ISSRseq: an extensible, low-cost, and efficient method for reduced representation sequencing

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ABSTRACT

1. The capability to generate densely sampled single nucleotide polymorphism (SNP) data is essential in diverse subdisciplines of biology, including crop breeding, pathology, forensics, forestry, ecology, evolution, and conservation. However, access to the expensive equipment and bioinformatics infrastructure required for genome-scale sequencing is still a limiting factor in the developing world and for institutions with limited resources.

2. Here we present ISSRseq, a PCR-based method for reduced representation of genomic variation using simple sequence repeats as priming sites to sequence inter-simple sequence repeat (ISSR) regions. Briefly, ISSR regions are amplified with single primers, pooled, and used to construct sequencing libraries with a low-cost, efficient commercial kit, and sequenced on the Illumina platform. We also present a flexible bioinformatic pipeline that assembles ISSR loci, calls and hard filters variants, outputs data matrices in common formats, and conducts population analyses using R.

3. Using three angiosperm species as case studies, we demonstrate that ISSRseq is highly repeatable, necessitates only simple wet-lab skills and commonplace instrumentation, is flexible in terms of the number of single primers used, is low-cost, and can generate genomic-scale variant discovery on par with existing RRS methods that require high sample integrity and concentration.

4. ISSRseq represents a straightforward approach to SNP genotyping in any organism, and we predict that this method will be particularly useful for those studying population genomics and phylogeography of non-model organisms. Furthermore, the ease of ISSRseq relative to other RRS methods should prove useful for those conducting research in undergraduate and graduate environments, and more broadly by those lacking access to expensive instrumentation or expertise in bioinformatics.

KEYWORDS

ISSRseq, reduced representation sequencing, *Corallorhiza*, population genomics

INTRODUCTION

Reduced representation sequencing methods (RRS), in concert with high-throughput sequencing technologies, have revolutionized research in agriculture, conservation, ecology, evolutionary biology, forestry, population genetics, and systematics (Altshuler et al., 2000; Van Tassell et al., 2008; Davey et al., 2011; Narum et al., 2013; Lemmon and Lemmon, 2013; Meek and Larson, 2019). These methods generate broad surveys of genomic diversity by reducing the amount of the genome to be sequenced, allowing multiple samples to be sequenced simultaneously (Peterson et al., 2012; Franchini et al., 2017). While there exists a bewildering array of RRS methods (Campbell et al., 2018), the most commonly employed are various forms of restriction-site associated DNA sequencing (RAD; Baird et al., 2008) and genotyping-by-sequencing (GBS; Elshire et al., 2011). Both involve fragmenting the genome with one or more restriction enzymes, ligating adapters and unique barcode sequences, pooling, optionally size-selecting the fragments for optimal sequencing length, amplifying resulting fragments, and sequencing typically with Illumina short-read technology (Davey et al., 2011). Flexibility in these methods lies principally in the selection of one or more restriction enzymes; e.g. one popular method, double-digest RAD sequencing, uses both a ‘rare-cutting’ and ‘common-cutting’ enzyme (Peterson et al., 2012).

While methods such as GBS and RAD are increasingly commonplace, they remain challenging for many researchers worldwide, e.g. in the developing world and/or primarily undergraduate institutions. Further, these methods require substantial amounts of high quality, high molecular weight DNA to be successful, which prove to be complications when working with historical samples with highly degraded DNA (Graham et al., 2015; but see Jordon-Thaden et al., 2020), ancient DNA (Billerman and Walsh, 2019), and taxa for which extracting sufficient amounts of high-quality DNA is difficult (Fort et al., 2018; Lienhard and Schäffer, 2019). More recently, researchers have turned to sequence capture protocols of genomic DNA libraries, using biotinylated probes that are either pre-synthesized or made in-lab from the products of various RRS methods (e.g. RAD fragments, exomes, amplified fragment length polymorphisms; Suchan et al., 2016; Hoffberg et al., 2016; Schmid et al., 2017; Puritz et al., 2017; Li et al., 2019). Two advantages drive interest in these methods: 1) they extend the utility of e.g. restriction-based methods to degraded historical or even ancient DNA specimens by focusing Illumina library preparation on genomic DNA instead of individual RRS libraries for each sample; and 2) they

may mitigate the effects of restriction-site or locus dropout among samples, as they rely on hybridization of genomic DNA to probes already constructed from one or more high-quality samples instead of individual restriction digests of each sample (e.g. Lang et al., 2020).

RRS methods are still technically challenging and economically infeasible for many researchers who may lack specific expertise in molecular biology, bioinformatics, and/or have limited access to expensive computational resources or sophisticated and often dedicated instrumentation. Thus, the need for simple, straightforward, and economical methods for generating genome-scale variation remains. An alternative class of methods focuses on amplicon-based sequencing (Campbell et al., 2018, Ericksson et al., 2020). One such method is MIG-seq (Suyama and Matsuki, 2015), which generates amplicons using primers comprising simple sequence repeat (SSR) motifs in multiplex, and sequences the inter-simple sequence repeat (ISSR) fragment ends. Briefly, ISSR involves the use of primers that match various microsatellite regions in the genome (Zietkiewicz et al., 1994); the primers often include 1-3 bp specific or degenerate anchors to preferentially bind to the ends of these repeat motifs (Gupta et al., 1994; Zietkiewicz et al., 1994) or consist entirely of a SSR motif (Bornet and Branchard, 2001). MIG-seq uses ‘tailed,’ barcoded, ISSR-motif primers in a 2-step PCR protocol, first with the amplification of ISSR, and the second with common primers matching the tails to enrich these amplicons, followed by size selection and sequencing on the Illumina platform. This method has the advantage of not requiring formal Illumina library preparation (i.e. shearing of genomic DNA and ligation of barcoded sequencing adapters), as the adapters and barcodes are included in the tailed ISSR-motif primers, thus reducing the cost and labor associated with conducting many library preps.

While MIG-seq has been cited by more than 90 studies to date (e.g. Tamaki et al., 2017; Park et al., 2019; Gutiérrez-Ortega, 2018; Takata et al., 2019; Eguchi et al., 2020), many questions remain with regard to its efficiency and reproducibility. First, the use of long, tailed, ISSR primers raises the possibility of primer multimerization and unpredictability of binding specificity (eight forward and eight reverse primers in multiplex, as has typically been implemented). Second, as originally implemented, the protocol requires 96 unique forward-indexed primers, each 61 bp in length. The cost of synthesizing such lengthy primers equates to thousands of US dollars spent up-front, which may be prohibitive for many researchers, though

these costs could be mitigated somewhat via dual indexing and the use of shorter adapter sequence tails. Third, MIG-seq only produces sequence data from the ISSR amplicon fragment ends, e.g. as is done with rDNA metabarcoding, potentially missing significant levels of variation by not sequencing the entire amplified fragments. The numbers of SNPs reported in studies using MIG-seq range from a few hundred (Suyama and Matsuki, 2015) to thousands (Eguchi et al., 2020), although missing data comprised the majority (81.4%) of the SNP matrix generated via the latter study. Data of this quantity and completeness can be useful for basic population genetics or phylogeographic studies, but are inadequate for studies requiring densely sampled polymorphisms across the genome (e.g. Quantitative Trait Locus mapping, genomic scans of adaptive variation, pedigree analysis). Indeed, Suyama and Matsuki (2015) state: "...the number of SNPs is fewer in our method [than in RAD-seq] (e.g. ~1,000 vs. ~100,000 SNPs), which means low efficiency in terms of the cost per SNP and sequencing effort."

Here we present ISSRseq, a novel RRS method that is straightforward, extensible, and relatively inexpensive using single-primer ISSR amplicons. Briefly, our approach is to: produce single-ISSR primer amplicons (as opposed to using tailed, highly multiplexed primer pairs); pool amplicons from multiple primers per sample; conduct low-cost, fragmentase-based Illumina library preparation; and sequence entire ISSR regions on the Illumina platform (Fig. 1). Further, we supply a user-friendly set of BASH scripts that together comprise an analysis pipeline for user-customized data quality control and analysis, SNP outputs in formats commonly used for population genomics and phylogeography, and an easy to use script template for downstream population genomic analyses in R. Given the use of enough single-primer amplicons, our method produces comparable levels of variation to RAD or GBS, and orders of magnitude more variation than MIG-seq while minimizing missing data. We present case studies of the utility of this method in *Populus deltoides*, a species with a relatively small and completely sequenced genome, and two mycoheterotrophic orchid species with large, uncharacterized genomes: *Corallorhiza bentleyi* Freudenst. and *C. striata* Lindl. All lab research was carried out by undergraduate students, demonstrating that this method is amenable and accessible to those with limited experience in molecular biology.

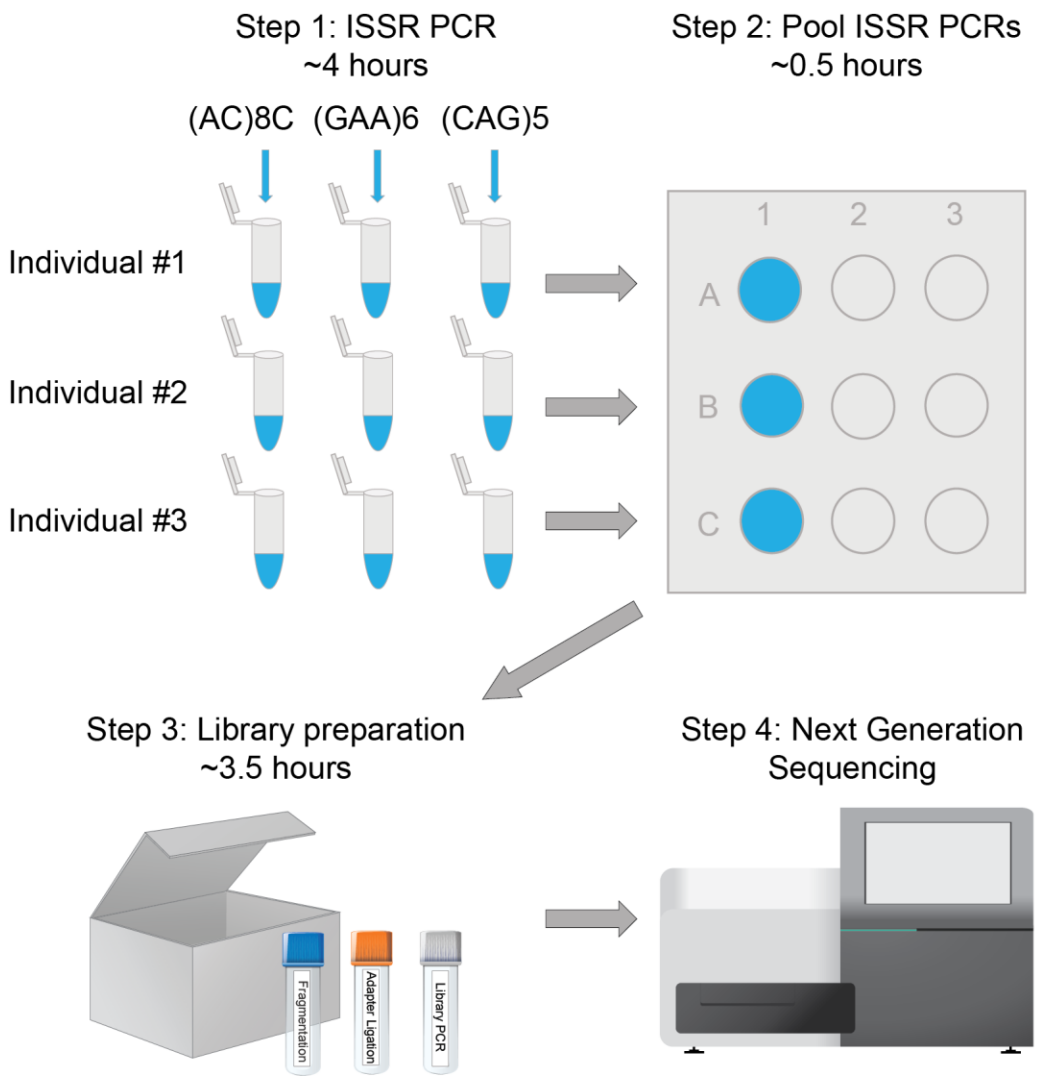

Figure 1. Critical steps of ISSRseq, including the approximate time projected for each estimated for library preparation of 48 samples with 4 primer sets.

MATERIALS AND METHODS

Lab Methods

Sample Collection and DNA extraction

We collected leaf tissue of *Populus deltoides* accession WV94 from a plantation located at the West Virginia University Agronomy Farm (39.658889, -79.905278) in Morgantown, West Virginia (Macaya-Sanz et al., 2017). Genomic DNA was extracted using a DNeasy plant mini kit (Qiagen, Hilden, Germany; Cat. No. 69104). We collected 37 individuals from six *C. bentleyi* localities and 87 individuals from 28 *C. striata* localities, as in Fama et al. (2020, unpublished data) and Barrett et al. (2011; 2018; Table 1). Samples of *C. bentleyi* were collected in

Allegheny, Bath, and Giles Counties, Virginia, USA; and from Monroe County, West Virginia, USA (Table 1). Samples of *C. striata* were collected from ten US states and two Canadian provinces (British Columbia and Manitoba, Table 1). Sampling of *C. striata* was focused on the western USA and in particular on California, where the taxonomic status of populations in this complex is uncertain (Barrett et al., 2011; 2018). For both species of *Corallorhiza*, approximately 0.2 g of perianth or ovary tissue was removed with a sterile scalpel (so as not to include seed material) and DNA was extracted using a CTAB DNA extraction protocol, modified to 1/10 volume (Doyle and Doyle, 1987).

Table 1. Sampling regions and localities for *C. striata* individuals and sampling counties and localities for *C. bentleyi* individuals.

Region/County	Sampling locality	Number of individuals
C. striata		
Coast Ranges Cascades	Ashland County, OR	2
	Lane County, OR	2
	Marin County, CA	5
	Santa County, Cruz, CA (1)	3
	Santa County, Cruz, CA (2)	4
	Sonoma County, CA (1)	2
	Sonoma County, CA (2)	1
Sierra Nevada	Fresno County, CA	7
	Mariposa County, CA	3
	Nevada County, CA	1
	Placer County, CA	5
Var. *striata* N USA	Cache County, UT	5
	Idaho County, ID	4
	Lewis and Clark County, MT	5
	Lewis County, WA	2
	Natrona County, WY	4
	Skamania County, WA	2
	Thompson-Nicola Regional District, BC	2
	Whinnipeg Region, MAN	2
Var. *vreelandii* SW USA	Graham County, AZ	4
	Otero County, NM	4
	Ouray County, CO	5
	Tooele County, UT	5
	Utah County, UT	4
C. bentleyi		
Alleghany	RG	5

Bath	CG	14
Giles	BS	3
	OR	5
	WR	5
Monroe	PM	5

ISSR Primers and PCR conditions

Twenty-one ISSR primers were selected from the UBC Primer Set #9 (University of British Columbia, Canada; paper available on GitHub www.github.com/btsinn/ISSRseq), with the addition of five unanchored primers designed by the authors (Table 2). After several rounds of initial optimization, reactions were set up in 10 µl volumes with: 5 µl 2x Apex PCR Master Mix (Genesee Scientific, San Diego, California, USA; Cat. No. 42-134), 0.5 µl 5M Betaine (Fisher Scientific, Waltham, Massachusetts, USA; Cat. No. AAJ77507AB), 0.5 µl of each single primer at 10 µM starting concentration (one primer per reaction; Integrated DNA Technologies, Coralville, Iowa, USA), 3 µl nuclease-free ultrapure water, and 1µl template DNA (diluted to 20 ng/µl prior to PCR with Tris-EDTA pH 8.0). PCR reactions were set up as single master mixes with individual ISSR primers and aliquoted to 96-well PCR plates. Conditions were as follows for each primer: 5 min at 95°C; 30 cycles of 95°C (30s), 50°C (30s), and 72°C (1m); and a final extension of 10m at 72°C. Caution was taken during PCR amplification to avoid human, microbial, or plant-based contamination; all lab surfaces were disinfected with 10% sodium hypochlorite solution prior to amplification.

Table 2. List of ISSR primers (Gupta et al., 1994) used in this study for the three test species. Numbers listed under 'ISSR primer' correspond to the UBC Primer Set 9 names (paper available on GitHub www.github.com/btsinn/ISSRseq).

ISSR primer	Motif	*P. deltoides*	*C. bentleyi*	*C. striata*
813	(CT)8T	X	X	
814	(CT)8A	X	X	
815	(CT)8G	X	X	
817	(CA)8A	X	X	
820	(GT)8T	X	X	
824	(TC)8G	X	X	
826	(AC)8C	X	X	X

834	(AG)8YT	X	X	X
836	(AG)8YA	X	X	X
840	(GA)8YT	X	X	X
843	(CT)8RA	X	X	
845	(CT)8RG	X	X	
848	(CA)8RG	X	X	
855	(AC)8YT	X	X	
856	(AC)8YA	X	X	X
857	(AC)8YG	X	X	
858	(AC)8RT	X	X	
859	(TG)8RC	X	X	
860	(TG)8RA	X	X	
868	(GAA)6	X	X	X
873	(GACA)4	X	X	
caa5	(CAA)5	X	X	X
cag5	(CAG)5	X	X	X
gtt5	(GTT)5	X	X	
gat5	(GAT)5	X	X	
cat5	(CAT)5	X	X	

Library preparation and sequencing of ISSR amplicon pools

PCR products in initial trials were visualized on 1% agarose gels and quantified via nanodrop spectrophotometry after cleaning with Axygen Axyprep Fragment Select beads (Corning, Tewksbury, MA, USA) and two 80% ethanol washes on a magnetic plate to remove excess PCR reagents. Then, PCR products were pooled for individual accessions across all primer amplification reactions and diluted with TE buffer to 5 ng/ul, and the original pools stored at -80°C as backups. Library preps were conducted with the QuantaBio sparQ DNA Frag and Library Prep Kit (QuantaBio, Beverly, Massachusetts, USA, Cat. No. 95194-096), a relatively inexpensive, rapid library kit that uses a fragmentase to shear genomic or amplified DNA. The library preparation protocol is described in detail elsewhere (supplementary materials; GitHub www.github.com/btsinn/ISSRseq). Briefly, we scaled 50 µl library preparation volumes to 20 µl (thus a 96-reaction kit yields 240 preps at ~$11 USD/prep). The sparQ Frag and Library Prep Kit conducts fragmentation and end repair in a single step followed by Y adapter ligation (Glenn et

al. 2016). We then performed a magnetic bead cleanup, total library amplification with barcoded Illumina iTru primers (Glenn et al. 2016), and a final bead cleanup. Fragmentase time was optimized in earlier trials, and consistently gave the best target library sizes for Illumina sequencing at 3.5 minutes (by contrast, for high molecular weight DNA fragmentation is suggested to be set at 14 minutes). Three µl of each library was run on an agarose gel with GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher; Cat. No. SM1331) followed by quantification via Qubit fluorometry with the dsDNA Broad Range assay (Invitrogen, Carlsbad, California, USA; Cat. No. Q32850). Amplicons for each accession were pooled at equimolar ratios. Size selection of an aliquot of the final pool was conducted using magnetic beads, but users could also conduct two selective bead cleanups or even gel excision. Fragment size range and intensity were quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and final library quantification was conducted via quantitative PCR at the West Virginia University Genomics Core Facility. The final library size ranged from 250-550 bp. Pooled, indexed ISSR amplicons were sequenced using 2 × 150 bp Illumina MiSeq (reagent kit v2) for *C. bentleyi*. For *C. striata*, three sequencing runs were conducted: one lane each of 2 × 100 bp and 2 × 50 bp on a HiSeq1500 at the WVU-Marshall Shared Sequencing Facility, and one run of 2 × 150 bp (reagent kit v2) Illumina MiSeq at the WVU Genomics Core Facility.

Bioinformatics and Downstream Analyses

Our bioinformatic analysis pipeline consists of five BASH scripts for use on UNIX-based systems, each with customized options, allowing simple but flexible parameter adjustment to fit the needs of a particular dataset or project (Fig. S1). We also supply an R script template to conduct various population genomic analyses. These scripts are not meant to dictate how ISSRseq data are treated or analyzed by users, but rather they are meant to serve as an accessible example of analysis potential for these data. Below, we detail the workflow iteratively and in the order of script usage. One of the BASH scripts, ISSRseq_ReferenceBased.sh, is optional if the user wishes to map to a reference genome, or to a previously assembled set of ISSRseq amplicons. BASH and R scripts are provided and a detailed wiki with usage examples can be found via the ISSRseq GitHub repository (www.github.com/btsinn/ISSRseq).

1) ISSRseq_AssembleReference.sh

Read Pre-processing.

BBDUK (38.51, available from: https://sourceforge.net/projects/bbmap/files/) is used to remove adapters and priming sequences from reads, quality trim, and exclude GC-rich or poor reads for each sample. Additionally, we used the kmer trimming feature of BBDUK to remove ISSR motifs used as primer sequences from the ends of reads. Kmer length was set to 18 and the 'mink' flag set to 8. The use of the 'mink' flag allowed for the removal of matched kmers down to a length of 8 bp from that of the supplied priming sequences (Table 2). We also enabled trim by overlap ('tbo') and 'tpe,' which in tandem allowed for adapter trimming by leveraging read overlap and in such an event trimmed both read pairs to the same length to ensure adapter removal. Trimmed reads for which the average quality score was below 10 or length was less than 50 bp were excluded, with the exception of the HiSeq 50bp reads. Hard trimming of read ends was not conducted when combining MiSeq 100 & 150 bp, and HiSeq 50 bp data generated for *C. striata*.

Reference Assembly. We then used ABySS-pe (version 2.2.4, Jackman et al., 2017) to assemble the trimmed reads from the user-specified reference sample using a kmer length of 91. BBDUK was then used to trim the assembled contigs in the same fashion as was used for read trimming, but with the GC content filter set to 35% and 65% and with the entropy filter enabled and set at 0.85. A reference index and sequence dictionary were created by SAMtools 'faidx' (version 1.7-13-g8dee2a2, Li et al., 2009) and the Picard tool 'CreateSequenceDictionary' (version 2.22.8; Broad Institute), respectively.

Contaminant Filtering. Next, we used the trimmed and filtered reference contigs as queries in BLASTn (version 2.6.0, Camacho et al., 2009) to identify putative contaminant loci by using the human genome, and those of 153 genomes of organisms that could be expected as common contaminants of plant samples (see supplementary materials), as subjects with an e-value cutoff of 0.00001. Contigs with e-values below this cutoff are excluded from the final reference. The user also specifies a plastid genome, and/or any other genome of interest, to be used as a negative reference. Contigs that can be mapped to the negative reference by BBMap (version 38.51, available from: https://sourceforge.net/projects/bbmap/files/) and also excluded from the

reference assembly. Contigs identified as putative contaminants or representing the negative reference are written to a FASTA file.

2) ISSRseq_CreateBams.sh

Read Mapping and BAM Creation. BBMap (version 38.51) was used to map trimmed reads from each sample to the assembled contigs using default mapping settings and killbadpairs enabled. SAMtools was used to sort and index the BAM (Cock et al., 2015) files of each sample. We then used the Picard tools MarkDuplicates (version 2.22.8; Broad Institute), to mark PCR and optical duplicate reads, and BuildBamIndex (version 2.22.8; Broad Institute) to re-index these final BAM files.

3) ISSRseq_AnalyzeBAMs.sh

Variant Calling and Filtering. We used the state-of-the-art variant calling pipeline GATK4 (version 4.1.8, McKenna et al., 2010) to call, filter, and jointly score variants amongst all samples simultaneously. HaplotypeCaller (Poplin et al., 2017) identified potential variant sites and called variants from locally-reassembled portions of each BAM, with --linked-de-bruijn-graph, --native-pair-hmm-use-double-precision, and -ERC GVCF modes enabled. GVCF files from each sample were then combined into a single VCF file with CombineGVCFs. GenotypeGVCFs was then used to perform joint variant scoring on the combined VCF file. Scored variants for downstream analysis were restricted to biallelic SNPs and INDELs, which were then hard-filtered guided by GATK Best Practices hard filtering recommendations (DePristo et al., 2011): "AF > 0.01 && AF < 0.99 && QD > 2.0 && MQ > 40.0 && FS < 60.0 && SOR < 3.0 && ReadPosRankSum > -8.0 && MQRankSum > -12.5 && QUAL > 30.0". Since the workflow exists as a BASH script, users can customize any of the variant filtering parameters to suit their needs. Additionally, we conducted sensitivity tests by also analyzing datasets where the minimum variant QUAL score was set to 60 which we refer to as QUALX2 datasets, below.

4) ISSRseq_CreateMatrices.sh

Matrix Creation. VCF2PHYLIP (version 2.0; Ortiz, 2019) was used to coerce the minor allele and hard-filtered SNP variants into nexus, phylip, and binary SNP formats with varying matrix

inclusion thresholds corresponding to the minimum number of samples a variant was identified from.

5) ISSRseq_ReferenceBased.sh [Optional]

This script processes input reads and prepares the necessary file structure for the use of the pipeline with a pre-existing reference, for example, if the user has a sequenced genome or previously generated *de novo* assembly of ISSR amplicons at their disposal. Unlike ISSRseq_AssembleReference.sh, this script does not conduct contaminant filtering or trim both ends of the sequence reads since the input reads are not used for *de novo* assembly of the reference. Users should remove organellar and other non-target contigs from the reference prior to using this script. The output directory can then be used for steps 2 - 4 outlined above.

Reference, coverage, and missing data comparisons for* C. striata *datasets

In order to test the effects of using different Illumina sequencing strategies (i.e. sequencing effort and read length), we collected three datasets for the same 87 accessions of *C. striata*: Illumina MiSeq 2 × 150 bp, HiSeq 2 × 50bp, and HiSeq 2 × 100 bp (see above). We were specifically interested in the total number of SNPs, mean coverage depth, and percent missing data for each of the three individual datasets plus a 'combined' dataset using all three. We mapped reads as above to a common reference based on the combined dataset, with the goal of producing the most complete reference for mapping reads from individual datasets.

Population Genetic Analyses in R

Data preparation

In order to estimate potential issues of genotyping error skewing population statistics, for *C. bentleyi* we analyzed both the GATK hard-filtered and QUALX2 datasets. Additionally, prior to population genetic analyses, loci in SNP matrices that had missing data for a high number of individuals were filtered out of datasets using *max-missing* command in the VCFtools (0.1.15) software package (Danecek et al. 2011). We set *max-missing* to a threshold of 0.1 and 0.2 respectively for the *C. bentleyi* and *C. striata* datasets. All population genetic analyses were carried out using packages in the R software environment (R Core Team 2019). An example R script is provided in the ISSRseq GitHub repository (www.github.com/btsinn/ISSRseq).

Analysis of Molecular Variance (AMOVA) and F-statistic Estimation

We used AMOVA to assess population structure by partitioning the genetic variation based on predefined population categories using the Poppr package (Kamvar et al. 2015; '*poppr.amova'* function). We performed a permutation test for 1,000 iterations to test phi statistics for significance ('*randtest'* function). We also estimated commonly used population statistics including observed heterozygosity (H_o), observed gene diversities (H_s), inbreeding coefficient (F_{is}), and fixation index (F_{st}) across all loci using the hierfstat package (Goudet 2005; '*basic.stats*' function) for both species. The hierfstat package was also used to estimate pairwise F_{st} ('*genet.dist*' function) among population categories.

Principal Components Analysis (PCA)

PCA was conducted using the pcadapt package (Privé et al. 2020; '*pcadapt*' function;) with linkage disequilibrium clumping size and threshold set to 200 and 0.1, respectively. The pcadapt package also allowed us to detect genetic marker outliers that are putatively involved in local adaptation. We extracted the q-values from the analysis and identified outlier SNP positions based on an alpha cutoff of 0.05. Finally, we used the adegenet package (Jombart & Ahmed 2011) to perform a discriminant analysis of principal components (DAPC) to assign sub-population membership probabilities to individual plants ('*dapc*' function).

RESULTS

Sequencing and Reference Assemblies

C. striata complex

PCR using eight SSR primers (Table 2) successfully generated amplicons from 81 of the 87 accessions. The total combined sequencing read pool comprised 310,379,211 read pairs, of which 239,142,938 remained after trimming and filtering (Table 3). We selected accession 253e_CA as the reference individual since it is the sample for which we recovered the greatest number of reads (28,935,452 read pairs). *De novo* assembly resulted in 17,143 contigs longer than 100 bp, comprising a reference assembly totaling 3,628,930 bp with an N50 of 212 bp and GC content of 47.24%, excluding 555 contigs that either mapped to a genome of a putative

contaminant or to any plastome sequenced from the *C. striata* complex (JX087681.1, NC_040981.1, MG874039.1, NC_040978.1) or that of *C. bentleyi* (NC_040979.1). The reference assembly comprised 14,164 contigs shorter than 250 bp, 450 contigs longer than 500 bp, and 34 contigs longer than 1 kb; the longest contig was 2,032 bp. The mean coverage depth of filtered variants scored in accession 253e_CA using this reference was 123.29x. Contigs of putative contaminant or plastid loci totaled 144,068 bp with an N50 of 230 bp, GC content of 44.17%, and a maximum contig length of 1,399 bp. This reference assembly was used for all comparisons of variant scoring for *C. striata*, below.

Table 3. Comparison of the analysis of Illumina MiSeq 150 bp PE, and Illumina HiSeq 50 bp and 100 bp PE reads analyzed singly and in combination using the same de novo reference assembly for the *C. striata* complex.

	MiSeq 2 x 150	**HiSeq 2 x 50**	**HiSeq 2 x 100**	**Combined**
# raw read pairs / # post-trim	9899895 / 13435422	151025536 / 97981594	149453780 / 138080666	310379211 / 239142938
Total HaplotypeCaller Variants	249697	328726	560669	641667
Filtered SNPs	8177	12694	28401	25904
Mean filtered variant QUAL score	1636.36	499.96	2982.01	4685.16
bp/SNP (total filtered SNPs)	410.42	280.03	122.10	140.10
Mean coverage depth/locus/ accession	3.81	5.55	10.67	14.82
SD coverage depth	3.48	7.30	12.42	16.71
Mean coverage depth/locus /accession*	4.09	5.94	11.44	15.88
SD coverage depth*	3.44	7.41	12.52	16.82
SNPs/%missing data (min 1)*	8177/54.8%	12694/51.1%	28401/42.7%	25904/40.0%

SNPs/%missing data (min 10)*	7219/49.5%	11808/47.9%	26506/38.9%	24168/35.9%
SNPs/%missing data (min 50)*	2412/20.7%	3991/19.7%	13387/18.2%	13762/17.7%
SNPs/%missing data (min 80)*	261/3.1%	627/2.3%	3209/2.1%	3503/2.1%

C. bentleyi

Amplicons were successfully generated from 37 *C. bentleyi* accessions using 26 primers shown in Table 2. The total combined sequencing read pool comprised 40,949,418 read pairs, of which 25,523,858 survived trimming and filtering. Accession B8 was chosen as the reference individual, as it was the accession for which we recovered the greatest number of reads (913,462 read pairs). *De novo* assembly recovered 16,813 contigs longer than 100 bp, comprising a reference assembly totaling 3,928,602 bp in length with an N50 of 234 bp and GC content of 46.43%, excluding 686 contigs that either mapped to a genome of a putative contaminant or to the plastome of *C. bentleyi* (NC_040979.1). The majority of reference contigs were less than 250 bp in length (11,894), while 3,748 were longer than 250 bp, 1,003 were longer than 500 bp, and 168 were longer than 1 kb; the longest contig was 2,390 bp. The mean coverage depth of filtered variants scored in accession B8 using this *de novo* reference was 16.42x. Putative plastid or contaminant contigs totaled 259,774 bp with an N50 of 463 bp, GC content of 43.24%, and a maximum contig length of 2,436 bp.

***Populus deltoides* WV94**

We used all 26 SSR primers used in *C. bentleyi* to conduct PCR amplification of one *P. deltoides* clone (WV94), which was multiplexed along with *C. bentleyi* accessions. The raw sequencing pool comprised 589,403 read pairs, of which 390,992 survived trimming. Kmer trimming of adapter and/or SSR primer sequences occurred on 51.84% of reads. The chromosome-level assembly of *P. deltoides* (445, version 2.0; https://phytozome.jgi.doe.gov) comprises 403,296,128 bp, with 3.5% N nucleotide calls, and GC content of 33.3%. Trimmed reads covered 4,376,825 bp or 1.09% of the genome (437,660 reads). Visual observation of BAM files qualitatively suggested that SSRs were amplified relatively evenly throughout the genome (Fig. S2). Trimmed reads mapped to a median of 1.04% (range = 0.62% to 1.85%; SD = 0.34) of each chromosome (Table S1). A positive correlation between chromosome length and percent of each

chromosome covered by mapped trimmed reads was recovered by linear regression ($r^2 = 0.412$, p-value = 0.003; Fig. S3), congruent with our visual assessment of SSR amplification. Contrary to this positive correlation was read mapping to Chromosome 8, where 53,666 reads covered 1.85% of its total length, the largest of such values, despite its standing as the eighth longest chromosome. Mean coverage depth of filtered variants across all chromosomes was 36.71x.

Overall levels of variation in Populus and Corallorhiza.

C. striata

Analysis of individual MiSeq and HiSeq sequencing runs of PCR amplicon pools and their combination resulted in variable numbers of raw variants, variant quality scores, variant coverage depth, and number and missingness of final filtered variants (Table 3). SNPs identified by HaplotypeCaller ranged from 249,697 using just the MiSeq 150 bp PE sequencing run to 641,667 using those data combined with the HiSeq 50 bp PE and HiSeq 100 bp PE sequencing runs. Hard filtering of HaplotypeCaller variants using SelectVariants retained 8,177 SNPs (443.78 bp/SNP) for the MiSeq 150 bp run, 12,694 (285.88 bp/SNP) from the HiSeq 50 bp run, 28,401 (127.77 bp/SNP) for the HiSeq 100 run, and 25,904 (140.10 bp/SNP) when combining all read pools. Mean quality of total filtered variants was lowest (499.69) for variants scored using solely the HiSeq 50 bp dataset and highest (4,685.16) for those scored using the combined read pool. Mean coverage depth per filtered variant across accessions ranged from 3.81 to 14.82 for the MiSeq and combined read pools, respectively. Exclusion of six samples, for which sequencing produced few reads, increased filtered variant depth for the MiSeq 150 run to 4.09 and that of the combined read pool to 15.88. Missing data was tested using cutoffs of 1, 10, 50, and 80 as the number of individuals in which a filtered variant was scored. The single accession minimum SNP matrix resulting from analysis of the MiSeq 150 read pool contained 54.8% missing data, while missing data comprised 40.0% of the same matrix resulting from analysis of the combined sequencing runs while containing 31.6% more SNPs. Higher variant quality, coverage, and concomitant reduction in missingness of data guided our decision to use the analysis of the combined read pool for downstream analyses. 24,078 variants remained in the *C. striata* SNP matrix after missingness filtering using VCFtools conducted prior to analysis in R.

C. bentleyi

HaplotypeCaller identified 236,694 variants of which 51,618 were retained after filtering. SNPs comprised 47,851 of filtered variants or 76.11 bp/SNP. The mean coverage and quality score of filtered variants across all samples were 9.75 and 314.13, respectively. The reduced mean coverage and variant quality scores for these data led us to additionally hard filter variants using a minimum variant quality threshold of 60, which is double that suggested by GATK hard filtering recommendations, which we refer to as the QUALX2 dataset. The QUALX2 dataset comprised 27,750 SNPs, representing a 58% reduction, and a 141.57 bp/SNP ratio which is within 2 bp of that produced by via analysis of the combined sequencing read pool from *C. striata*. Contrastingly, doubling the minimum variant quality score threshold for variants scored using the *C. striata* combined read pool resulted in the loss of only 2,826 SNPs or a 10.9% reduction. Although the mean SNP quality score among the *C. bentleyi* samples was lower than that of SNPs scored from *C. striata*, missing data comprised only 10% of total filtered SNPs, 8.6% scored from at least 10 accessions, 7.0% scored from a minimum of 20 accessions, and 4.5% scored from 30 of the 37 accessions. After filtering with VCFtools prior to population genetic analyses in R, 51,132 variants remained in the hard-filtered *C. bentleyi* SNP matrix, 27,513 variants remained in the QUALX2 *C. bentleyi* SNP matrix.

P. deltoides WV94

Of the 8,134 indels and SNPs called by HaplotypeCaller in the *P. deltoides* WV94 clone we sequenced, 1,040 SNPs passed hard filtering. Variants were identified on all chromosomes (Fig. S3), and all filtered variants were heterozygous.

AMOVA and population statistics

For the *C. bentleyi* dataset, which contained 51,132 variants, we found that 97.3% of genetic variation was explained within individuals which was significantly less than expected by chance (phi = 0.027, sigma = 957, p-value < 0.001), while 2.32% was explained among sampling localities within county which was significantly greater than expected by chance (phi = 0.023, sigma = 22.9, p-value < 0.005), and 0.351% of variation was explained among county (phi = 0.004, sigma = 3.46, p-value = 0.326) (Table 4). Average observed heterozygosity and observed gene diversity was 0.093 and 0.089, respectively (Table 5). The F-statistic coefficients for the *C.*

bentleyi sampling locality grouped populations that had low inbreeding coefficients and genetic distances (Fig. 2a). Analysis of the additionally filtered QUALX2 dataset, which contained 27,513 variants, was congruent with the findings presented above; 96.7% of genetic variation was explained within individuals which was significantly less than expected by chance (phi = 0.033, sigma = 736, p-value < 0.001), while 2.91% was explained among sampling localities within county which was significantly greater than expected by chance (phi = 0.029, sigma = 22.1, p-value < 0.004), and 0.377% of variation was explained among county (phi = 0.004, sigma = 2.87, p-value = 0.414) (Table 4). Patterns of average observed heterozygosity (0.138), observed gene diversity (0.130) and F-statistic coefficients varied little for the QUALX2 dataset (Table 5).

Table 4. Analysis of molecular variance (AMOVA) output with fixation index for *Corallorhiza* populations.

Species	# Markers	b/w region-county	b/w loc	w/in ind	Φ_{CT}	Φ_{SC}	Φ_{IT}
C. bentleyi	51,132	0.351	2.32	97.3	0.004	0.023^{***}	0.027^{**}
C. bentleyi	27,513	0.377	2.91	96.7	0.004	0.029^{***}	0.033^{**}
C. striata	24,078	45.4	8.44	46.2	0.453^{***}	0.154^{***}	0.538^{***}

Markers indicate SNP variants in the dataset. Columns 3-5 are percent variation explained by each hierarchical level; 'a/m regions-county' = among regions (*C. striata*) or county (*C. bentleyi*); 'a/m loc' = among sampling localities within regions or county; and 'w/in ind' = within individuals. Columns 6-8 are values of fixation index (Φ) and their significance: * $P < 0.005$, ** $P < 0.001$. 'Φ_{CT}' = among regions or county; "Φ_{SC}" = among sampling localities within regions or county, and "Φ_{IT}" = within individuals.

Table 5. *Corallorhiza* population statistics table for *C. bentleyi* sampling locality and *C. striata* region.

Species	# Markers	H_o	H_s	F_{is}	F_{st}
C. bentleyi	51,132	0.093	0.089	-0.047	0.009
C. bentleyi	27,513	0.138	0.130	-0.063	0.012
C. striata	24,078	0.083	0.097	0.144	0.223

H_o = observed heterozygosity, H_s = observed gene diversities, F_{is} = inbreeding coefficient, F_{st} = fixation index

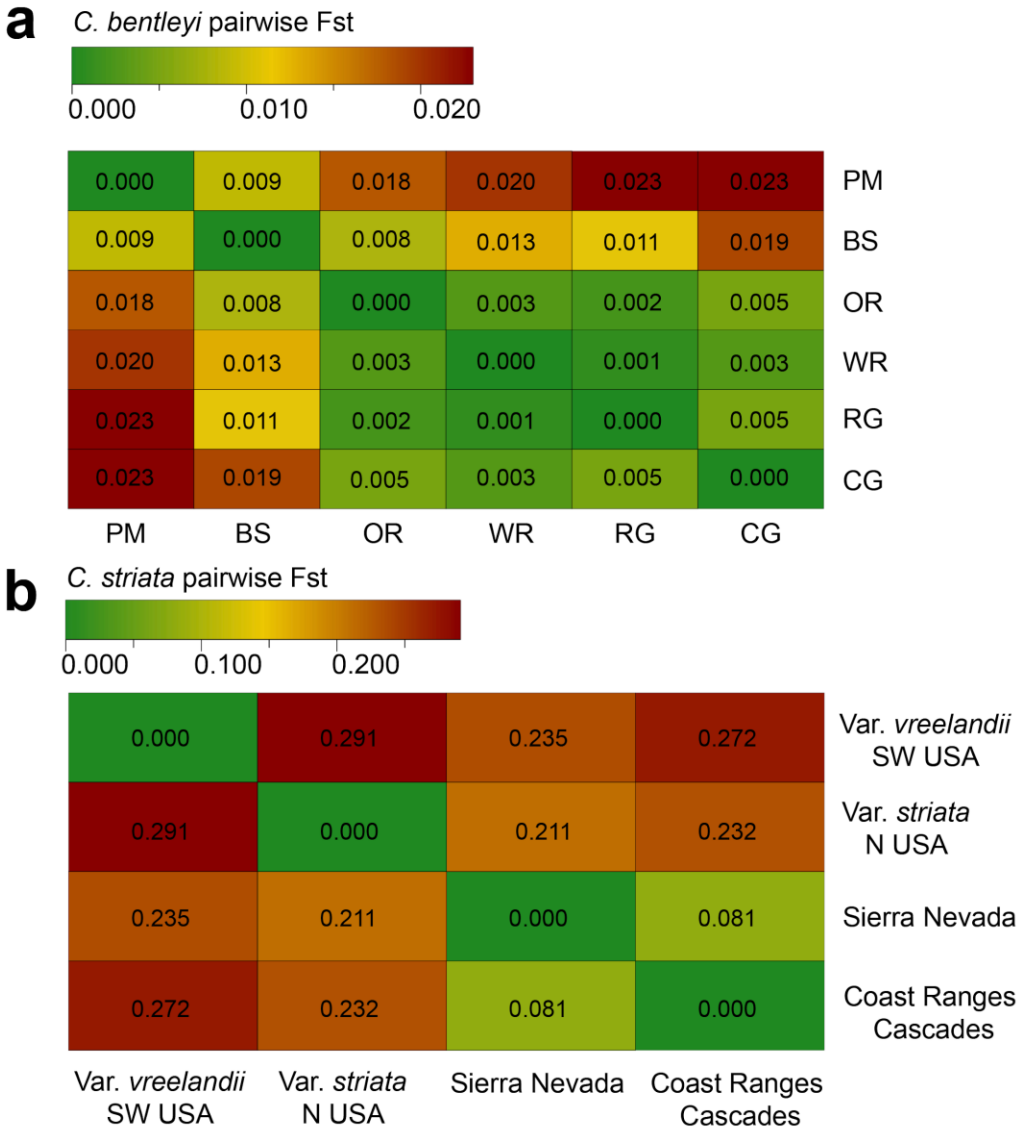

Figure 2. Pairwise F_{st} values comparing **a**. *C. bentleyi* sampling localities and **b**. *C. striata* regions. Red indicates high relative differentiation.

For *C. striata*, we found that 46.2% of genetic variation was explained within individuals which was significantly less than expected by chance (phi = 0.538, sigma = 130, p-value < 0.001), while 8.44% was explained among sampling localities (phi = 0.154, sigma = 23.7, p-value < 0.001), and 45.3% of variation was explained among regions (phi = 0.453, sigma = 127, p-value < 0.001) which were both significantly greater than expected by chance (Table 4). Average observed heterozygosity and observed gene diversity was 0.083 and 0.097, respectively. The F-statistic coefficient values were moderately high for the *C. striata* when treating geographic region at the level of subpopulation (Table 5, Fig. 2b).

PCA results

We retained 3 PC axes for the *C. bentleyi* analysis which explained a total of 29.2% of the variation in the genetic dataset (Fig. 3a). Discriminant analysis of principal components revealed the number of clusters appropriate for assigning membership probability based on principal components was 2 for *C. bentleyi* (Fig. 3b). In the *C. striata* analysis we retained 4 PC axes which explained 58.0% of the total genetic variation (Fig. 3c). *C. striata* sample membership was best explained by four clusters (Fig. 3d). Of the 51,132 genetic variants identified in *C. bentleyi*, only 130 were identified to be outlier loci (q-value < 0.05; Fig. 4a), while 625 of the 24,078 genetic variants called from *C. striata* were identified as outliers (q-value < 0.05, Fig. 4b).

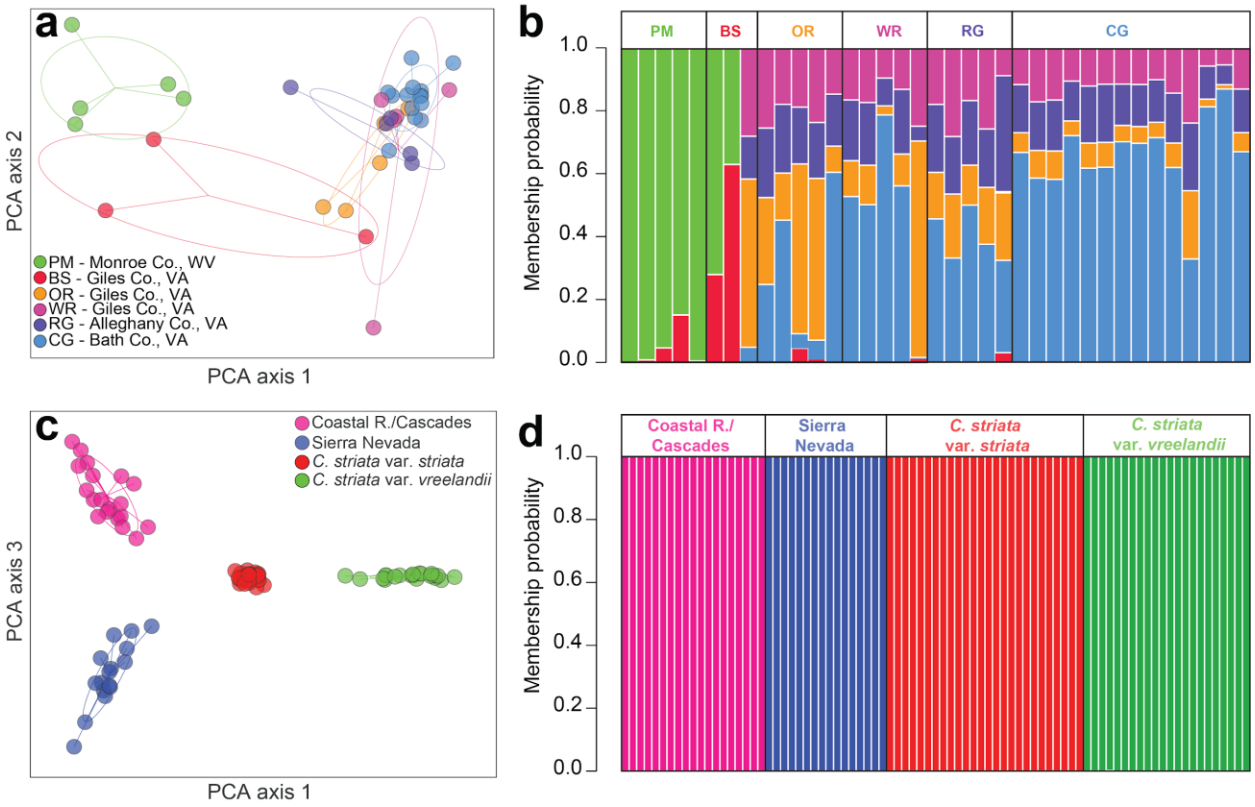

Figure 3. Population structure analyses of *Corallorhiza bentleyi* (n = 37 accessions) and the *C. striata* complex (n = 81 accessions). **a**. Discriminant Analysis of Principal Components (DAPC) dotplot and **b**. DAPC membership probabilities for *C. bentleyi* from 51,132 SNPs. **c**. DAPC dotplot and **d**. barplot for *C. striata* complex from 24,078 SNPs.

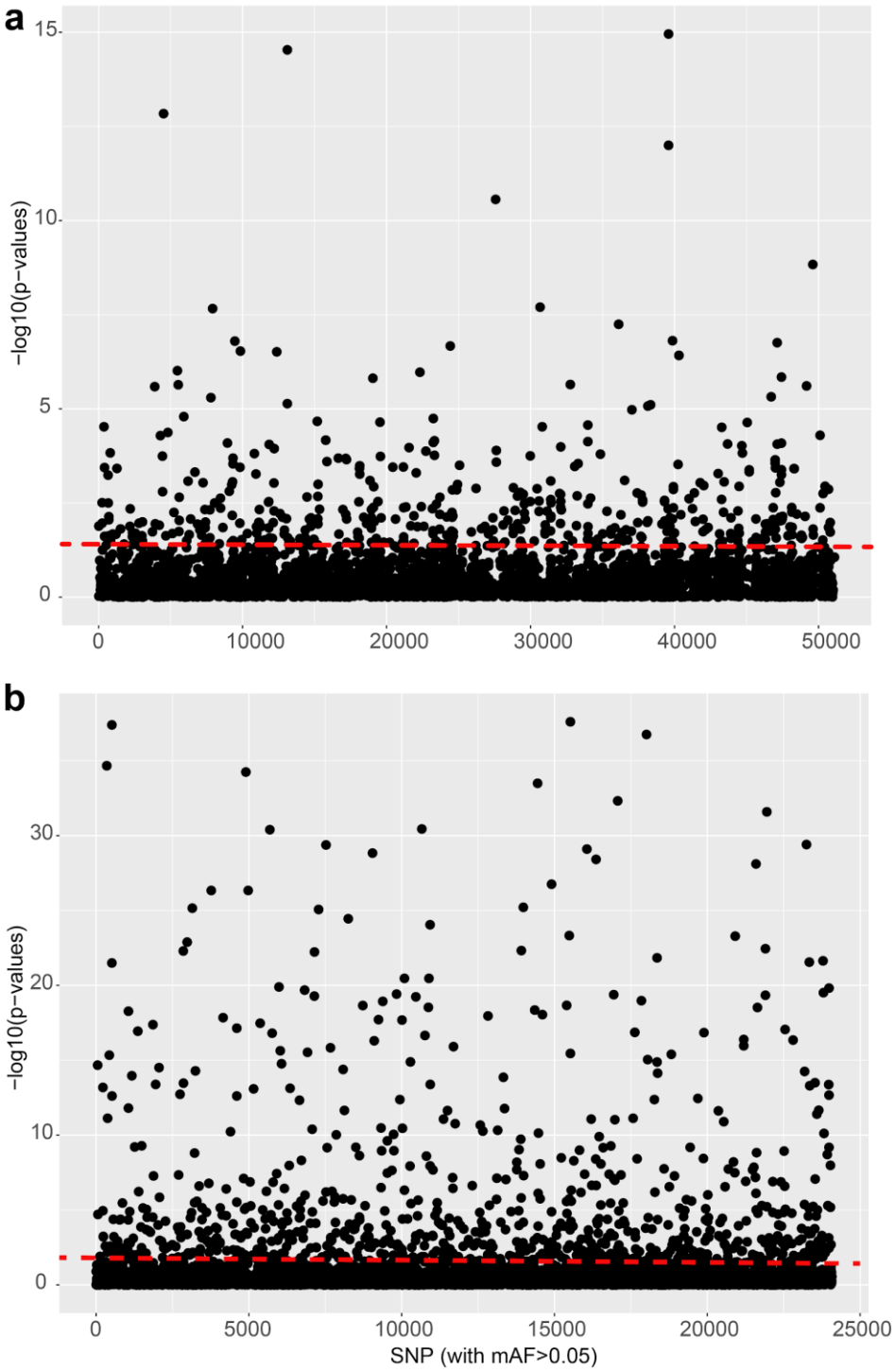

Figure 4. Manhattan plots depicting PCAdapt detection of outlier loci for **a**. *C. bentleyi* and **b**. *C. striata*.

DISCUSSION

C. striata

Population genetic analyses of ISSRseq-generated SNPs correspond well to previously published phylogeographic patterns recovered using targeted sequence capture of plastid loci (Barrett et al., 2018) and population genetic estimates using nuclear markers (Barrett and Freudenstein, 2011). For example, on the basis of three nuclear introns Barrett and Freudenstein (2011) estimated mean Φ_{CT} as 0.45, using some of same accessions used in the present study, and we estimated the same parameter at 0.453 using 24,078 SNPs generated by ISSRseq. Furthermore, F-coefficients and the results of AMOVA and DAPC suggest the presence of population subdivision and geographic partitioning of genetic variation in like fashion with that found using previous AMOVA, STRUCTURE analyses (Barrett and Freudenstein, 2011) and phylogenomic inference (Barrett et al., 2018). The congruence of the results of independent analyses using thousands of SNPs identified using ISSRseq with those of previous studies suggests that these data found using our novel method: 1) are appropriate for commonly used analyses; and 2) contain reliable population genomic signal.

C. bentleyi

ISSRseq revealed a strong signal of excess heterozygosity ($F_{is} \leq 0$) in *C. bentleyi*, a species known from approximately one dozen sites in just two states. Long-term clonality is expected to result in excess heterozygosity (Balloux et al., 2003) and *C. bentleyi* individuals exist as subterranean rhizomatous masses and are cleistogamous to varying degrees. Another possible explanation for more heterozygous variants in *C. bentleyi* could be an elevated rate of false positive variants called due to lower sequencing coverage (9.75 vs 14.82) and lower mean variant quality scores (314 vs 4685) for *C. bentleyi* than *C. striata*. Due to a lack of sequenced nuclear genomes for any *Corallorhiza* species (*Corallorhiza* genomes range from 6.35-16.10 Gb; Bai et al., 2012) we are unable to test whether the elevated number and heterozygosity of variants in *C. bentleyi* relative to *C. striata* are due to methodology or biology. Instead, we propose below that excess heterozygosity in *C. bentleyi* is congruent with what we know of the natural history of the species, and is more likely the result of a biological process than a pervasive, species-specific methodological artifact.

ISSRseq corroborated results of a traditional ISSR investigation study of *C. bentleyi* conducted previously by our group. Fama et al. (unpublished data) scored ISSR bands visually for two

primers used in this study and found that 89% of molecular variance occurred within populations. Using ISSRseq, we likewise found that the majority of genetic variation was explained within sampling localities, with only 2.32% of the genetic variation explained by sampling locality for our 51,132 SNP dataset and 2.91% for our more conservatively filtered 27,513 SNP dataset. Perhaps most strikingly, despite a greater number of variants *C. bentleyi* also had fewer missing data, indicating that false positives would have to be called consistently within each sample and then jointly across multiple individuals and then remain through hard filtering. The relatively low amount of missing data recovered was not surprising given the reproducibility of independent PCRs as assessed visually using gel electrophoresis (Fig. S5). Adjusting stringency of variant filtering did not appreciably impact our findings suggesting that either: 1) the workflow used here did not generate large proportions of false SNPs, or 2) that the downstream analyses used were robust to false SNPs in our matrices. Indeed, even when we removed any variant for which the QUAL score was less 60, analysis of the remaining variants did not change our overall conclusions from our population genetic analyses. Additional evidence of the recovery of population genomic signal in these data was our identification of fixed homozygous sites exclusive to cleistogamous individuals. We find the general congruence of visually scored ISSR banding patterns analyzed as dominant markers with our more sensitive analyses of ISSRseq data to be additional corroboration of our new method.

***Populus deltoides* WV94**

Our analysis of a *Populus deltoides* WV94 clone demonstrated that the loci sequenced and variants identified by ISSRseq are located throughout the genome, and variants were not obviously clustered in particular chromosomes or their centromeres or telomeres (Figs S2 & S4). As expected, we observed that the depth of final filtered SNPs was greater (36.71x) than the median depth of coverage for each chromosome (1.04%), which is to be expected with any reduced representation sequencing method.

ISSRseq is efficient, extensible, and low-cost

ISSRseq generates genomic variants on a scale that is comparable to other established RRS methods, while minimizing time and wet-lab complexity. Assuming the availability of two PCR machines and familiarity with basic wet laboratory techniques, users can go from extracted DNA

to an Illumina sequencing library for 48 samples and 4 primer sets within an eight-hour workday. This timeframe and capacity can be greatly increased for users who use 96 or 384 well plates to conduct PCR.

ISSRseq is suitable for undergraduate students and users with minimal lab experience or resources, since only a thermocycler and commonplace equipment such as a DNA quantitation device and affordable neodymium magnets used for PCR cleanup with magnetic beads are necessary prior to sequencing. Indeed, the ISSRseq data analyzed here were generated by undergraduates, and ISSRseq projects have been conducted in an undergraduate course at West Virginia University. Additionally, the use of a commercially-available, fragmentase-based sequencing library preparation kit means that users can receive support directly from a company, rather than relying on personal communications with authors. While we know that some advanced users might prefer a "home-brew" sequencing library preparation protocol, we found the efficiency and reliability of a commercially-available kit that can prepare a library in about 2.5 hours to be fitting for our purposes. That said, a sizable fraction of the cost of ISSRseq, as currently implemented, is the use of a library preparation kit. Our publication of this method stands as a proof of concept, rather than a prescription, and we suspect that advanced users of ISSRseq could further reduce the per sample cost of this method.

The recovery of loci generated amongst samples sequenced using RAD-like RRS approaches is impacted by mutations to restriction sites, DNA sample impurities and degradation, and user error or random variation during size selection (Andrews et al., 2016). ISSRseq generates loci for downstream analysis via PCR rather than careful size selection of restriction-fragmented DNA via gel excision or Pulsed-Field electrophoresis, and we expect that locus dropout due to user error or DNA quality should be minimal relative to RRS methods such as ddRAD. In line with this expectation, missing data accounted for only 10% of the 47,851 SNP data matrix we generated for *C. bentleyi* and only 4.5% missing data when we required a SNP to be called in 30 of 37 individuals. As a point of reference, Tripp et al. (2017) used RADseq to identify 302,987 SNPs in *Petalidium* spp. (Acanthaceae), however the number of SNPs included in their data matrices (1,568 to 53,792, depending on parameter choice) was only greater than that recovered using ISSRseq when the missingness cutoff was greater than 60%. While variable SNP recovery

may have marginal impacts for phylogenetic applications (Eaton et al., 2017; Tripp et al., 2017; Piwczyński et al., 2020), missing data are known to bias estimation of population genetic parameters, such as N_e and F statistics (Marandel et al., 2020). The use of PCR for the generation of loci analyzed using ISSRseq means that locus dropout is minimized across DNA samples of relatively lower concentration and/or molecular weight than is preferable when using many existing RRS methods.

Methodological Considerations, Suggestions, and Caveats

Not surprisingly, sequencing depth is an important consideration when using ISSRseq just as with using other RRS methods. Conducting fewer ISSR PCRs using diverse SSR motifs is one option to maximize the efficiency of sequencing depth and number of samples that can be multiplexed during sequencing. For example, we recovered similar cumulative assembled amplicon length in *C. striata* as *C. bentleyi* despite using 8 PCRs and 26 PCRs for each, respectively. This is likely due to the fact that many of the primers used for *C. bentleyi* comprised similar SSR motifs but had different anchors, whereas we used fewer primers of differing SSR motifs in *C. striata*, which together reflect the diversity of SSR motifs amplified from the latter species. We also found that 100 bp, paired-end sequencing of the same library on a single HiSeq 2500 nearly tripled the mean sequencing depth per locus (4.09x vs 11.44x) and more than quadrupled the number of variants scored (8,177 vs 28,401) over a single lane of paired-end 150 bp sequencing on the MiSeq. Given these results, we suggest that users select primers representing diverse and dissimilar SSR motifs that produce the most amplicons and prioritize read number over read length. Furthermore, we recommend bead-based exclusion of the shortest PCR amplicons prior to library preparation to reduce sequencing of short amplicons likely comprising mostly low complexity sequence. Following these recommendations should also save on PCR reagent cost and worker time.

Although joint variant calling should be able to minimize false positives, we do recommend that future researchers consider conducting more stringent variant filtering that incorporates a control sequence to empirically determine variant filtering parameters. For example, GATK is able to leverage machine learning of known variants to recalibrate variant quality scores and determine appropriate filtering parameters for genomes that are already well characterized (DePristo et al.,

2011). The implementation of more sophisticated variant filtration techniques will no doubt reduce noise in ISSRseq data.

As a final caveat we would like to acknowledge that we have not tested the impact of phylogenetic distance or reference choice on locus recovery or variant calling. Phylogenetic distance between samples and a given reference genome has been shown to differently impact variant scoring depending on the chosen genotype caller (Duchen and Salamin, 2020). HaplotypeCaller, and therefore the supplied pipeline, may not be the best choice for users interested in using ISSRseq to generate SNP matrices for deep phylogenetic inference. However, researchers are encouraged to use the BASH scripts we provide to guide them in designing a workflow that fits the needs of their study.

Potential Applications

In addition to studies of local adaption, population genomics, and phylogenetic inference, ISSRseq could prove a useful technique to those interested in RRS using longer loci, agricultural crop or livestock authentication, forensics, and microbiome studies. For example, the use of PCR to amplify loci for sequencing means that the theoretical maximum of locus length is limited only by the DNA polymerase used during the PCR step. By modifying the PCR conditions and reagents, users could conduct long range amplifications to recover loci that are thousands of base pairs in length. These long loci could facilitate robust estimates of linkage disequilibrium and allele phasing in species without pre-existing genomic resources, and for the construction of gene trees for phylogenomic studies, allowing the use of many multilocus coalescent analyses (ASTRAL, Bayesian species delimitation, etc.). Furthermore, due to the ubiquity of SSRs in genomes, ISSRseq could be used to conduct RRS for the investigation of microbiomes, symbioses, and even simultaneous sequencing of hosts and one or more pathogens.

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AUTHORS' CONTRIBUTIONS

CFB conceived ISSRseq. BTS, SJS, CFB, and SPD designed the laboratory and bioinformatic methodology and protocols. BTS, SJS, MVS, and NMF conducted laboratory work. CFB conducted all field work. BTS, SJS, CFB, and SPD analyzed the data. BTS, SJS, and CFB led the writing of the manuscript. All authors contributed to manuscript drafts and provided their consent for publication.

DATA AVAILABILITY

Data matrices are available from the Dryad Digital Repository (doi:XXX) sequencing reads available via NCBI (accession #s). Scripts used and usage instructions are available via GitHub (www.github.com/btsinn/ISSRseq).

Supplementary Documents

ISSRseq wetlab protocol

Document of supplementary tables and figures

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SUPPLEMENTAL TABLES & FIGURES

Supplementary Table 1. Descriptive statistics of *P. deltoides* WV94 and mapping of trimmed reads.

Chromosome	Length (bp)	Reads Mapped	bp covered	% bp covered	
1	53086243	52775	534218	1.006321	
2	26748850	27372	166950	0.624139	
3	22915696	16653	144066	0.6286783	
4	24223889	24175	256558	1.0591115	
5	26443402	28379	366857	1.3873291	
6	28326727	41552	295106	1.0417935	
7	16146797	5441	144415	0.8943879	
8	21710561	53666	402354	1.8532639	
9	13407431	30241	188690	1.4073539	
10	21864811	16827	240152	1.0983493	
11	18311809	9054	172726	0.9432492	
12	15564500	19837	152948	0.9826721	
13	17534440	22999	208538	1.1893052	
14	17670024	11811	110966	0.6279901	
15	15256731	20625	152503	0.9995785	
16	14755466	12147	261082	1.7693918	
17	16616056	3820	143324	0.8625633	
18	17191025	31245	231940	1.3491924	
19	15521670	9041	203432	1.3106322	
totals	403296128	437660	4376825	1.0852633	Avg. % covered
				1.0417935	Median % covered
				0.3354717	SD

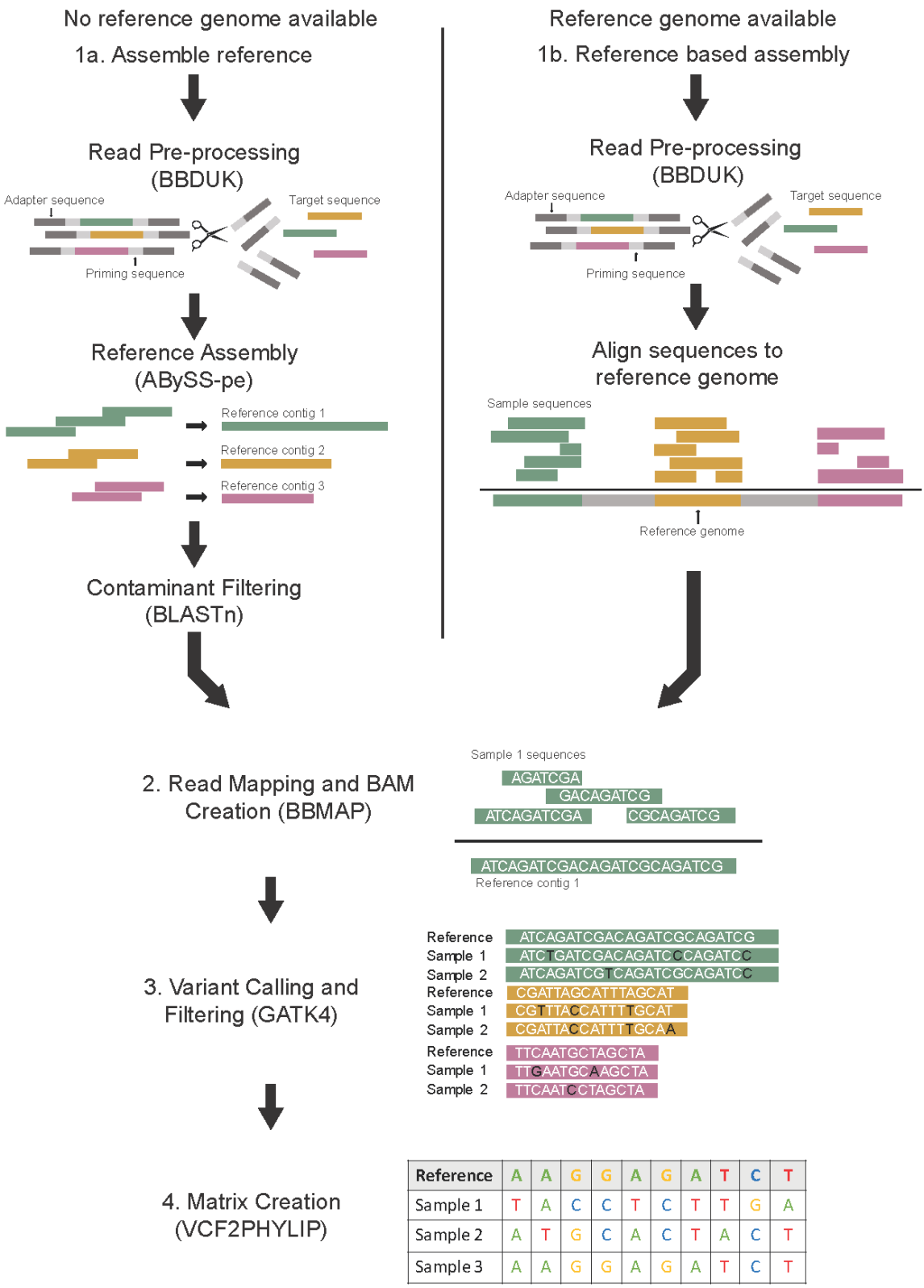

Figure S1. Flowchart for bioinformatic analysis pipeline with dependencies indicated in parentheses.

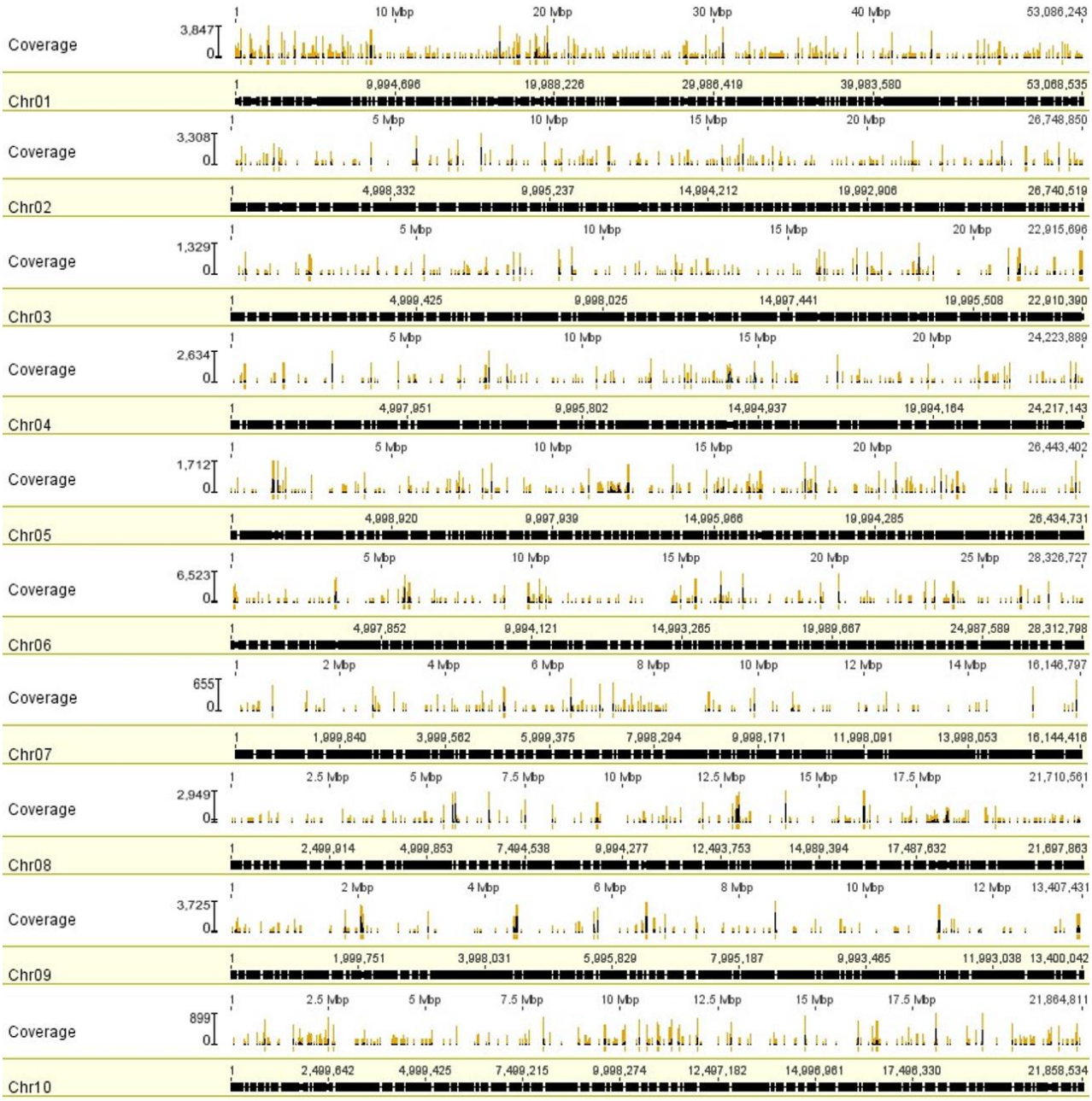

Figure S2. Mapping of trimmed reads to *P. deltoides* WV94 genome. Log-transformed coverage is shown above each chromosome, with regions of greater than 50x coverage depth denoted in yellow below the coverage histogram.

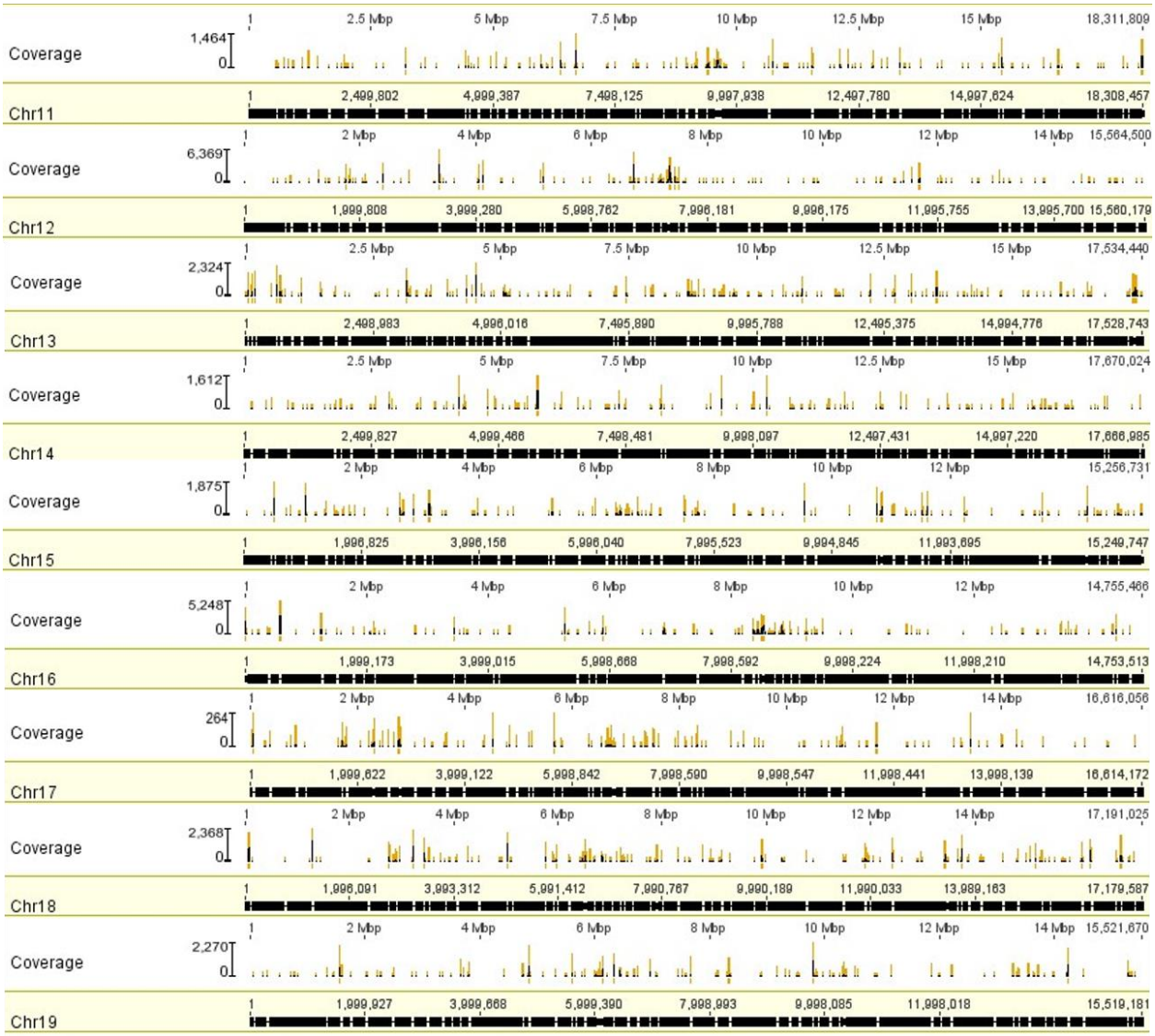

Figure S2 (cont.). Mapping of trimmed reads to *P. deltoides* WV94 genome. Log-transformed coverage is shown above each chromosome, with regions of greater than 50x coverage depth denoted in yellow below the coverage histogram.

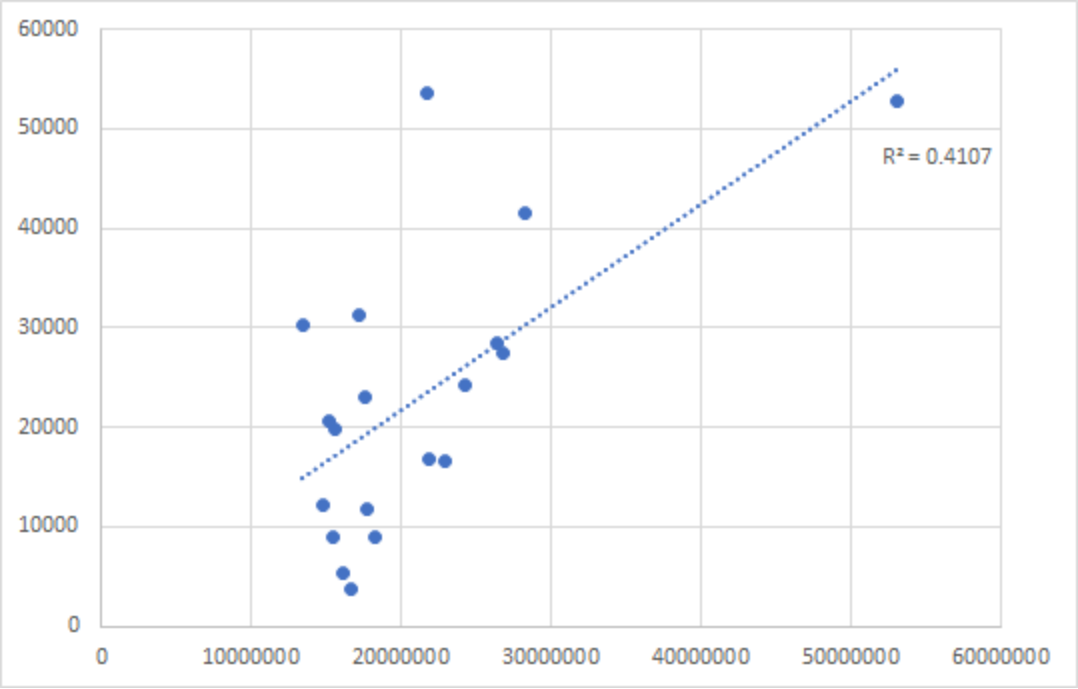

Figure S3. Trimmed reads mapped vs *P. deltoides* WV94 chromosome length. The dotted line represents the linear best fit (R^2 = 0.4107; p-value = 0.003).

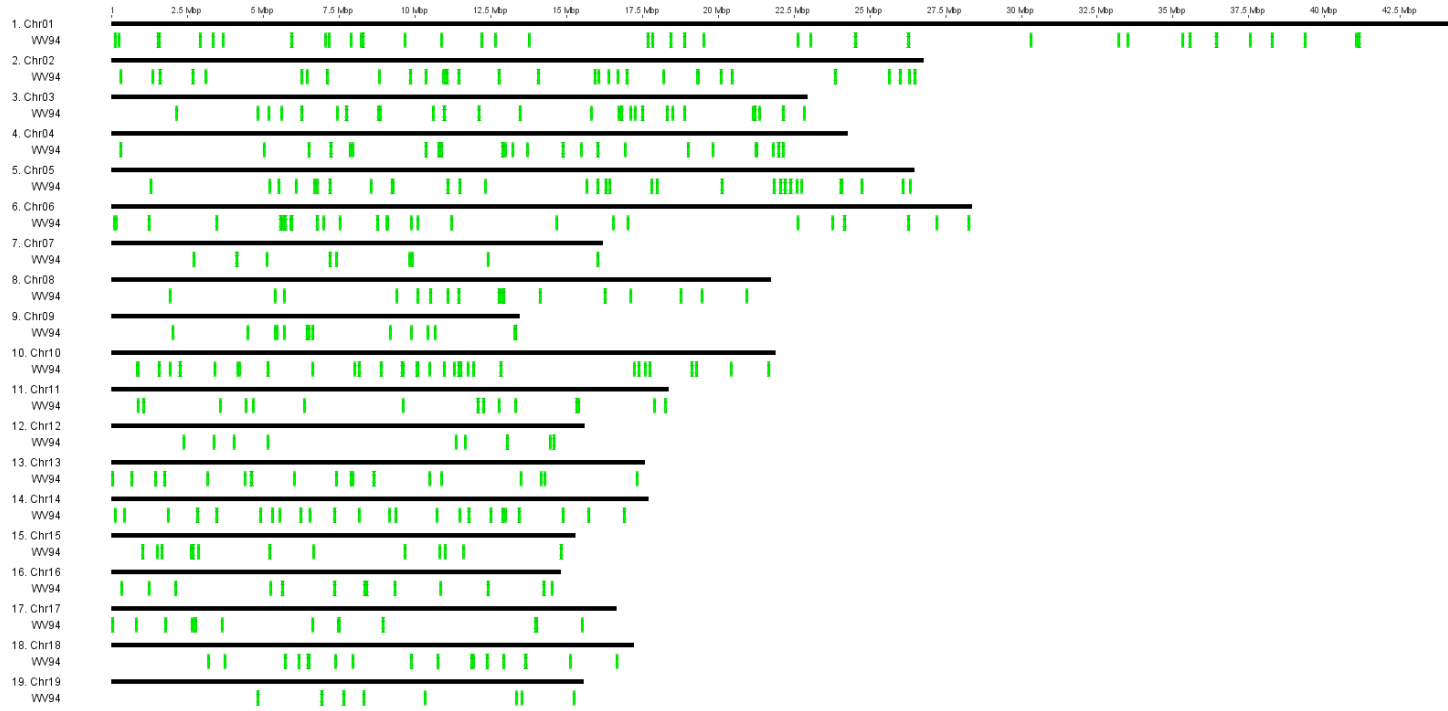

Figure S4. SNP variants annotated on the genome of *Populus deltoides* WV94. Variant positions are denoted with a green box below their position on each chromosome.

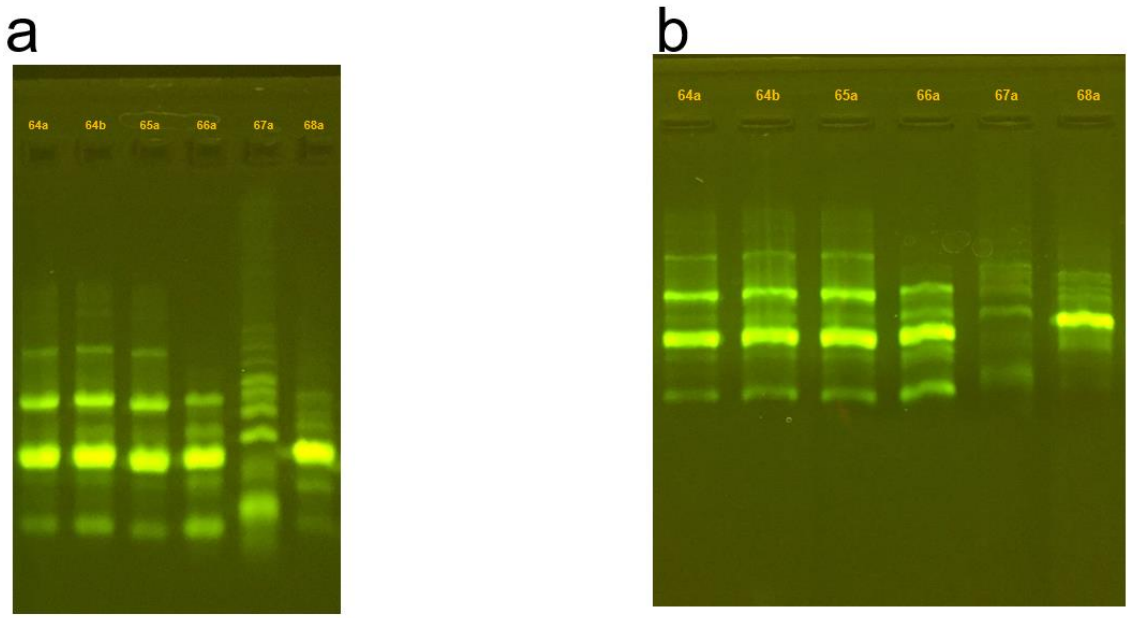

Figure S5. Gel electrophoresis (1% agarose) of PCRs conduced on A) 27 March and B) 31 May 2018 using ISSR Primer 868 on C. bentleyi samples: 64a, 64b, 65a, 66a, 67a, 68a (lanes labeled.) PCR products were allowed to separate differently by running gel A over a longer time period.

Supplementary File: Sources and identifiers of genomes used to filter putative contaminant loci.

Mycorrhizal Fungi (one from each genus available)

https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Mycorrhizal_fungi

Acephala macrosclerotiorum EW76-UTF0540 v1.0: Project: 1034881, Acema1

Amanita muscaria Koide v1.0: Project: 403202, Amamu1

Boletus coccyginus 2016PMI039 v1.0: Project: 1234182, Bolcoc1

Cantharellus anzutake C23 v1.0: Project: 1006245, Cananz1

Cenococcum geophilum 1.58 v2.0: Project: 402530, Cenge3

Ceratobasidium sp. anastomosis group I; DN8442 v1.0: Project: 1019533, CerAGI

Choiromyces venosus 120613-1 v1.0: Project: 1016739, Chove1

Clavulina sp. PMI_390 v1.0: Project: 1021554, ClaPMI390

Cortinarius austrovenetus TL2843-KIS7R v1.0: Project: 1193804, Coraus1

Gautieria morchelliformis GMNE.BST v1.0: Project: 1019425, Gaumor1

Geopyxis carbonaria DOB1671 v1.0: Project: 1127117, Geocar1

Gigaspora rosea v1.0: Gigro1

Gyrodon lividus BX v1.0: Project: 1016731, Gyrli1

Hebeloma cylindrosporum h7 v2.0: Project: 402607, Hebcy2

Hyaloscypha finlandica PMI_746 v1.0: Project: 1113379, Hyafin1

Hydnum rufescens UP504 v2.0: Project: 1019557, Hydru2

Hysterangium stoloniferum HS.BST v1.0: Project: 1019429, Hyssto1

Kalaharituber pfeilii F3 v1.0: Project: 1060175, Kalpfe1

Kurtia argillacea OMC1749 v1.0: Project: 1231371, Kurarg1

Laccaria amethystina LaAM-08-1 v2.0: Project: 1006253, Lacam2

Lactarius akahatsu QP v1.0: Project: 1177532, Lacaka1

Lactifluus cf. subvellereus BPL653 v1.0: Project: 1108981, Lacsub1

Leucangium carthusianum GMNB180 Co-culture: Project: 1191024, Leuca1

Melanogaster broomeianus MBLB.BST v1.0: Project: 1019457, Melbro1

Meliniomyces bicolor E v2.0: Project: 1008686, Melbi2

Morchella importuna CCBAS932 v1.0: Project: 1024000, Morco1

Multifurca ochricompacta BPL690 v1.0: Project: 1106235, Muloch1

Oidiodendron maius Zn v1.0: Project: 403613, Oidma1

Paxillus adelphus Ve08.2h10 v2.0: Project: 1006419, Paxru2

Piloderma croceum F 1598 v1.0: Project: 402979, Pilcr1

Pisolithus albus SI12 v1.0: Project: 1133208, Pisalb1

Rhizophagus cerebriforme DAOM 227022 v1.0: Rhice1

Rhizopogon salebrosus TDB-379 v1.0: Project: 1039526, Rhisa1

Rhizoscyphus ericae UAMH 7357 v1.0: Project: 1006265, Rhier1

Russula brevipes BPL707 v1.0: Project: 1106237, Rusbre1

Scleroderma citrinum Foug A v1.0: Project: 404724, Sclci1

Sebacina vermifera MAFF 305830 v1.0: Project: 403638, Sebve1

Serendipita vermifera ssp. bescii NFPB0129 v1.0: Project: 1053109, Sebvebe1

Sphaerosporella brunnea Sb_GMNB300 v2.0: Project: 1108957, Sphbr2

Suillus americanus EM31 v1.0: Project: 1052751, Suiame1

Terfezia boudieri ATCC MYA-4762 v1.1: Project: 1018931, Terbo2

Thelephora ganbajun P2 v1.0: Project: 1050999, Thega1

Tirmania nivea G3 v1.0: Project: 1108979, Tirniv1

Tricholoma matsutake 945 v3.0: Project: 404726, Trima3

Tuber aestivum var. urcinatum v1.0: Tubae1

Tulasnella calospora AL13/4D v1.0: Project: 404728, Tulca1

Wilcoxina mikolae CBS 423.85 v1.0: Project: 1016515, Wilmi1

Xerocomus badius 84.06 v1.0: Project: 1019549, Xerba1

Plant Pathogenic Fungi

https://genome.jgi.doe.gov/portal/Plant_pathogens/Plant_pathogens.download.html

Alternaria alternata ATCC11680: Alalt1

Ascochyta rabiei ArDII: Ascra1

Atropellis piniphila CBS 197.64 v1.0: Project: 1134530, Atrpi1

Blumeria graminis f. sp. hordei 5874 v1.0: Project: 1145186, Bgh5874

Botryosphaeria dothidea: Botdo1

Botrytis cinerea v1.0: Botci1

Cercospora zeae-maydis v1.0: Project: 401984, Cerzm1

Choanephora cucurbitarum NRRL2744 (50) v1.0: Project: 1099013, Chocucu1

Cladosporium fulvum v1.0: Clafu1

Cochliobolus carbonum 26-R-13 v1.0: Project: 403760, Cocca1

Colletotrichum abscissum IMI 504890: Colab1

Corynespora cassiicola CCP v1.0: Project: 1019537, Corca1

Cronartium quercuum f. sp. fusiforme G11 v1.0: Project: 401997, Croqu1

Cryphonectria parasitica EP155 v2.0: Project: 16952, Cryphonectria

Cytospora chrysosperma CFL2056 v1.0: Project: 1110761, Cytch1

Dactylonectria estremocensis MPI-CAGE-AT-0021 v1.0: Project: 1103627, Daces1

Diaporthe ampelina UCDDA912: Diaam1

Didymella zeae-maydis 3018: Didma1

Diplodia seriata DS831: Dipse1

Dothidotthia symphoricarpi v1.0: Project: 1011345, Dotsy1

Elsinoe ampelina CECT 20119 v1.0: Project: 1064684, Elsamp1

Elytroderma deformans CBS183.68 v1.0: Project: 1166950, Elyde1

Entoleuca mammata CFL468 v1.0: Project: 1117716, Entma1

Erysiphe pisi Palampur-1 v2.0: Project: 1176372, Erypi2

Eutypa lata UCREL1: Eutla1

Exobasidium vaccinii MPITM v1.0: Project: 1016319, Exova1

Fomitiporia mediterranea v1.0: Project: 402518, Fomme1

Fusarium fujikuroi IMI 58289: Fusfu1

Gaeumannomyces graminis var. tritici R3-111a-1: Gaegr1

Golovinomyces cichoracearum UCSC1 v1.0: Project: 1055992, Golci1

Gremmeniella abietina DAOM 170408 v1.0: Project: 1104310, Greab1

Grosmannia clavigera kw1407: Grocl1

Heterobasidion annosum v2.0: Project: 16080

Leptosphaeria maculans: Lepmu1

Macrophomina phaseolina MPI-SDFR-AT-0080 v1.0: Project: 1103647, Macpha1

Magnaporthe oryzae 70-15 v3.0: Magor1

Magnaporthiopsis poae ATCC 64411: Magpo1

Melampsora allii-populina 12AY07 v1.0: Project: 1026279, Melap1finSC

Microbotryum lychnidis-dioicae p1A1 Lamole: Micld1

Microthyrium microscopicum CBS 115976 v1.0: Project: 1011369, Micmi1

Mixia osmundae IAM 14324 v1.0: Project: 404192, Mixos1

Moniliophthora perniciosa FA553: Monpe1

Mycosphaerella graminicola v2.0: Project: 16205

Nectria haematococca v2.0: Project: 16208, Necha2

Neocosmospora boninensis NRRL 22470 v1.0: Project: 1034400, Neobo1

Neofusicoccum parvum UCRNP2: Neopa1

Neonectria ditissima R09/05: Neodi1

Oliveonia pauxilla MPI-PUGE-AT-0066 v1.0: Project: 1103641, Olipa1

Ophiostoma piceae UAMH 11346: Ophpic1

Paraconiothyrium sporulosum AP3s5-JAC2a v1.0: Project: 1029422, Parsp1

Phaeoacremonium aleophilum UCRPA7: Phaal1

Phaeomoniella chlamydospora UCRPC4: Phach1

Phakopsora pachyrhizi MG2006 v1.0: Project: 1097278, Phapa1

Phellinus igniarius CCBS 575 v1.0: Project: 1108911, Pheign1_1

Phoma multirostrata 7a v1.0: Project: 1150440, Phomu1

Phyllosticta citriasiana CBS 120486 v1.0: Project: 1011301, Phycit1

Pseudocercospora (Mycosphaerella) fijiensis v2.0: Project: 16189

Pseudoidium neolycopersici MF1 Metagenome Minimal Draft: Project: 1145388, Psene1

Puccinia coronata avenae 12NC29: PuccoNC29_1

Pyrenophora teres f. teres: Pyrtt1

Rhizoctonia solani AG-1 IB: Rhiso1

Saccharata proteae CBS 121410 v1.0: Project: 1011317, Sacpr1

Sclerotinia sclerotiorum v1.0: Sclsc1

Septobasidium sp. PNB30-8B v1.0: Project: 1047727, Sepsp1

Septoria musiva SO2202 v1.0: Project: 401987, Sepmu1

Setomelanomma holmii CBS 110217 v1.0: Project: 1019737, Setho1

Setosphaeria turcica Et28A v2.0: Project: 401988, Settu3

Sporisorium reilianum SRZ2: Spore1

Stemphylium lycopersici CIDEFI-216: Stely1

Taphrina populi-salicis CBS419.54 v1.0: Project: 1166952, Tappo1

Teratosphaeria nubilosa CBS 116005 v1.0: Project: 1019765, Ternu1

Testicularia cyperi MCA 3645 v1.0: Project: 1040186, Tescy1

Urocystis occulta CBS 102.71 v1.0: Project: 1185590, Uroocc1

Ustilago maydis 521 v2.0: Ustma2_2

Venturia inaequalis: Venin1

Verticillium alfalfae VaMs.102: Veral1

Zopfia rhizophila v1.0: Project: 1011325, Zoprh1

Zymoseptoria ardabiliae STIR04_1.1.1: Zymar1

Human Genome

ftp://ftp.ensembl.org/pub/release-100/fasta/homo_sapiens/dna/

Homo_sapiens.GRCh38.dna_rm.primary_assembly.fa.gz