abstract

Oak gallwasps (Hymenoptera, Cynipidae, Cynipini) are one of seven major animal taxa that commonly reproduce by cyclical parthenogenesis (CP). A major question in research on CP taxa is the frequency with which lineages lose their sexual generations, and diversify as purely asexual radiations. Most oak gallwasp species are only known from an asexual generation, and secondary loss of sex has been conclusively demonstrated in several species, particularly members of the holarctic genus *Andricus*. This raises the possibility of widespread secondary loss of sex in the Cynipini, and of diversification within purely parthenogenetic lineages. We use two approaches based on analyses of allele frequency data to test for cryptic sexual generations in eight apparently asexual European species distributed through a major western palaearctic lineage of the gallwasp genus *Andricus*. All species showing adequate levels of polymorphism (7/8) showed signatures of sex compatible with cyclical parthenogenesis. We also use DNA sequence data to test the hypothesis that ignorance of these sexual generations (despite extensive study on this group) results from failure to discriminate among known but morphologically indistinguishable sexual generations. This hypothesis is supported: 35 sequences attributed by leading cynipid taxonomists to a single sexual adult morphospecies, *Andricus burgundus*, were found to represent the sexual generations of at least six *Andricus* species. We confirm cryptic sexual generations in a total of 11 *Andricus* species, suggesting that secondary loss of sex is rare in *Andricus*.

Keywords: cyclical parthenogenesis, cynipid, heterogony, Hymenoptera, sex

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Introduction

It has long been realized that obligately asexual reproduction is characterized by short-term advantages and long-term costs (Williams 1975; Maynard Smith 1978). Asexual mutants have a transient advantage over a sexual ancestor through escape from the twofold cost of sex, and

the maintenance of favourable gene combinations (Maynard Smith 1978). The cost is the prevention of selectively advantageous intergenomic recombination, which promotes adaptation to and persistence in novel and rapidly changing environments (Williams 1975; Maynard Smith 1978; Bell 1982; Ladle 1992; Kumpulainen *et al.* 2004; Agrawal 2006) and purges weakly deleterious mutations from the genome (Kondrashov 1988). Two predictions follow: (i) that transition from sexual to asexual reproduction should occur frequently because of the short-term success

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of asexual lineages over their sexual progenitors; and (ii) that asexual lineages will suffer rapid extinction as the disadvantages of asexual reproduction become apparent. From a phylogenetic perspective, this leads to the prediction that obligately asexual lineages should be polyphyletic and young (e.g. Belshaw *et al.* 1999; Delmotte *et al.* 2001, 2003; Pongratz *et al.* 2003). However, the existence of ancient asexual lineages (Chaplin & Hebert 1997; Normark *et al.* 2003; Welch *et al.* 2004; Heethoff *et al.* 2007) fuels the ongoing debate on why sexual reproduction is the rule, rather than the exception (Burt 2000; Otto & Nuismer 2004; Agrawal 2006), and on how some taxa cope with the costs associated with long-term asexual reproduction (Normark *et al.* 2003).

Cyclically parthenogenetic taxa play a particularly important role in this debate. Their existence implies that there must be a short-term advantage to maintaining a sexual generation; if not, such taxa would be expected to make a rapid transition to obligate asexual reproduction ('balance argument' of Maynard Smith 1978). Cyclical parthenogenesis (CP) has been described in over 15 000 species in three animal phyla, including the monogonont rotifers, the digenean trematodes, and multiple lineages of Arthropods (White 1973; Hebert 1987; King *et al.* 2005). The latter include the cladoceran water fleas (Crustacea: Cladocera), some gallwasps (Hymenoptera; the sister group tribes Pediastidini and Cynipini of the family Cynipidae), and isolated lineages of gall midges (Diptera: Cecidomyiidae), true bugs (Homoptera: Adelgidae and Aphididae), and beetles (Coleoptera) (Moran 1992; Stone *et al.* 2002; Frantz *et al.* 2006; Havill & Foottit 2007). Oak and sycamore gallwasps are unique among CP arthropods in that their sexual and apomictic asexual generations are produced in strict alternation (Stone *et al.* 2002; Atkinson *et al.* 2003). Life cycles of all other CP taxa comprise multiple asexual generations punctuated by occasional sexual generations, with male production often triggered by a change in environmental conditions (e.g. Innes & Hebert 1988; King *et al.* 2005; Frantz *et al.* 2006).

Most CP lineages also contain secondarily derived purely asexual lineages and secondary loss of sex has occurred repeatedly in some groups (Hebert 1987; Moran 1992; Little & Hebert 1997; Delmotte *et al.* 2001, 2003; Stone *et al.* 2002). The transition from CP to purely asexual life cycles has occurred in a range of ways, including: (i) development of populations in habitats lacking the environmental cues needed for male production (Eastop 1986; Fenton *et al.* 1998; Dedryver *et al.* 2001; Wilson & Sunnucks 2006), (ii) lineage origin through mating between distantly related species (Jourdan *et al.* 1995), (iii) polyploid lineage formation through the hybridization of two different species (Beaton & Hebert 1988; Dufresne & Hebert 1994; Ward *et al.* 1994; van Herwerden *et al.* 1999), and (iv) spread of a dominant sex-linked meiosis suppresser allele (Blackman 1972; Innes & Hebert 1988). As predicted above, known secondarily

asexual taxa within CP lineages are generally polyphyletic and young (Crease *et al.* 1989; Delmotte *et al.* 2001, 2003).

The gallwasp tribes Pediastidini (sycamore gallwasps, three species) and Cynipini (oak gallwasps, around 1000 species in 27 genera worldwide) comprise one of the richest CP animal lineages. Their diversity prompts two questions: first, how common is secondary loss of sex in this lineage, and second, do secondarily purely asexual gallwasps constitute clades of species, or represent dispersed isolated events distributed through the gallwasp phylogeny? The oak gallwasps are a potentially promising group in which to find widespread secondary loss of sex. Most are known only from females whose morphology matches them to the apomictic generations of known CP species (Melika *et al.* 2000; Nieves-Aldrey 2001; Atkinson *et al.* 2003), including all members of the genera *Aphelonyx* (five species), *Atrusca* (> 40 species), *Holocynips* (four species), *Odontocynips* (one species), *Philonix* (seven species), *Phylloterax* (seven species), *Pseudoneuroterus* (one species) and *Zopheroterax* (six species) (Melika & Abrahamson 2000). In the European oak gallwasp fauna, which represents the best-studied globally, the most speciose genus *Andricus* contains 81 species, only 28 of which are known to have a CP life cycle and 36 of which are known only from an asexual generation (Melika *et al.* 2000, 2007; Nieves-Aldrey 2001; Csóka *et al.* 2005).

For the vast majority of such 'asexual only' species, presence/absence of a sexual generation has yet to be confirmed, and obligate asexuality cannot be assumed. However, loss of sex from CP life cycles has been demonstrated experimentally for at least eight palaeartic oak gallwasp species, including five *Andricus* species (Adler 1877; Csóka *et al.* 2005; Abe 2007). Maynard Smith's (1978) 'balance argument' is particularly appropriate for *Andricus* because in two species (*A. quadrilineatus* and *A. paradoxus*), individual asexual generation females are able to give rise to both sexual and asexual females (Adler 1877; Csóka *et al.* 2005), while in two others (*A. pseudoflos* and *A. targionii*) asexual species are sympatric with their CP ancestors (Abe 2007). The underlying mechanism of alternation of generations in oak and sycamore gallwasps, and of loss of the sexual generation, is unknown (Atkinson *et al.* 2002; Stone *et al.* 2002). Bacterial symbionts such as *Wolbachia* that can cause loss of sex in a range of arthropods (including other gallwasp tribes; Plantard *et al.* 1998, 1999) infect some *Andricus* gallwasps, but never cause loss of sex (Rokas *et al.* 2001, 2002; Abe & Miura 2002). Demonstration that oak cynipids can sustain purely asexual life cycles raises the question of how many 'asexual only' species are genuinely obligately parthenogenetic, and how many are in fact cyclical parthenogens with an unknown sexual generation. All regional gallwasp faunas contain orphan sexual taxa that represent candidates for closure of CP life cycles with known asexual taxa. However, the richness of such orphan sexuals lags far behind that of orphan asexuals.

In the past, CP gallwasp life cycles have been demonstrated by technically challenging rearing experiments (e.g. Adler 1877; Folliot 1964). The requirement of many gallwasp species for mature oak tissues and diapause makes them very difficult to rear under controlled conditions, and a high proportion of field rearings fail (see Folliot 1964). Known sexual and asexual generations are otherwise difficult to pair as a single life cycle because the adults and galls of the two generations are morphologically very different, and historically have often been placed in different genera (Adler 1877; Folliot 1964). The asexual generation galls are large, complex and long-lived, while the sexual generation galls are usually small and structurally simple, often cryptic and short-lived (e.g. Stone *et al.* 1995, 2002; Nieves-Aldrey 2001). The sexual galls are thus harder to find and can lack obvious distinguishing characters. This could lead to undersampling and misidentification of sexual generations, and may explain why many fewer species are known only from a sexual rather than an asexual generation.

Here we use two approaches to resolve the life cycle type of multiple gallwasp species known only from a single asexual generation. First, we use three allele frequency-based methods to test for cryptic sex in seven *Andricus* species currently known only from an asexual generation. These species all belong to a single monophyletic western palaeartic *Andricus* lineage (Fig. 1) dominated by apparently purely asexual taxa. We then use phylogenetic analyses of DNA sequence data to test the hypothesis that members of a single geographically widespread sexual taxon — *Andricus burgundus* Giraud, 1859 — in fact comprise the cryptic sexual generations of multiple asexual taxa. *Andricus burgundus* induces small, structurally simple thin-walled galls (Fig. 2) on catkins of oaks (genus *Quercus* subgenus *Quercus*) in the section *Cerris*. This combination of gall structure, location and host association represents a possible ancestral state for the *Andricus* lineage under study (Cook *et al.* 2002), and it is reasonable to posit that it may have been conserved during radiation of the clade, and so be shared as the sexual generation phenotype of multiple species.

Materials and methods

Study species and sample sites

Asexual generation females of seven 'asexual only' *Andricus* species — *A. caputmedusae* (Hartig, 1843), *A. coriarius* (Hartig, 1843), *A. glutinosus* (Giraud, 1859), *A. lucidus* (Hartig, 1843), *A. mayri* Mayr 1882 (= *A. panteli* Kieffer, 1896), *A. quercustozae* (Bosc, 1792) and *A. seckendorffi* (Wachtl, 1879) — were reared from large gall populations collected at sites across the Western Palearctic from Morocco to Turkey (Table 1) in 1998 and 1999, and reared under quarantine in Edinburgh, UK. The galls of *A. coriarius*, *A. lucidus*, *A. mayri* and *A. seckendorffi*

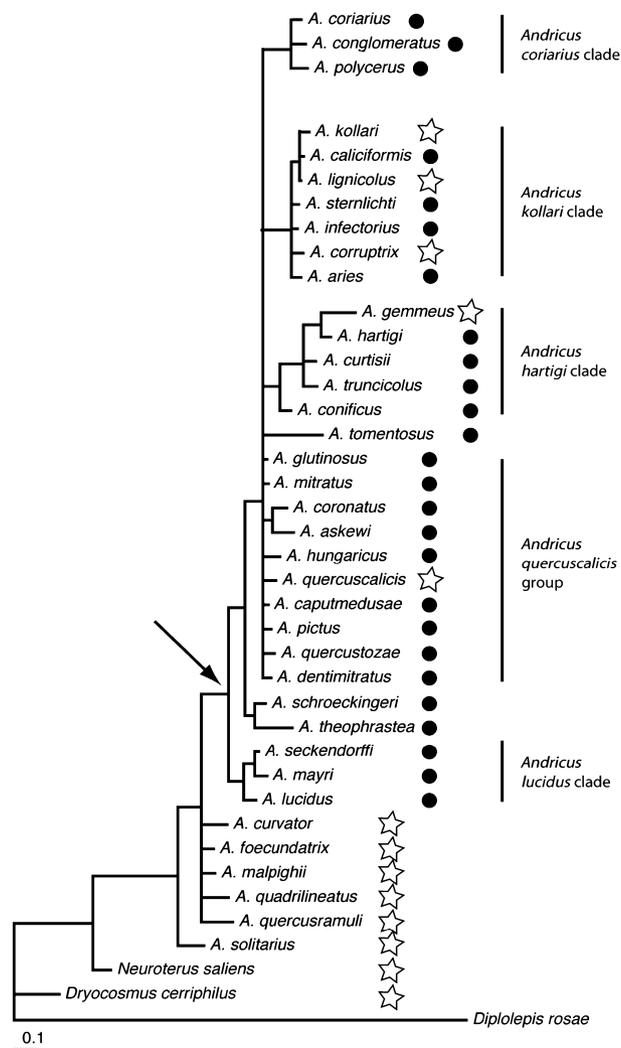


Fig. 1 A phylogeny of selected western palaeartic oak gallwasps, including all species in the host-alternating clade known either from cyclical parthenogenesis (CP) lifecycles or only from an asexual generation. The phylogeny was generated using Bayesian estimation for a 433-base pair fragment of the mitochondrial cytochrome *b* gene. The relationships match those generated in other analyses using a fragment of the nuclear long wavelength opsin gene (Cook *et al.* 2002), and clade names follow previous work (Stone & Cook 1998; Cook *et al.* 2002; Rokas *et al.* 2003b). Species known only from an asexual generation at the start of this research are marked with a filled circle, and known CP species with a white star. The arrow indicates the most recent common ancestor of the host-alternating *Andricus* clade. All nodes in the phylogeny have a posterior probability ≥ 0.95 .

are multilocular (they contain multiple offspring of one or more foundresses; Atkinson *et al.* 2002), and these galls were reared individually. The galls of *A. caputmedusae* Hartig 1843, *A. glutinosus* (Giraud 1859), and *A. quercustozae* (Bosc, 1792) are unilocular (contain a single wasp), and field samples from each site, representing the offspring of many



Fig. 2 The aggregated catkin galls of *Andricus burgundus* on Turkey oak, *Quercus cerris*. Scale bar = 1 mm. © Dr György Csóka.

females, were reared from their galls en masse. Emerging wasps were frozen and stored at -80°C . Due to the different analytical treatments used for multilocular and unilocular galls (see below), we selected only one population for each multilocular gall species (Table 1). In contrast, samples of 12–50 individuals were reared from 16 populations of *A. quercustozae* ($n = 463$) and *A. caputmedusae* ($n = 570$) and seven populations of *A. glutinosus* ($n = 325$).

All of these species have been placed by previous work within the host-alternating clade of the genus *Andricus*, whose life cycles involve sexual generation galls on oaks in the section *Cerris*, and asexual generation galls on oaks in the section *Quercus sensu stricto* (Cook *et al.* 2002) (Fig. 1). To allow sequence-based identification of currently unpaired sexual and asexual generations, we collected and reared singly galls of four 'sexual only' species (*A. burgundus* Giraud, 1859, *A. crispator* Tschek, 1871, *A. multiplicatus* Giraud, 1859, and *A. singularis* Mayr, 1870) from Turkey oak, *Quercus cerris* in Mátrafüred, Hungary. An additional four adult individuals of *A. burgundus* were reared from galls collected on cork oak, *Quercus suber*, from Somiollo, near Madrid, Spain. Two individuals of *A. hystrix* Kieffer 1897, an asexual-only species, were reared from galls collected from *Quercus pubescens* near Szentkut, Hungary.

Allozyme analysis

Individual wasps were scored for allelic variants at 13 polymorphic loci using cellulose acetate electrophoresis

(Zip-zone, Helena Laboratories) and substrate-staining protocols described by Richardson *et al.* (1986). All 13 loci have been used in previous studies on oak gallwasps with known CP life cycles, and found to meet predictions of Hardy–Weinberg and linkage equilibrium (Stone & Sunnucks 1993; Stone *et al.* 2001, 2007). The running buffers for each locus and the loci that were polymorphic in each species are given in Table 2.

Here we use two groups of techniques to discriminate between patterns of allele frequency variation predicted for wholly asexual and CP taxa. For one group of species, we test for departure of gene frequencies from predictions of population-wide Hardy–Weinberg equilibrium and linkage equilibrium between loci expected under sexual reproduction (Maynard Smith *et al.* 1993; Sunnucks *et al.* 1997; Stone *et al.* 2001; Wilson *et al.* 2006). For a second, we discriminate between expectations of the genetic similarity between distinct multilocus genotypes reared from a single gall with and without sex (Atkinson *et al.* 2002).

Unilocular galls: detecting deviations from linkage and Hardy–Weinberg equilibria. Cyclically parthenogenetic gallwasps show strict alternation between asexual and sexual generations. The autumn asexual generation comprises the daughters of mated females, while the spring sexual generation comprises males and females produced by parthenogenesis (Atkinson *et al.* 2002, 2003). Because the asexual generation is produced sexually, in large and stable populations we expect selectively neutral allele frequencies sampled from the asexual generation of a cyclically parthenogenetic species to approximate Hardy–Weinberg and linkage equilibria. This pattern is observed in populations with known CP life cycles (Stone *et al.* 2001, 2007). In contrast, neither equilibrium is expected under obligate asexual reproduction (Suomalainen *et al.* 1987; Maynard Smith *et al.* 1993). Detection of these equilibria in species currently known only from an asexual generation is thus strong evidence for the existence of an unknown sexual generation.

We analysed genotypic data from populations of three species: *A. caputmedusae* (16 populations), *A. glutinosus* (seven populations) and *A. quercustozae* (16 populations) (Table 1). We tested for deviations from Hardy–Weinberg and linkage equilibrium using GENEPOP (Raymond & Rousset 1995), correcting threshold significance levels for multiple comparisons using a Dunn–Sidak correction (Ury 1976). GENEPOP uses the exact test of Guo & Thompson (1992) to compare the observed data against a distribution calculated under the null hypothesis of random union of gametes (for Hardy–Weinberg equilibrium) or alleles (for linkage equilibrium). For comparisons at the species level, we used the multi-sample test of Raymond & Rousset (1995) to sum results for each species over loci (for linkage equilibrium) and across populations (for Hardy–Weinberg equilibrium).

Table 1 The locations of sampled populations and numbers of galls for each asexual species of oak gallwasp studied. Only one population was used for each species with multilocular galls, and table cells show the number of singly reared galls (with the number of wasps that emerged in parentheses). Sample site information for the sexual generation species is provided in the main text

Country	Population	Location	Unilocular galls			Multilocular galls			
			<i>A. caputmedusae</i>	<i>A. glutinosus</i>	<i>A. quercustozae</i>	<i>A. coriarius</i>	<i>A. lucidus</i>	<i>A. mayri</i>	<i>A. seckendorffi</i>
Austria	Vienna	48°13'N, 16°22'E	40	50					
Czech Republic	Valtice	48°45'N, 16°45'E	27						
France	Bordeaux	44°50'N, 00°34'W			12				
Hungary	Balatonfüred	46°40'N, 17°19'E	42						
Hungary	Eger	47°53'N, 20°28'E	44	34					
Hungary	Karcag	47°19'N, 20°53'E		41					
Hungary	Mátrafüred	47°50'N, 19°57'E		50					
Hungary	Sopron	47°40'N, 16°35'E	14	50	40		37(365)		
Hungary	Szeghalom	47°01'N, 21°09'E	28	50	40				
Hungary	Szentendre	47°40'N, 19°02'E		50					
Hungary	Tatabanya	47°31'N, 18°25'E			10				
Hungary	Veszprem	47°06'N, 17°54'E	44		40				
Italy	Bombiana	44°12'N, 10°57'E	43		16				
Italy	Chiusi	43°02'N, 11°57'E	30						
Italy	Greve	43°35'N, 11°19'E	44		39				
Italy	Massa Maritima	43°03'N, 10°53'E	17		27				
Italy	Poppi	43°43'N, 11°46'E	44		39				
Italy	Rieti	42°24'N, 12°51'E	26		22				
Italy	San Venanzo	42°52'N, 12°16'E			27				
Morocco	Azrou	33°27'N, 05°14'W			39				
Spain	Prado del Rey	36°48'N, 05°33'W			17				
Turkey	Aglasun	37°39'N, 30°32'E	44		15			17(353)	
Turkey	Beysehir	37°40'N, 31°43'E	42						
Turkey	Egirdir	37°52'N, 30°51'E	41						
Turkey	Gezende	36°32'N, 33°09'E			40				
Turkey	Madenli	38°08'N, 31°01'E			40	11(228)			21(356)
		Total populations	16	7	16	1	1	1	1
		Total individuals	576	325	463	228	365	353	356

Table 2 Enzyme loci scored in seven *Andricus* species known only from asexual generations. All 13 loci were screened in each species, and loci that were polymorphic are indicated by an asterisk. The systems were run on buffers as follows: 1 = 40 mM sodium phosphate pH 6.3 (Stone & Sunnucks 1993), 2 = 0.1 M Tris-EDTA-maleate-MgCl₂ pH 7.6 (Richardson *et al.* 1986; buffer F), 3 = 25 mM Tris-Glycine pH 8.5 (Richardson *et al.* 1986; buffer I)

Enzyme locus	Buffer	<i>Andricus caputmedusae</i>	<i>Andricus coriarius</i>	<i>Andricus glutinosus</i>	<i>Andricus lucidus</i>	<i>Andricus mayri</i>	<i>Andricus quercustozae</i>	<i>Andricus seckendorffi</i>
AK	1			*		*	*	
αGPD1	1	*			*		*	
αGPD2	1						*	
GOT-s	2	*					*	
GOT-m	2	*	*				*	
GPI	2	*	*	*	*		*	*
HK	3	*			*		*	*
MDH-s	2							
MDH-m	2						*	
ME	2						*	
PEP-b	1			*			*	
6PGD	2		*	*	*	*	*	*
PGM	3			*		*	*	*
Total loci		5	3	5	4	3	12	4

Multilocular galls: genotype similarity between occupants of a single gall. We use a novel method based on a comparison of observed data with the expected similarity of multilocus genotypes (MLG) within and between galls under sexual and asexual reproduction. Asexual reproduction in oak cynipids results in siblings having identical MLGs to each other and to their mother (Atkinson *et al.* 2002, 2003). In species lacking a sexual generation, a single asexual generation gall will thus only yield more than one MLG when it contains the offspring of more than one female (i.e. it is multiply-founded, Atkinson *et al.* 2002, 2003). If we assume that egg-laying females select their oviposition site randomly with respect to their genotype, then the MLGs from a single gall are expected to be no more similar to each other than equivalent numbers of MLGs selected from the population at random. In contrast, if the individuals within a single gall are produced sexually, then siblings will have related but not necessarily identical MLGs. These MLGs are expected to be more similar to each other than to genotypes drawn at random from the population (assuming that the population is unstructured). Under sexual reproduction, the similarity of genotypes emerging from one gall is expected to fall as the number of foundresses per gall and mates per female increases (Atkinson *et al.* 2002). However, as long as some of the individuals emerging from each gall are full or half sisters, MLGs should always be more similar within a single gall than between galls.

In order to carry out a statistical test to distinguish between the two scenarios, we reduce the full data set for each gall to a single copy of each distinct MLG within it. We assume that no female has progeny in more than one sampled gall (which we regard as acceptable for galls sampled at random from large populations, as here, given the ~20 eggs carried by individual sexual generation *Andricus* females; Stone *et al.* 2002). We then calculate the difference, D , between the average number of alleles shared by the genotypes within galls and the average number of alleles shared by genotypes in different galls:

$$D = \frac{1}{n_w} \sum_{ij,k} S_{ij,k} - \frac{1}{n_b} \sum_{ij,kl} S_{ij,kl}$$

where $S_{ij,kl}$ is the number of alleles shared between genotypes i and j in galls k and l , and n_w and n_b are the total number of comparisons within and between galls, respectively. Galls with a single MLG cannot contribute to the average number of alleles shared within galls although they are used in between-gall comparisons. The null distribution of the statistic D is obtained by recalculation following random permutation of genotypes among galls (maintaining the total number of distinct MLGs sampled for each gall). Because genotypes compared between galls may be identical, but those compared within galls cannot be, the null expectation of the statistic is slightly negative, making the test conservative. The P -value (one-tailed) for

the test-statistic is the proportion of permuted data sets with a D statistic equal to, or greater than, the observed value.

DNA sequencing and phylogenetic analyses

DNA was extracted using the DNeasy Tissue kit (QIAGEN catalogue no. 69504), following the manufacturer's protocol for insect DNA extraction. A 433bp fragment of the mitochondrial cytochrome b gene was amplified using the primers CB1 (forward) 5'-TATGTACTACCATGAGGACAAATATC-3' and CB2 (reverse) 5'-ATTACACCTCCTAATTTATTAGGAAT-3' (Stone & Cook 1998; Rokas *et al.* 2001; Stone *et al.* 2001). Twenty-five microlitre polymerase chain reactions (PCRs) were carried out in a PTC-200 DNA Engine (MJ Research) using 1 U *Taq* polymerase (Promega), 2.5 μ L 10 \times *Taq* buffer, 1.5 μ L MgCl₂ (25 mM), 0.5 μ L dNTPs (10 mM), 0.35 μ L primers (20 pmol), 1.0 μ L template DNA and 18.85 μ L distilled water. PCR products were purified using shrimp alkaline phosphatase (USB Corporation). PCR products were sequenced directly using ABI BigDye Terminator chemistry on ABI automated sequencers (Applied Biosystems), and in both directions to minimize PCR artefacts, ambiguities and base-calling errors. All new sequences are deposited in GenBank (Accession nos: '*A. burgundus*', EU100336–EU100369; *A. crispator*, AF539560; *A. hystrix*, EU100370, EU 100371; *A. multiplicatus*, DQ217996; *A. singularis*, EU100372).

All sequences were the same length and were checked for an open reading frame using SEQUENCHER 4.1 (Gene Codes) to identify nuclear pseudogenes (cf. Rokas *et al.* 2003a) and could be unambiguously aligned with those for a comprehensive set of western palaearctic species in *Andricus* and other oak gallwasp genera downloaded from GenBank (see Appendix SI, Supplementary material). This set comprises all the known species (CP, asexual-only and sexual-only) found in previous analyses to lie in the host-alternating *Andricus* clade (Cook *et al.* 2002; Rokas *et al.* 2003b). Phylogenies were rooted using a rose gallwasp, *Diplolepis rosae*, as in previous analyses, and two sequences for other oak gallwasp genera (*Dryocosmus* and *Neuroterus*) were added to reduce the potential impact of long branch effects (Stone & Cook 1998; Cook *et al.* 2002; Rokas *et al.* 2003b). A nexus format alignment for the cytochrome b sequences is available online as Appendix SII, Supplementary material.

Phylogenetic relationships were estimated using Bayesian inference in MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001). Sequence data were partitioned by codon, and appropriate molecular models for each codon were identified by comparing harmonic mean likelihoods for alternative models in MRBAYES using Bayes factors (Kass & Raftery 1995). The models selected were GTR + I for the first codon position, and GTR + I + G for the second and third codons. We used two independent runs of 4 million iterations, with a heat parameter value of 0.05 to achieve adequate levels of parameter exchange between hot and cold chains in each run.

Table 3 Results from tests for Hardy–Weinberg and linkage equilibria. The threshold probability is that used under the Dunn-Sidak correction for multiple comparisons

Species	No. of polymorphic loci	Hardy–Weinberg equilibrium			Linkage equilibrium		
		No. of populations	Threshold probability	Populations showing significant departures	No. of pairwise comparisons	Threshold probability	Significant comparisons
<i>A. quercustozae</i>	12	16	0.00063	0	54	0.00016	0
<i>A. caputmedusae</i>	5	16	0.00063	1	49	0.00021	0
<i>A. glutinosus</i>	5	7	0.0015	0	10	0.0007	0

Table 4 A summary of results for multilocus genotype (MLG) tests for the four *Andricus* species inducing multilocular galls. Observed *D* is the observed difference between the average similarity between MLGs within and between galls. The *P* value is the probability (calculated by permutation) of observing these values of *D* under random assortment of MLGs into galls

Species	<i>A. coriarius</i>	<i>A. mayri</i>	<i>A. lucidus</i>	<i>A. seckendorffi</i>
Location of sample	Madenli (Turkey)	Aglasun (Turkey)	Sopron (Hungary)	Madenli (Turkey)
No. of galls collected	11	17	37	21
No. of wasps screened	228	353	365	356
No. of polymorphic loci	3	3	3	4
Average heterozygosity/locus	0.322	0.364	0.439	0.365
Observed <i>D</i>	0.13	0.33	0.72	1.13
<i>P</i> value	0.085	0.004	0.000	0.000

Convergence was assessed through the standard deviation of split frequencies between the two runs (using a 'rule of thumb' threshold of 0.01; Huelsenbeck & Ronquist 2001) and visual examination of plots of parameter values in TRACER 1.4 (Rambaut & Drummond 2003). Parameter values were sampled from the cold chain every 1000 iterations. Parameter values and posterior probabilities for nodes were sampled after a burn-in of 3.5 million iterations. We used Bayes factors to test the hypothesis of monophyly for *Andricus burgundus* by comparing the harmonic mean likelihood for an unconstrained analysis with the likelihood for an analysis in which *A. burgundus* monophyly was enforced. The threshold natural log (ln) of the Bayes factor value for statistical significance was taken as 20, as recommended by Kass & Raftery (1995) for phylogenetic data.

Results

Detection of deviations from linkage and Hardy–Weinberg equilibria

Allele frequencies for 38 of a total of 39 *Andricus* populations of *A. caputmedusae*, *A. glutinosus* and *A. quercustozae* were in Hardy–Weinberg equilibrium (Table 3; allele frequencies for all sites and species are given on line as Appendix SIII, Supplementary material). None of the pairwise comparisons between loci in any of these species showed significant linkage disequilibrium (Table 3; full results for

all pairwise locus comparisons are given online as Appendix SIV, Supplementary material). *A. caputmedusae*, *A. glutinosus* and *A. quercustozae* all show signatures compatible only with cyclical parthenogenesis.

Genotypic similarity between occupants of a single gall

For all four multilocular *Andricus* species (*A. coriarius*, *A. lucidus*, *A. mayri* and *A. seckendorffi*), the average number of alleles shared by MLGs within galls was greater than that between galls (Table 4). For *A. lucidus*, *A. mayri* and *A. seckendorffi*, this difference is significantly greater than expected by chance, implying that multiple MLGs within a single gall are sexually generated siblings rather than the result of multiple founding events by purely asexual females. We conclude that *A. lucidus*, *A. mayri* and *A. seckendorffi* are cyclically parthenogenetic species with cryptic sexual generations. Although the same may be true for *A. coriarius* ($P = 0.085$ for this species, Table 4), this approach is not conclusive for this species.

Polyphyletic status of *Andricus burgundus*

Thirty-five *Andricus burgundus* sequences yielded 17 cytochrome *b* haplotypes. These were widely distributed through the host-alternating *Andricus* lineage (Fig. 3), and monophyly of *A. burgundus* was rejected with a high ln Bayes factor (524). The distribution of *A. burgundus* sequences suggests that

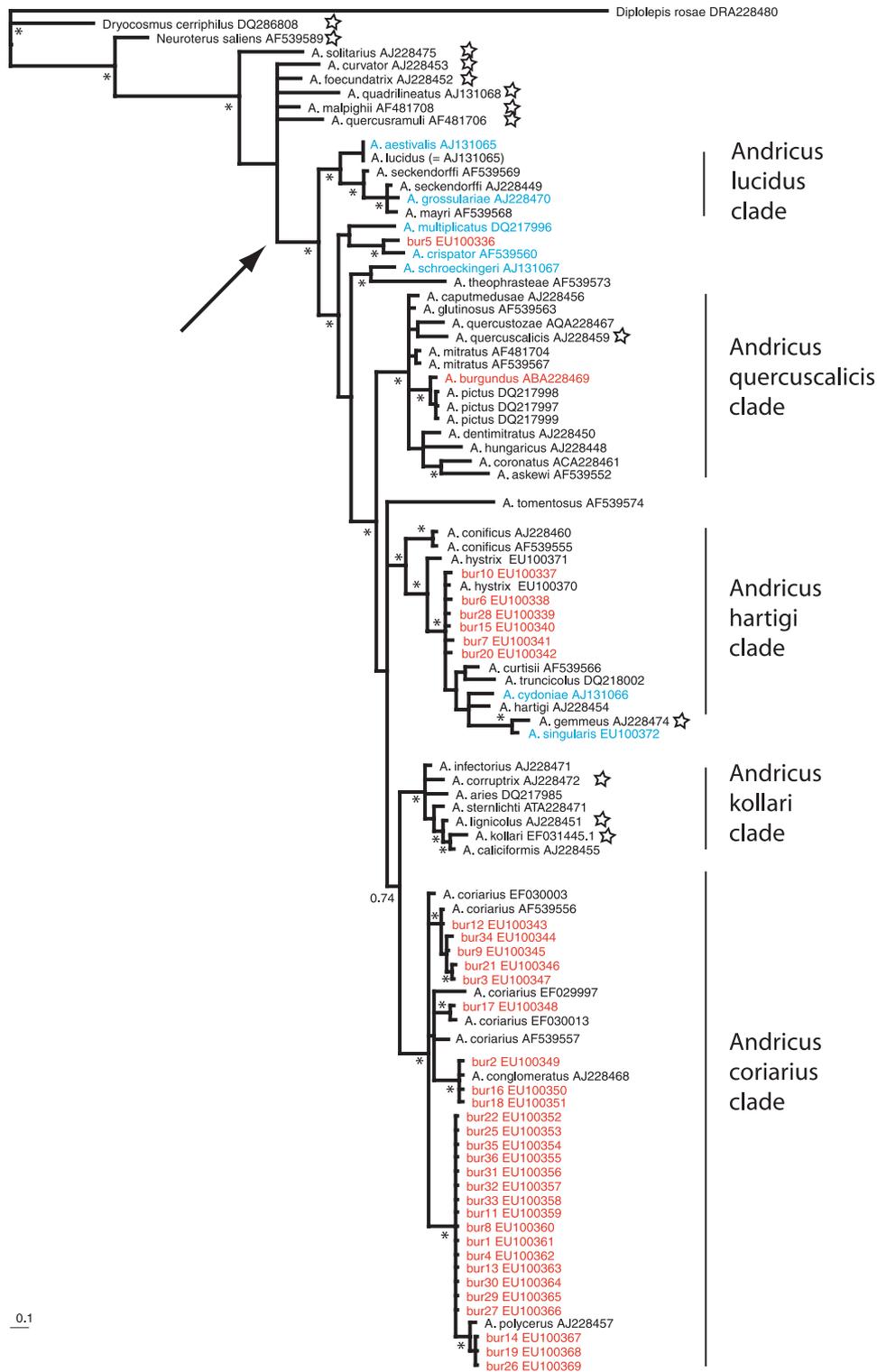


Fig. 3 A phylogeny of the host-alternating *Andricus* clade, including taxa known only from a sexual generation (with taxon labels in blue) and multiple accessions of the sexual generation species *Andricus burgundus* (with taxon labels in red). Species known to be cyclical parthenogens prior to this study are indicated by a white star, and all other taxa are known only from an asexual generation. Clade names follow previous work (Stone & Cook 1998; Cook *et al.* 2002; Rokas *et al.* 2003b). The arrow indicates the most recent common ancestor of the host-alternating *Andricus* clade. Values at nodes indicate posterior probabilities in Bayesian reconstruction, and an asterisk indicates values ≥ 0.95 .

this taxon represents the sexual generation of at least six *Andricus* species. In five cases, a putative asexual partner taxon can be identified on the basis of high sequence similarity (Fig. 3). Twenty-seven sequences representing 10 haplotypes were placed into the *A. coriarius* clade, and *A. burgundus* haplotypes were clustered with strong support with each of the asexual-only taxa *A. conglomeratus*, *A. coriarius* and *A. polycerus* (Fig. 3). Five sequences from Spanish *A. burgundus* yielded a single haplotype and clustered with strong support with the sympatric asexual-only taxon *Andricus pictus*. Six sequences representing five haplotypes showed high sequence similarity with the sympatric asexual-only species *Andricus hystrix*, in the *A. hartigi* clade (Fig. 3). Finally, one sequence was placed in a clade containing two other sexual-only taxa, *A. multiplicatus* and *A. crispator*, without candidate partner asexual-only taxa.

Phylogenetic placement of other known sexual-only taxa

Four sexual-only taxa were placed in clades with sympatric asexual-only species. *A. aestivalis* was paired with the asexual-only taxon *A. lucidus* with a posterior probability of 1 (Fig. 3), implying that these taxa comprise the two generations of a single CP species. *Andricus grossulariae* was placed in a clade containing the asexual-only taxa *A. mayri* and *A. seckendorffi* with a posterior probability of 1, suggesting that it is the sexual generation of one of these taxa. *Andricus cydoniae* was placed in the *Andricus hartigi* clade, and could represent the sexual generation of either of the two sympatric asexual-only taxa, *A. hartigi* and *Andricus truncicolus*. *Andricus singularis* was placed in the same group of *Andricus* species, in a strongly supported sister group relationship with *Andricus gemmeus*. *A. gemmeus* is known to have a CP life cycle whose sexual generation gall (a small, pip-like gall partially inside lateral buds on shoots) is nothing like the gall induced by *A. singularis* (a larger fleshy gall in leaf buds). *A. singularis* must represent either the sexual generation of another species (known or unknown) in the *A. hartigi* clade, or an alternative gall morphology for the sexual generation of *A. gemmeus*.

Three relatively abundant sexual-only species in the Hungarian fauna did not cluster with any known sympatric asexual-only taxon. *A. multiplicatus* and *A. crispator* are placed in a clade containing one of the *A. burgundus* sequences (Fig. 3) and are relatively distant phylogenetically from any of the sampled asexual-only taxa. *Andricus schroeckingeri* was placed with strong support as a sister taxon to the asexual only species *Andricus theophrastea*. The latter species has a highly distinctive gall (a fluffy bag up to 5 cm long surrounding a very hard, free-rolling inner larval chamber) and is unknown in Hungary, where *A. schroeckingeri* is native. *A. schroeckingeri* probably represents another orphan sexual generation whose asexual counterpart has yet to be discovered.

Discussion

Cryptic sexual generations are widespread in Andricus gallwasps

Our results imply that 11 *Andricus* species previously known only from asexual generations are in fact CP species. Allele frequency data strongly infer the existence of sexual generations for six putatively purely asexual *Andricus* taxa: *A. caputmedusae*, *A. glutinosus*, *A. lucidus*, *A. mayri*, *A. quercustozae* and *A. seckendorffi*. Sequence data identify *Andricus burgundus* as the sexual generation counterpart of a further five species with which it is sympatric: *A. conglomeratus*, *A. coriarius*, *A. hystrix*, *A. pictus* and *A. polycerus*. The sexual generation bud gall of *A. hystrix* has been confirmed independently by rearing experiments (Folliot & Pujade-Villar 2006). Sequence data further identify the sexual generation of *A. lucidus* as the known sympatric sexual-only species *A. aestivalis*, and suggest that the sexual generation of either *A. mayri* or *A. seckendorffi* is the known sympatric sexual-only species *A. grossulariae*. Subsequent rearing experiments (Walker 2002) have confirmed the pairings between *A. aestivalis* and *A. lucidus*, and between *A. grossulariae* and *A. mayri*. Our results provide no evidence for life cycle simplification through loss of the sexual generation in host-alternating *Andricus*. The distribution of newly demonstrated CP life cycles through this lineage suggests that although individual host-alternating *Andricus* species may yet be shown to have lost their sexual generation, the same is unlikely to be true for clades. There is thus no evidence in this genus of diversification of purely asexual lineages (in contrast to the bdelloid rotifers: Normark *et al.* 2003; King *et al.* 2005). The possibility remains that secondarily asexual lineages have diversified in the palaeartic genera *Aphelonyx* and *Pseudoneuroterus* and the Nearctic genera *Atrusca*, *Holocynips*, *Odontocynips*, *Philonix*, *Phylloterax*, and *Zopheroterax*, for which no candidate sexual generations are currently known (Melika & Abrahamson 2000; Stone *et al.* 2002). The approaches demonstrated here represent a time- and labour-efficient approach to confirming the reproductive mode of these little-studied genera.

Work on other gallwasp genera known only or primarily from females is required before we know whether cryptic sexual generations are the rule in these taxa. The rarity of secondarily obligately asexual oak gallwasps to date suggests either that selection acts against loss of sex, or that constraints make loss of sex difficult. The latter view is supported somewhat by the fact that gallwasp lineages whose default reproductive mode is sexual reproduction rather than cyclical parthenogenesis, particularly the rose gallwasps (tribe Diplolepidini) and herb gallwasps (tribe Aylacini), show widespread parthenogenesis in response to *Wolbachia* infection (Plantard *et al.* 1998, 1999). In contrast, although

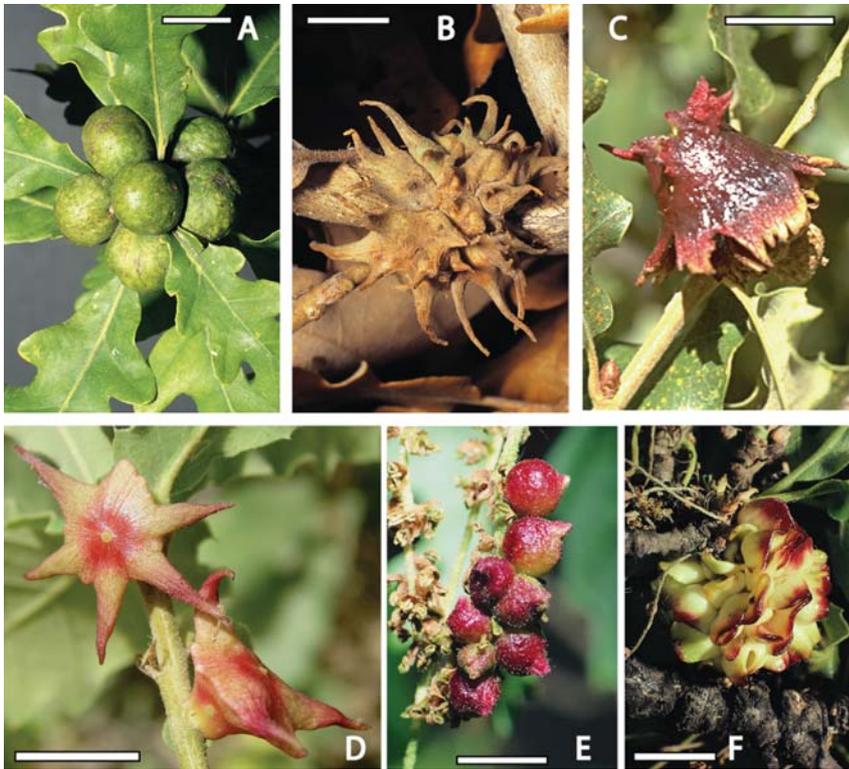


Fig. 4 (A–D) Asexual generation galls of four species inferred by our work to have morphologically indistinguishable sexual generations of the *Andricus burgundus* type. (A) *Andricus glutinosus*. (B) *A. coriarius*. (C) *A. pictus*. (D) *A. polycerus*. (E–F) Large, morphologically complex *Andricus* sexual generation galls. (E) *A. grossulariae*. (F) *A. lucidus*. Scale bars are 1 cm.

some oak gallwasps are infected with *Wolbachia*, they never show parthenogenesis in response (Rokas *et al.* 2001, 2002), and the oak gallwasps that do show proven obligate parthenogenesis that have been screened are not infected with *Wolbachia* (Abe & Miura 2002). Selection in favour of maintenance of sex could potentially be imposed by arms race co-evolution by natural enemies (van Valen 1973; Bell 1982; Ladle 1992), particularly the parasitoid and inquiline Hymenoptera that impose high mortality and local extinction on oak gallwasps (Stone & Schönrogge 2003; Csóka *et al.* 2005).

Sexual and asexual generations show contrasting patterns of phenotypic diversity

The polyphyly of *A. burgundus* shows that the apparent rarity of sex in this lineage is due in large part to our inability to distinguish sexual generation adults and their galls. This in turn reflects contrasting patterns of phenotypic evolution between asexual and sexual generations in this *Andricus* lineage. While asexual generation gall phenotypes are large, complex and structurally diverse (Stone & Cook 1998), sexual generation galls (as exemplified by *A. burgundus*) are often small, structurally simple, and phylogenetically conserved. This contrast is shown for a selection of the asexual generations inferred to have galls and sexual adults of the *A. burgundus* type in Fig. 4. Similarly, while the asexual generation *Andricus* females can be distinguished

on the basis of morphology (Eady & Quinlan 1963; Melika *et al.* 2000; Nieves-Aldrey 2001; Melika & Abrahamson 2002), failure of taxonomists with considerable experience in cynipid research (GM, JLNA) to detect diagnostic variation in *A. burgundus* is compatible with greater morphological conservatism in the sexual generation. Cynipid gall traits are under gallwasp control (Stone & Cook 1998; Cook *et al.* 2002; Stone & Schönrogge 2003), and both gall traits and adult morphology represent the extended phenotypes of gallwasp genes. There is thus a general contrast between phenotypic diversification in the asexual generation, and phenotypic uniformity in the sexual generation. This suggests in turn that there is either a contrast in the selective pressures shaping diversity in the two generations, or in constraints limiting the potential of the two generations to respond to selection. Sexual generation galls in the host-alternating *Andricus* can be large and structurally complex (e.g. *A. grossulariae*, *A. lucidus*; Fig. 4), suggesting that alternative adaptive strategies rather than constraints may underlie the contrast between generations. The structurally contrasting galls of asexual and sexual generation in the *Andricus* clade are attacked by very different communities of parasitoid wasps (e.g. Schönrogge & Crawley 2000; Stone *et al.* 1995) small size/crypsis/rapid development (sexual generation galls) and large size/structural complexity/slower development may represent alternative strategies for escape from these natural enemies (Stone & Schönrogge 2003; Vamosi 2005). Formal comparison of patterns in the

evolution of sexual and asexual generations must await a more in-depth analysis.

Newly discovered sexual generations imply the existence of unknown asexual generations

A striking feature of our analysis is the inference that there must be as-yet undiscovered asexual generation galls. No oak gallwasps are known to have a life cycle consisting only of a sexual generation, and the most parsimonious interpretation is that the unpartnered *A. burgundus* lineage, *A. multiplicatus*, *A. crispator* and *A. schroeckingeri* are orphan sexual generations whose asexual counterparts have yet to be discovered. The known richness of asexual generation oak galls in central Europe rose rapidly as study on them began in the second half of the 19th century, but of 59 recognized CP or asexual-only European *Andricus* species, only four have been described in the last hundred years (Melika *et al.* 2000; Stone *et al.* in press). The inference from our phylogeny is that four asexual partner taxa are required for the orphan sexuals. Only one candidate asexual taxon not included in our analysis, *Andricus superfetationis* (Giraud, 1859), is known from central Europe that could potentially be a member of the host-alternating *Andricus* lineage. Uniquely for the western Palaearctic fauna, this species induces galls on the outer wall of the acorn cup. This gall structure is sufficiently distinct from sampled *Andricus* species groups that it could represent the asexual counterpart to one of *A. multiplicatus*, *A. crispator*, *A. schroeckingeri* or the closely allied lineage of *A. burgundus*. Even if one of these pairings is correct, further asexual generation taxa are required to pair up the remaining orphan sexuals. The unpaired sexuals – particularly *A. multiplicatus*, are often quite common, and our prediction is that the unknown asexual taxa must be extremely cryptic (for example, inducing galls that are concealed within acorns or develop on roots) to have escaped discovery for so long.

Utility of allele-frequency based methods for detection of cryptic sexual generations

Our analysis of similarities of multilocus genotypes recovered from single galls assumes that the high degree of similarity between discrete MLGs within as opposed to between galls is a signature of sexual reproduction. Similar signatures could potentially be generated by other processes. Both misscoring of alleles and *de novo* mutation could result in recording different MLGs for sisters generated apomictically (Atkinson *et al.* 2003), which should have identical genotypes. Under both of these scenarios, the most likely pattern would be for the MLGs to differ by a single allele but this was not observed and the rate of misscoring or mutation would have to be extremely high to generate the patterns seen. A second type of artefact

could result if galls assumed to represent a single panmictic population are actually derived from discrete subpopulations between which gene flow is limited. The similarity of MLGs from different galls would thus be reduced and support for sexual reproduction would be strengthened erroneously. We minimized this potential problem by sampling galls from one single discrete population for each species. This approach is of widespread potential value in clarifying reproductive mechanisms wherever the offspring of single females commonly develop gregariously in a single resource. Potential candidates include other gall inducing lineages (Raman *et al.* 2005) and many groups of dipteran and hymenopteran parasitoids (Luck *et al.* 1993; Godfray 1994).

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This work forms part of an ongoing series of projects on the molecular ecology of oak gallwasps, led by Graham Stone (population genetics, phylogenetics). Rachel Atkinson, Antonis Rokas, Alex Hayward and Zoltan Ács have applied molecular population genetic and phylogenetic approaches to many aspects of gallwasp reproductive biology, population structure, and evolution. György Csóka works on all aspects of the health of forests in Hungary, with particular interest in insect pests. George Melika and José-Luis Nieves-Aldrey have a long-term interest in the biology and taxonomy of gallwasps. Richard Bailey has a wide-ranging interest in the trophic and spatial structure and the dispersal dynamics of natural communities. Caroline Buckee now focuses on the evolutionary dynamics of host-parasite interactions, particularly malaria. Gil McVean studies multiple areas in evolutionary biology and population genetics, particularly the analysis of recombination from population genetic data, the relationship between linkage disequilibrium and properties of the underlying genealogy, and methods for inferring genealogical history from DNA sequence data.

Supplementary material

The following supplementary material is available for this article:

Appendix S1 Genbank accession numbers for all previously published sequences used in the phylogeny in text Figure 3, including outgroups.

Appendix S2 A nexus format alignment of the cytochrome *b* sequences contributing to the phylogeny in text Figure 3.

Appendix S3 Allele frequencies for polymorphic loci, observed heterozygosity (H_{obs}) and heterozygosity expected under the assumption of Hardy–Weinberg equilibrium (H_{exp}) in each of 3 unilocular asexual gallwasp taxa.

Appendix S4 Summaries of analyses of linkage disequilibrium in the 3 unilocular asexual gallwasp taxa *Andricus caputmedusae*, *A. glutinosus*, and *A. quercustozae*.

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