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Systematic relationships of *Pityopus californicus* inferred from large ribosomal subunit (26S) rRNA gene sequences

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SYSTEMATIC RELATIONSHIPS OF *PITYOPUS CALIFORNICUS* INFERRED FROM LARGE RIBOSOMAL SUBUNIT (26S) rRNA GENE SEQUENCES

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ABSTRACT.—*Pityopus californicus* is a rare mycoheterotrophic herb that occurs in coniferous and mixed forests of western North America. Previous authors have speculated that *Pityopus californicus* is not a true species but is a recurring hybrid. The reputed parental candidates of *P. californicus* include the closely related *Pleuricospora fimbriolata, Hemitomes congestum,* and *Monotropa hypopithys.* However, a phylogenetic analysis of large ribosomal subunit (26S) rRNA gene sequences suggests that *Pityopus californicus* is sister to *Monotropa hypopithys* and not a recurring hybrid.

Key words: Pityopus californicus, *Monotropoideae, Ericaceae, plant systematics.*

Commonly called pine foot, *Pityopus californicus* is a rare mycoheterotrophic herb that obtains fixed carbon from basidiomycete ectomycorrhizal *Tricholoma* fungi (Bidartondo and Bruns 2001). It occurs in coniferous and mixed forests at 30–1840 m elevation from the Sierra Nevada, Cascade Range, and Coastal Range of California and the Coastal Range of Oregon (Wallace 1975).

Pityopus californicus was originally placed in the genus *Monotropa* by Eastwood (1902). She noted that its erect habit distinguishes it from *Monotropa hypopithys* to which it is most closely allied. Small (1914) moved it into his newly named genus *Pityopus* and listed *Monotropa californica* as a synonym. Later, Domin (1915) reduced it to a variety of *Monotropa hypopithys.* The currently accepted combination was published by Copeland (1935).

Nevertheless, Copeland (1935) expressed concern that *Pityopus californicus* may not be a true species. He noted that the only material difference between *Monotropa hypopithys* and *Pityopus californucus* is in the placentation that is axile in the former and parietal in the latter. Copeland (1935) noted that collection data indicated that *Pityopus californicus* is commonly associated ecologically with the closely related *Pleuricospora fimbriolata, Hemitomes congestum,* and *Monotropa hypopithys,* and he intimated that perhaps *Pityopus californicus* is a recurring hybrid between 2 of these.

Through a personal communication from Gary Wallace, author of a comprehensive monograph of the Monotropoideae (1975), Cullings (2000) reported that Wallace hypothesized that *Pityopus californicus* may be the result of hybridization between *Hemitomes congestum* and *Pleuricospora fimbriolata*. Cullings pointed out that previous systematic analyses placing *Pityopus californicus* with either *Hemitomes congestum* or *Pleuricospora fimbriolata* suggest that Wallace's hypothesis may be correct.

The purpose of this study was to examine the systematic relationships of *Pityopus californicus.* This investigation was based on an analysis of large ribosomal subunit (26S) rRNA gene sequences. The 26S gene was used in this study because it has been shown to exhibit a level of divergence that is informative within the Monotropoideae (Cullings 1994, Bidartondo and Bruns 2001, Neyland 2004, Neyland and Hennigan 2004).

METHODS

Vouchers and GenBank accessions for the taxa included in this study are listed in Table 1. The ingroup consists of representatives from all North American genera within the subfamily Monotropoideae (Ericaceae). The outgroup consists of representatives from other indicated subfamilies in Ericaceae (Table 1). Taxonomy follows Kron et al. (2002).

An approximate 578 base-pair DNA segment of the 26S gene for each representative listed in Table 1 was analyzed in this study. This segment, which spans base positions 380–958

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TABLE 1. Taxa analyzed in this study. All ingroup representatives are from subfamily Monotropoideae (Ericaceae) (sensu Kron et al. 2002). Outgroup representatives were selected from the indicated Ericaceae subfamilies. Multiple representatives of a particular taxon are distinguished by accession numbers. Vouchers for each taxon sequenced in the present study are indicated and housed at McNeese State University (MCN). GenBank accession numbers are indicated for each sequenced segment. All other sequences are from Bidartondo and Bruns (2001) and were retrieved from GenBank.

Taxon	Voucher	GenBank accession
INGROUP		
Monotropa hypopitys L.		
2037	Neyland 2037	AF543835
2052	Neyland 2052	AY166966
2046	Neyland 2046	AY166968
Monotropa uniflora L.		
1954	Neyland & Hennigan 1954	AF540062
2066	Neyland 2066	AY221084
2082	Neyland 2082	AY488114
Pityopus californica (Eastw.) Copeland f.		
I1Ump	—	AF351917
OR2124	—	AF351916
Pleuricospora fimbriolata Gray		
Ump7	—	AF351928
Blo2267	—	AF351929
Allotropa virgata Torr. & Gray ex Gray	—	AF351919
Monotropsis odorata Schwein. ex Ell.	—	AF351922
Moneses uniflora (L.) Gray	Neyland 2079	AY566296
Chimaphila maculata (L.) Porsh	Neyland 2049	AY294625
Pterospora andromedea Nutt.	Neyland 2078	AY368156
Hemitomes congestum A. Gray		
Silt11	—	AF351920
HecA14	—	AF351921
Sarcodes sanguinea Torr.	—	AF351933
OUTGROUP		
Lyonia lucida (Lam.) K.		
Subfamily Vaccinoideae	Neyland 2095	AY561836
Vaccinium elliottii Chapm.		
Subfamily Vaccinoideae	Neyland 1189	AY561835
Rhododendron canescens (Michx.) Sweet		
Subfamily Ericoideae	Neyland 659	AY561837
Arctostaphylos uva-ursi (L.) Spreng.		
Subfamily Arbutoideae	Neyland 2094	AY596455

in *Nicotiana tabacum* (GenBank Accession AF479172), is characterized by conserved segments and variable expansion segments designated as D2, D3, and D4 by Kuzoff et al. (1998).

Total DNAs were extracted from tissue using the CTAB method of Doyle and Doyle (1987). DNA sequences were amplified via polymerase chain reaction (PCR; Mullis and Faloona 1987) with combinations of forward and reverse primers referenced in Neyland (2002). Amplification was achieved with Tfl enzyme (Epicentre Technologies, Madison, WI, USA) using the following thermocycling protocol: a hot start at 94°C for 3 minutes; 30 amplification cycles of 94°C for 1 minute, 55°C for 1 minute; 72°C for 3.5 minutes, a terminal extension phase at 72°C, and an indefinite terminal hold at 4°C. The double-stranded PCR product was purified with QIAquick (Qiagen, Hilden, Germany) using the manufacturer's protocol. Two μL of each sample was electrophoresed in a 1.0% agarose mini-gel for quantification against a known standard. Automated sequencing was conducted on an ABI Prism 377 Sequencer with XL Upgrade (housed at Louisiana State University, Baton Rouge, LA, USA) using ABI Prism, Big Dye Terminator cycle sequencing protocol (P.E. Applied Biosystems, Foster City, CA, USA). Sequences have been deposited in the GenBank database (Table 1).

DNA sequences were used to infer the systematic relationships of *Pityopus californicus* through a maximum parsimony phylogenetic analysis using the heuristic search algorithm

TABLE 2. Absolute nucleotide pairwise differences between selected representatives included in this analysis.

Representative	1	2	3	4	5	6	7	8	9
1. *Monotropa hypopithys* 2037	—								
2. *Monotropa hypopithys* 2052	10	—							
3. *Monotropa hypopithys* 2046	14	5	—						
4. *Pleuricospora fimbriolata* Ump7	57	50	50	—					
5. *Pleuricospora fimbriolata* Bio2267	58	51	51	0	—				
6. *Hemitomes congestum* HecA14	45	40	44	42	42	—			
7. *Hemitomes congestum* Silt11	44	39	43	41	41	0	—		
8. *Pityopus californicus* I1Ump	31	23	28	43	43	30	30	—	
9. *Pityopus californicus* OR2124	31	23	28	43	44	31	30	0	—

with Phylogenetic Analysis Using Parsimony (PAUP version 4.0b10) software (Swofford 2002). Searches employed 1000 random stepwise addition replications. All characters including transitions and transversions were weighted equally. Gaps were treated as missing data.

A maximum likelihood search of 1000 replicates was also performed with the same data and outgroup used in the parsimony analysis. Starting trees were obtained through stepwise addition. In this search the transition/transversion ratio was designated at 3.4, based on the empirical value obtained in the parsimony search. Branches ≤1 were collapsed.

Disk copies of aligned sequences are available from the author. As a measure of clade stability or robustness, bootstrap support (Felsenstein 1985) was calculated. Ten thousand bootstrap replications were employed in this analysis (MulTrees option in effect).

Additionally, DNA sequences between *Pityopus californicus* and other related sympatric members of the Monotropoideae were examined in a point-by-point, pairwise analysis to determine if *Pityopus californicus* is the result of recurring hybridization. If this were the case, then *Pityopus californicus* sequences would have no nucleotide character states or point insertions unique with respect to those parents. That is, allelic forms of the 26S gene not found in either parent would not be expected to appear in a recurring hybrid.

RESULTS

Sequences were aligned by visual inspection. Of the 578 characters included in the data set, 109 (18.9%) were informative. Gaps were introduced to accommodate 12 single-point insertions/deletions (INDELS) in the data set. INDELS were not treated as informative characters. The largest absolute distance between any 2 members in the data set was 69 between *Sarcodes sanguinea* and *Monotropa hypopithys* (2037). Absolute distances between *Pityopus californicus* and *Hemitomes congestum*, *Monotropa hypopithys*, and *Pleuricospora fimbriolata* were 30–31, 23–31, and 43–44 respectively (Table 2). The number of unambiguous transitions and transversions numbered 165 and 48 respectively. Therefore, transitions outnumbered transversions by a factor of about 3.4 to 1.

The maximum parsimony analysis resulted in the recovery of the single most parsimonious tree of 264 steps with a consistency index of 0.7045 and a retention index of 0.8074 (Fig. 1). An examination of the phylogram strongly suggests that *Pityopus californicus* is sister to *Monotropa hypopitys* (bootstrap = 99%). The *Monotropa hypopithys–Pityopus californicus* clade is sister to *Hemitomes congestum* (Fig. 1). *Pleuricospora fimbriolata* is more distantly related (Fig. 1).

From the maximum likelihood analysis, the score of the best tree found has a log likelihood value of –2304.8050. Topology of the maximum likelihood tree (Fig. 2) is identical to that of the maximum parsimony tree (Fig. 1). Additionally, bootstrap support for each branch in the maximum parsimony tree (Fig. 1) is comparable to that of the maximum likelihood tree (Fig. 2).

Unequivocal point differences (including transitions, transversions, and INDELS) between *Pityopus californicus* sequences and the sequences of its reputed parents are illustrated in Tables 3–5. Unequivocal in this sense means that the specific nucleotide state or INDEL is identical in all representatives of each taxon. The nucleotide sequence of *P. californicus* exhibits unequivocal differences between it and its reputed parents (Tables 3–5).

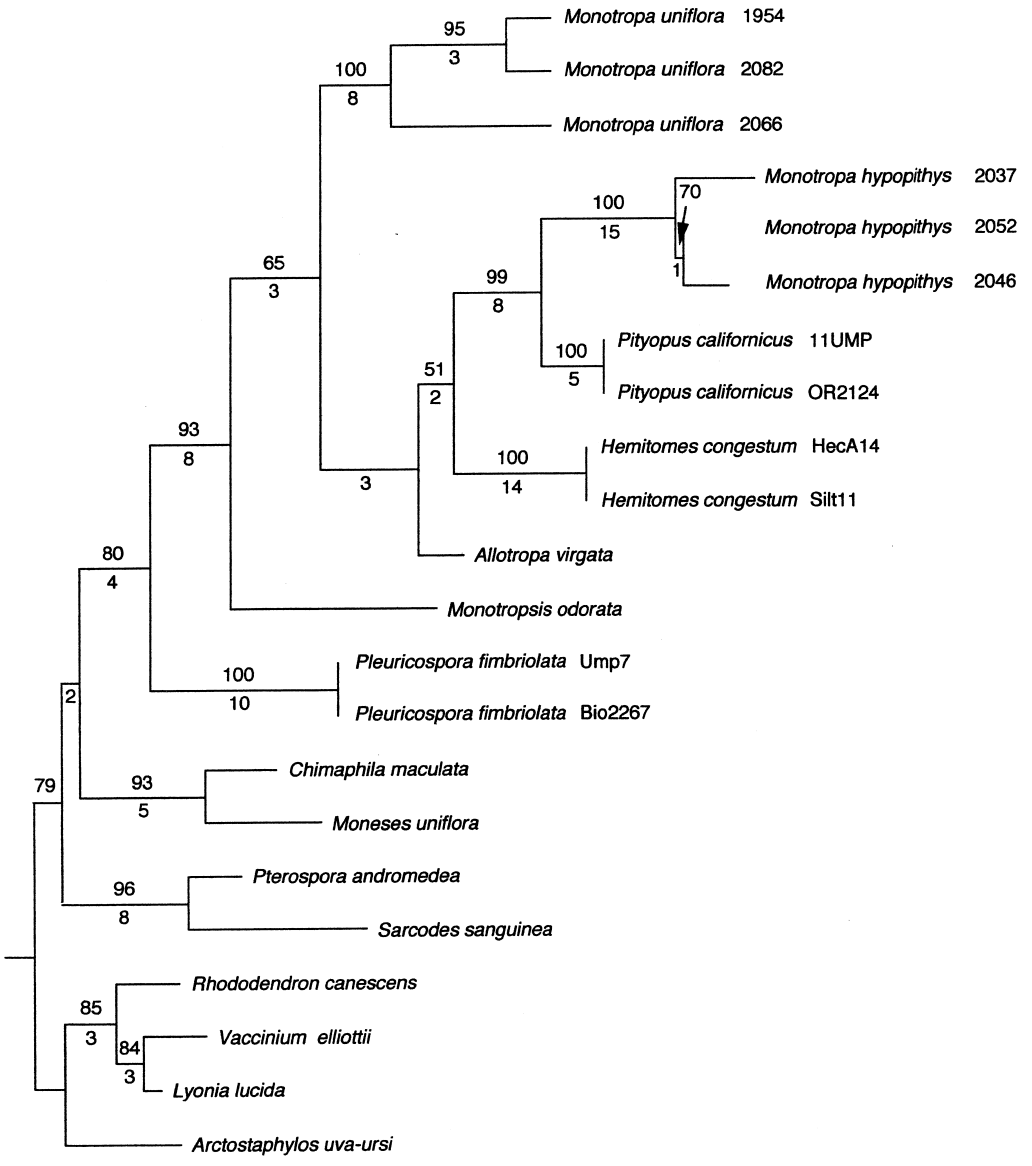

Fig. 1. Phylogram of the single most parsimonious tree discovered from a heuristic search using 26S rRNA gene sequences. The number of unequivocal synapomorphies is indicated below each branch. Bootstrap values are indicated above each branch.

DISCUSSION

The phylogenetic position of *Pityopus californicus* strongly suggests that this taxon is closely related to but distinct from *Monotropa hypopithys* (Figs. 1, 2). Therefore, by the criteria embodied in the phylogenetic species concept (Eldridge and Cracraft 1980, Nixon and Wheeler 1990), *P. californicus* appears to be a true species that arose from a common ancestor between it and *Monotropa hypopithys.* The sister relationship between *Pityopus californicus* and *Monotropa hypopithys* is supported by other studies. For example, results from the phylogenetic study by Bidartondo and Bruns (2001) indicated that *Pityopus californicus* is sister to North American *Monotropa hypopithys* when plastid *rps*2 data were used. In their phylogeny inferred from nrDNA (combined 28S and ITS sequences), Bidartondo and

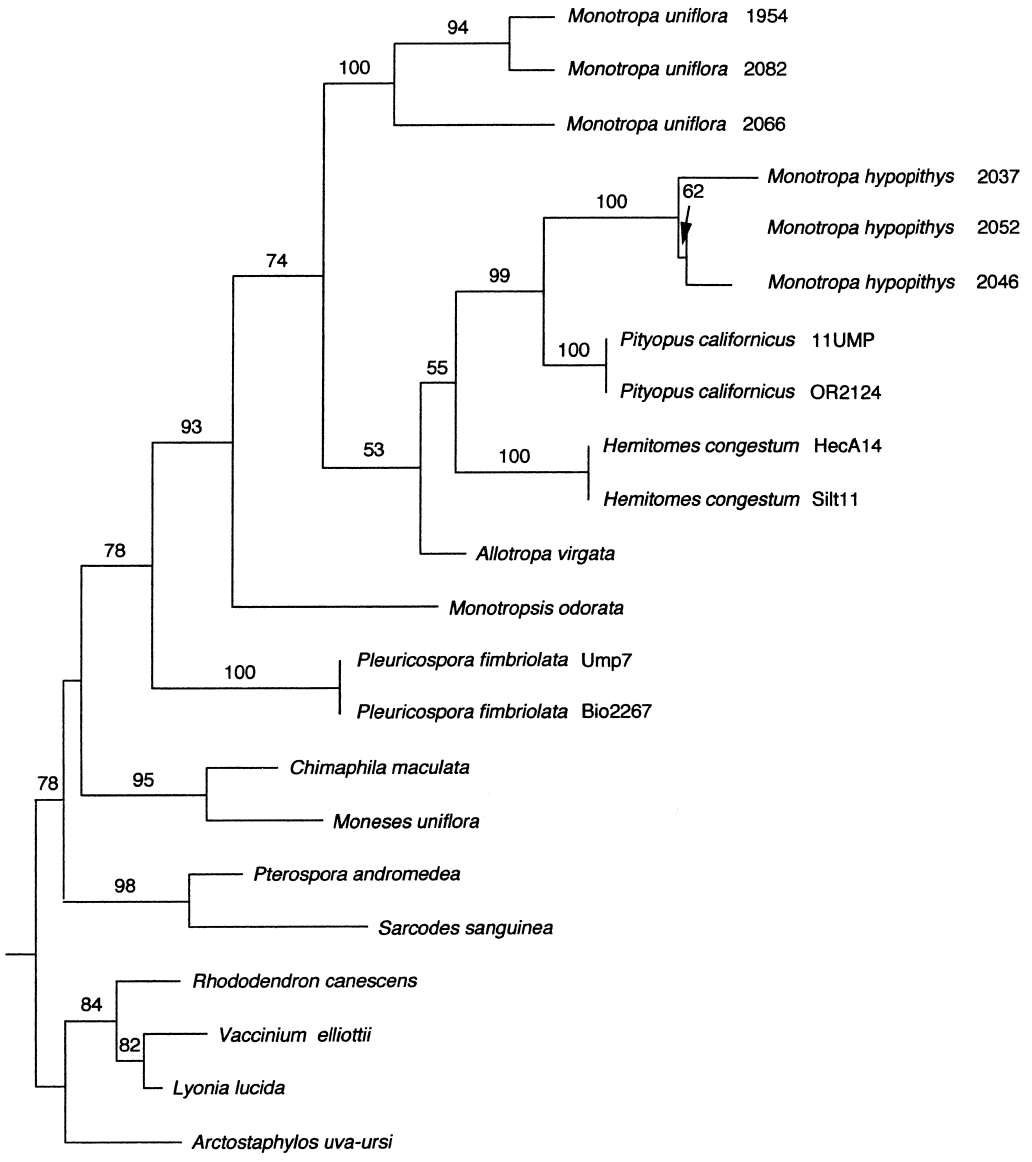

Fig. 2. The best tree from a maximum likelihood search. Bootstrap values are indicated above each branch.

Bruns (2001) positioned *Pityopus californicus* as sister to both American and Eurasian representatives of *Monotropa hypopithys.*

An earlier phylogeny inferred from partial 28S rRNA gene sequences by Cullings (1994) indicated that *Pityopus californicus* is sister to a clade composed of *Hemitomes congestum, Allotropa virgata,* and *Monotropa uniflora.* However, due to possible misidentification of specimens used in that analysis, Cullings (2000) stated that no taxonomic conclusions regarding members of the Monotropoideae should be drawn from those data.

The number of unequivocal nucleotide point differences between *Pityopus californicus* and its reputed parents (Tables 3–5) suggests that *Pityopus californicus* is not the product of current ongoing hybridization. That is, nucleotide states not found in either parent would not be expected to appear in a recurring hybrid.

Although the sequence data suggest that *Pityopus californicus* is not the product of

TABLE 3. Specific unequivocal point differences between indicated taxa. Position indicates the point in the sequence data set.

Position	Point difference		
	Monotropa	*Hemitomes*	*Pityopus*
45	C	C	T
100	C	C	T
119	—	—	A
176	G	T	C
223	C	C	T
236	T	T	C

TABLE 4. Specific unequivocal point differences between indicated taxa. Position indicates the point in the sequence data set.

Position	Point difference		
	Pleuricospora	*Monotropa*	*Pityopus*
45	C	C	T
100	C	C	T
119	—	—	A
154	T	A	G
176	T	G	C
190	C	C	T
223	C	C	T
236	T	T	C
361	C	C	T

TABLE 5. Specific unequivocal point differences between indicated taxa. Position indicates the point in the sequence data set.

Position	Point difference		
	Pleuricospora	*Hemitomes*	*Pityopus*
40	C	C	T
45	C	C	T
100	C	C	T
117	G	G	T
119	—	—	A
138	C	A	T
147	C	C	T
176	T	T	C
178	G	G	T
190	C	C	T
222	C	C	T
223	C	C	T
236	T	T	C
242	C	C	T
366	C	C	T

recurring hybridization, the data do not suggest necessarily that *Pityopus californicus* could not have arisen originally as the result of hybridization in the past. Such hybrid speciation leading to reticulate evolution is well documented (e.g., Anderson 1953, Stebbins 1959, Riesenberg and Ellstrand 1993, Arnold and Hedges 1995), and it may occur through either amphiploidy or introgression. If, for example, unreduced pollen and ovules from members of a parental population crossed and produced fertile autopolyploid offspring that were reproductively isolated from the parental population, then this could have lead to an intraspecific hybrid that evolved into the present *Pityopus californicus.* Over time, the mutational differences between the 2 populations would accrue in their DNA sequences.

Alternatively, *Pityopus californicus* may be an allopolyploid between 2 species. For this to have occurred, gametes from one species must have pollinated those of the other and a rare doubling of the genome must have occurred prior to embryonic development. This would have produced an F_1 interspecific hybrid that at once was both fertile and reproductively isolated from the parental population. It is estimated that between 25% and 50% of all plant species are allopolyploids (Niklas 1997).

Under the process of introgression, a possible scenario for the evolution of *Pityopus californicus* would have proceeded with the crossing of gametes between 2 species that led to a generally infertile interspecific F_1 hybrid population. If, however, the F_1 back-crossed with one of the parents and produced fertile F_2 that had different adaptive traits than the parental population, then the hybrid population could have evolved into the present *Pityopus californicus.*

Evidence supplied by Bidartondo and Bruns (2001) suggests an additional distinction between *Pityopus californicus* and its reputed parents. Specifically, the putative basidiomycete symbionts associated with *Pityopus californicus* are different from those found in *Hemitomes congestum, Pleuricospora fimbriolata,* and *Monotropa hypopithys.* Because of the apparent extreme specificity between fungal host and mycoheterotrophic members of subfamily Monotropoideae (Bidartondo and Bruns 2001), it appears unlikely that if *Pityopus californicus* is a recurring hybrid, it would form a symbiotic relationship with a fungal host not shared by either of it parents.

Whether or not *Pityopus californicus* is a true species by the criteria embodied in the

biological species concept (Dobzhansky 1937, Mayr 1942, Stebbins 1950) has yet to be determined. Even if *Pityopus californicus* is an allopolyploid, hybrid populations are not considered true species unless they maintain their unique biological identity in successive generations (Niklas 1997). That is, by biological species concept criteria, *Pityopus californicus* may not be considered a true species unless it can interbreed and yield viable fertile offspring but cannot breed successfully with members of other species. However, because recognized plant species often can freely hybridize in nature and in controlled experiments (Niklas 1997), the biological species concept as it applies to plants has been rigorously challenged (Raven 1980). It remains unresolved whether and to what extent *Pityopus californicus* is reproductively isolated from its sympatric relatives.

Future studies addressing these issues may focus on the ecological relationship between *Pityopus californicus* and its sympatric relatives. For example, a cross-pollination study may indicate whether and to what extent *Pityopus californicus* is reproductively isolated from its sympatric relatives. This type of study likely will prove problematic due to the exacting physiological requirements necessary to germinate seeds of mycoheterotrophs and grow them to maturity.

If indeed *Pityopus californicus* is the product of a distant hybridization event, then a cytological study may prove informative. For example, a comparison between the karyotypes of *Pityopus californicus* and its closest relatives may help determine whether or not this species arose through hybridization.

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