

California Conservation Genomics of the *Azolla* symbiosis Rothfels, UC Berkeley

1. *Synopsis:*

Azolla is a genus of tiny freshwater ferns with obligate, endosymbiotic nitrogen-fixing cyanobacteria, which has extraordinary economic potential in biofertilizer technology, phytoremediation, carbon sequestration, and as a biofuel (Brouwer et al. 2014). In addition, introduced species of *Azolla* rank among the globe's most ecologically noxious weeds, while other species are potentially of conservation concern. *Azolla* is also of great importance for evolutionary biology due the insights it provides into the origins and consequences of heterospory, and its potential as a model system for studying the evolution of symbioses and organelles.

We have a strong interest in the patterns of variation in *Azolla* in California, where up to five described species (*A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*, and *A. rubra*) and at least one additional undescribed species may co-occur (Rothfels and Li, unpublished), some potentially as introductions, and others as rare natives.

We are interested in the spatial variation of this diversity within California at three levels:

1. The species/taxonomic level (which species are present, how can they be distinguished, what are their distributions and abundances);
2. The population genomics level (where are centers of genomic diversity, what are the patterns of connectivity, are particular populations or lineages—of either the natives or the invasives—genomically depauperate);
3. And at the level of the microbiome (do patterns of microbial variation mirror those of the host, do invasive *Azolla* have distinct microbiomes that might explain their ecological success).

For each population sampled for CCGP, we will photo-document a set of plants with a standardized protocol (to capture whole-organism morphology), fix samples for micromorphological characterization (SEM), preserve samples for microbiome isolation and sequencing, and silica-dry material, in addition to taking a herbarium voucher.

We will thus be able to synthesize the CCGP results with comparable morphological and microbiome datasets to produce a broad multi-level picture of *Azolla* diversity within California.

2. *Proposed target species:*

Azolla has an extremely small genome for a fern, providing an unique opportunity to get deep resequencing data for a broad sample of accessions, an enterprise that would be cost-prohibitive for other fern species. From the perspective of the CCGP it is ecologically complementary to other proposed samples, being a freshwater aquatic that is widely distributed across the state, including in the Central Valley. Furthermore, as a member of the sister lineage to vascular plants *Azolla* helps address a major phylogenetic in the CCGP sample.

Azolla also helps address a functional-ecology gap central to the CCGP. Specifically, the *Azolla* dispersal unit is a haploid spore, and both their fertilization and dispersal are abiotically-

mediated (there are no biotic intermediates, such as animal pollinators or dispersers). In addition to their reliance on abiotically-mediated fertilization and dispersal, free-sporing vascular plants differ from seedplants in fundamental characteristics of their life cycles: in these plants both “generations” (the diploid sporophyte and the haploid gametophyte) are free-living, multicellular, and sessile—they unable to remove themselves from unfavorable environmental and must be able to tolerate those conditions as a fully-functional organism in both the diploid and haploid states. As a result, genetic diversity across the landscape is more closely coupled with climate and microsite in these lineages, making them ideal models for landscape genomics.

Azolla species are important for conservation planning both as potential invasives that need monitoring and control, and as rare natives that warrant conservation attention. More generally, as species of freshwater habitats, they are important as indicators of the health of these ecosystems, which are under particular pressure in water-conscious California. *Azolla* in California are usually treated in *A. filiculoides* and *A. microphylla*, but this treatment is likely inadequate; we will target all *Azolla* in California.

Table 1: Target Species

Class	Order	Family	Species
Polypodiopsida	Salviniales	Salviniaceae	<i>Azolla filiculoides</i>
Polypodiopsida	Salviniales	Salviniaceae	<i>Azolla microphylla</i> s.l.

3. ***Sampling design:***

We will sample across the species’ range in California, which spans 11 of the 19 ecoregions (see the Species Page). As a genus of freshwater aquatics, *Azolla* will help fill in geographic gaps in the otherwise predominantly terrestrial sample of CCGP taxa, including strong representation in the Central Valley.

4. ***Preferred research approach:***

We will provide high-quality DNA extractions (each documented with a herbarium voucher and linked to other research materials, as described in the Synopsis) of wild-collected samples collected during dedicated CCGP fieldwork. These samples will be submitted to the UCLA core facility, for library prep. under the CCGP protocol.

The primary research goal is for these data to be integrated into the CCGP’s broad synthesis of genomic diversity across California. In addition, Carl Rothfels, Fay-Wei Li, and Michael Song intend to produce a related manuscript characterizing the spatial patterns of microbiome variation, and synthesizing those with the patterns inferred for the host. Depending on preliminary results, we may also characterize *Azolla* morphology (including SEM imaging), and perform morphometric analyses, with the goal of providing end-users (conservation managers, botanical professionals, etc.) with a useable resource for identifying the Californian *Azolla* species in the field.

5. **Research Team:**

Table 2: Research Team

Member	Current title	Affiliation	Role
Dr. Carl Rothfels	Curator of Ferns and Lycophytes at UC/JEPS, Assistant Prof. Integrative Biology	UC Berkeley	PI
Dr. Fay-Wei Li	Assistant Professor	Boyce Thompson Institute	PI
Michael Song	PhD Candidate	UC Berkeley	team member
Forrest Freund	PhD Candidate	UC Berkeley	team member

Carl Rothfels will coordinate sample acquisition and associated project logistics (including supervising and mentoring the graduate student research assistant). Fay-Wei Li will advise on sampling priorities, and guide subsequent taxon-specific publications resulting from these data. Michael Song will focus on microbiome analysis, and Forrest Freund will be responsible for fieldwork and DNA extractions.

6. **Budget:**

Table 3: Budget

Activity	Cost
1. Field work	
Transportation	\$2400
Food	\$900
Camping	\$700
Total requested for field work	\$4000
2. Bench work	
CTAB extractions (x150)	\$200
Library prep. (\$30 x 150)	\$4500
Sequencing (.75Gb x 10x x \$7/Gb x 150 at 70% efficiency)	\$11235
Total requested for bench work	\$15935
3. Personnel	
One semester GSR	\$22086
Total requested for personnel	\$22086
4. Incidentals	
Incidentals for field research	\$400
Incidentals for lab research	\$340
Total requested for incidentals	\$740
Total requested	\$42,761

Budget justification:

1. Field work

The requested fieldwork budget will cover ~4200 miles of travel to allow sampling throughout the distribution of *Azolla* species at the standard UC mileage rate. We will use personal vehicles for land travel.

2. Bench work and sequencing

Extractions will be conducted in the Rothfels lab in the Department of Integrative Biology at UCB. Library prep. expenses (\$30 each) are based on the UCLA core facility's CCGP rate. The sequencing estimate is based on 10x coverage, a .75 Gb genome size, and a conservative estimate of 70% efficiency (we expect approx. 25% of the reads to be from the microbiome rather than from the *Azolla*; F.-W. Li, pers. comm.).

3. Personnel

Funds are requested for a one-semester graduate student researcher (GSR). The GSR will perform the field and labwork.

4. Incidentals

Requested funds for incidentals for fieldwork include materials for collecting leaf tissue for DNA analysis, such as vials and silica desiccant, as well as materials for collecting herbarium vouchers. Vouchers will be deposited in UC/JEPS. Requested funds for incidentals for lab work will be used for miscellaneous but essential lab items, such as tips, gloves, tubes, and beads for grinding, as well as additional reagents required for DNA extraction.

Species Page:

1. Species: The *Azolla* diversity that occurs in California is often treated under two species, but this classification likely under-represents the true number of species present (Smith and Murdock 2012).

Common Mosquito Fern. *Azolla filiculoides* Lam.

Mexican Mosquito Fern. *Azolla microphylla* Kaulf.

2. Genome size and brief description of available genomic resources: *Azolla filiculoides* is the first fern to have its genome sequenced (0.75 Gb; Li et al. 2018) with genomic resources available at <https://www.fernbase.org/> including a genome sequence from *Salvinia cucullata*, a member of its sister genus.

3. Known range of *Azolla* in California encompasses 11 Ecoregions: Central California Coast; Central Valley Coast Ranges; Great Valley; Mojave Desert; Mono; Northern California Coast; Northern California Coast Ranges; Sierra Nevada; Sierra Nevada Foothills; Southern California Coast; Southern California Mountains and Valleys.

4. Researchers' experience: Carl Rothfels is the Curator of Ferns and Lycophytes at UC/JEPS, and has extensive experience researching the biology and evolution of ferns and lycophytes. Michael Song is working on microbiome biology for his dissertation and has experience with both field collections and genomics. Forrest Freund has extensive experience with Californian fieldwork and DNA extraction/sequencing. Fay-Wei Li is an expert on fern genomics, and led the project to sequence the *Azolla* genome.

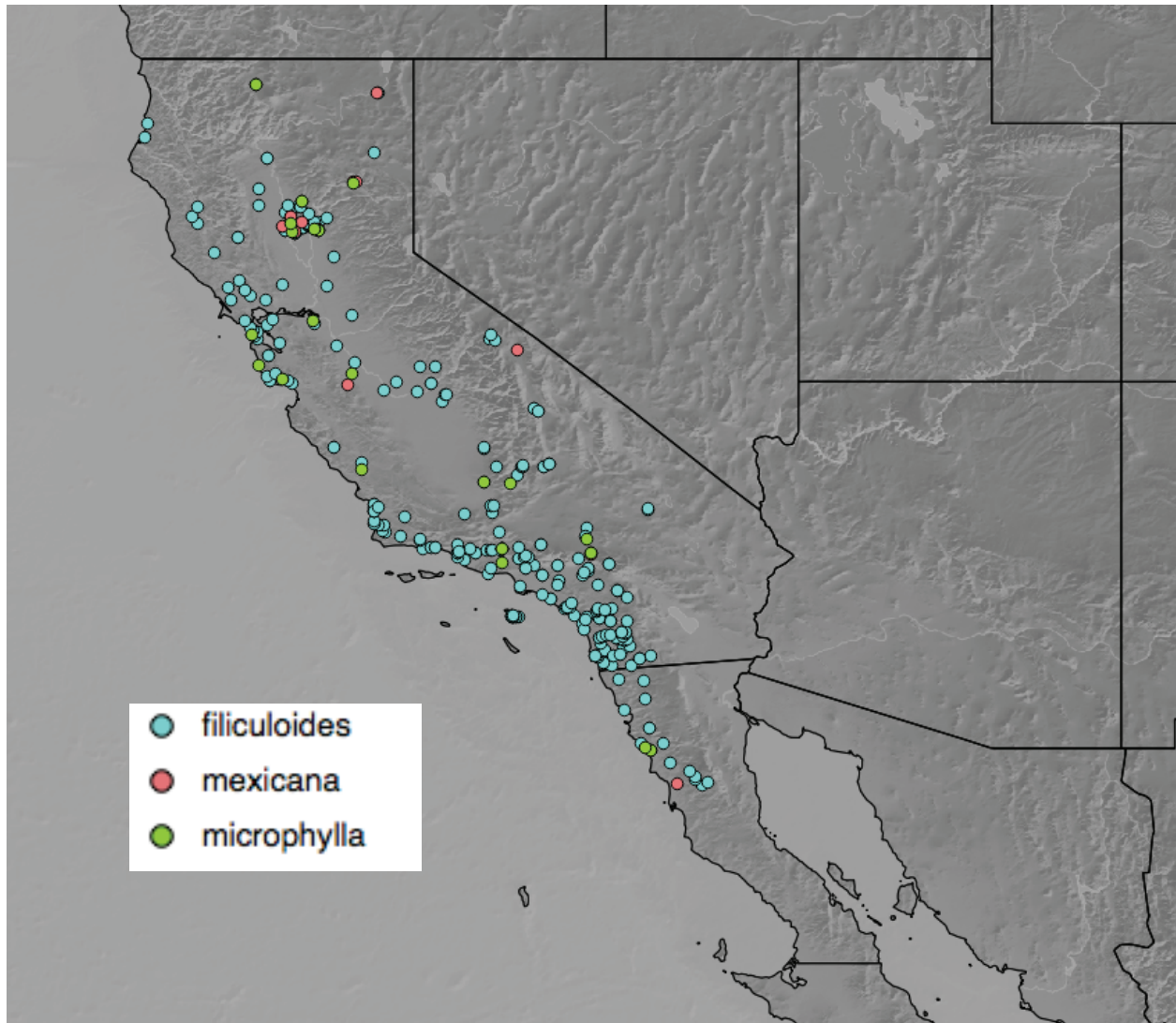
5. Samples in hand and anticipated: See attached template.

6. Anticipated sampling design: Sampling will be approximately uniform across the California range of *Azolla*; see attached Excel file.

7. Timeline for sample acquisition: The sampling will be completed by fall 2021.

8. Special considerations: No special considerations are necessary.

9. Realistic timeline for benchwork and fieldwork: Fieldwork and benchwork (extractions, quantification) will be completed by fall 2021. Fieldwork will be concentrated in the Spring 2021 semester, with the potential for summer fieldwork to fill in any gaps. Extractions will be completed during the summer, too.



Geographic range of *Azolla* species in California. Points indicate collections available through the Consortium of California Herbaria.

References:

- Brouwer, P., Bräutigam, A., Külahoglu, C., Tazelaar, A. O. E., Kurz, S., Nierop, K. G. J., et al. (2014). *Azolla* domestication towards a biobased economy? *New Phytologist*
- Li, F.-W., Brouwer, P., Carretero-Paulet, L., Cheng, S., Vries, J., Delaux, P.-M., et al. (2018). Fern genomes elucidate land plant evolution and cyanobacterial symbioses. *Nature Plants*, 1–16.
- Sessa, E. B., Banks, J. A., Barker, M. S., Der, J. P., Duffy, A. M., Graham, S. W., et al. (2014). Between two fern genomes. *GigaScience*, 3(15), 1–7
- Smith, A.R. & Andy Murdock 2012, *Azolla*, in Jepson Flora Project (eds.) Jepson eFlora, /eflora/eflora_display.php?tid=10288, accessed on February 29, 2020.