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# Challenges to developing a reference sequence database using mass parallel sequencing and lichen herbarium specimens: a case study for microlichens of the Australian Capital Territory

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## Abstract

Molecular species identification is a powerful tool in taxonomy, biodiversity research and ecology, in particular for groups of organisms with limited diagnostic features. This tool relies on the development of high-quality reference sequence databases, and such databases can be built using collection specimens and mass parallel sequencing. Here, lichen herbarium specimens and mass parallel sequencing were used to generate reference ITS sequences for microlichens from the Australian Capital Territory (ACT) and to develop a reference sequence database. The preliminary database was then tested on un-identified specimens collected during the 2018 ACT BushBlitz expedition. Challenges met during both database development and molecular species identification suggest that these processes will not be straightforward for microlichens, due to the high number of sequences generated for non-target species (lichen-associated fungi, co-occurring lichenised species and sample cross-contaminants).

***Keywords*:** Australia, ITS barcode, PacBio amplicon sequencing, collection specimens

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## Introduction

Lichens, symbiotic associations between a fungus and a microalga (either a green alga or a cyanobacterium, or both), come in all sizes and shapes. Their vegetative structure, called a thallus, ranges from a few millimetres to tens of centimetres in extent. Microlichens, or lichens with small and/or thin thalli, mostly include crustose, squamulose or granulose species, whereas macrolichens, or lichens with large and often bushy thalli, mostly include foliose and fruticose species. Such as for macrolichens, several factors make microlichens difficult to identify to the species level using morphological characters. Diagnostic features are sparse and may be lacking in immature specimens, or misleading in old specimens or specimens growing in atypical conditions. Even when present, critical diagnostic features, such as the ascus tip and ascospore structure, are often difficult to observe and may require the preparation of numerous cross-sections for microscopic examinations. Additionally, morphological convergence and high infraspecific morphological variability are common in lichens, both for vegetative and reproductive structures, making morphological species delimitation and, as a result, morphological identifications, rather difficult, in particular when conducted in the field. Due to their small sizes, all these issues are compounded for microlichens, and their taxonomy lags behind compared to the taxonomy of macrolichens.

In the Australian Capital Territory (ACT), lichens have historically been well studied thanks to the work of local lichen taxonomists (e.g. McCarthy & Elix 2016; Elix & MacCarthy 2018; McCarthy & Elix 2020, 2021). As listed in the Census of Plants of the ACT v. 4.1 (30 August 2019), 521 lichen species and infraspecific taxa occur in the territory, many of which were microlichens. Lichens occur in various ecosystems, from the dry soils of open *Eucalyptus* woodlands to exposed alpine rocky outcrops in Namadgi. Macrolichen genera well represented in the region are *Xanthoparmelia* and *Cladonia*, and diverse microlichen genera including *Buellia* and *Lecanora*. For lichen species identification, various keys and monographs are available, either as part of the volumes of the Flora of Australia (ABRS, Canberra and CSIRO, Melbourne) or as journal or online publications (e.g. Elix *et al.* 2017 for buellioid taxa; McCarthy 2012 for *Verrucaria*; McCarthy *et al.* 2020 for *Rhizocarpon*). A lichen checklist for Australia and its island territories is also available online (McCarthy 2020). In addition to high quality morphological data, chemistry data are available for a large number of species and specimens thanks to the work of Elix and others (e.g. Archer & Elix 1999; Elix 2009; Elix *et al.* 2009).

Although morphological and chemical data are available for many Australian lichens, including microlichens from the ACT, DNA sequence data is still sparse. Few molecular revisions have either targeted (e.g. Crespo *et al.* 2010; Lumbsch *et al.* 2010; Gueidan & Elix 2022) or included (e.g. Leavitt *et al.* 2018; Barcenas-Peña *et al.* 2021) Australian taxa, but most genera have not yet been the subject of molecular work. Well curated lichen material stored in Australian herbaria could however be used to produce such data, as methods to generate sequences from herbarium specimens are becoming more and more effective. These methods include protocols to obtain sequences from old herbarium specimens (Sohrabi *et al.* 2010; Redchenko *et al.* 2012; Kistenich *et al.* 2019). For example, using Ion Torrent sequencing technology, sequence data were obtained from lichen specimens up to 150-year-old (Kistenich *et al.* 2019). These new methods also allow the processing of large number of specimens at once, including 96 lichen specimens in Gueidan *et al.* (2019) and 384 lichen specimens in Gueidan and Li (2022). The potential of using lichen herbarium specimens for molecular taxonomy have however not yet been fully realised in Australia.

Reference sequence databases are important resources for various fields of science, including systematics, ecology, biodiversity and eDNA research. For fungi, the marker ITS was chosen as the fungal universal barcode early on (Schoch et al. 2012), and several well curated databases now provide access to large numbers of well-curated and high-quality ITS sequences, including UNITE (Kõljalg *et al.* 2013; Nilsson *et al.* 2018) and RefSeq (Schoch *et al.* 2014; O'Leary *et al.* 2015). Large taxon gaps however still exist in these databases (Orok *et al.* 2012; Kõljalg *et al.* 2013; Crous *et al.* 2014; Nilsson *et al.* 2018), and dried and living fungal collections have been proposed as possible solutions to generating ITS sequences from large number of species, specimens or strains (Yahr *et al.* 2016; Gueidan *et al.* 2019; Gueidan & Li 2022; Crous *et al.* 2014).

Here, as a first step towards the development of an ACT microlichen sequence database, we use CANB specimens (including types) and both traditional and next generation long-read sequencing techniques to generate ITS sequences for microlichens from the ACT. These sequences are then used to create a preliminary version of a sequence database for molecular identification purpose. This preliminary database is tested on several un-identified specimens collected during the 2018 ACT BushBlitz expedition and challenges to both generating sequences for a reference database and using this database to identify specimens to the species level are discussed.

## Methods

### Taxon selection and digitisation

The Census of Plants of the Australian Capital Territory (ACT) v. 4.1 (30 August 2019) was used to generate a list of microlichens from the ACT (Suppl. file 1a). Numerous specimens collected from the ACT are available from CANB (5,819 specimens, including 56 types), and from other Australian herbaria. Species with foliose, fruticose and dimorphic (foliose and fruticose) thalli were removed from the 521 listed lichen species in the ACT. A total of 254 species and infraspecific taxa with crustose to squamulose thalli (and sometime small foliose) were selected (Suppl. file 1b). After updating the list with new records and species, synonymies, re-identifications, and new combinations (Suppl. file 1c), the list included 246 taxa. Specimens of these 246 taxa were searched for in the CANB herbarium, and 99% of those were represented by at least one specimen in CANB. When available and large and recent enough for DNA work (in this case, collected after 1960), specimens from the ACT or NSW were chosen in priority over specimens from other Australian states and territories or overseas material. Twelve taxa could not be sampled, either because of the lack of specimens or available specimens were unsuitable (Suppl. file 1d). For the remaining 234 taxa, suitable specimens could be found in CANB (Suppl. file 2). A total of 394 CANB specimens representing these 234 taxa (including some multiple collections of the same taxon) were digitised using an Aptus Leaf II 12 camera and the Capture One software.

### DNA extraction, amplification and sequencing

Specimens were sampled under a stereomicroscope using either (1) sterile tweezers or (2) clean razor blades and weighing paper. The ITS sequences were generated using two different approaches. Most samples were sequenced with a long-read amplicon approach, as described in Gueidan and Li (2022). In short, lichen material was ground using Lysing Matrix A tubes (MP Biomedicals) and a Precellys tissue lyser (Bertin Instruments) and the DNA extracted with the Invisorb DNA Plant HTS 96 kits (Stratec Molecular). A two-step amplification was then conducted, first using the ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) primers tailed with the PacBio M13 primers (first step of amplification), then using the PacBio M13 primers with unique index combinations (second step of amplification). The PCR products were cleaned, normalised and pooled, then sent for sequencing to Macrogen (Seoul, Korea), where a SMRTbell 1–2Kb amplicon library was prepared. One SMART cell of a Sequel I (V3) platform (Pacific Biosciences) was used to sequence the pooled sample, which included other PCR products from this study for a total of 548 samples. ITS sequences were obtained for additional specimens using a phenol-chloroform DNA extraction modified from Zolan and Pukkila (1986), as described in Gueidan *et al.* (2007). The ITS barcode was amplified using ITS1F and ITS4 primers and sequences were generated using Sanger sequencing at Macrogen, as described in Gueidan and Elix (2022).

### ITS sequence editing and sequence database building

Sanger sequences were assembled and edited using Sequencher v. 5.4.6 (Gene Codes Corporation). For PacBio sequences, a BAM file of Circular Consensus Sequences (CCSs) was provided by Macrogen. The data was demultiplexed using the lima command in SMRT Tools v. 7.0.1 (Pacific Biosciences). The files were then converted to FATSQ files using BAMTools v. 2.5.1 (Barnett *et al.* 2011). CCSs were denoised using DADA2 v. 1.14 (Callahan *et al.* 2016) as described in Gueidan and Li (2022). Resulting sequence variants were converted to FASTA files using BAMTools and each file subjected to a blastn query using BLAST+ v. 2.12.0 (Altschul *et al.* 1990;

Camacho *et al.* 2009) against the NCBI nt database using a max_target_seqs of 3. For samples for which sequence variants were generated for the target species, only the main sequence variant was included in the database. For samples for which no sequence variants were generated for the target species, CCSs were converted to FASTA and subjected to the same blastn query to identify target CCSs. For these samples, all available target and non-chimeric CCSs were used as entries in the sequence database. All available sequences were added to a FASTA file. The sequence database was built using the makeblastdb command using BLAST+ v. 2.13.0.

### Testing the molecular identification of BushBlitz microlichen specimens

To test the utility of this preliminary version of the ACT microlichen sequence database, eleven herbarium accessions representing 27 unidentified individuals of microlichens collected during the 2018 ACT BushBlitz expedition were used here (Table 1). Together with a broader sampling of a total of 140 BushBlitz microlichen specimens, they were digitised, sampled, extracted and sequenced using the PacBio amplicon protocol as described above. For the digitisation, as several specimens comprised multiple individuals (ie, multiple species occurring on the one fragment of substrate), generated specimen images were edited with Photoshop (Adobe) to indicate the sampled areas (Fig. 1). For these selected test specimens, resulting sequence variants were then subjected to two blastn searches with BLAST+ v. 2.13.0, one using the NCBI nt database, the other our created custom ACT microlichen database. Sequence identity matches superior to 95% were then compared to potential morphological identification of their respective specimens. Leica stereo- and compound microscopes and chemistry spot test were used to confirm the match between morpho-chemical and molecular identifications.

## Results

Out of the 394 specimens used to generate ITS sequences for the custom ACT microlichen database, sequence data were obtained for 375 specimens. Among these, generated sequences of 151 samples could be unambiguously matched to their corresponding target taxon because their BLAST result matched the target species or genus with 80% similarity or more (Suppl. file 2). These sequences were used to create the first version of the database. For all others, additional work will be required to identify if the unmatching generated sequences were the result of mis-identification, mis-sampling, unclear species boundaries or sample cross-contaminations. Combined with data available from GenBank and data generated using Sanger sequencing, the first version of the database includes 347 sequences, representing 151 specimens from 99 microlichen species found in the ACT. The ACT microlichen ITS sequence database v.1 is available at https://doi.org/10.25919/qzaq-4182 [CSIRO Data Access Portal]. Additionally, ITS sequences will be deposited in GenBank once confirmed by more detailed and group-focused molecular taxonomic studies, as carried out in Gueidan & Elix (2022).

This sequence database was used in parallel to the NCBI nt database to identify specimens collected during the 2018 ACT BushBlitz expedition. The results for eleven accessions are presented in Table 1. For each of these accessions, one to several lichen individuals were sampled, for a total of 27 samples. For 15 of these samples, the blast results matched the lichen samples found in the accession (ie, morphological identifications confirmed molecular identifications). For four samples, the blast results likely matched the sample but, due to the small size of the thallus or the absence of fruiting bodies, the morphological identification could not fully confirm the molecular identification. For four samples, the molecular identification did not match the morphological identification (the species identified with molecular data could not be found on the accession). For all other samples (4), the blast result was below 95% identity and therefore unlikely to represent a usable match.

## Discussion

Here, we used lichen herbarium specimens kept at CANB to generate ITS sequences for microlichens from the ACT. In order to reduce cost and effort, we processed samples in batches of 96 samples and used multiplexing for sequencing. Although material availability was not an issue (ie, most ACT microlichen species had usable specimens in CANB), mostly thanks

**Table 1**. Blast results (species match and percentage identity) and morphological identifications for 27 samples obtained from eleven 2018 ACT BushBlitz accessions. Main sequence variants (SVs) were used for the NCBI nt and the custom database blast searches.

| | Collection number | Herbarium accession | SVs NCBI nt database blast result (% identity) | SVs custom database blast result (% identity) | Morphological confirmation |
|---|---|---|---|---|---|
| 1 | CG2447a | CANB910330 | *Trapelia placodioides* 95.8% | *Trapelia placodioides* 96.4% | *Trapelia placodioides* confirmed |
| 2 | CG2447b | | *Verrucaria rosula* 92% | *Verrucaria nigrescens* 87.7% | match with low similarity (< 95%) |
| 3 | CG2447c | | *Japewia tornoensis* 84% | *Lecidea fuscoatrula* 82.4% | match with low similarity (< 95%) |
| 4 | CG2495 | CANB910378 | *Ramboldia petraeoides* 97% | *Ramboldia petraeoides* 98.4% | *Ramboldia petraeoides* confirmed |
| 5 | CG2414 | CANB910297 | *Trapelia elacista* 92.3% | *Trapelia crystallifera* 100% | *Trapelia crystallifera* confirmed |
| 6 | CG2434a | CANB910317 | *Trapelia elacista* 98.6% | *Trapelia atrocarpa* 99.1% | *Trapelia atrocarpa* confirmed |
| 7 | CG2434b | | *Trapelia tristis* 90% | *Trapelia concentrica* 99.2% | *Trapelia concentrica* confirmed |
| 8 | CG2434c | | *Verrucaria rosula* 93% | *Endocarpon helmsianum* 89% | match with low similarity (< 95%) |
| 9 | CG2434d | | *Trapelia placodioides* 95.8% | *Trapelia placodioides* 96.4% | *Trapelia placodioides* confirmed |
| 10 | CG2482a | CANB910365 | *Lecanora farinacea* 98% | *Lecanora farinacea* 99.4% | *Lecanora farinacea* confirmed |
| 11 | CG2482b | | *Ramboldia petraeoides* 98% | *Ramboldia petraeoides* 98.9% | *Ramboldia petraeoides* confirmed |
| 12 | CG2428a | CANB910311 | *Trapelia placodioides* 95.4% | *Trapelia placodioides* 95.2% | *Trapelia placodioides* confirmed |
| 13 | CG2428b | | *Trapeliopsis granulosa* 96% | *Trapeliopsis granulosa* 97.8% | mismatch, species not seen |
| 14 | CG2428c | | *Buellia subarmeniaca* 92% | *Buellia halonia* 86% | match with low similarity (< 95%) |
| 15 | CG2433a | CANB910316 | *Caloplaca* sp. 88% | *Caloplaca arandensis* 99.1% | mismatch, *Caloplaca* but not *C. arandensis* |
| 16 | CG2433b | | *Trapelia lilacea* 93.9% | *Trapelia pruinosa* 99.1% | *Trapelia pruinosa* likely (apothecia not seen) |
| 17 | CG2433c | | *Rhizocarpon nidificum* 99% | *Rhizocarpon geographicum* 99.6% | *Rhizocarpon geographicum* likely (apothecia not seen) |
| 18 | CG2433d | | *Aspicilia epiglypta* 90% | *Aspicilia cinerea* 99.7% | *Aspicilia cinerea* confirmed |
| 19 | CG2445a | CANB910328 | *Ramboldia petraeoides* 97% | *Ramboldia petraeoides* 98.3% | mismatch, species not seen |
| 20 | CG2445b | | *Trapelia placodioides* 95.8% | *Trapelia placodioides* 96.4% | *Trapelia placodioides* confirmed |
| 21 | CG2476a | CANB910359 | *Rhizocarpon smaragdulum* 96% | *Rhizocarpon vigilans* 94.2% | mismatch, *Rhizocarpon* but not *R. vigilans* |
| 22 | CG2476b | | *Ramboldia petraeoides* 97% | *Ramboldia petraeoides* 98.2% | *Ramboldia petraeoides* confirmed |
| 23 | CG2423a | CANB910306 | *Ramboldia petraeoides* 98% | *Ramboldia petraeoides* 98.6% | *Ramboldia petraeoides* confirmed |
| 24 | CG2423b | | *Rhizocarpon badioatrum* 94% | *Rhizocarpon bicolor* 99.1% | *Rhizocarpon bicolor* likely (apothecia not seen) |
| 25 | CG2423c | | *Rhizocarpon geographicum* subsp. *frigidum* 97% | *Rhizocarpon geographicum* 99.8% | *Rhizocarpon geographicum* likely (apothecia not seen) |
| 26 | CG2523a | CANB918145 | *Lecanora farinacea* 100% | *Lecanora farinacea* 97.9% | *Lecanora farinacea* confirmed |
| 27 | CG2523b | | *Ramboldia petraeoides* 98% | *Ramboldia petraeoides* 98.8% | *Ramboldia petraeoides* confirmed |

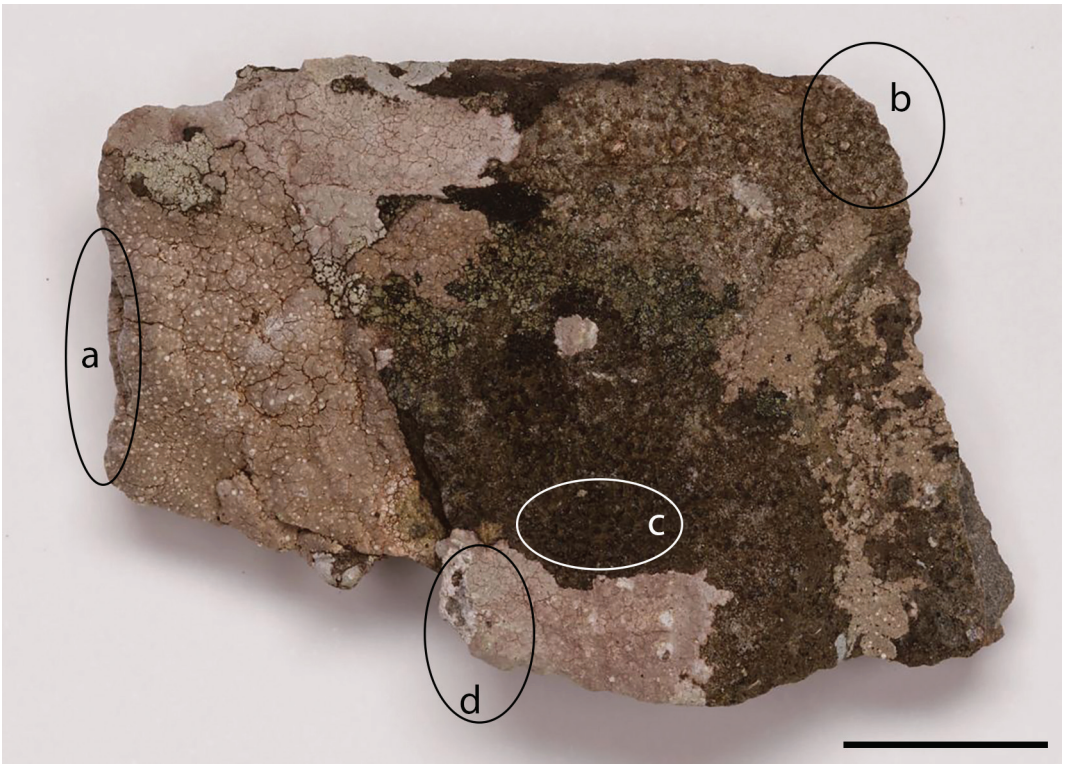


**Figure 1**. Annotated photograph of the 2018 ACT BushBlitz specimen CANB910317, showing the four sampled lichen thalli. The areas sampled for DNA extractions were encircled and labelled. The presence of three species of *Trapelia* (a. *T. atrocarpa*; b. *T. concentrica*; d. *T. placodioides*) was confirmed by molecular and morphological data. The presence of a species of Verrucariaceae was suggested by both molecular and morphological data, but with no close species match. Scale bar = 1 cm.

to the legacy collections of ACT-based lichenologists, other challenges were met, which are discussed below.

First, reliability in the available herbarium material is critical for building reference sequences. Although most used CANB specimens were accurately identified, some appeared to be mis-identified and led to unmatching molecular results. More importantly, in addition to mis-identification, is the issue of unclear species boundaries. For Australian lichens, most species boundaries are based on morpho-chemical data, with no molecular data available for confirming, for example, if a Northern hemisphere species indeed occurs in Australia or if this taxon should be described as new Australian-endemic species. Similarly, molecular data is critical to draw attention to morphologically similar but phylogenetically divergent species (convergent species), or species with particularly wide infraspecific variability. As a result, unless a lichen group has already been revised using both morpho-chemical and molecular data, the direct use of herbarium specimens to generate ITS sequences that reliably and accurately represent species is not realistic. Morpho-chemical and molecular revisions focussing on particular genera, subgenera or group of species, such as the one recently done on *Trapelia* (Gueidan & Elix 2022), will have to be done in parallel to specimen mass parallel sequencing, to clarify, or at least start clarifying, species boundaries. This will be a significant endeavour, which will require time and taxonomic expertise.

Second, reliability in the laboratory processes to generate the data is also critical to building reference sequences. Here, to reduce cost and time, a mass parallel/multiplexing sequencing approach was used, allowing to process large numbers of samples at once. This approach started with the sampling of material from a large number of herbarium specimens, a tedious process involving the removal of material from the substrate with a tweezer or a razor blade under

a stereomicroscope. As microlichens often co-occur with other species on the same fragment of substrate, and some of these species are morphologically very similar, mis-sampling has also been the cause of mis-matching ITS sequences in this study. Additionally, even if the correct lichen thallus is sampled, if any material of neighbouring species is accidentally sampled and then preferentially amplified, sequencing can result in mis-matching sequences. To make things more difficult, lichen thalli harbor a high diversity of associated fungi, which will also be sequenced in the same process. For lichens, these are major issues which have to be added to the more common issues of sample cross-contamination (contamination between wells during the amplification steps) and index cross-talk (index mis-assignment after mass parallel sequencing). As a result, the generation of ITS sequences for lichens is not a straightforward process and will necessitate significant efforts of manual curation of the data. This manual data curation will make the application of automated laboratory and bioinformatic pipelines particularly challenging for this group of organisms.

As for the molecular identification of specimens using the generated reference sequence database, the same issues apply. Resulting sequences include at best the sequence of the target taxon, but also sequences of neighbouring species, associated lichen-inhabiting fungi, and possible cross-contaminants. It is therefore again not a straightforward process and will require input from a taxonomic expert. Fully automated molecular identification processes will therefore be difficult to apply at this stage. The reliability of molecular identifications will also depend on solid morpho-chemical and molecular revisions of Australian lichen taxa. These revisions will require time and taxonomic expertise, but once species concepts are clarified, reference sequence databases are a powerful tool to accelerate and confirm species identifications. This was demonstrated here by the higher BLAST identity percentages obtained with our ACT microlichen custom sequence database as compared to the percentages obtained with the current NCBI nt database. It was also evident for the recently revised Australian *Trapelia* species (Gueidan & Elix 2022): all unidentified *Trapelia* material collected during the 2018 ACT BushBlitz expedition were correctly identified to the species level thanks to their ITS sequences and our newly generated sequence database for ACT microlichens.

Whether carried out in the field or in the laboratory, species identifications using molecular sequences is a powerful tool to the study of taxonomy, ecology and biodiversity, especially for organisms with limited diagnostic features. It however relies on the development of high-quality reference sequence databases. Although generating these references from collection specimens may be a straightforward process for some organisms, it has some challenges for lichens, mostly due to unclear species concepts, but also to other issues such as mis-identification, mis-sampling and cross-contamination. As a result, initial molecular taxonomic revisions of Australian material from all the main lichen genera will be necessary before mass parallel sequencing of herbarium specimens can be used to generate reliable reference sequence databases.

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**Supplementary file 1**. Lichenised fungal species and infraspecific taxa from the ACT, as listed in the Census of Plants of the Australian Capital Territory v. 4.1 (30 August 2019). There are four worksheet tabs:
a. list of all species as published in v. 4.1;
b. list of all ACT microlichen species;
c. list of all ACT microlichen species after name update;
d. list of ACT microlichen species for which specimens were available in CANB.

**Supplementary file 2**. List of specimens used to generate ITS sequences for the ACT microlichen ITS sequence database, with voucher information.

Supplementary files 1 and 2 are appended to the online listing of this paper, accessible via:
https://www.rbg.vic.gov.au/science/journal/