

# Taxonomic Classification of *Diplazium molokaiense*, a Fern Endemic to Hawaii, Using Molecular and Morphological Characters

Sarah Jaksich<sup>1</sup>, Hank Oppenheimer<sup>2</sup>, and P. Roxanne Kellar<sup>1</sup>

<sup>1</sup>Department of Biology, University of Nebraska at Omaha, Omaha, NE 68182

<sup>2</sup>Plant Extinction Prevention Program, University of Hawaii at Manoa, Honolulu, HI 96822

## ABSTRACT

*Diplazium molokaiense* is an endangered fern species endemic to Hawaii. There are only an estimated 61 individuals left in the wild. Taxonomic classification of this species is an important element to its conservation. Using the following morphological characters: sori size and shape, scale size and shape, and frond shape, *D. molokaiense* was preliminarily placed into the *Diplazium* genus. Six regions (*rbcL*, *matK*, *atpA*, *atpB*, *trnLF*, and *rps4-trnS*) of the chloroplast genome were amplified and sequenced using Sanger-sequencing. Sequences for outgroup taxa and 20 *Diplazium* species were downloaded from GenBank and used to infer a phylogeny. Our data provide evidence for the phylogenetic placement of *D. molokaiense* and can aid in its conservation.

## INTRODUCTION

*Diplazium molokaiense* (Fig. 1) is an extremely rare fern species endemic to Hawaii. The species is listed as critically endangered on the IUCN Red List, indicating that *D. molokaiense* is at the highest risk of extinction (Wood 2006). A 2006 survey conducted by co-author HO, estimated only 61 individuals remain on the Hawaiian Islands. *Diplazium molokaiense* has not been placed on the tree of life, meaning that its taxonomic position has not been determined and very little is known about its relationship with other fern species. Understanding the taxonomic classification of this species will be beneficial to conservation efforts and, ultimately, to the survival of the species.



**Figure 1.** *Diplazium molokaiense* (Photo by PRK)

*Diplazium molokaiense* is found along the East Honomanu Stream in Kula Forest Reserve, East Maui on a concave wall below overhanging rocks (Wood 2006) and in volcanic fissures accessible only by helicopter and mountain hiking (authors HO and RK). Their location has made it difficult for researchers to reach and study them. Threats to *D. molokaiense*'s habitat include: destruction by feral pigs, predication by seed-eating rats, flash floods, and competition with non-native plant taxa (Wood 2006). Human involvement and deforestation also threaten *D. molokaiense* and other native flora.

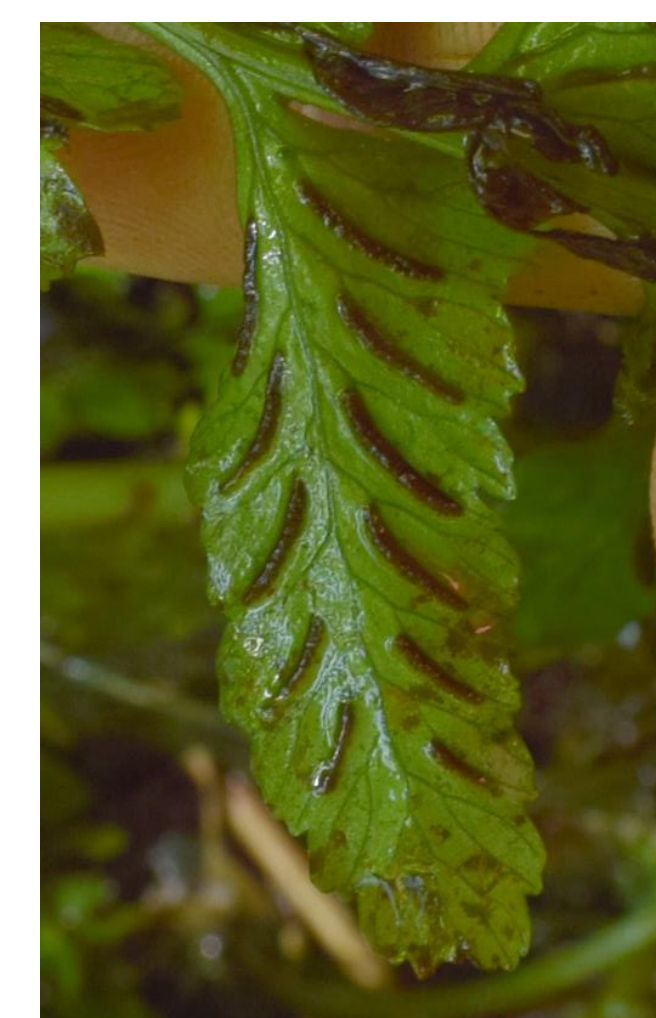
The *Diplazium* genus is extremely diverse, with a variety of morphologies (Wei et al. 2013), making it very difficult to infer a phylogeny by morphological characters alone. However, species in *Diplazium* do have distinctive patterns of sori shape and arrangement as well as scale color and shape (Wei et al. 2013 and Wei et al. 2015). Comparing frond shape and size is not sufficient. A phylogeny inferred from chloroplast genome markers results in greater confidence in evolutionary relationships. Including both coding and noncoding regions helps with alignment and distinguishing closely related species.

## METHODS

Morphological: Because of the critical status of the species, we were not allowed to remove fern tissues from the field for study in the lab; therefore, we visited the ferns in the field and took multiple photographs for further study. Additionally, we studied images of herbarium specimens and written descriptions by Wood (2006). Particular attention was paid to scale shape and color as well as sori shape. *D. molokaiense* has toothless, lanceolate scales that are brown/gray and concolorous (Fig. 2). The leaves are imparipinnate (Fig. 1), and the sori are elongated and flattened (Fig. 3).

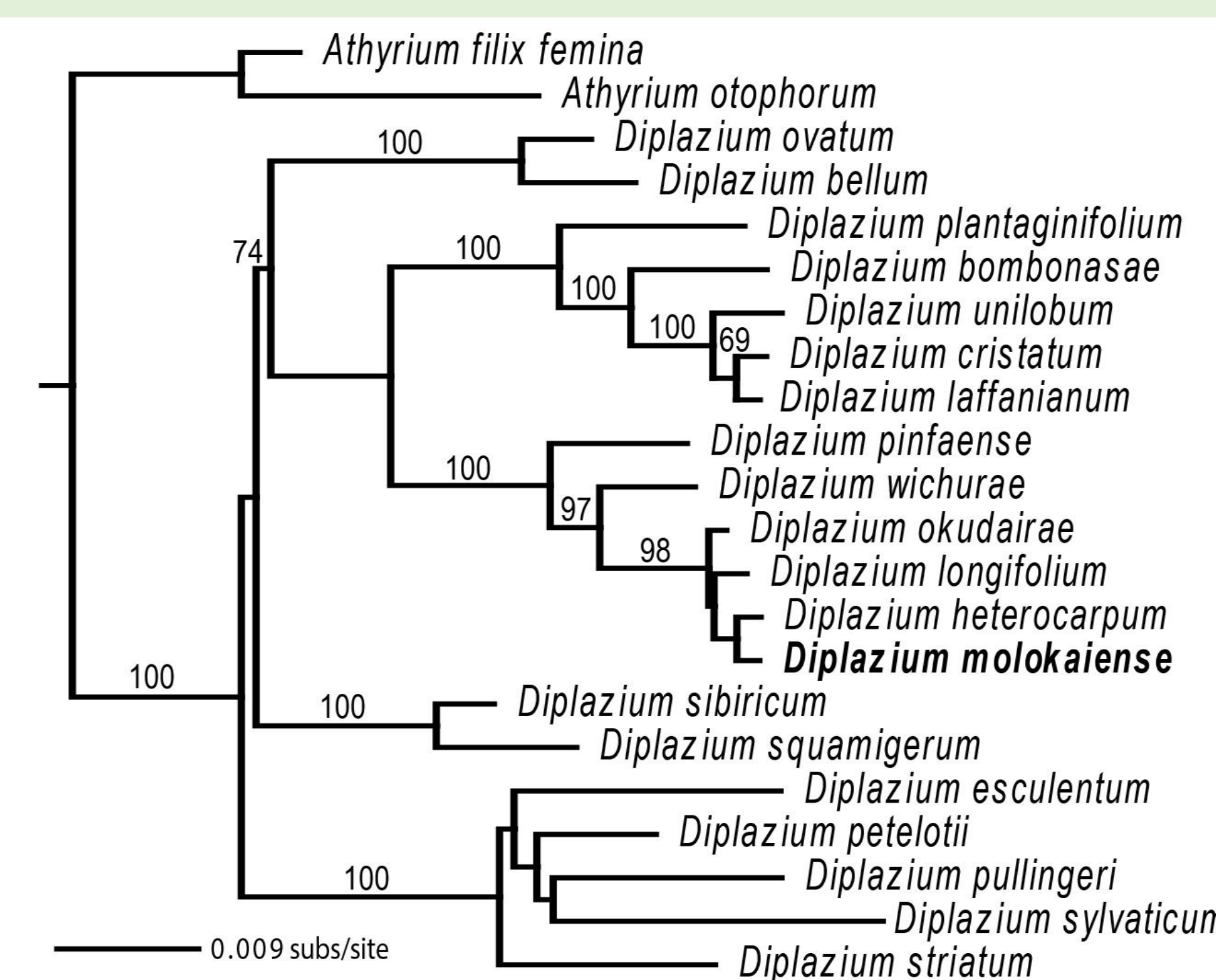


**Figure 2.** *Diplazium molokaiense* scales (Photo by PRK)



**Figure 3.** *Diplazium molokaiense* sori (Photo by PRK)

Molecular: We obtained dried frond material from a specimen in Maui. DNA was extracted using IBI Scientific Genomic (Dubuque, IA) DNA Mini Kit. The six regions – four plastid genes (*atpA*, *atpB*, *matK*, and *rbcL*) and two intergenic spacers (*rps4-trnS* and *trnL-F*) were amplified with PCR and purified using Exo-SAP. The resulting DNA was sent to the Genomics Core Facility at the University of Nebraska Medical Center for sequencing with Genewiz. The sequences were uploaded to Geneious. Sequences for 18 additional *Diplazium* species and two *Athyrium* (outgroup) species were downloaded from Genbank (Houser et al. 2016; Wei et al. 2013) and uploaded to Geneious as well. *Athyrium* is a closely related genus and a suitable outgroup. The sequences were aligned and concatenated using MAFFT (Katoh et al. 2013) and used to estimate a maximum likelihood phylogeny (Fig. 4) using RAxML (Stamatakis, 2014).



**Figure 4.** Maximum likelihood (-lnL = 11475.41) phylogeny of 20 *Diplazium* species based on six chloroplast markers. *Diplazium molokaiense* is highlighted in bold text. Numbers above branches indicate bootstrap support (BS) values. Branches without BS values collapsed in the 50% strict consensus tree. *Athyrium filix femina* and *Athyrium otophorum* are outgroups

## RESULTS

Combining results from two lines of evidence – morphological and molecular – our data place *Diplazium molokaiense* in Main Clade II (Fig. 4; Wei et al. 2013). The placement is based on sori shape, scale shape/color, geographic distribution, and six chloroplast markers. *D. molokaiense* is sister to *Diplazium heterocarpum*, but with only weak support.

## CONCLUSIONS & FUTURE DIRECTIONS

- Morphological and molecular characters place *Diplazium molokaiense* in Main Clade II and is sister to *Diplazium heterocarpum*.
- D. molokaiense* has never been placed in the tree of life before, and new information can aid in the conservation and propagation of the species.
- Sequences of additional DNA regions are needed to boost support for some clades in the phylogeny.
- Diplazium esculentum*, *Diplazium sandwichianum*, and *Diplazium arnottii* are fern species also found in Hawaii. DNA from frond tissue from these species are being extracted and sequenced. A more robust *Diplazium* phylogeny will further conservation and knowledge of Hawaiian *Diplazium* species.

## REFERENCES

- Houser, D., From, M., Landry, M., Copeland, A., & Kellar, P. R. (2016). Systematics of *Diplazium laffaniana* (Athyriaceae), a Fern Species Endemic to Bermuda. *American Fern Journal*, 106(3), 206–222.
- Katoh, K., Misawa, K., Kuma, K., and Miyata, K. (2013) MAFFT: A novel method for rapid multiple sequence alignment based on four Fourier transform. *Nucleic Acids Research* 30: 3059–3066.
- Stamatakis, A. (2014) RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* open access.
- Wei, R., Xiang, Q., Schneider, H., Sundue M. A., Kessler, M., Kamau, P. W., Hidayat, A., & Zhang, X. (2015). Eurasian origin, boreotropical migration and transoceanic dispersal in the pantropical fern genus *Diplazium* (Athyriaceae). *Journal of Biogeography*, 42, 1809–1819.
- Wei, R., Schneider, H., & Zhang, X. (2013). Toward a new circumscription of the twinorous-fern genus *Diplazium* (Athyriaceae): A molecular phylogeny with morphological implications and infrageneric taxonomy. *Taxon*, 62(3), 441–457.
- Wood, K. R. (2006). Conservation status of the Hawaiian endemic fern *Diplazium molokaiense* (Athyriaceae) in Honomanu, East Maui, Hawaii. *Endangered Species Research*, 2, 1–5.

## ACKNOWLEDGEMENTS

We thank the Funding for Undergraduate Scholarly Experiences (FUSE) and the University Committee on Research and Creative Activity (UCRCA) grant awarded by UNO and associates from the Leeward Haleakalā Watershed Restoration Partnership (LHWRP) in Maui for field assistance. The UNMC DNA Sequencing Core Facility receives partial support from the Nebraska Research Network In Functional Genomics NE-INBRE P20GM103427-14, The Molecular Biology of Neurosensory Systems CoBRE P30GM110768, The Fred & Pamela Buffett Cancer Center - P30CA036727, The Center for Root and Rhizobium Innovation (CRR1) 36-5150-2085-20, and the Nebraska Research Initiative.

