Molecular identification of Andricus species (Hymenoptera

Molecular identification of *Andricus* species (Hymenoptera: Cynipidae) inducing various oak galls in central Zagros of Iran

Majid Tavakoli[a], Samad Khaghaninia[a], George Melika[b], Graham N. Stone[c], Asadollah Hosseini-Chegeni[d,e]

[a] *University of Tabriz, Faculty of Agriculture, Department of Plant Protection, Tabriz, Iran*

[b] *Plant Health and Molecular Biology Laboratory, National Food Chain Safety Office, Directorate of Plant Protection, Soil Conservation and Agri-environment, Budaorsi str. 141-145, Budapest 1118, Hungary*

[c] *Institute of Evolutionary Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3FL, United Kingdom*

[d] *Department of Plant Protection, Faculty of Agriculture, University of Lorestan, Khorramabad, Iran*

[e] *Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran*

Corresponding authors: Asadollah Hosseini-Chegeni, Department of Plant Protection, Faculty of Agriculture, University of Lorestan, Lorestan, Iran. Tel: +98 916 669 5140, E-mail: hosseinichegeni@gmail.com

Abstract

This study uses an integrated approach to address the taxonomic status of six different and problematic oak galls and their inducing wasps sampled from two sites in the Central Zagros Mountains (Lorestan province) in western Iran. Our aim was to establish whether morphologically similar but different galls are induced by the same or distinct gall inducers. The gallwasp specimens were identified morphologically to species, and their genomic DNA extracted. We used PCR and Sanger sequencing to amplify three fragments comprising cytochrome oxidase subunit I (*COI*), cytochrome b (*cytB*), and a multi-gene fragment of ribosomal DNA (rDNA) including partial 5.8S, complete internal transcribed spacer 2 (*ITS2*), and partial 28S rRNA. We find that a pair of structurally similar but differently coloured galls are induced by the sexual generation of *Andricus grossulariae,* while another similar pair are induced by the asexual generation of *A. sternlichti.* In contrast, we find that two similar galls that differ in some structural details and in developmental phenology are induced by two

closely related but different gall wasps; one is the sexual generation of *A. cecconii*, while the second is a new but closely related sexual generation *Andricus* sp.

Keywords: Galling wasps, Gall morphology, Phylogeny tree, *Andricus*, Zagros Iran

Introduction

Oak gallwasps (Hymenoptera: Cynipidae: Cynipini) induce structurally complex plant galls and are distributed in all continents except Antarctica. The highest species richness of these wasps is found in temperate regions of the northern hemisphere. Cynipid gallwasps have the highest species richness among gall inducing invertebrates after cecidomyiid gall midges, and induce some of the most complex and beautiful plant galls . Cynipid wasp taxonomy was revised in 2006 to include 12 tribes (rather than the previous 6) based on phylogenetic analysis derived from molecular, morphology, and life history data . The tribe Cynipini associated with plants in the family Fagaceae is the most species-rich , and currently contains 954 species in 41 genera . Over 86% of cynipid gallwasps are found on Fagaceae trees, especially oaks in the genus *Quercus* . Species in the tribes Cynipini and Pediaspidini are usually cyclical parthenogens, with alternating sexual and asexual generations in a single year. Generally, each generation induces a gall on a specific plant species or a group of very closely related species, and the gall induced by each gallwasp species and generation has a unique color and form .

The systematics of cynipids has gradually evolved over the last decade. New genera have been described and old names synonymized and/or transferred between genera that were originally based on the host-plant relationships and other biological data . Adult morphology-based taxonomy is almost unable to distinguish species in some Cynipini gallwasp genera reliably, particularly in the species-rich and holarctic genera *Andricus*, *Callirhytis* and *Dryocosmus*. Field identification of cynipids is thus currently often based on the gall structure induced by the gallwasp . Further, gall morphological characteristics and host plant relationships provide essential supporting information for the diagnosis of adult insects . However, there may be highly different gall characteristics among closely related plant species due to geographical changes in the vegetation . Potential inaccuracies in the identification and taxonomy of cynipid gallwasps have created problems for subsequent

studies . The adults of some cynipids represent sets of morphologically indistinguishable cryptic species , and in some cases gall structures also fail to discriminate species (Stone et al. 2008).

Molecular methods such as DNA barcoding are now increasingly used to complete and/or confirm the morphological identification of species. In this regard, it is claimed that up to 97% of species are correctly identified by this method . The use of molecular techniques along with morphological studies of the adult cynipid and its associated gall(s), the identity of the host plant, the geographic sampling area, and even the location of the gall on the plant host contribute to the diagnosis and description of new species . Moreover, capturing the cynipid wasps (the inducer and any associated inquilines) that live in the galls greatly facilitates identification.

Oaks (*Q. brantii* in *Quercus* section Cerris and *Q. infectoria* in section *Quercus* sensu stricto) are two of the most valuable and abundant tree taxa in the Zagros Mountains of Iran. The oaks host multiple gallwasp species, particularly in the genus *Andricus* . This study uses DNA sequence data for mitochondrial and nuclear loci (Nicholls et al 2012) to resolve the taxonomic status of the wasps emerging from three pairs of similar gall phenotypes that differ in subtle but consistent elements of size, colour and adult emergence phenology. We focus on yellow and green colour morphs of the asexual generation “Mazouj” galls induced by *A. sternlichti* on *Q. infectoria*, on red and yellow colour morphs of the grape-like sexual generation galls induced by *A. grossulariae* on *Q. brantii,* and on two structural forms (walnut nut-like and fig-like) of the sexual generation galls induced by *A. cecconii* on *Q. brantii*. The question is whether the gall phenotypic differences indicate genetic differentiation between gall causer lineages, or instead indicate phenotypic plasticity in the gall form induced by a single wasp lineage.

Material and Methods

Morphological study

Sample collection and rearing

Developing galls of each of the six target gall types were identified at two sites (Aleshtar and Khorramabad) in the central Zagros Mountains of Lorestan province (Table 2). We recorded the date at which gall development on host oaks began in the field and collected samples of mature galls of the spring sexual and autumn asexual generations in 2016 and 2017. Sampled galls were reared in plastic containers with adequate ventilation and transparent walls under laboratory conditions (22-5 ˚C, 60-70% relative humidity). Each container was examined daily and the time of wasp emergence from the gall was recorded. The emerged wasps were anesthetized at –10 ˚C, then collected with a minute brush and stored in 70% ethanol for further examination.

Wasp and gall determination

Gallwasp specimens were identified to species under a stereomicroscope (Wild-Heerbrugg M8) using existing identification keys (Pujade-Villar et al. (2003), Melika (2006), Ronquist et al. (2008), and Melika et al. (2010). To identify and prepare the wasps, photos of body parts (head, thorax, wing, antenna, and abdomen) of 5-6 specimens were captured and examined individually from various aspects. Gall morphology was characterized by general features, color, surface projections, and the number of larval cells. Images of gall and wasps were prepared using a Sony camera. Image processing was performed using Adobe Photoshop CS4 software. Voucher specimens are retained in Lorestan Agricultural and Natural Resources Research Center.

Molecular study

DNA extraction, PCR, and sequencing

Genomic DNA of each species was extracted using CTAB according to Doyle and Doyle (1987) with minor modifications. A fragment of cytochrome oxidase subunit I (*COI*), cytochrome b (*cytB*), and a multigene fragment located of ribosomal DNA (rDNA) comprising partial 5.8S, a complete internal transcribed spacer 2 (*ITS2*), and partial 28S rRNA (for this study, abbreviated as rDNA) were amplified by polymerase chain reaction (PCR). The list of primer sequences used in this study is summarized in Table 1. These sequences were chosen based on extensive previous use in gallwasp DNA barcoding and phylogenetics for *COI* , *cytB* , and rDNA . We use both mitochondrial and nuclear sequences because of the potential for reticulate evolution of mitochondrial loci through hybridization,

which is known to occur between closely related gallwasps (Nicholls et al 2012). PCR reactions were carried out in a thermocycler (MyCycler™ Thermal Cycler-Bio-Rad®, U.S.A) using a touchdown temperature profile of 5 min at 95℃, 11 X [60 s at 95℃, 30 s at 60℃, 45 s at 72℃], 24 X [60 s at 95℃, 30 s at 50℃, 45 s at 72℃], and 5 min at 72℃. PCR for each 25 µl final volume reaction was performed using 12.5 µl RedMaster PCR 2X (Ampliqon®, Denmark), 1 µl from each primer (10 pM), 4 µl gDNA template (100 ng/µl), and 6.5 µl ddH_20. The PCR products were visualized using 1% agarose gel electrophoresis and the desired bands were purified using the GF-1 Gel DNA Recovery Kit (Vivantis®, Malaysia). Finally, the purified PCR products were submitted to Faza-Biotech® Inc. (Iran) for sequencing.

Alignment, genetic distances, and phylogenetic tree

PCR-derived sequences were edited manually using FinchTV (ver. 1.4) and were compared using the BLAST option in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All new sequences have been submitted to GenBank (see Results). To structure our phylogenetic analyses, we selected out-groups following Smith (1994) and Wenzel (2002), with representatives from sister groups as well as successively more distant lineages. We used GenBank sequences for three selected gene sequences (*COI*, *cytB,* and rDNA) for four additional *Andricus* species, three of which are in the same host-alternating clade as the study taxa and so part of the ingroup (*A. caputmedusae, A. kollari* and *A. quercustozae*), and one of which is part of the outgroup within the gall inducing tribe Cynipini (*A. curvator*). We use the inquiline gallwasp *Synergus umbraculus* as a more distant out-group .

The sequences were aligned using SeaView4 as a concatenated dataset including three *COI*, *cytB*, and rDNA sequences for each sample (with edited lengths of 520, 329 and 391 base pairs respectively, total 1240 base pairs). Genetic distances among sequences were calculated using the Maximum Composite Likelihood (MCL) model in MEGA7 . To construct a phylogenetic tree with a combined data set, the partition homogeneity test (PHT) was applied to the sequences using PAUP v.4.0B10 . Since the *P value* for this test was non-significant (P>>0.05), the *COI*, *cytB,* and rDNA sequences of each wasp species were combined. The three gene combined alignment was created using MEGA7 and the phylogenetic relationships between taxa were inferred using Bayesian inference (BI) in BEAST v.2.5.1 . We selected an appropriate molecular model using jModeltest v.2.1.7 , identified as the Jukes-Cantor (JC) substitution model with a –lnL score of 2695.55. BI employs Markov chain Monte Carlo

(MCMC) algorithms and infers a most credible tree given the posterior probabilities of alternative tree topologies. The clades in the phylogenetic tree were arranged and labelled based on posterior probability support value, the genetic distance within- and between the clade members, and different characteristics of gall morphology (shape and color) and biology (the time of gall formation and adult gall maker emergence).

Results

Morphological study

Andricus sternlichti Bellido, Pujade-Villar & Melika, 2003 and its gall

The asexual generation of this gall, known as the Mazouj, is an important source of tannins in human trade, and is commonly observed in two colour forms: one is pale-yellow brown (Figure 2A), and the second is green with paler points on its surface (Figure 2C). Where the galls are abundant, occasional pale green intermediate colour forms are observed (Figure 2B). Both galls are spherical, reaching a maximum diameter of 15mm (though usually smaller), with blunt surface projections several millimeters long. Each gall contains a single larval cell, and the outer gall tissues are very hard and woody when mature. The females of the wasp induce galls on the lateral and terminal buds of the shoots of *Q. infectoria*, the only host for this species in the Zagros. To date, no sexual generation of this gall wasp has been identified. Adult gall wasps emerge from these galls from mid-September until mid-November. Our analysis of adult morphology and DNA sequence (detailed below) found the wasps that emerged from both colour morphs to be *A. sternlichti* Bellido, Pujade-Villar & Melika, 2003 (Figure 1A).

Andricus grossulariae Giraud, 1859 and its gall

The sexual generation galls of *A. grossulariae* are induced on the catkin of *Q. brantii* at the beginning of spring in early May. *Andricus grossulariae* has a host alternating lifecycle between oak sections *Quercus* sensu stricto and section Cerris, and is found only in areas with both *Q. infectoria* and *Q. brantii* or their hybrids. Relatively large for a catkin gall at up to 5mm in diameter, each gall is attached to the catkin axis and nearly spherical, with a small apical point. Each gall has a single larval cell with a surrounding internal air space. The galls are usually found in clusters. These galls exist in red and yellow colour forms. Early in

development, the red form is green, soft and covered in velvety pubescence. Once mature, the galls lose their pubescence, develop a shiny surface and turn a brilliant red. Ultimately, the gall becomes reddish-black, woody and hard (Figure 2F). In contrast, the yellow form galls are initially yellow (Figure 2E) and remain the same color until the last stage. Our morphological and molecular analyses (detailed below) show the inducers of both gall colour morphs to be *A. grossulariae* Giraud, 1859 (Figure 1B).

Andricus cecconii Kieffer, 1901 and a new closely-related *Andricus* species.

In the Zagros Mountains there are two structurally similar galls that could be identified as *A. cecconii*. Both develop on the catkins of *Q. brantii* (Figures 2C and 2D), forming a large multi-chambered and more or less spherical cluster, with a complex folded surface. In both, the catkin axis is often slightly thickened and strengthened when a gall is present. The two forms differ as follows. In one form, which we term fig-like, the galls that make up the cluster have a velvety surface and a shape similar to a Saturn peach fruit (Figure 2C). Each gall in the cluster contains one or two larva chambers and is connected by a stalk to the catkin axis. The galls are found singly or more often in dense spherical or elongate clusters on the *Q. brantii* catkin. Initially the gall is soft and green, becoming red or reddish brown, hard and woody when mature. This gall begins development from early to late May, and can be found for 3-4 months. The adults usually emerge from early to mid-July. The second form has a similar overall structure, but has an outer surface similar to a walnut kernel rather than a fig (Figure 2D). Each gall in the cluster is pale yellow or yellowish even when mature (not reddish), with a smooth and shiny (and not velvety) surface. Each gall contains 1-2 larval chambers. The outer part of each individual gall in the cluster is more or less circular with a central depression. This gall begins development in May, but the adults emerge in late May, before those of the previous gall type. This gall has a shorter life span than the fig type, at 2 to 2.5 months. Based on our morphological and molecular analyses, the galls of the first type (Figure 2C) are induced by sexual generation wasps of *A. cecconii* Kieffer, 1901 (Figure 1C). To date, no asexual generation is known for this wasp. Based on the molecular (rDNA), and biological traits (the time of adult emergence, gall morphology), we believe that the second gall morphology (Figure 2D) is a new sexual generation *Andricus* species closely related to *A. cecconii*. The adult morphology (Figure 1D) will be formally described in the future. As for *A. cecconii,* no asexual forms of this wasp are currently known.

Molecular analysis

PCR and sequences

Amplicons for *COI* (520bp), *cytb* (329bp) and rDNA (391bp) were each obtained for 7 adult *Andricus* wasps (Genbank accession numbers *COI*: KY819129-34 and MK030141, *cytb*: MK039705-11, rDNA: MK032872, MK036376, MK059975-8 and MK059983).

Phylogenetic analysis

The phylogenetic tree generated from the combined data set of *COI*, *cytB,* and rDNA is shown in Figure 3. Phylogenetic relationships among the selected taxa were fully resolved with high posterior probability (1.0 for all nodes). The *Andricus* taxa in our study were divided among three distinct clades (Figure 3) separated by genetic distances ranging from 2% to 8% (Table 3). Sequences for the insects emerging from the alternative colour forms of *A. sternlichti* differed by only 0.5%, compatible with induction of these galls by a single gallwasp species. Similarly, sequences for the insects emerging from the alternative colour forms of *A. grossulariae* differed by only 0.5%, also compatible with induction of these galls by a single gallwasp species.

For the two *cecconii*-like gall morphologies, *ITS* shows 3% sequence divergence between insects emerging from the typical fig-like *A. cecconii* gall (*A. cecconii*, Figure 2C) and those emerging from the walnut-like form (*Andricus* spp., Figure 2D), while across all three genes there is 1% sequence divergence (Table 3). Within *Andricus*, *A. cecconii* and *Andricus* sp. are more closely related to *A. grossulariae*, while *A. sternlichti* is more closely related to *A. kollari*, *A. caputmedusae* and *A. quercustozae* (Figure 3, Table 3). As expected based on previous work , *A*. *curvator* is placed basally to the other *Andricus* species.

Discussion

Morphological analysis

Cynipid gall wasps are an interesting group for evolutionary studies . In this study, we examined in detail the status of six cynipid galls of uncertain status using an integrated approach incorporating adult morphology, sequence data, and biological information (gall morphology, phenology and host plant-wasp relationships) . We found red and yellow grape-like galls to be induced by *A. grossulariae*, yellow and green Mazouj gall to be induced by *A. sternlichti*, and the fig-like and walnut-like galls on catkins of *Q. brantii* to be induced by *A.*

cecconii and a probable new but closely-related *Andricus* species. Prior to our study, these gall types were thought to be induced by only three cynipid species, i.e., *A. grossulariae*, *A. sternlichti*, *A. cecconii* for grape-like, Mazouj and walnut nut-like/fig-like galls, respectively (Bellido et al. (2003), Melika (2006), Tavakoli et al. (2008), and Sadeghi et al. (2009). Our study confirms the results of previous work on *A. grossulariae* and *A. sternlichti* based on molecular confirmation of gallwasp species. However, we believe the previously-grouped gall types of *A. cecconii* to be induced by two different wasp species. This finding is based on molecular divergence between the causing gall wasps, and differences in gall morphology and adult emergence phenology. Assuming that the difference between the two taxa is real (see below), the new species will be described in future.

Our findings show that variability in the color and form of galls does not necessarily reflect a difference in the identity of the gall inducing wasp. Generally, gall morphological characteristics are extended phenotypes of the causing gallwasps, and can provide useful taxonomic characters . However, in some cases the galls of different species are similar enough that this situation is reversed, and the inducers can only be identified using adult morphology . Moreover, sometimes, the structure of galls created by a gallwasp species can vary considerably among different species of oaks . Thus, the gall characteristics can only be used as an additional source of useful data for the determination of gallwasp species.

Phylogenetic analysis

The main idea of the current research was based on the question of whether each *Andricus* gallwasp could produce two forms of galls. We addressed this issue through analysis of *Andricus* species using 1240 bp of sequence data from two mitochondrial genes (*COI*, *cytB*) and nuclear ribosomal DNA from representative galling wasps of each gall form. These sequence data support induction of the two different gall forms in *A. grossulariae* and *A. sternlichti* by a single inducer taxon in each case, but the existence of two distinct species inducing the *A. cecconii* gall type. Our data are thus consistent with the general pattern that closely-related gallwasps often induce structurally similar galls. It is interesting that we found sequence divergence for rDNA to be greater than that for the two mitochondrial loci. Usually the opposite is true for these specific markers in gallwasps (Nicholls et al. 2012). We hypothesize that this pattern may have arisen through hybridization on secondary contact between two lineages that diverged in allopatry (Nicholls et al. 2012). Deep splits within gallwasp species are a common feature of gallwasp faunas to the west of the Zagros, either

side of the Anatolian Diagonal in Turkey (e.g. Rokas et al. 2003, Dinç and Mutun 2011, 2019, Mutun 2010, 2011, 2016), together with cryptic species richness (Challis et al. 2007). It is possible that the relationship between *A. cecconii* and the new *Andricus* sp. is the result of a similar process in the Zagros. More in depth sampling of molecular variation is desirable, to rule out the possibility that observed differences in our small sample size are the result of incomplete sorting of ancestral polymorphism rather than genuine species-level divergence.

A wide range of genetic distance from 2% to 8% was found among taxa; e.g., 5% between *A. sternlichti* with *A. cecconii* and *A. grossulariae* and 3% between *A. cecconii* with *A. grossulariae*. Though limited in taxon selection, our analysis also supports previous work in grouping *Andricus* species into clades, with *A. inflator* as an outgroup to those species that involve alternation of generations between host oaks in the sections Cerris and *Quercus* sensu stricto, i.e. *Q. brantii* and *Q. infectoria* respectively in the Zagros (Stone and Cook 1998; Cook et al. 2002; Rokas et al. 2003; Stone et al. 2009). We therefore predict that the asexual generations of *A. cecconii* and the related new species will develop in galls on *Q. infectoria*.

Conflict of interest

The authors declare no competing personal or financial interests.

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Table 1- Gene fragments and PCR primers used in molecular analysis

Gene	Size (bp)	Forward primer		Reverse primer	
		Name	Sequence (5′ → 3′)	Name	Sequence (5′ → 3′)
COI	520	C1-J-1718*	GGAGGATTTGGAAATTGATTAG	C1-N-2191*	CCCGGTAAAATTAAAATATAAACTTC
cytB	329	F-cytb**	TGATTATGAGGAGGATTTAGAGT	R-cytb**	CATTCAGGTTGAATATGAATTGG
rDNA	391	F-rDNA***	GGTCCACGGATACAATTCC	R-rDNA***	TCAAACAACCGTCCATAA

According to Simon et al. (1994); ** and *** were designed in this study

Table 2. Sampling information for the *Andricus* wasps collected in this study

Wasp	No. of collected wasp	Gall type*	Type of wasp generation	*Quercus* host	Locality (county)	GPS coordinates (EN)
A. sternlichti	80	YM	Asexual	*Q. infectoria*	Aleshtar	47°58'19" 33°45'10"
A. sternlichti	35	GM	Asexual	*Q. infectoria*	Aleshtar	47°55'10" 33°45'25"
A. sternlichti	65	IM	Asexual	*Q. infectoria*	Khorramabad	48°33'37" 33°20'16"
A. grossulariae	115	YG	Sexual	*Q. brantii*	Aleshtar	47°56'59" 33°48'06"
A. grossulariae	72	RG	Sexual	*Q. brantii*	Aleshtar	47°58'19" 33°45'10"
Andricus sp.	63	WNl	Sexual	*Q. brantii*	Aleshtar	47°55'10" 33°45'25"
A. cecconii	70	SPl	Sexual	*Q. brantii*	Khorramabad	48°33'37" 33°20'16"

* Yellow Mazouj (YM), Intermediate (green yellowish) Mazouj (IM), Green Mazouj (GM), Walnut nut-like (WNl), Saturn peach-like (SPl), Yellow grape (YG), Red grape (RG).

Table 3. Estimates of genetic distance (%) among *Andricus* sequences for the combined *COI*, *cytB*, and rDNA dataset

Clades**	***A. sternlichti***	***A. cecconii***	***Andricus* sp.**	***A. grossulariae***	*A. kollari*	*A. caputmedusae*	*A. curvator*	*A. quercustozae*
A. sternlichti	0							
A. cecconii	5							
***Andricus* sp.**	5	1						
A. grossulariae	5	3	3	0				
A. kollari	2	6	6	5				
A. caputmedusae	5	7	7	6	6			
A. curvator	7	6	6	6	7	8		
A. quercustozae	5	7	7	6	6	5	8	

Calculated based on the final dataset including 386, 285 and 168 positions in *COI*, *cytB,* and rDNA (with gaps), respectively; Bootstrapping with 1,000 replicates; all positions containing gaps and missing data were eliminated. Within-clade divergences are shown as diagonal (in highlight). ** According to clades of phylogenetic tree (Figure 3); Clades of this study are presented as bold text.

Figure 1. Gall wasp species: A) *Andricus sternlichti* asexual female, B) sexual female *Andricus grossulariae*, C) sexual female *Andricus cecconii*, and D) sexual female *Andricus* sp. Body parts are (clockwise from top right): forewing; antenna; head from above; mesosoma in lateral view; metasoma in lateral view; scutum and scutellum in dorsal view; head in anterior view. The whole body habitus is shown in the center of each of A-D.

Figure 2. Gall morphologies in this study: A) Yellow Mazouj. B) Intermediate (yellow-green) Mazouj. C) Green Mazouj (A-C induced by asexual generation *Andricus* sternlichti). D) Walnut nut-like gall of *Andricus* sp. E) Fig-like gall of *Andricus cecconii*. F) Yellow grape, and G) Red grape galls induced by sexual generation *Andricus grossulariae*.

Figure 3. Phylogenetic relationships among *Andricus* taxa derived from Bayesian inference (BI) analysis of combined data for *COI*, *cytB*, and rDNA; numbers below each node show posterior probability value in BI analysis (1,000 replicates). Taxon labels give the species name followed by (in turn) GenBank accession numbers for *COI*, *cytB*, and rDNA in parentheses; taxa sequenced in the present study are highlighted in bold. Galls and their inducing wasps are shown on the right side of the tree. The phylogeny is rooted with *Synergus umbraculus*.