Whole Genome Amplification as an Innovative Approach for Genomic Studies in Lichenized Fungi

Cristóbal Ivanovich (cristobal.ivanovic@gmail.com)
University of Frankfurt: Goethe-Universitat Frankfurt am Main https://orcid.org/0000-0002-2810-0084
Juraj Paule
Senckenberg Forschungsinstitut und Naturmuseum Frankfurt: Senckenberg Forschungsinstitut und Naturmuseum
Elisa Lagostina
Senckenberg Forschungsinstitut und Naturmuseum Frankfurt: Senckenberg Forschungsinstitut und Naturmuseum
Scott LaGreca
Duke University
Steve Leavitt
Brigham Young University
Lucia Muggia
University of Trieste: Universita degli Studi di Trieste
Zdeněk Palice
Czech Academy of Sciences: Akademie ved Ceske republiky
Sergio Pérez-Ortega
Real Jardín Botánico: Real Jardin Botanico
Christian Printzen
Senckenberg Forschungsinstitut und Naturmuseum Frankfurt: Senckenberg Forschungsinstitut und Naturmuseum

Research

Keywords: DNA, LECANORALES, LICHENS, MITOGENOME, MYCOBIONT

Posted Date: June 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1725113/v1

Abstract

Molecular sequence data has transformed the field of lichen taxonomy and provided crucial insight into evolutionary relationships. However, DNA obtained from standard extraction methodologies may not yield sufficient high-quality DNA to successfully perform high-throughput sequencing. Furthermore, standard DNA extraction protocols often, require the destructive use of large portions of lichen thalli and apothecia.

For crustose lichens, in particular, obtaining enough high-quality DNA for subsequent genomic sequencing becomes challenging considering the small amount of source material. A further problem arises by the presence of non-target microorganisms, often co-symbionts within the lichen thalli, which can decrease the coverage of sequence reads from the mycobiont and photobiont of interest. In this paper, we present Whole Genome Amplification (WGA) as a suitable technique to recover large quantities of high-quality DNA from limited amounts of lichen thallus material or fractions of a single apothecium and minimize the quantity of non-target reads. Two WGA kits were tested: Qiagen Repli-G Single-Cell Kit and Jena Bioscience Direct WGA kit, and results were compared to CTAB-extracted DNA. DNA obtained from the three methods, was used to assemble and annotate a total of 18 mitochondrial genomes from the order Lecanorales. Of these, ten mitogenomes were fully and eight mitogenomes were partially assembled. The resulting mitogenomes showed consistent gene position patterns and phylogenetic placement was largely concordant with data previously published. We demonstrate that this alternative method can be reliably used for obtaining enough high-quality DNA material for genomic research on lichens.

Introduction

Lichens are a mutualistic symbiotic association between a major fungal partner (mycobiont) and a population of microalgae or cyanobacteria (photobiont). Classical lichen systematics relies on morphological and chemical characters of the fungal partner (taxonomically diagnostic secondary metabolites). Nevertheless, phenotypical traits of the mycobiont are often scarce and may be plastic, e. g. due to abiotic factors (Pérez-Ortega et al., 2012) or photobiont partner shifts, leading to unreliable systematic classifications (Lücking et al., 2021; Muggia et al., 2014).

The advent of sequencing technologies and the incorporation of sequence data have provided powerful tools to help resolve long-standing challenges in lichen taxonomy. Over the past two decades, multi-locus datasets have become the standard for phylogenetic reconstructions and systematic assessment. However, published phylogenies sometimes contain poorly supported nodes and unresolved clades, or incongruences among single-locus trees (Degnan & Rosenberg, 2009). Now, a wide array of modern genome sequencing approaches facilitates the generation of genome-scale datasets, providing huge amounts of data to help resolve the current shortcomings of single and multi-locus phylogenetics (Lücking et al., 2021; Pizarro et al., 2019). Genome-wide data is also increasingly used to study species delimitations (Lutsak et al., 2020) and functional aspects of the lichen symbiosis (e.g., Grube et al., 2014; Spribille et al., 2016; Tagirdzhanova et al., 2021). However, the integration of new phylogenomic methods, brings about new challenges for lichen-specific research.

Standard DNA extraction practices

One common problem in DNA-sequence-based studies with lichenized fungi is the difficulty to obtain sufficient DNA for high-throughput genome sequencing using standard extraction protocols, for example cetyltrimethylammonium-bromide (CTAB) extraction or commercially available DNA extraction kits. The yields of these kits are usually below 5 ng/µl. This issue can be explained, in part, by the nature of the fungal cell wall, consisting of specific polysaccharides such as alkali-insoluble/soluble hexoses and chitinous amino sugars (Honegger & Bartnicki-García, 1991). These can be difficult to digest, thus limiting the success of isolating total genomic DNA available for downstream applications. DNA isolation from lichens therefore requires homogenization of lichen mycelia prior to the DNA extraction steps. Most common protocols include deep-freezing (using liquid nitrogen) and grinding the lichen thalli or ascomata with sterile sand, glass beads or a pestle, and subsequent or simultaneous incubation in lysis buffer. Grinding can be facilitated also by a high-power bead mill homogenizer.

Another issue related to lichens are secondary metabolites. About 1000 lichen substances are known, although the function of many of them is still unknown (Ranković & Kosanić, 2015). These metabolites may inhibit polymerase activity in PCR reactions when carried over into DNA elutions (Ekman, 1999). Washing lichen samples in acetone and drying them prior to homogenization usually enhance downstream amplification success.

Protocols for second and third-generation sequencing usually require a minimum amount of DNA for library preparation higher than those sufficient for Sanger sequencing. In order to reach the required DNA amounts with standard DNA extraction procedures, multiple extractions are often pooled. This not only increases the time and lab resources, but also the amount of thalli required. In the case of species with small thalli, extractions from multiple thalli may be necessary, increasing the likelihood of studying genetically heterogeneous DNA samples. Under these circumstances, destructive sampling for DNA extraction might not be feasible for species with particularly small, inconspicuous or underdeveloped thalli, or regionally rare, endemic or threatened taxa.

It is usually outright impossible to obtain sufficient amounts of material from small crustose taxa and difficult to generate it from mycobiont cultures, since most lichen-forming fungi grow at extremely slow rates in the lab. For example, we attempted to CTAB-extract cultured material of *Myriolecis hagenii* (Ach.) Śliwa, Zhao Xin & Lumbsch and *Lecanora horiza* (Ach.) Röhl., but this yielded so little DNA (29 ng and 91 ng respectively, after several rounds of CTAB extraction), that they were unsuitable for sequencing.

Whole Genome Multiple Displacement Amplification

The Whole Genome Multiple Displacement Amplification (WGA) procedure relies on the usage of randomized hexameric primers, and a Phi-29 polymerase (Blanco et al., 1989; Dean et al, 2001). Phi-29 polymerase is an enzyme that carries out an isothermal amplification at room temperature and has a 3´->5´ proofreading activity, with strong strand displacement activity (Garmendia et al., 1992, Pugh et al., 2008). In WGA, primers randomly bind to the genomic material, and the polymerase subsequently extends the regions until it reaches an already amplified portion. The portion gets partially detached as the

polymerase advances. New primers bind and polymerases start extending on the detached portion, thus creating “branched” structures (Fig. 1). Final amplicons range between 50–100 kb (Huang et al., 2015).

This method can enhance the amount of DNA necessary for later applications especially from extremely low amounts of lichen portions. WGA has been already proven successful on protozoan *Leishmania* (Imamura et al., 2020) and in cultured fungal strains of *Claroideoglomus* (Montoliu-Nerin et al., 2020)

Here, we present results of the WGA approach for obtaining high amounts of DNA from small fragments of lichen forming fungi and lichen thalli, followed by Illumina whole-genome sequencing. We compare *de novo* (complete and partially) assembled and annotated mitochondrial genomes generated from a standard extraction method and the amplicons generated thorough WGA, and perform a phylogenetic reconstruction based on the marker regions from novel primers designed after the resulting mitochondrial genomes.

Material & Methods

Sample Selection

We selected representatives of major Lecanoraceae clades, according to Zhao et al., (2016). The fungal family Lecanoraceae comprises several hundred crustose species with mostly thin thalli and apothecia of ca. 1 mm diameter. A total of eighteen specimens from fourteen different species were analysed. In order to compare DNA amounts and concentrations obtained from standard CTAB extractions with our WGA protocol, we also included axenic thallus cultures of two different species (Table 1). Four specimens belonging to Parmeliaceae and Physciaceae, sister families to the Lecanoraceae, were also included in the analysis. Additionally, fourteen mitogenomes were obtained through the NCBI portal, encompassing taxa belonging to Lecanorales (Table 2) and compared with the newly obtained data.

Table 1

Summary of taxa used in this study, with voucher information, year of collection, DNA amounts, DNA concentration, mitogenome length, final assembly status raw read pairs count, GC content and Genbank codes. Specimens of *Japewia tornoensis*, *Lecanora confusa* and *Miriquidica deusta* could not be assembled. C *horiza* and *Myriolecis hagenii* were considered but we failed to obtain enough DNA to reach a minimum threshold required by the sequencing company. Conce *Physcia caesia*, *Pseudephebe pubescens*, *Usnea antarctica* and *U. aurantiacoatra* were obtained through Qbit.

Taxon	Envelope code	Year of Collection	Total Amount (ng)	DNA Concentration Tapestation (ng/ul)	DNA Concentration Novogene (ng/ul)	Lenght (bp)	Assembly status	Method	Raw Read Pairs	GC%	Genb Acce
Lecanora bermudensis	FR-0264718	2017	3060	375	204		incomplete	SC-kit	54272042	30,2	Six scaf
Lecanora cadubriae	FR-0279001	2018	1900	160	100	43271	complete	JBD-Kit	37099414	32,7	ON1
Lecanora carpinea	FR-0264946	2016	461	38,2	57,6	35169	incomplete	SC-Kit	54808923	30,8	ON1
Lecanora intumescens	FR-0279044	2016	134	4,16	8,96	35049	complete	JBD-Kit	50071587	30,6	ON1
Lecanora polytropa	Hb. McCune 37633	2018	1796	99	99,8	40863	incomplete	SC-Kit	44905435	29,5	ON1
Lecanora pseudargentata	Cultures 1 to 9; 16 & 17		655	19,5	26,2	51996	complete	CTAB	32674536	30,3	ON1
Lecanora rupicola	FR-0264951	2016	192	187	120	36421	incomplete	SC-Kit	47235845	30,7	ON1
Lecanora subcarnea	FR-0279045	2016	194	16,6	12,9	20375	incomplete	SC-Kit	41632013	32,5	ON1
Lecanora subintricata	Cultures 13 to 15		629	19,3	26,2	53733	complete	CTAB	36072789	30,9	ON1
Lecanora subintricata	Cultures 13 to 15		2560	291	160	53739	complete	JBD-Kit	35586234	30,9	ON1
Lecanora subintricata	FR-0261121	2017	4080	225	204	54304	complete	JBD-Kit	40384465	30,7	ON1
Lecanora subintricata	FR-0261122	2017	1856	532	116	54056	complete	SC-Kit	32378642	30,8	ON1
Lecanora subintricata	FR-0279002	2017	2052	511	114	52389	incomplete	JBD-Kit	44922601	30,9	ON1
Lecanora subsaligna	FR-0279046	2018	1881	237	99	59221	complete	JBD-Kit	31415755	30,7	ON1
Lecanora varia	FR-0261120	2017	937	45,1	55,1	32782	complete	JBD-Kit	36725268	29,7	ON1
Lecidella elaeochroma	FR-0261123	2017	1029	64,2	68,2	32214	incomplete	SC-Kit	40483845	28,6	ON1
Myriolecis dispersa	FR-0264963	2016	1908	75	106	36909	incomplete	JBD-Kit	41974611	29,7	ON1
Physcia caesia	FR-0279004	2015	3650	36,7		37905	complete	CTAB	6606423	28,3	ON1
Protoparmeliopsis muralis	FR-0264962	2016	1734	109	102	62641	complete	JBD-Kit	37342254	29,5	ON1
Pseudephebe pubescens	FR-0279003	2015	940	9,4		83143	complete	CTAB	4116013	30,7	ON1
Usnea antarctica	FR-0264581	2015	2940	29,4		87363	incomplete	CTAB	1549379	40,2	ON1
Usnea aurantiacoatra	FR-0264585	2015	1750	17,5		80823	complete	CTAB	877941	40,3	ON1

†- *L. bermudensis* assembly resulted in disjointed 6 scaffolds. Each scaffold was submitted individually to GenBank, their accession codes being: scaffold 1 = ON118978; scaffold 3 = ON118979; scaffold 4 = ON118980; scaffold 5 = ON118981; scaffold 6 = ON118982.

Table 2
Summary of mitogenomes obtained through GenBank and compared with the mitogenomes obtained in this study.

Taxa	Genbank Accession	Size (nt)	GC%
Alectoria fallacina	MG711470	75417	31,9
Bacidia sorediata	MK294991	35525	27,5
Cladonia macilenta	MK318967	46553	29
Cladonia petrophila	MG941021	53100	29,3
Cladonia rangiferina	KY460674	59116	29,6
Imshaugia aleurites	KY360311	32029	30,5
Lecanora cinereofusca	MH359410	32657	31,2
Lecanora saxigena	MH359409	56579	28,9
Lecanora strobilina	KU308740	39842	29,2
Parmotrema stuppeum	KY362439	108024	30,3
Parmotrema ultraluscens	MG807882	79456	30,5
Pseudevernia consocians	MG696867	76322	30,9
Ramalina intermedia	MK321681	30678	30,3
Usnea subgracilis	MG720066	94464	43,6

Culture isolation of Lecanora mycobionts

The isolation of the lichen mycobiont followed the protocol of Yamamoto et al., (2002) and it was carried out on single collections of *Lecanora pseudargentata* Lumbsch and *L. subintricata* (Nyl.) Th. Fr. Approximately 2 mm^2 fragments of lichen thalli were dissected with a sterile razor blade and a few areoles and one apothecium were taken by slightly scratching the thalli from the substrate without including the latter. The fragments were washed three times for 15 minutes with sterile water, followed by a wash with 500 µl of Tween 80 diluted 1:10 for 30 minutes. A final washing step was performed by rinsing the thallus fragments three times for 15 minutes with sterile water. The clean fragments were ground in sterile water under laminar flow bench and tiny pieces of the fragmented thallus were picked individually with a sterile bamboo stick and transferred into agar tubes. Five different media were used to promote the growth of the mycobiont: Trebouxia medium (TM, Ahmadjian 1987), Lilly & Barnett (LB, Lilly & Barnett 1951), Sabouraud (SAB, Pagano et al., 1957–58), Potato Dextrose agar (PDA, ApplChem A5828), and Malt Yeast-extract (MY, Lilly and Barnett 1951). We inoculated two slant agar tubes of the same medium for each sample for a total of 10 inocula from each lichen individual. The tubes were incubated in a growing chamber under the following conditions: 17°C, 20 µmol fot*m − 2*s − 1, with a light/dark cycle of 14/10 h. After six to eight months, the inocula had reached a diameter of about 1–3 mm, and were taken for DNA extraction to check their identity by sequencing the ITS locus and for subcultures. Subcultures were then set on Petri plates using the same growth media in which the inocula were isolated successfully. Three subcultures were prepared for each strain and maintained alive.

DNA Extraction

Two methods were employed for obtaining DNA: CTAB extraction and WGA.

CTAB extraction was performed on thallus cultures of *Lecanora pseudargentata* and *Lecanora subintricata*. Cultured mycelia were collected for each plate in standard Eppendorf tubes. The CTAB protocol was modified after Doyle and Doyle 1987; Cullings 1992; and Fernández-Mazuecos et al., 2018 (complete protocol in Supplement 1). To obtain enough DNA for library preparation, multiple replicates of the CTAB extractions were carried out. Eleven replicates were made from cultures of *Lecanora pseudargentata*, and eight replicates from *L. subintricata*. The resulting eluates for each species were pooled into two tubes, one per species, and reduced by vacuum centrifugation to 50 µl of volume per tube (Table 1, DNA Concentrations).

Genomic DNA from *Pseudephebe pubescens* (L.) M. Choisy, *Physcia caesia* (Hoffm.) Fürnr., *Usnea antarctica* Du Rietz and *U. aurantiacoatra* (Jacq.) Bory was extracted from 20 mg of thalli, pre-treated in acetone for 30 seconds, deep frozen in liquid nitrogen and homogenized with sterile mortar and pestle. A modified CTAB extraction (Cubero et al, 1999, Cubero & Crespo, 2002, Lagostina et al., 2017) was applied.

Whole Genome Amplification

For the WGA, horizontal cross sections of apothecia of selected specimens (Table 1) were prepared using a HYRAX KS 34 cryotome (Zeiss, Jena, Germany). The first 20 µm of the upper portion of the hymenium were discarded as a measure to discard potential hyphae of non-target parasitic fungi present. The three subsequent slices of 20 µm were stored in PCR tubes in sterile distilled water. Two different WGA kits were used: the Qiagen Repli-G Single-Cell Kit (Qiagen GmbH, Düsseldorf) (“SC-kit”), and Jena Bioscience Direct (Jena Bioscience GmbH, Jena) WGA kit (“JBD-kit”). Both kits perform PCR using random oligosequences as primers and using Phi–29 polymerase. Amplification procedure followed manufacturers protocols, with the following modification: Amplification lasted for a minimum of 16 hours at 30°C in the heating block.

DNA concentrations and quality were assessed using a TapeStation 2200 System (Agilent, Santa Cruz, USA) at the “Zentrum für translationale Biodiversitätsgenomik” (LOEWE-TBG) and by Novogene (HK) Co., Ltd.

Sequencing

Illumina paired-end libraries were generated using the NEBNext® DNA Library Prep Kit following the manufacturer's recommendations and indices were added to each sample. The genomic DNA was randomly fragmented to a size of 350 bp. Whole-genome sequencing was performed with Illumina HiSeq, and 10 GB of raw data was obtained per sample; paired-end reads were 150 bp long. Library preparation and sequencing were carried out by Novogene (HK) Co., Ltd.

For *Physcia caesia*, *Pseudephebe pubescens*, *Usnea antarctica* and *U. aurantiacoatra*, whole genome sequencing was carried out as previously described (Lagostina et al., 2017), on Illumina MiSeq v3 by LGC Genomics GmbH (Berlin, Germany) resulting in 300 bp paired-end reads.

Data treatment

Mitogenomes of 22 samples were assembled *de novo* using the pipeline GetOrganelle v1.7.1 (Jin et al., 2020) using 30 cycles, 21, 45, 65, 85, and 105 kmer values and “fungus_mt” as a reference genome database. The pipeline filters organelle-associated reads using a baiting and iterative mapping approach, conducts *de novo* assembly, disentangles the assembly graph, and finally produces all possible configurations of circular organelle genomes (Jin et al., 2020). It starts with the recruitment of initial target-associated reads by using Bowtie2 (Langmead & Salzberg 2012) and, taking target genome as the seed, the initial target-associated reads (seed-mapped reads) are treated as “baits” to get more target-associated reads through multiple extension iterations. Then, the total target-associated reads are *de novo* assembled into a FASTA assembly Graph (FASTG) file using SPAdes (Bankevich et al., 2012). The FASTG file is subsequently used to calculate all possible paths of the complete target organelle genome based on the graph characteristics and the coverages of the contigs. The results of the assemblies were visualized with Bandage v0.8.1 (Wick et al., 2015). Successfully *de novo* assembled mitogenomes were annotated with GeSeq (Tillich et al., 2017) and tRNAscan-SE v2.0.7 (Lowe & Eddy, 1997) using published *Lecanora* (GenBank NC_042184.1, NC_042183.1, NC_030051.1) and *Usnea* (NC_035940.1, NC_039633.1) mitogenomes as a reference. The mitogenomes were visualized using OGDRAW (Greiner et al., 2019) and manually curated in Geneious Prime 2019.2.1 (https://www.geneious.com).

Primer development

Coding regions of the annotated and assembled mitogenomes were extracted and aligned to each other. Eight pairs of primers, two per region were developed from atp6, cob, cox3, and nad1. These primers were tested on DNA extractions of 23 specimens from 20 different species of *Lecanora* sensu lato. PCR conditions were explored and optimized for each primer pair. After tests, the four optimal pairs of mitochondrial primers were used for amplifying parts of the regions atp6, cob, cox3 and nad1 for various Lecanoraceae (Table 3). Sanger sequencing was performed by Macrogen Europe B.V. (Amsterdam, the Netherlands). Alignment and primer development was performed in Geneious Prime.

Table 3
Mitochondrial primers pairs developed for Lecanoraceae after our resulting mitogenomes.

Name	Length	Direction	Sequence (5´ -> 3´)	GC%	Hairpin Tm	Self Dimer Tm	Tm
atp6F2	23	forward	ACTAACATAGGACTTTACTTAAC	30.4	None	None	51.4
atp6R2	24	reverse	ACCTGAAAATGCTATAATAAAAGC	29.2	39.3	None	54.0
cobF2	22	forward	AGTCATCCTTTGCTAAAATTGG	36.4	None	None	55.0
cobR2	20	reverse	TGTTCTAGGAGCTTTATATG	35.0	None	None	49.3
cox3F2	18	forward	CAGGTGTTTTAACAATGC	38.9	41.2	None	49.6
cox3R2	22	reverse	TAAAAACAAGAACCATATGTAC	27.3	None	1.2	50.2
nad1F1	19	forward	TGCAAAGAAGATTAGGTCC	42.1	None	None	52.2
nad1R1	20	reverse	CAAATAAGAACAATGCTAGC	35.0	None	8.1	50.5

Results comparison

For the mitogenomes obtained from CTAB extractions and WGA products, DNA amounts and concentrations of the respective eluates/amplification products, were related to assembly completeness, GC% content, assembly sizes and coding region position.

We assessed the amplification accuracy of the kits by analysing genetic distances between samples of the same species (using different extraction/amplification protocols). Pairwise distances were measured among mitogenomes of *Lecanora subintricata* extracted and amplified with the different protocols to assess possible amplification errors by the kits. Pairwise distance together with GC% content, assembly and coding region lengths and position for all the mitogenomes were calculated and visualized in Geneious Prime.

Also, comparison of the consistency of a phylogenetic reconstruction based on loci recovered from the resulting mitogenomes versus already published data was performed.

Phylogenetic reconstruction

A maximum likelihood (ML) phylogenetic tree based on four mitochondrial loci from the mitogenomes was constructed to compare the placement of the analyzed taxa with published phylogenies of Lecanorales, and assess statistical branch support values for a combined analyses based on these four loci.

Consensus sequences of the coding regions of the loci atp6, cob, cox3 and nad1 were first aligned through GUIDANCE2 (Landan & Graur 2008; Penn et al., 2010; Sela et al., 2015), removing unreliably aligned positions with a score below 0.999.

The ML tree was constructed with W-IQ-Tree (Trifinopoulos et al., 2016) by using the default settings of an ultrafast bootstrap analysis, 1000 bootstrap repetitions, and automatic substitution model selection. A custom partition was elaborated considering third-codon positions. We eliminated ambiguously aligned columns, and an intron of 275 bp at the cox3 locus in *Usnea aurantiacoatra* as well as 5 intronic regions within nad1 present in only 1 to 9 species from total the alignment. Selected substitution models are summarized on Table 4. *Physcia caesia* (Physciaceae), *Ramalina intermedia* (Delise ex Nyl.) Nyl. and *Bacidia sorediata* Lendemer & R.C. Harris (Ramalinaceae) were chosen as outgroup.

Table 4
IQTree substitution models selected for each third-position codon per locus.

	atp6	cob	cox3	nad1
Codon pos. 1	TPM3 + F + G4	TIM2 + F + I + G4	K3P + I + G4	GTR + F + I + G4
Codon pos. 2	TVM + F + R2	K3Pu + F + I + G4	TVM + F + R3	HKY + F + I + G4
Codon pos. 3	TIM3 + F + I + G4	TIM2 + F + I + G4	TIM2 + F + I + G4	TPM3u + F + R3

The resulting tree was visualized in TreeGraph 2.0 (Stöver & Müller 2010) and graphically enhanced in Biorender (BioRender.com). All other figures were created with BioRender.com.

WGA yields

Both WGA commercial kits yielded high DNA concentrations, enough for whole genome sequencing purposes (Table 1). DNA amount ranged between 134 and 4080 ng, with a mean value of 1568 ng. DNA concentrations varied between 9 ng/µl to 204 ng/µl, with a mean value of 102 ng/µl.

The mean DNA amounts obtained with the SC-kit were slightly lower than those obtained with the JBD-kit (1227 vs. 1910 ng, respectively, corresponding to DNA concentrations of 97 and 105 ng/µl). The CTAB extractions from cultures yielded on average 642 ng DNA (26 ng/µl) each (Table 5).

Table 5
Average values of DNA amounts, concentration, read pairs, and GC content resulting from the genomes obtained from the different methods analyzed.

Method	DNA Amounts (ng)	DNA Concentration Tapestation (ng/ul)	DNA Concentration Novogene (ng/ul)	Raw Read Pairs	GC%
CTAB	642	21,97	26,20	13649513,50	33,45
JBD-Kit	1909,56	184,14	105,45	39502465,44	30,58
SC-Kit	1226,86	187,43	96,93	45102392,14	30,56

Sequencing

Sequencing and high-quality data selection of the specimens sequenced on Illumina MiSeq yielded between 877,841 (*Usnea aurantiacoatra*) to 6,606,423 reads (*Physcia caesia*).

The specimens sequenced on Illumina HiSeq yielded between 31,415,755 (*Lecanora subsaligna* M. Brand & van den Boom) and 54,808,923 (*L. carpinea* (L.) Vain.) reads. On average, the number of reads generated after amplification with the JBD-kit was slightly lower than that generated with the SC-kit (39,502,462 vs. 45,102,392 reads, Table 5). The mean number of reads generated from CTAB extractions was 13,649,514.

Mitochondrial genomes

A total of 18 mitochondrial genomes were assembled for Lecanoraceae, ten of which were fully assembled and eight only partially assembled. The fully assembled mitochondrial genomes ranged between 32,782 bp in *Lecanora varia* (Hoffm.) Ach. and 62,647 bp in *Protoparmeliopsis muralis* (Schreb.) M. Choisy. GC content ranged between 28.6% in *Lecidella elaeochroma* (Ach.) M. Choisy and 32.7% in *Lecanora cadubriae* (A. Massal.) Hedl. In comparison, the mitogenome sizes of the specimens belonging to the sister families Cladoniaceae, Parmeliaceae and Ramalinaceae ranged between 30,678 bp in *Ramalina intermedia* and 108,024 bp in *Parmotrema stuppeum* (Taylor) Hal). GC content within these families ranged between 27,5% in *Bacidia sorediata* and 43.6% in *Usnea subgracilis* Vain.

Fifteen coding regions were detected atp6, atp8, atp9, cob, cox1, cox2, cox3, nad1, nad2, nad3, nad4, nad4L, nad5, nad6 and rps3, plus two mitochondrial ribosome regions (LSU and SSU). However, atp9 was only present in species of Cladoniaceae, Parmeliaceae and Ramalinaceae, but not in the mitogenomes of Lecanoraceae. Several of the aforementioned regions were missing in the incompletely assembled mitogenomes. With regards to tRNA genes, between 26 and 31 genes were detected in the completely assembled mitogenomes of Lecanoraceae, with sizes ranging between 31 to 86 bp (Table 6).

Table 6

Summary of mitochondrial coding regions, mitochondrial ribosomal DNA, tRNAs and their sizes per taxon. abs = absent; fr-=fragmen

Taxa/Locus	atp6	atp8	atp9	cob	cox1	cox2	cox3	nad1	nad2	nad3	nad4	nad4L	nad5	nad6	rps3	LS
Alectoria fallacina	579	147	285	1158	1464	750	714	1095	abs	abs	1482	270	2082	abs	1374	18
Bacidia sorediata	666	153	225	1158	1527	753	810	969	1704	426	1473	261	1968	690	1287	23
Cladonia macilenta	789	147	225	1152	1665	831	810	1089	1812	390	1419	273	1971	693	1320	35
Cladonia petrophila	792	147	225	1128	1644	726	810	1086	1797	390	1464	270	2019	693	1893	32
Cladonia rangiferina	789	147	225	1155	5421 (fr)	843	810	1155	1557	390	1686	270	2031	768	1902	18
Imshaugia aleurites	441	147	abs	2172	1716	747	810	1089	1707	384	1524	270	1995	648	1194	25
Lecanora bermudensis	abs	147	abs	1714	1026	1441	abs	abs	1691	468	1621	270	1946	abs	930	79
Lecanora cadubriae	780	147	abs	1149	1575	936	810	1104	1713	366	1194	270	2013	666	1353	19
Lecanora carpinea	441	147	abs	1152	1752	723	810	1146	1701	528	1407	270	1959	636	1314	18
Lecanora cinereofusca	792	147	abs	1224	1647	726	810	1113	1707	363	1482	270	1959	636	1221	28
Lecanora intumescens	780	147	abs	1149	1620	696	810	1089	1713	420	1353	270	1956	636	1416	85
Lecanora polytropa	780	147	abs	1152	1602	729	810	1089	1845	417	1419	270	2127	636	1401	55
Lecanora pseudargentata	780	147	abs	1233	1731	684	810	1182	171	372	1533	270	2043	636	1419	17
Lecanora rupicola	441	147	abs	1335	1602	693	810	1146	1701	369	1389	270	1959	636	1188	38
Lecanora saxigena	774	147	abs	1155	1590	726	810	1110	1710	426	1476	270	1968	612	1317	31 (f
Lecanora strobilina	441	147	abs	2262	1737	726	810	1272	1713	1104	1116	abs	1959	600	1089	28
Lecanora subcarnea	420	147	abs	936	1029	558	690	864	996	480	1197	288	1701	408	759	53
Lecanora subintricata	780	147	abs	1239	1608	1014	1173 (fr)	1104	2124 (fr)	360	1497	270	1965	642	1053	17 (f
Lecanora subsaligna	441	147	abs	1086	1569	1110	810	1095	1704	360	2367	414 (fr)	2019	639‡	912	69
Lecanora varia	780	147	abs	1149	1749 (fr)	792	810	1131	1719	447	1353	270	1995	666	1497	83
Lecidella elaeochroma	780	147	abs	1359	1920	1005 (fr)	810	1212	1701	402	1440	270	1968	633	1389	65 (f
Myriolecis dispersa	780	147	abs	1380	1683	864	810	1134	1758	417	1419	270	2127	636	1389	55
Parmotrema stuppeum	444/441†	147	abs	1098	1713	726	816	999	1695	409	1579	270	1994	657	1374	31
Parmotrema ultraluscens	756	147	abs	1173	1623	726	807	1149	1695	420	1491	270	2019	657	1380	32
Physcia caesia	441	147	abs	1161	1641	780	1113	1230	1698	393	1476	270	1968	651	1317	17 (f

†.- Two almost identical copies

‡.- Two copies

§.- A 559 nt-long SSU short copy was identified

Taxa/Locus	atp6	atp8	atp9	cob	cox1	cox2	cox3	nad1	nad2	nad3	nad4	nad4L	nad5	nad6	rps3	LS
Protoparmeliopsis muralis	780	147	abs	1152	1584	744	810	1089	1701	408	1419	270	2331	636	897	2(
Pseudephebe pubescens	249	234 (fr)	abs	1074	183	705	807	1164	1695	381	2121 (fr)	270	1974	657	1404	32
Pseudevernia consocians	930	147	abs	1164	1590	780	774	1086	1770	390	1539	294	1914	645	1080	3(
Ramalina intermedia	765	150	225	1149	1605	741	810	999	1701	411	1479	270	1965	651	1203	25
Rhizoplaca shushanii	764	147	abs	1128 (fr)	797 (fr)	724	810	abs	1650	354	1524	270	1955	636	abs	1((f
Usnea antarctica	441	147	abs	1050	2871	927	1077	903	1761	363	1485	270	2277	660	609	7(
Usnea aurantiacoatra	441	147	abs	1422	2541	960	1071	990	1767	363	1485	270	2259	660	609	3(
Usnea subgracilis	756	147	abs	1209	1608	723	858	1098	1764	393	1533	270	1905	675	1185	3(
†.- Two almost identical copies																
‡.- Two copies																
§.- A 559 nt-long SSU short copy was identified																

CDS position structure among Lecanorales

Lecanoraceae showed a relatively constant coding-region order: cox1-nad4-rps3-cob-cox2-nad1-atp6-atp8-SSU-nad6-cox3-LSU-nad2-nad3-nad4L-nad5. However, exceptions to this order were found, such as the shift of the position of nad4 between atp6 and atp8 in *L. pseudargentata*, the presence of a fragment of the cox3 gene between cox1 and nad4 in *L. subintricata;* and the position of atp6 and atp8 between nad6 and cox3 in *L. subsaligna.* The Genbank sequences of *L. saxigena* Lendemer & R.C. Harris and *L. strobilina* (Spreng.) Kieff., showed however, a different order of genes (Fig. 3). The sequence of *L. cinereofusca* H. Magn. showed loci order consistent with the rest of the Lecanoraceae mitogenomes, with exception of the presence of an SSU fragment between nad5 and cox1. The three specimens of *Cladonia* showed the same order with no changes among each other, as that of the Lecanoraceae, with two changes: nad1 is placed between cox1 and nad4, and the presence of the gene atp9. The gene atp9 was also detected in *Alectoria fallacina* Motyka, *Ramalina intermedia*, and *Bacidia sorediata* belonging to Parmeliaceae and Ramalinaceae respectively. Species of Parmeliaceae, differed strongly in gene order. The specimens belonging to Ramalinaceae showed a partially conserved order. Only the genes cox3, nad4L and nad5 occupied different positions within the mitogenomes of *Bacidia sorediata* and *Ramalina intermedia*.

Lecanora subintricata alignment and distance values

Five mitogenomes of *L. subintricata* were aligned to each other and pairwise distance values were measured to assess possible amplification errors of the kits, resulting in an alignment of 54635 bp length. The mitogenome obtained from CTAB-extraction and the one amplified from the JBD-kit showed were identical. The mitogenome obtained from the SC-kit showed a distance of 552 bp to the CTAB mitogenome. The mitogenomes obtained from the vouchers FR-0279002 and FR-0261122 have a distance of 518 bp and 552 bp respectively. The mitogenome with the largest distance values of 937 bp to the CTAB mitogenome was that of voucher FR-0261121. Overall, only 45 SNPs were detected between different vouchers, whereas the rest of the dissimilarities are due to tandem-repeats, insertions/inversions or both. Absolute distance values within the mitogenomes of *L. subintricata* are summarized in Table 7.

Table 7
Genetic distance values of the aligned mitogenomes of *Lecanora subintricata.* Blue = specimen amplified with the SC-kit; green = specimens amplified with the JBD-kit

L. subintricata **Accession Codes**	**ON101757**	**ON101758**	**ON101754**	**ON101755**	**ON101756**
ON101757		0	552	518	937
ON101758			552	518	937
ON101754				500	547
ON101755					413
ON101756					

Phylogenetic analysis

The phylogenetic analysis was performed on a concatenated four-loci alignment of the four gene regions for which we developed primers. It comprises 33 specimens and has a length of 2843 characters (401 for atp6, 936 for cob, 686 for cox3 and 820 for nad1) with 1399 conserved sites, 1194 parsimony informative, and 250 non-informative sites. Figure 3 shows the topology of the ML phylogenetic tree based on the above-mentioned alignment. Most of the nodes receive high bootstrap support, save for three nodes in the backbone, one node in the Lecanoraceae clade and two nodes in the Parmeliaceae clade,

The tree showed four distinct clades consistent with published data on Lecanorales (Zhao et al, 2016). The phylogenetic relationships among the families appear unsupported. However, each family clade shows to be monophyletic with high support value. Not so surprisingly, *Lecanora cadubriae* appeared sister to the Cladoniaceae clade with high support.

Discussion

Efficiency of WGA

The high DNA yield of the WGA methods from small fragments of crustose lichens demonstrated above greatly facilitates the inclusion of crustose lichens in genomic studies and opens new perspectives for molecular genetic approaches to lichenology.

The DNA concentrations generated by WGA were on average 8.5 times higher than the DNA concentrations obtained by pooling CTAB extractions from mycobiont cultures (Table 1; Table 5). and between 23 and 33 times higher than in the unpooled extracts. In our opinion, the most important advantage is that the WGA amplified DNA extractions were directly generated from tiny fractions (20 µm cross sections of apothecia) of herbarium vouchers without the need to produce cell cultures. The species used for this study took more than a year to produce enough “thallus” for DNA extraction. Furthermore, the amount of material is not larger than that necessary for microscopical studies even of fragmentary type specimens.

Reliability of sequencing data based on WGA

A concern of ours was the introduction of amplification errors by the tested WGA kits resulting in false SNPs and negative effects on downstream analyses. However, the CTAB-based mitogenome and the JBD-Kit amplified mitogenome of the same voucher of *Lecanora subintricata* were identical. It can be safely assumed that during amplification, the JBD-Kit did not introduce false nucleotides.

The largest distance between aligned mitogenomes of different vouchers of *L. subintricata* (Table 7), was 937 nt, only 1.7% difference between the CTAB mitogenome and the mitogenome of FR-0279002. Only 45 of these (0.08%) were SNPs, well within the expected infraspecific variability in ascomycota (1,96%, SD 3.73, Nilsson et al, 2008). Most of the differences resulted from tandem-repeats or inversions.

Incomplete assemblies due to amplification bias

We were unable to completely assemble eight out of eighteen mitogenomes. From the incomplete mitogenomes, six were sequenced after amplification with the SC-kit; the other two incomplete mitogenomes were amplified with the JBD-kit. Amplification bias, as reported by Huang et al., (2015) is a problem generated by the very nature of the amplification process. As WGA is an exponential process, this can cause certain regions to be overamplified, and some regions to be under-amplified or not amplified at all. One reason for this bias is the inability of the polymerase to continue amplification after encountering a /GC-rich/highly repetitive region (Green & Sambrook, 2019; Orpana et al, 2012) due to secondary structure formation and the relatively low temperature at which WGA method is performed. Another possibility is due to stochasticity: i.e., primers may not bind to a particular region (Fig. 2). Thus, it is possible that amplification bias may be the reason behind the incompletely assembled mitogenomes. Furthermore, it is possible that the SC-kit produces a higher occurrence of this amplification bias, since six out of eight incompletely assembled mitogenomes were amplified with the SC-kit.

Loci positioning and overall structure within the mitogenomes obtained from Lecanoraceae species, are consistent with the annotated mitogenome of *Lecanora markjohnstonii* And. Stewart, E. Tripp & Lendemer published by Stewart et al., (2018). This mitogenome is the only published and annotated mitogenome available for *Lecanora* sensu lato. The mitogenomes of *L. cinereofusca*, *L. saxigena* and *L. strobilina*, albeit publicly available at GenBank, were not formally published, and thus comparing and discussing the strongly divergent gene order of *L. saxigena* (MH359409) and *L. strobilina* (NC030051) with our mitogenomes is unfortunately not possible.

Phylogeny of Lecanorales

The four-loci mitochondrial tree is largely consistent with the topologies previously published for Lecanorales (Pérez-Ortega et al., 2010; Ivanovich et al., 2021; Zhao et al., 2016). An exception is the position of *Lecanora cadubriae*, which appears as sister to the Cladoniaceae. DNA contamination of *L. cadubriae* was initially considered but subsequently discarded, as none of BLAST search results of *L. cadubriae* individual loci reached above 95% of identity. The tentative placement of *L. cadubriae* in *Lecanora* is discussed in Edwards et al., 2009. Further analyses on the phylogenetic position of the aforementioned taxon is out of the scope of this publication, and is going to be addressed in a future paper

All medial and terminal nodes in the tree show good support, thus it is possible that the high accumulation of substitutions on organelle DNA (Neiman & Taylor, 2009) makes the selected loci here better suited for phylogenetic classifications at lower levels, the delimitation of closely related taxa and studies at the population level. The backbone of the tree, on the other hand, lacks support in three nodes. It must be seen whether phylogenetic information from more conserved loci, such as mtSSU or nucLSU, can be used as a supplement to these mitochondrial loci to obtain better support also on the deeper nodes of the tree.

Outlook

Lücking et al. (2020, 2021) have recently summarized problems concerning species delimitation and recognition in lichenized and non-lichenized fungi. Among others, the failure of ITS as a universal DNA barcoding marker (Schoch et al. 2012) to reliably distinguish species in all clades. As Aime et al., (2021) and Lendemer (2021) pointed out, molecular data has become an integral part of fungal taxonomy no longer only employing the suggested universal fungal barcode but increasingly also taxon-specific barcoding loci. The WGA approach outlined here facilitates the generation of taxon-specific loci even for small crustose species.

Another possible use of WGA is to obtain suitable quantities of DNA for genomic sequencing even from scant herbarium type material, from which destructive sampling must be reduced to a minimum. Our results show that it is possible to generate genome-wide data from as little as a single 20 µm section of an apothecium. Due to degeneration of DNA in old herbarium vouchers, it may not always be possible to assemble whole genomes from these data, but even incomplete data may greatly stabilize the nomenclature of species distinguished by molecular methods. Because DNA sequence data on historical type material is usually lacking researchers currently rely on accurately determined specimens to assign DNA sequences to species names (Lücking et al, 2021). DNA sequence data from genomes of type material would finally allow to match newly generated sequences with older names thereby increasing the reliability of species identification and delimitation including the large percentage of misnamed fungal sequences already uploaded to NCBI (Hofstetter et al, 2019).

Abbreviations

atp6: ATP synthase membrane subunit 6

atp8: ATP synthase membrane subunit 8

atp9: ATP synthase membrane subunit 9

bp: Base Pairs

cob: cytochrome b

cox1: cytochrome c oxidase subunit I

cox2: cytochrome c oxidase subunit II

cox3: cytochrome c oxidase subunit III

CTAB: cetyltrimethylammonium-bromide

GC%: Guanine-Cytosine percentage of content

ITS: Nuclear ribosomal internal transcribed spacer

JBD-Kit: Jena Bioscience Direct WGA kit

LSU: mitochondrial large subunit ribosomal RNA

Mitogenome:Mitochondrial genome

ML: Maximum likelihood

mtSSU: mitochondrial small subunit ribosomal RNA

nad1: NADH dehydrogenase subunit 1

nad2: NADH dehydrogenase subunit 2

nad3: NADH dehydrogenase subunit 3

nad4: NADH dehydrogenase subunit 4

nad4l: NADH dehydrogenase subunit 4L

nad5: NADH dehydrogenase subunit 5

nad6: NADH dehydrogenase subunit 6

NCBI: National Center for Biotechnology Information

nt: nucleotide

nucLSU: Large subunit nuclear ribosomal RNA gene

PCR: Polymerase Chain Reaction

rps3: ribosomal protein S3

SC-Kit: Qiagen Repli-G Single-Cell Kit

SNP: single nucleotide polymorphism

SRA: Sequence Read Archive

tRNA: transfer RNA

WGA: Whole Genome Amplification

Declarations

Ethics approval and consent to participate

Not applicable.

Adherence to national and international regulations

We the authors declare that our research is compliant with the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity.

Consent for publication

Not applicable

Availability of data and materials

Raw reads were deposited in the NCBI Sequence Read Archive (SRA) database under the BioProject ID PRJNA793761. Annotated mitogenome assemblies were submitted to GenBank. Accession codes for raw reads and mitogenomes per specimen are summarized in Table 1. Four-loci alignment was deposited in DRYAD: doi:10.5061/dryad.905qfttnm

Competing interest

We the authors declare that we have no competing interests.

Funding

DFG grant PR 567/19–1 to CP. CI was partially supported by the Goethe Research Academy for Early Career Researchers (GRADE) during this research. ZP is being supported by the long-term research development grant n. RVO 67985939.

Author Contributions

CI conducted the research, wrote the draft, produced Tables and Figures. CI, EL & JP produced data. CI & JP analysed the data. SPO provided the modified CTAB methodology used to generate some of the data. CI, EL, SLG, SPO, LM, ZP & CP provided samples. CP designed the research and provided funding. All co-authors reviewed, corrected and provided commentary on the draft in several rounds.

Acknowledgements

This work benefited from the sharing of expertise within the DFG priority program SPP 1991 ‘Taxon-Omics’. We thank Heike Kappes of the Grunelius-Möllgaard Laboratory (Senckenberg Research Institute Frankfurt), Carola Greve and Damian Baranski (both LOEWE-TBG) for lab support, our students Iris Tuscherer and Lilith Weber for the help with sample preparation, DNA amplification and primer trials. We thank Grainger Bioinformatics Center at the Field Museum for their support with *Rhizoplaca* data analysis.

Authors' information

Juraj Paule: juri.paule@gmail.com

Elisa Lagostina: elisa.lagostina@gmail.com

Scott Lagreca: scott.lagreca@duke.edu

Steve Leavitt: steve_leavitt@byu.edu

Lucia Muggia: lucia_muggia@hotmail.com

Zdeněk Palice: zdenek.palice@ibot.cas.cz

Sergio Pérez-Ortega: sperezortega@rjb.csic.es

Christian Printzen: Christian.Printzen@senckenberg.de

References

1. Ahmadjian V (1987) Coevolution in Lichens. Annals of the New York Academy of sciences. 503:307–315. 10.1111/j.1749-6632.1987.tb40617.x. 1
2. Aime MC, Miller AN, Aoki T, Bensch K, Cai L, Crous PW, Hawksworth DL, Hyde KD, Kirk PM, Lücking R, May TW, Malosso E, Redhead SA, Rossman AY, Stadler M, Thines M, Yurkov AM, Zhang N, Schoch CL (2021) How to publish a new fungal species, or name, version 3.0. IMA Fungus 12(1):11. doi:10.1186/s43008-021-00063-1
3. Bankevich A, Nurk S, Antipov D, Gurevich A, Dvorkin M, Kulikov AS, Lesin V, Nikolenko S, Pham S, Prjibelski A, Pyshkin A, Sirotkin A, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19(5):455–477. doi: 10.1089/cmb.2012.0021
4. Blanco L, Bernad A, Lázaro JM, Martin G, Garmendia C, Salas M (1989) Highly efficient DNA synthesis by the phage φ29 DNA polymerase: symmetrical mode of DNA replication. J Biol Chem 264:8935–8940
5. Cubero OF, Crespo A, Fatehi J, Bridge PD (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium stored, lichenized, and other fungi. Plant Syst Evol 216:243–249. doi: 10.1007/BF01084401
6. Cubero OF, Crespo A (2002) Isolation of Nucleic Acids from Lichens. In: Kranner IC, Beckett RP, Varma AK (eds) Protocols in Lichenology. Springer Lab Manuals. Springer, Berlin, Heidelberg
7. Cullings KW (1992) Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. Mol Ecol 1:233–240. doi: 10.1111/j.1365-294X.1992.tb00182.x
8. Dean FB, Nelson JR, Giesler TL, Lasken RS (2001) Rapid amplification of plasmid and phage DNA using phi29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res 11:1095–1099. doi: 10.1101/gr.180501
9. Degnan JH, Rosenberg NA (2009) Gene tree discordance, phylogenetic inference and the multispecies coalescent. Trends Ecol Evol 24(6):332–340. doi: 10.1016/j.tree.2009.01.009
10. Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure from small quantities of fresh leaf tissue. Phytochemical Bull 19:11–15
11. Edwards B, Aptroot A, Hawksworth DL, James PW (2009) *Lecanora* Ach. in Luyken (1809). In: Smith CW, Aptroot A, Coppins BJ, Fletcher A, Gilbert OL, James PW, Wolseley PA (eds) The lichens of Great Britain and Ireland. British Lichen Society, London
12. Ekman S (1999) PCR optimization and troubleshooting, with special reference to the amplification of ribosomal DNA in lichenized fungi. The Lichenologist 31(5):517–531. doi: 10.1006/lich.1999.0226
13. Fernández-Mazuecos M, Mellers G, Vigalondo B, Sáez L, Vargas P, Glover BJ (2018) Resolving recent plant radiations: power and robustness of genotyping-by-sequencing. Syst Biol 67(2):250–268. doi: 10.1093/sysbio/syx062
14. Garmendia C, Bernad A, Esteban JA, Blanco L, Salas M (1992) The bacteriophage φ29 DNA polymerase, a proofreading enzyme. J Biol Chem 267:2594–2599
15. Green MR, Sambrook J (2019) Polymerase Chain Reaction (PCR) Amplification of GC-Rich Templates. Cold Spring Harbor Protoc. doi: 10.1101/pdb.prot09514
16. Greiner S, Lehwark P, Bock R (2019) OrganellarGenomeDRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. Nucleic Acids Res 47:W59–W64. doi: 10.1093/nar/gkz238
17. Grewe F, Lagostina E, Wu H, Printzen C, Lumbsch HT (2018) Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*. MycoKeys. 43:91–113. 10.3897/mycokeys.43.29093
18. Grube M, Berg G, Andrésson OS, Vilhelmsson O, Dyer PS, Miao VPW (2014) Lichen genomics: Prospects and Progress. In: Martin F (ed) The Ecological Genomics of Fungi. Wiley-Blackwell, Hoboken. doi: 10.1002/9781118735893.ch9
19. Hofstetter V, Buyck B, Eyssartier G, Schnee S, Gindro K (2019) The unbearable lightness of sequenced-based identification. Fungal Divers 96:243–284. doi: 10.1007/s13225-019-00428-3
20. Honegger R, Bartnicky-Garcia S (1991) Cell wall structure and composition of cultured mycobionts from the lichens *Cladonia macrophylla, Cladonia caespiticia*, and *Physcia stellaris* (Lecanorales, Ascomycetes). Mycol Res 95(8):905–914. doi: 10.1016/S0953-7562(09)80085-3
21. Huang L, Ma F, Chapman A, Lu S, Xie XS (2015) Single-Cell Whole-Genome Amplification and Sequencing: Methodology and Applications. Annu Rev Genom Hum Genet 16:79–102. doi: 10.1146/annurev-genom-090413-025352
22. Imamura H, Monsieurs P, Jara M, Sanders M, Maes I, Vanaerschot M, Berriman M, Cotton JA, Dujardin J-C, Domagalska MA (2020) Evaluation of whole genome amplification and bioinformatic methods for the characterization of *Leishmania* genomes at a single cell level. Scientific Reports, 14;10(1):15043. doi: 10.1038/s41598-020-71882-2
23. Jin J-J, Yu W-B, Yang J-B, Song Y, dePamphilis CW, Yi T-S, Li D-Z (2020) GetOrganelle: a fast and versatile toolkit for accurate de novo assembly of organelle genomes. Genome Biol 21(241). doi: 10.1186/s13059-020-02154-5
24. Landan G, Graur D (2008) Local reliability measures from sets of co-optimal multiple sequence alignments. Pacific Symposium on Biocomputing 13:15–24
25. Langmead B, Salzberg S (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. doi: 10.1038/nmeth.1923
26. Lendemer J (2021) Proposed best practices for taxonomic innovations in lichen and allied
27. Fungi A framework derived from analysis of more than 1,000 new taxa and new combinations.The Bryologist, 124(1):90–99. doi: 10.1639/0007-2745-124.1.090
28. Lilly VG, Barnett HL (1951) Physiology of the fungi. McGraw-Hill, New York

29. Lowe TM, Eddy SR (1997) tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res 25:955–964. doi: 10.1093/nar/25.5.955
30. Lücking R, Aime MC, Robbertse B, Miller AN, Ariyawansa HA, Aoki T, Cardinali G, Crous PW, Druzhinina IS, Geiser DM, Hawksworth DL, Hyde KD, Irinyi L, Jeewon R, Johnston PR, Kirk PM, Malosso E, May TW, Meyer W, Öpik M, Robert V, Stadler M, Thines M, Vu D, Yurkov AM, Zhang N, Schoch CL (2021) Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA Fungus. 11:14. 10.1186/s43008-020-00033-z
31. Lücking R, Leavitt SD, Hawksworth DL (2021) Species in lichen–forming fungi: balancing between conceptual and practical considerations, and between phenotype and phylogenomics. Fungal Divers 109:99–154. doi: 10.1007/s13225-021-00477-7
32. Lutsak T, Fernández-Mendoza F, Kirika P, Wondafrash M, Printzen C (2020) Coalescence-based species delimitation using genome-wide data reveals hidden diversity in a cosmopolitan group of lichens. Organisms Divers Evol 20:189–218. doi: 10.1007/s13127-019-00424-0
33. Montoliu-Nerin M, Sánchez-García M, Bergin C, Grabherr M, Ellis B, Kutschera VE, Kierczak M, Johannesson H, Rosling A (2020) Building *de novo* reference genome assemblies of complex eukaryotic microorganisms from single nuclei. Sci Rep 10:1303. doi: 10.1038/s41598-020-58025-3
34. Muggia L, Pérez-Ortega S, Fryday A, Spribille T, Grube M (2014) Global assessment of genetic variation and phenotypic plasticity in the lichen-forming species *Tephromela atra*. Fungal Divers 64:233–251. doi: 10.1007/s13225-013-0271-4
35. Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson K-H (2008) Intraspecific ITS Variability in the Kingdom Fungi as Expressed in the International Sequence Databases and Its Implications for Molecular Species Identification. Evolutionary Bioinf 4:193–201. doi: 10.4137/ebo.s653
36. Orpana AK, Ho TH, Stenman J (2012) Multiple Heat Pulses during PCR Extension Enabling Amplification of GC-Rich Sequences and Reducing Amplification. Bias Anal Chem 84(4):2081–2087. doi: 10.1021/ac300040j
37. Orange A, James PW, White FJ (2001) Microchemical Methods for the Identification of Lichens. British Lichen Society, London
38. Pagano J, Levin JD, Trejo W (1958) Diagnostic medium for differentiation of species of Candida. Antibiot Annual 5:137–143
39. Penn O, Privman E, Ashkenazy H, Landan G, Graur D, Pupko T (2010) GUIDANCE: a web server for assessing alignment confidence scores. Nucleic Acids Res 38:W23–W28. doi: 10.1093/nar/gkq443
40. Pérez-Ortega S, Fernández-Mendoza F, Raggio J, Vivas M, Ascaso C, Sancho LG, Printzen C, de los Ríos A (2012) Extreme phenotypic variation in *Cetraria aculeata* (lichenized Ascomycota): adaptation or incidental modification? Ann Botany 109(6):1133–1148. doi: 10.1093/aob/mcs042
41. Pizarro D, Dal Grande F, Leavitt SD, Dyer PS, Schmitt I, Crespo A, Lumbsch HT, Divakar PK (2019) Genome Biol Evol 11(3):721–730. doi: 10.1093/gbe/evz027. Whole-Genome Sequence Data Uncover Widespread Heterothallism in the Largest Group of Lichen-Forming Fungi
42. Pugh TJ, Delaney AD, Farnoud N, Flibotte S, Griffith M, Li HI, Qian H, Farinha P, Gascoyne RD, Marra MA (2008) Impact of whole genome amplification on analysis of copy number variants. Nucleic Acids Res 36(13):e80. doi: 10.1093/nar/gkn378
43. Ranković B, Kosanić M (2015) Lichen secondary metabolites. Springer International Publishing, Cham, Switzerland
44. Sela I, Ashkenazy H, Katoh K, Pupko T (2015) GUIDANCE2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. Nucleic Acids Res W7–W14 43(Web Server issue). doi: 10.1093/nar/gkv318
45. Spribille T, Tuovinen V, Resl P, Vanderpool D, Wolinski H, Aime MC, Schneider K, Stabentheiner E, Toome-Heller M, Thor G, Mayrhofer H, Johannesson H, McCutcheon JP (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353:488–492. doi: 10.1126/science.aaf8287
46. Stewart CRA, Lendemer JC, Keepers KG, Pogoda CS, Kane NC, McCain CM, Tripp EA (2018) *Lecanora markjohnstonii* (Lecanoraceae, lichenized Ascomycetes), a new sorediate crustose lichen from the southeastern United States. Bryologist 121(4):498–451. doi: 10.1639/0007-2745-121.4.498
47. Stöver BC, Müller KF (2010) TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics 11(7). doi: 10.1186/1471-2105-11-7
48. Tagirdzhanova G, Saary P, Tingley JP, Díaz-Escandón D, Abbott DW, Finn RD, Spribille T (2021) Predicted Input of Uncultured Fungal Symbionts to a Lichen Symbiosis from Metagenome-Assembled Genomes. Genome Biol Evol 13(4):evab047. doi: 10.1093/gbe/evab047
49. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE; a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res 44(W1):W232–W235. doi: 10.1093/nar/gkw256
50. Tillich M, Lehwark P, Pellizzer T, Ulbricht-Jones ES, Fischer A, Bock R, Greiner S (2017) GeSeq – versatile and accurate annotation of organelle genomes. Nucleic Acids Res 45:W6–W11. doi: 10.1093/nar/gkx391
51. Wick RR, Schultz MB, Zobel J, Holt KE (2015) Bandage: interactive visualization of de novo genome assemblies. Bioinformatics 31:3350–3352. doi: 10.1093/bioinformatics/btv383
52. Yamamoto Y, Kinoshita Y, Yoshimura I (2002) Culture of Thallus Fragments and Redifferentiation of Lichens. In: Kranner IC, Beckett RP, Varma AK (eds) Protocols in Lichenology. Springer Lab Manuals. Springer, Berlin, Heidelberg
53. Zhao X, Leavitt SD, Zhao ZT, Zhang LL, Arup U, Grube M, Pérez–Ortega S, Printzen C, Śliwa L, Kraichak E, Divakar PK, Crespo A, Lumbsch HT (2016) Towards a revised generic classification of lecanoroid lichens (Lecanoraceae, Ascomycota) based on molecular, morphological and chemical evidence. Fungal Divers 78:293–304. doi: 10.1007/s13225-015-0354-5

Figures

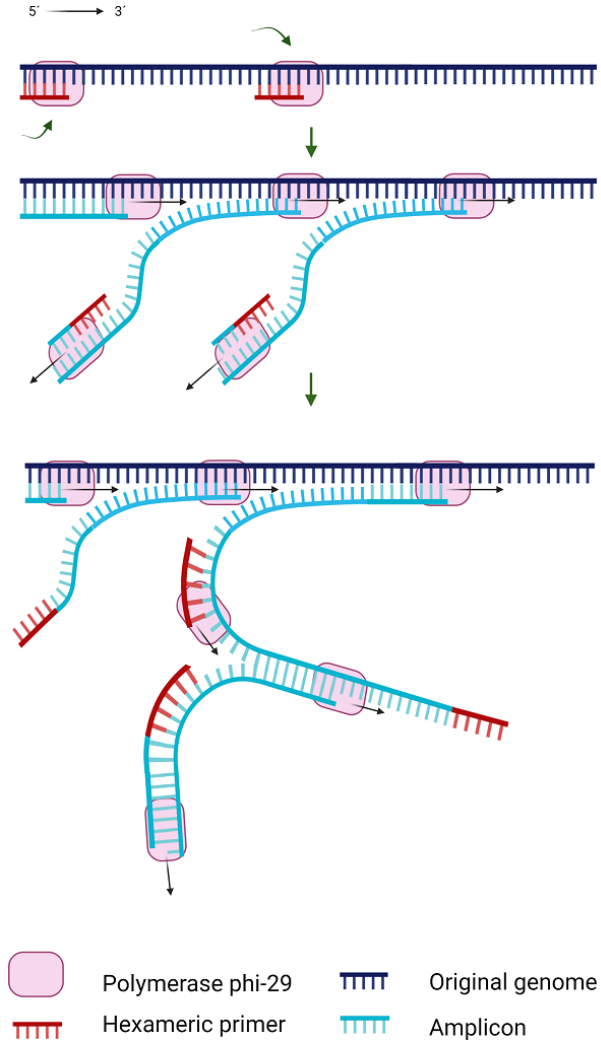

Figure 1

WGA-MDA. Diagram of Whole Genome Multiple Displacement Amplification. Random primers (red) bind to any available DNA under isothermal amplification. Phi-29 polymerase (pink), binds to the primer positions. As amplification and extension of the new strands happens, the Phi-29 polymerase is capable of displacing already-amplified strands. Primers also bind subsequently to the amplicons, exponentially augmenting the amount of DNA generated.

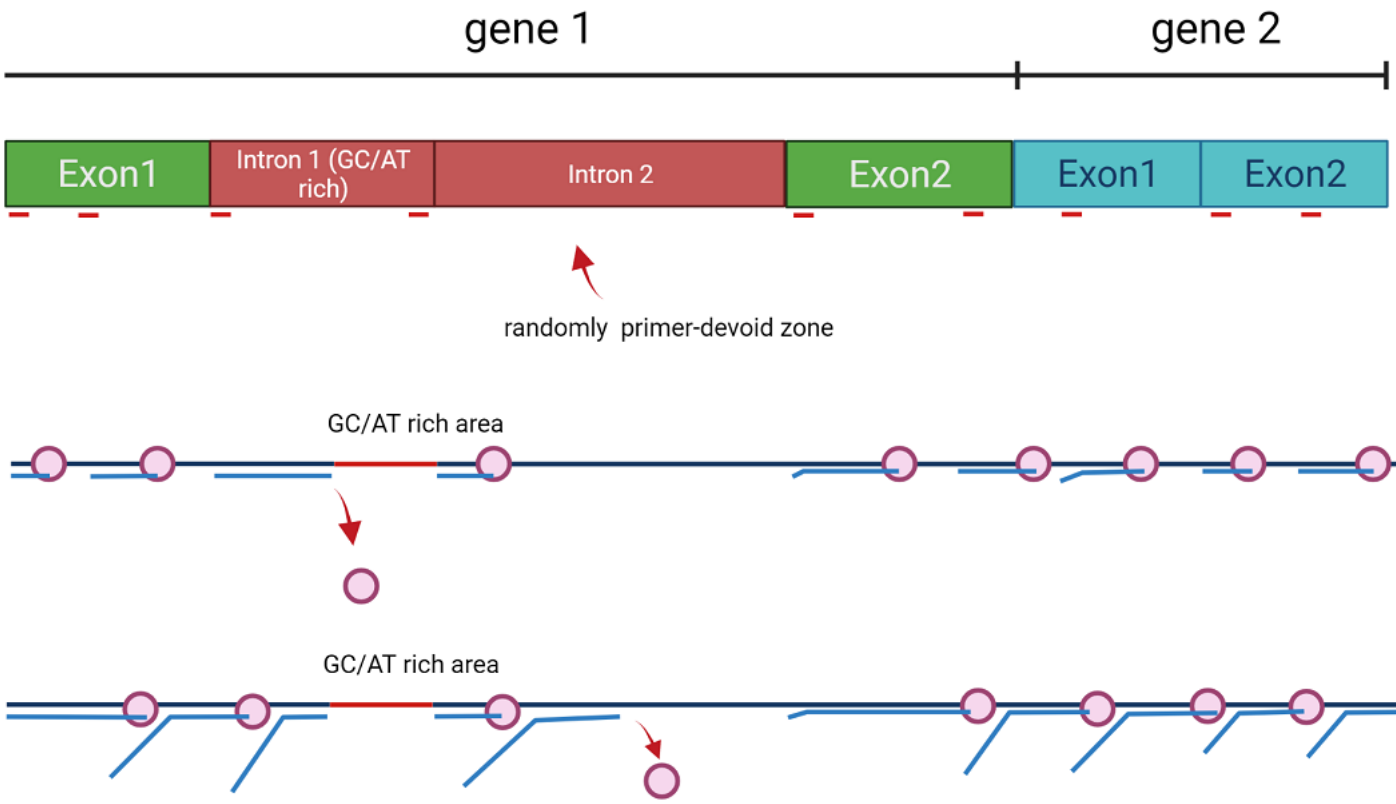

Figure 2

Diagram of WGA-MDA bias that appears to be the cause of incomplete read coverage. It can happen that 1) primers do not bind in an area due to stochasticity, or 2) the area has a large repetitive region where the primers can't bind, or else the polymerase cannot continue and becomes detached.

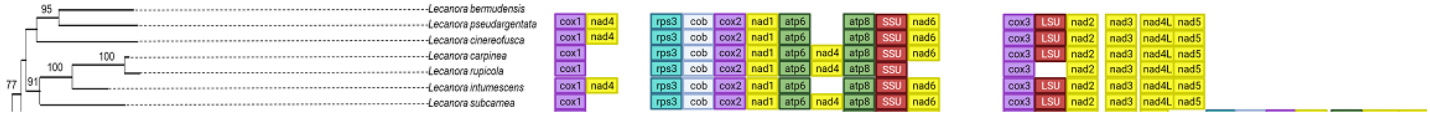

Figure 3

Mitochondrial synteny among Lecanorales ordered by taxon position on a 4-loci ML tree.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplement1.docx