

DNA-based identification of *Herbertus* species on Gough Island, South Atlantic Ocean

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Abstract – Based on phylogenetic analyses of nrITS sequences and accompanying morphological investigations, several accessions of *Herbertus* from the South Atlantic Gough Island were identified as *Herbertus runcinatus* or *Herbertus sendtneri*. The Gough Island accession of *H. sendtneri* incorporated in the molecular study is nested in a tropical American clade of *H. sendtneri*, and is placed sister to a Bolivian accession. Both species are new to Gough Island.

Jungermanniales / Herbertaceae / *Herbertus* / DNA taxonomy / Gough Island / nrITS

INTRODUCTION

Molecular studies have improved the taxonomy of liverworts at different ranks, e.g., families (Hentschel *et al.*, 2006), genera (Wilson *et al.*, 2007) or species (Feldberg *et al.*, 2004). These studies pointed to vast morphological homoplasy of morphological character states of liverworts and demonstrated the need of an integrative taxonomy that considers evidence from both morphology and molecules (Heinrichs *et al.*, 2009).

Herbertus S.Gray was once regarded as an ancient genus (Schuster, 2000); however, recent divergence time estimates (Heinrichs *et al.*, 2007) point to a Cenozoic origin of this genus, and speciation events in the Neogene (Feldberg *et al.*, 2007). *Herbertus* is one of the most difficult genera in terms of morphological circumscription of species (Feldberg *et al.*, 2004; Feldberg & Heinrichs, 2006) and may therefore benefit from the introduction of molecular methods. The problems in identifying *Herbertus* species are the consequence of limited morphological variation in combination with morphological variation within populations that possess largely similar sequences (Feldberg *et al.*, 2004).

A single representative of *Herbertus* has been recognized for the subantarctic and temperate islands of the eastern Southern Hemisphere by Grolle (2002). He lists *Herbertus capensis* (Steph.) Sim as the only *Herbertus* species in the region, a taxon that is now treated as a synonym of *H. dicranus* (Taylor)

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Trevis. (Hodgetts, 2008). Alternatively, Wace (1961) published *H. oldfieldianus* (Steph.) Rodway in his investigation of the vegetation of Gough Island. The latter identification was maintained in Wace & Dickson (1965) and Hänel *et al.* (2005).

Morphological investigation of several *Herbertus* specimens from Gough Island revealed the presence of two morphologically divergent phenotype groups. However, assignment of these groups to species proved problematic using solely morphological evidence. To overcome the problem of morphological ambiguity (Feldberg *et al.*, 2004), two recent collections from Gough Island were used for a molecular phylogenetic study. This study allowed for an assignment of the Gough Island accessions to species clades.

MATERIAL AND METHODS

DNA-extraction, sequencing and phylogenetic analyses

Two morphologically diverging specimens of *Herbertus* from Gough Island (Gremmen 2000-170 and 2000-904, PRC, duplicates in GOET) were used for the molecular investigation. DNA extraction and sequencing of the nuclear ribosomal internal transcribed spacer region (nrITS) followed the protocols described in Feldberg & Heinrichs (2006). The new sequences were manually inserted in the nrITS sequence alignment of Feldberg *et al.* (2007) using BioEdit version 5.0.9 (Hall, 1999). A subset of the nrITS sequences from Feldberg *et al.* (2007) was used for the phylogenetic analyses (Table 1).

Table 1. Geographic origins, voucher numbers, and GenBank/EMBL accession numbers of the taxa investigated. If not otherwise indicated, vouchers are at GOET. New sequences in bold.

<i>Taxon</i>	<i>Origin</i>	<i>Voucher</i>	<i>Acc.-Numb. nrITS</i>
<i>Mastigophora diclados</i>	Reunion	<i>Schneider H05-01</i>	AM259580
<i>Triandrophyllum subtrifidum</i>	Bolivia	<i>Churchill et al. 22800</i>	AJ972455
<i>H. aduncus</i>	British Columbia	<i>Schofield 86245</i>	AM259582
<i>H. aduncus</i> subsp. <i>hutchinsiae</i>	British Isles	<i>Rycroft 01015</i>	AJ783340
<i>H. delavayi</i>	Bhutan	<i>Long 28864</i>	AJ972450
<i>H. delavayi</i>	British Colombia	<i>Schofield 83644</i>	AM259583
<i>H. delavayi</i>	British Isles	<i>Rycroft 030725A</i>	AJ783341
<i>H. dicranus</i>	Ethiopia (1)	<i>Miehe & Miehe 2524</i>	AJ783343
<i>H. dicranus</i>	Ethiopia (2)	<i>Miehe & Miehe 2435</i>	AM259584
<i>H. dicranus</i>	Indonesia	<i>Gradstein 10243</i>	AJ783344
<i>H. dicranus</i>	Japan	<i>Kurita 51 (HIRO)</i>	AJ972452
<i>H. dicranus</i>	Madagascar	<i>Pócs et al. 90116/O</i>	AJ783346

Table 1. Geographic origins, voucher numbers, and GenBank/EMBL accession numbers of the taxa investigated. If not otherwise indicated, vouchers are at GOET. New sequences in bold.

<i>Taxon</i>	<i>Origin</i>	<i>Voucher</i>	<i>Acc.-Numb. nrITS</i>
<i>H. dicranus</i>	Réunion	<i>Schneider H05-02</i>	AM259587
<i>H. dicranus</i>	Rwanda	<i>Solga RWA-1117</i>	AM259589
<i>H. dicranus</i>	Thailand	<i>Schäfer-Verwimp & Verwimp 23850</i>	AJ972451
<i>H. juniperoideus</i> subsp. <i>pensilis</i>	Bolivia	<i>Heinrichs et al. 3998</i>	AM113607
<i>H. juniperoideus</i> subsp. <i>acanthelius</i>	Bolivia	<i>Heinrichs et al. 4000</i>	AM113600
<i>H. juniperoideus</i> subsp. <i>acanthelius</i>	Ecuador	<i>Gradstein & Mandl 10144</i>	AJ783339
<i>H. juniperoideus</i> subsp. <i>bivitattus</i>	French Guiana	<i>Holz FG-00-201</i>	AM113598
<i>H. juniperoideus</i> s.str.	Ecuador	<i>Schäfer-Verwimp 23346</i>	AM113605
<i>H. juniperoideus</i>	Madagascar	<i>Pócs 9444/D (EGR)</i>	AM259592
<i>H. oldfieldianus</i>	New Zealand (1)	<i>Glenny 9584</i>	AM259594
<i>H. oldfieldianus</i>	New Zealand (2)	<i>Engel 18886</i>	AM259595
<i>H. runcinatus</i>	Chile (1)	<i>Rycroft 031123-1120</i>	AM259596
<i>H. runcinatus</i>	Chile (2)	<i>Rycroft 031202-1125</i>	AM259597
<i>H. runcinatus</i>	Gough Island	<i>Gremmen 2000-170</i>	FN582125
<i>H. sendmeri</i>	Austria	<i>Heinrichs 4377</i>	AJ783347
<i>H. sendmeri</i>	Azores	<i>RG2402</i>	AJ783349
<i>H. sendmeri</i>	Bhutan	<i>Miehe 00-380-01</i>	AM259598
<i>H. sendmeri</i>	Bolivia (1)	<i>Churchill et al. 22797</i>	AM259599
<i>H. sendmeri</i>	Bolivia (2)	<i>Heinrichs et al. 4002</i>	AJ783351
<i>H. sendmeri</i>	Bolivia (3)	<i>Heinrichs et al. 4004</i>	AJ783352
<i>H. sendmeri</i>	Costa Rica	<i>Holz & Schäfer-Verwimp CR 00-77</i>	AJ783354
<i>H. sendmeri</i>	Ecuador	<i>Heinrichs et al. 4418</i>	AM113611
<i>H. sendmeri</i>	Gough Island	<i>Gremmen 2000-904</i>	FN582126
<i>H. sendmeri</i>	Malaysia	<i>Gradstein 10353</i>	AJ972453
<i>H. sendmeri</i>	Panama	<i>Dauphin et al. 1576</i>	AJ783355
<i>H. stramineus</i>	British Isles	<i>Rycroft 00039</i>	AJ783356

Phylogenetic trees were inferred using maximum likelihood (ML) criteria implemented in PAUP* 4.0b10 (Swofford, 2003). Modeltest 3.06 (Posada and Crandall, 1998) was employed to determine the nucleotide substitution model

with the smallest number of parameters that best fits the data. The hierarchical likelihood ratio tests selected the TrN model (Tamura & Nei, 1993) with gamma shape parameter for among-site variation. This model was implemented in the ML analysis. The analysis was carried out as a heuristic search with 3 random-addition sequence replicates. Clade support was assessed by non-parametric bootstrapping with 200 replicates (Felsenstein, 1985). Bootstrap percentage values (BSP) above 70 were regarded as good support (Hillis and Bull, 1983).

Morphological investigation

Two morphological groups were identified in the *Herbertus* collections from Gough Island using standard light microscopy. Based on the identification of the two DNA vouchers representing these groups, the collections from Gough Island were assigned to species.

RESULTS

The ML analysis yielded a single optimal topology with $lnL = -4072,0731$ (Fig. 1). A robust clade with two accessions of *H. runcinatus* (Taylor) Trevis. and the Gough Island specimen Gremmen 2000-170 is placed sister to the rest of *Herbertus*. A clade with *Herbertus oldfieldianus* from New Zealand and accessions of *H. juniperoideus* (Sw.) Grolle from tropical America and Madagascar is resolved in a well supported sister relationship with a clade made up by *H. sendtneri* (Nees) Lindb., *H. aduncus* (Dicks.) S.Gray, *H. stramineus* (Dumort.) Trevis., *H. delavayi* Steph. and *H. dicranus*. The well supported *H. sendtneri* clade comprises accessions from Asia, Europe including Macaronesia, as well as Central and tropical South America. The Gough Island accession Gremmen 2000-904 is nested in a Neotropical subclade of *H. sendtneri* and placed sister to an accession from the Bolivian Andes. Based on the outcome of the molecular investigation, other representatives of the two morphological groups were assigned to either *H. runcinatus* or *H. sendtneri*:

Herbertus specimens examined (from Gough Island)

***Herbertus runcinatus* (Taylor) Trevis.:** S-slope of South Peak, loose groups in bryophyte mat in heathland on mountain slope, 530 m, 15 Sep 1999 coll. *N.J.M. Gremmen 99-168* (PRC); S of Tafelkop, undergrowth in fernbush, slightly sheltered, very damp, 300 m, 7 Jun 2000 coll. *N.J.M. Gremmen 2000-0170* (GOET, PRC); top of South Peak, on shallow, wet organic soil, slightly sheltered by boulders, 800 m, 16 Sep 2000 coll. *N.J.M. Gremmen 2000-823* (PRC).

***Herbertus sendtneri* (Nees) Lindb.:** Main Glen, Michael's Ridge, 1150 ft., 21 Jan 1956, coll. *N.M. Wace 579*, det. S.W. Arnell as *H. oldfieldianus* (cf. Wace, 1961: 353; BM, UPS); Tafelkop, growing in small numbers in large hepatic mats on very wet peat, 500 m, 14 Sep 1999 coll. *N.J.M. Gremmen 99-138* (PRC); Tafelkop, extensive mats in bryophyte dominated mire on very wet peat, 500 m, 14 Sep 1999 coll. *N.J.M. Gremmen 99-141* (PRC); N-side of Tafelkop, large mats on the lower part of rock outcrops surrounded by mire, 500 m, 15 Sep 1999 coll. *N.J.M. Gremmen 99-161* (F, PRC); Waterfall Camp, mat of mixed hepatics on very wet peaty soil in seepage area, 750 m, 16 Sep 2000 coll. *N.J.M. Gremmen 2000-873* (F); Waterfall Camp, dense mats on very wet peaty deposit on vertical rockface in seepage area,

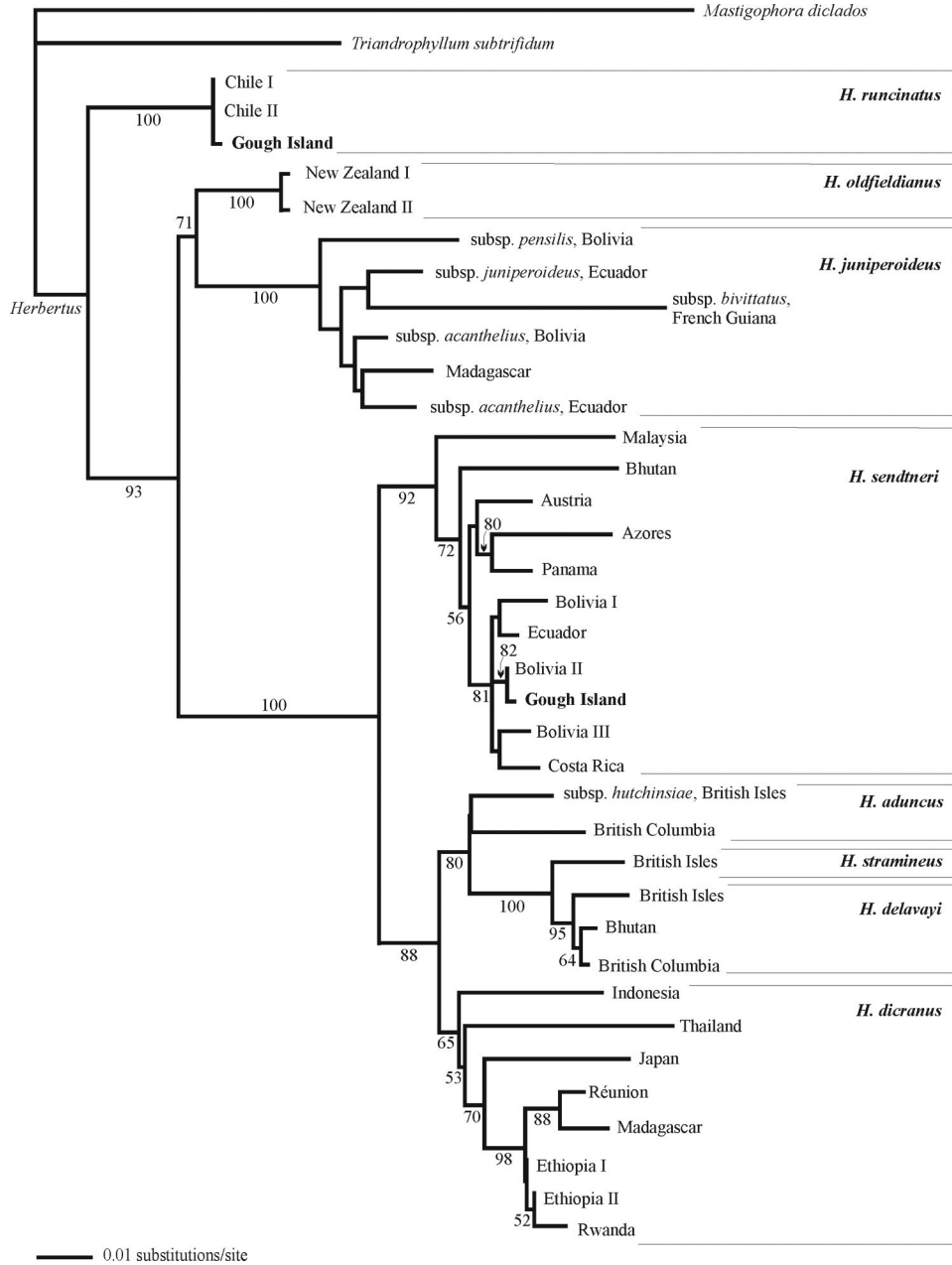


Fig. 1. Phylogram recovered in maximum likelihood analysis of the nrITS dataset. Bootstrap percentage values >50 are indicated at branches. The specimens from Gough Island are identified as *Herbertus runcinatus* (Gremmen 2000-170) and *H. sendtneri* (Gremmen 2000-904).



Fig. 2. *Herbertus runcinatus*, Gough Island, Gremmen 2000-170 (GOET).

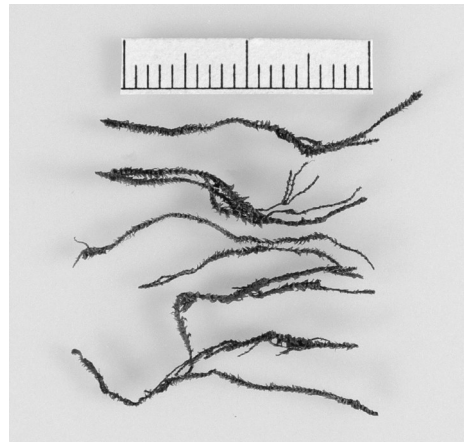


Fig. 3. *Herbertus sendtneri*, Gough Island, Gremmen 2000-904 (GOET).

750 m, 16 Sep 2000 coll. *N.J.M. Gremmen* 2000-878 (F); Waterfall Camp, small groups in hepatic mat on very wet peaty deposit on vertical rockface in seepage area, 750 m, 16 Sep 2000 coll. *N.J.M. Gremmen* 2000-880 (F); Waterfall Camp, extensive, deep turfs on peaty deposits overlying rock, wet, slightly sheltered, 750 m, 16 Sep 2000 coll. *N.J.M. Gremmen* 2000-904 (GOET, PRC); Tafelkop, in bryophyte dominated bog vegetation, 500 m, 27 Sep 2005 coll. *N.J.M. Gremmen* 2005-G967 (PRC).

Key to *Herbertus* on Gough Island

1. Plants robust, leaves densely imbricate, lobes curved in one direction, vitta bifurcating just below leaf sinus. *Herbertus runcinatus* (Fig. 2)
2. Plants slender, leaves laxly imbricate, lobes somewhat squarrose, vitta bifurcating at 1/3-3/4 up the lamina. *Herbertus sendtneri* (Fig. 3)

DISCUSSION

The nrITS sequence from the specimen Gremmen 2000-904 strongly resembles tropical American haplotypes of *H. sendtneri*, and is therefore assigned to this species. Morphologically, the Gremmen accession resembles *H. dicranus* rather than *H. sendtneri* because the leaves are only weakly papillose, the tip cells of the leaf lobes are usually not strongly elongate, and the basal leaf appendages are poorly developed. However, in nrITS phylogenies both species are well separated, and serve as an example of cryptic speciation (Feldberg *et al.*, 2004). The similar morphology of separate lineages of *Herbertus* with parallel variation of closely related populations (Feldberg *et al.*, 2004) hampers morphological identification of representatives of this genus. The difficulties in recognizing morphological characters of *Herbertus* species appear, e.g., in the different *H. dicranus* concepts of Hodgetts (2003, 2008). Neither morphological approaches nor phylogenies based on variable molecular markers such as nrITS allow for a

decision on the number of populations that represent a single metapopulation (*i.e.* a biological species); however, it is likely that the Gough accessions here assigned to *H. sendtneri* are related to a population of the high mountains of tropical America. The Gough Island accession Gremmen 2000-170 that is nested in the Chilean *H. runcinatus* clade fully matches the morphology of this taxon and can be assigned to *H. runcinatus* based on morphological and molecular evidence. The investigated accessions include robust, stiff *Herbertus* plants with densely imbricate leaves and a vitta that bifurcates just below the leaf sinus.

Based on our investigation, we accept *H. runcinatus* and *H. sendtneri* for the liverwort flora of Gough Island and tentatively exclude *H. oldfieldianus* from this flora.

Muñoz *et al.* (2004) and Sanmartín *et al.* (2007) proposed wind as a long-distance dispersal vehicle in the Southern Hemisphere. Gough Island is in the direct path of the roaring forties, and shares many floristic elements with Chile, and subantarctic islands (Wace, 1961). It is therefore not surprising that the Gough Island accessions of *Herbertus* are closely related to South American *Herbertus* populations. Elix & Gremmen (2002) similarly observed a significant South American element in the Gough Island Parmeliaceae flora, besides pantemperate or cosmopolitan taxa.

The present study adds to growing evidence that molecular studies allow for deeper insights into relationships of populations of liverworts than investigations based solely on morphological evidence (Hentschel *et al.*, 2007; Fuselier *et al.*, 2009; Heinrichs *et al.*, 2009). Detailed sampling of *Herbertus* accessions representing the morphological variation and the range of the genus is needed to improve both taxonomy and knowledge on the biogeography of this genus.

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