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BRIEF COMMUNICATION

HAPTANTHUS STORY: REDISCOVERY OF ENIGMATIC FLOWERING PLANT FROM HONDURAS[1]

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- *Premise of the study*: Finding a plant or animal that was previously considered extinct is a fortunate (but rare) event in biology. *Haptanthus hazlettii* was collected from Honduras (Central America) in 1980, but numerous attempts to re-collect it have failed. Reproductive organs of *Haptanthus* are unique among angiosperms and make the search for phylogenetic relations difficult. Unfortunately, all attempts to extract DNA from the existing sample were unsuccessful.
- *Methods*: In 2010, we organized a small expedition to Honduras and were able to re-collect this plant, extract DNA from dried samples, and sequence the barcoding region of *rbcL.*
- *Key results and conclusions*: We obtained phylogenetic trees with reliable support for the placement of *Haptanthus* as a new member of Buxaceae (boxwood family).

Key words: angiosperms; Buxaceae; Haptanthaceae; *Haptanthus*; Honduras; phylogeny.

To date, representatives of almost all families of flowering plants have been studied with molecular phylogenetic tools. *Haptanthus hazlettii*, an enigmatic Central American tree, was one of the remaining exceptions. It was discovered, nearly accidentally, in 1980 (Doust and Stevens, 2005) and described as a new species and genus in 1989 (Goldberg and Nelson, 1989). In 2001, the new monotypic family Haptanthaceae was proposed for this genus (Nelson, 2001). Armen Takhtajan in his last book considers Haptanthaceae as the only family of uncertain position among angiosperms (Takhtajan, 2009). Unfortunately, only two herbarium specimens of *Haptanthus* existed, and all attempts to extract DNA failed. Five expeditions were organized with the goal of re-collecting the plant, but they brought no results. Because the locus classicus of *Haptanthus* was converted into pastures, the plant was considered "likely extinct" (Doust and Stevens, 2005).

Unique morphological features prevented the establishment of a stable phylogenetic position for *Haptanthus*. The plant has an extensively modified inflorescence with a central carpellate flower and two subtending clusters of staminate organs of questionable nature. These organs have been interpreted either as single stamens or as modified staminate flowers with two adnate stamens and probably two sepals (Doust and Stevens, 2005). Staminate and carpellate organs are practically naked, with minute basal bracts. Several taxa have been proposed as candidate related groups (Goldberg and Alden, 2005), but morphological support for these relations has been problematic.

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MATERIALS AND METHODS

In April 2010, we organized a small expedition to northern Honduras. After several days of intensive searching, we found one living tree of *Haptanthus hazlettii* Goldberg & C. Nelson 2 km from the estimated locus classicus, in the tropical rain forest growing on a steep slope (300 m above sea level) near the river Matarras (Atlantida, Honduras). The location was only a few meters away from an edge of a new plantation. Exact GPS coordinates were supplied to botanists of Lancetilla Botanical Garden (Tela, Honduras), and 1 month later they found a few more *Haptanthus* trees (L. Bejarano, Lancetilla Botanical Garden, personal communication).

DNA sequencing—Several silica-gel–dried samples were collected, DNA was extracted, and the region of the *rbcL* gene (which has proven to be an excellent tool for revealing higher-level phylogenetic relations among angiosperms) suggested as a candidate for plant barcoding (Hollingsworth et al., 2009; Consortium for the Barcode of Life, 2010) was sequenced. DNA was extracted by using a MO BIO PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). Dry leaf material (~0.07 g) was powdered by using a sterile mortar and pestle and then was processed in accordance with the supplied protocol. Polymerase chain reaction was carried out as follows. The reaction mixture in a total volume of 25 μL contained 5 μL of 5× NEB PCR Master Mix (New England BioLabs, Ipswich, Massachusetts, USA), 0.5 μL of 10 μM rbcLa_f and rbcLa_rev primers, 1 μL of highly diluted (1 : 1000) DNA solution from the extraction above and 18 μL purified water. Samples were incubated in a thermal cycler according to the recommended protocol (David Erickson, Smithsonian Institute): 94°C for 4 min, followed by 5 cycles of 94°C for 1 min; 55°C for 1 min, 72°C for 1 min, 30 cycles of 94°C for 1 min; 54°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. Single-band PCR products were purified by using an UltraClean PCR Clean-up Kit (MO BIO) according to the manufacturer's instructions. Purified PCR products then were sequenced in both directions with the rbcLa_f and rbcLa_rev primers (David Erickson, Smithsonian Institute). DNA sequences were determined by a 3730xl DNA sequencer (Applied Biosystems, Carlsbad, California, USA). Sequences

TABLE 1. GenBank accession numbers for sequences used.

GenBank Accession No.	Species name	Family (APG III*)
GQ248538	*Acorus calamus*	Acoraceae
EU002281	*Liquidambar styraciflua*	Altingiaceae
EU002274	*Berberidopsis corallina*	Berberidopsidaceae
DQ182333	*Buxus sempervirens*	Buxaceae
146762264: 58596-60023	*Buxus microphylla* (chloroplast, complete genome)	Buxaceae
AF197588	*Sarcococca confusa*	Buxaceae
AF093733	*Styloceras laurifolium*	Buxaceae
AF093718	*Pachysandra procumbens*	Buxaceae
L12640	*Chloranthus japonicus*	Chloranthaceae
L11216	*Cornus mas*	Cornaceae
AF061994	*Didymeles perrieri*	Didymelaceae
GQ997181	*Dillenia indica*	Dilleniaceae
DQ790164	*Erythropalum scandens*	Erythropalaceae
EU002279	*Gunnera manicata*	Gunneraceae
AF081068	*Fortunearia sinensis*	Hamamelidaceae
AF206791	*Magnolia tripetala*	Magnoliaceae
EU213503	*Myrothamnus flabellifolia*	Myrothamnaceae
GQ997596	*Nelumbo nucifera*	Nelumbonaceae
M77034	*Nymphaea odorata*	Nymphaeaceae
EF450315	*Piper nigrum*	Piperaceae
AY858644	*Platanus orientalis*	Platanaceae
EU676946	*Polygonum aviculare*	Polygonaceae
U79180	*Leucadendron laureolum*	Proteaceae
AY395557	*Ranunculus acris*	Ranunculaceae
GQ997513	*Meliosma* aff. *cuneifolia*	Sabiaceae
GQ248681	*Populus tremula*	Salicaceae
GQ998840	*Trochodendron aralioides*	Trochodendraceae
AF119174	*Vitis rotundifolia*	Vitaceae
FJ976143	*Leea indica*	Vitaceae

*Angiosperm Phylogeny Group III system.

then were assembled and edited with Sequencher 4.5 (Genes Codes Corporation, Ann Arbor, Michigan, USA). The resultant sequence was deposited to NCBI GenBank (accession # HQ634681).

Phylogenetic analysis—We obtained a 599-bp fragment, from which we constructed a phylogenetic tree, using 29 other taxa in the analysis (Table 1). All sequences were aligned with ClustalX (Thompson et al., 1997) by using gap opening cost = 9, gap extension cost = 0.05, and IUB weight matrix, followed by manual adjustments. The most parsimonious trees based on DNA sequences were obtained by heuristic search using tree bisection-reconnection (TBR) branch swapping on starting trees generated by random sequence addition (1000 replications) using PAUP* 4.0.10b (Swofford, 2003). Bootstrap values were evaluated by 500 replications by using a heuristic search with simple sequence addition, TBR branch swapping, and MULTREES on. A sequence of *Nymphaea odorata* was used as an outgroup on the basis of widely accepted hypotheses of angiosperm phylogeny. The best-fit model of evolution for the data sets was chosen through a likelihood ratio test with the program MrModeltest 2.2 (Nylander, 2004). Models obtained were used for Bayesian analyses with the Markov chain Monte Carlo method (MCMC); MCMC chains were run for 500 000 generations, sampling every 100th generation, resulting in 20 000 trees. The first 1250 trees were discarded as burn-in, and the remaining trees were summed to calculate the posterior probabilities.

RESULTS

The resulting trees from both MP and maximum likelihood (ML) analyses agreed well with results obtained in other studies of angiosperm phylogeny (Fig. 1). We found that *H. hazlettii* belongs to the Buxaceae clade on the phylogenetic tree with reliable bootstrap and strong Bayesian support. However, MP and ML methods were contradictory in regard to the position of *Haptanthus* within Buxaceae. Whereas the MP tree supported the placement as sister to *Sarcococca-Pachysandra-Styloceras*

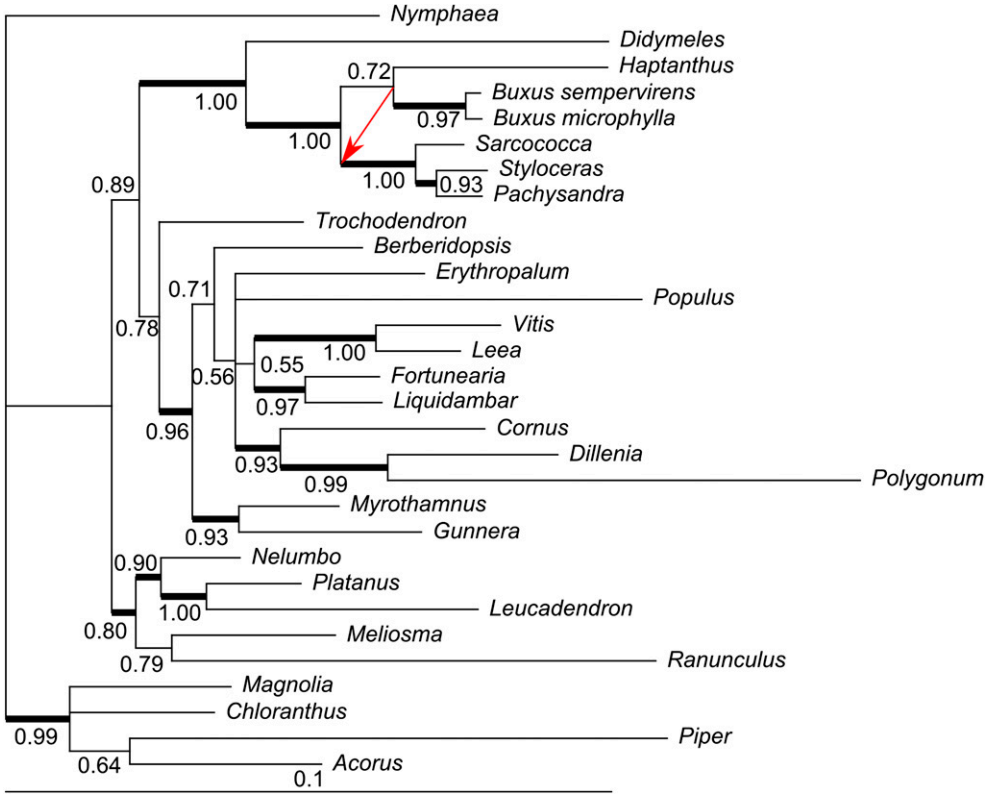

Fig. 1. Phylogram showing the position of *Haptanthus hazlettii* on angiosperm phylogenetic tree. Consensus tree is from Mr. Bayes (500 K replicates); nodes with Bayesian support >0.8 are in bold. Red arrow shows the alternative placement of *Haptanthus* on most parsimonious tree from PAUP*.

Fig. 2. Field photograph of general structure of *Haptanthus hazlettii* inflorescences.

group (revealed in all representative phylogenetic analyses of Buxaceae; e.g., von Balthazar et al., 2000, and von Balthazar and Endress, 2002), ML provided a position sister to the *Buxus* clade (Fig. 1). The immediate sister group to the expanded Buxaceae is *Didymeles*; this position also has been obtained in all previous phylogenetic analyses.

DISCUSSION

The position of *Haptanthus* as a new member of the boxwood family—a small group of basal eudicot genera, was previously predicted by using some morphological characters, especially wood structure, plinerved leaf venation, anatomy of outer integument, and elongated and curved stigmatic area (Doust and Stevens, 2005). This placement sheds some light on the interpretation of the unique reproductive structures of *Haptanthus*. Madagascan *Didymeles* is known to have paired stamens, and *Buxus* inflorescences with central female flowers and lateral male flowers, being much less reduced, resemble those of *Haptanthus* (Fig. 2). Preliminary observations in nature provide support for insect pollination, which is also common in Buxaceae. However, parietal placentation and anatomy of staminate organs (flowers or stamens) of *Haptanthus* remain unique in Buxaceae; more detailed research is needed to clarify the homology of these structures. Information about fruits is still missing; the plant flowered in April, but by the end of July, no fruits were observed (L. Bejarano, Lancetilla Botanical Garden, personal communication).

It is possible that more robust placement of *Haptanthus* inside Buxaceae could be obtained when more DNA data become available. Anatomy, morphology, and flower ontogeny of *Haptanthus* also require future research, which is now achievable because of ongoing efforts toward conservation of this endangered plant.

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