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From one to five putative species: an unexpected high genetic diversity in *Usnea flavocardia* (lichenized Ascomycetes, *Parmeliaceae*) with the discovery of a new clade within the subgenus *Usnea* s.str.

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Abstract. In this study, we analyzed the genetic diversity within *Usnea flavocardia*, a widespread species found in all continents except Antarctica. The species is characterized by a shrubby thallus growth form, the presence of soralia, a yellow central axis and/or the presence of red dots on the cortex. Using ITS rDNA and two protein-coding genes (*mcm7* and *rpb1*) in a multispecies coalescent (MSC) approach, we showed that *U. flavocardia* comprises five different lineages, four of which can be considered as putative new species. Each of the five lineages, except one, is characterized by specific chemical compounds. Within the outgroup that was used in this study, we furthermore showed that *U. gaudichaudii* and *U. eulychniae*, both endemic to Chile, constitute a new major clade in the subgenus *Usnea* s.str.

Key words: fatty acids, lichens, multi-species coalescent, species delimitation, STACEY, thin layer chromatography, ITS, *mcm7*, *rpb1*

Introduction

In lichenized Ascomycetes species, delimitation has always been challenging due to the often-extreme variability characterizing these organisms. Because lichens are perennial, long-lived organisms, they are especially subject to changing environmental conditions affecting their external morphology in multiple ways (Pintado et al. 1997; Nayaka et al. 2009; Vondrák et al. 2010; Leavitt et al. 2011; Pérez-Ortega et al. 2012; Muggia et al. 2013). In large foliaceous and fruticose lichens, delineating species boundaries using morphology only, as it was the case in early times of lichen systematics, has often been a puzzle for lichen taxonomists (e.g., Printzen 2009). Later, the use of chemical substances produced by these symbiotic organisms resolved many problems, but added new challenges at the same time (Culberson 1969; Brodo 1978, 1986; Egan 1986). In the last 30 years, the use of molecular markers has revolutionized systematics and lichen taxonomy in which most revisions published today are primarily based on molecular data (Miadlikowska & Lutzoni 2000; Leavitt et al. 2011; Otálora et al. 2014; Spribille et al. 2014; Zhao et al. 2016; Kistenich et al. 2018; Barcenas-Peña et al. 2023; Davydov et al. 2024). However, the road ahead is long and still full of obstacles and challenges even using molecular markers. For instance, recognizing independent evolutionary lineages can be problematic since evolutionary processes, such as selection, introgression and gene flow, as well as incomplete lineage sorting (ILS) might lead to misinterpretation while delimiting species (Naciri & Linder 2015). At least one of the former processes, ILS, that strongly impacts species delimitation with recent divergence histories, is now considered within the multispecies coalescent model (MSC, Yang & Rannala 2010) as implemented in several software programs used for species delimitation based on DNA sequence data (Jones et al. 2015; Jones 2017).

Usnea Adans constitutes a strongly supported monophyletic lineage within the family *Parmeliaceae* (Crespo et al. 2007). The genus is characterized by the presence of usnic acid in the cortex and by shrubby to pendant

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thalli with radially symmetrical branches containing a central elastic axis made of chondroidal tissue (Clerc 1998; Ohmura 2001). Until the late 20th century, the world monograph of Motyka (1936–1938) was considered as the cornerstone of the genus systematics, with 451 accepted species, of which 229 (52%) were newly described. Although several taxonomic treatments were published during the last decades (Awasthi 1986; Clerc 1998, 2004, 2011; Halonen et al. 1998; Stevens 1999; Ohmura 2001, 2012; Hinds et al. 2007; Rodriguez et al. 2011; Truong et al. 2011, 2013b; Truong & Clerc 2012, 2013; Herrera-Campos 2016; Gerlach et al. 2017, 2020; Bungartz et al. 2018; Clerc & Otte 2018), the genus *Usnea* remains one of the most difficult genera in lichen systematics. For instance, the high phenotype variability of many *Usnea* species (Swinscow & Krog 1978; Clerc 1987, 1998) has led to more than 1,200 described species worldwide. Today, the estimated number of *Usnea* species varies from 350 to over 400 taxa (Nadel & Clerc 2022). According to previous phylogenies, the genus *Usnea* is divided into 4 clades named USNEA-1 to USNEA-4 (Truong et al. 2013a; Lücking et al. 2020). In addition, phylogenetic analyses carried out within the *Usnea cornuta* group highlighted the importance of secondary chemistry to delimit species by showing that several clades were characterized by a specific chemotype (Gerlach et al. 2019, 2020). This led to the description of several new species, all of them being recognized by subtle morphological and anatomical characters previously overlooked in this aggregate (Gerlach et al. 2020). Therefore, taxonomic significance of chemotypes, as well as the existence of previously undetected, subtle morphological and anatomical characters in many *Usnea* species remain to be investigated in light of DNA analysis. Lücking et al. (2020) mentioned that only 30% of the known accepted species in *Usnea* s.lat. were so far sequenced with both taxonomic and geographic bias. In this context, it is clear that we are still far from knowing the final word on the number of species within this genus.

In this study, we aim to clarify several issues in *Usnea flavocardia* Räsänen using molecular tools. *Usnea flavocardia* is easily recognized by its short, shrubby sorediate thallus, its yellow pigmented medulla around the central axis and/or the presence of cortical red dots (Clerc 2007). Clerc (1984) described *Usnea wirthii* P. Clerc as a species containing psoromic acid based on material collected in south-western France by the Swiss lichenologist Eduard Frey. Later, Isabelle Tavares from Berkeley University drew Clerc's attention to the fact that Räsänen (1936) had already described a species from central-southern Chile named *Usnea flavocardia* characterized by a yellow central axis, as in *U. wirthii*, but with norstictic acid in the medulla instead of psoromic acid. Clerc (2004) consequently reduced *U. wirthii* to synonymy with *U. flavocardia*. However, looking at the important chemical diversity within the group, as well as discovering further herbarium material recently collected in the Andes, we suspected that the systematics of this group is much more complex than currently understood. The aims of this article are (1) to test that all the specimens *U. flavocardia* group fall within USNEA-3 as suggested by Truong et al. (2011), but only based on one sample; (2) to determine whether the observed chemical diversity corresponds to an equivalent genetic diversity and whether the psoromic (*U. wirthii*) and the norstictic (*U. flavocardia*) strains are genetically identical, thus supporting the synonymy proposed by Clerc (2004); and (3) to take the opportunity of our broad sampling to place two endemic species from Chile within the *Usnea* main clades. This study presents the results of a worldwide study of the *Usnea flavocardia* group based on chemical and molecular data.

Materials and methods

Taxon sampling

We sampled tissue from 45 *Usnea* specimens identified as belonging to the *U. flavocardia* group following Clerc (1998, 2007). All of the former specimens were characterized by a short, shrubby, sorediate thallus with a yellow pigmented medulla around the central axis and/or the presence of cortical red dots. Seventeen specimens contained psoromic acid and were identified as *U. wirthii*. Eleven specimens contained norstictic acid and were identified as *U. flavocardia* s.str. An additional 17 specimens had a different chemistry (see Fig. 1).

Our sampling covered a broad geographical area including Australia (3), Brazil (3), Canada (1), the Canary Islands (4), Chile (15), Costa Rica (2), the Czech Republic (1), France (2), Portugal (3), New Zealand (2), Switzerland (2), Taiwan (2), Tanzania (1), the Netherlands (1) and the USA (3). All of the Chilean samples were collected during a field trip conducted in 2019 by PC, DR and IP. The remaining *U. flavocardia* samples were provided by some of the other authors of this paper (TG, AG, RH and YO). Altogether, the sampling included 77 specimens (26 species) selected to represent all clades defined by Truong et al. (2013a) in the subgenus *Usnea* s.str. This sampling allowed to test for the belonging of the *U. flavocardia* group to USNEA-3 with regards to its sister group USNEA-4 (Truong et al. 2013a). Taking advantage of this large sampling, we added two endemic *Usnea* species collected in the Atacama Desert, *U. gaudichaudii* Motyka (3 specimens) and *U. eulychniae* Follmann (1 specimen) to determine their phylogenetic position within subgenus *Usnea* s.str. Finally, *U. durietzii* (2 specimens for which only ITS was retrieved from GenBank) was also included to test whether and how the latter species, which also displays a reddish spotted pigmentation in the basal part of its main branches, is related to *Usnea flavocardia*. The voucher material sequenced by Wirtz et al. (2006) consists of several dozen thalli corresponding to two different species, one of which is indeed *U. durietzii*. Unfortunately, no thallus was marked as having been sequenced and uncertainty therefore remains as to the identity of the sequenced specimens. All information about the 128 specimens used in this study is given in Table 1. All specimens newly sequenced are preserved in the G fungarium.

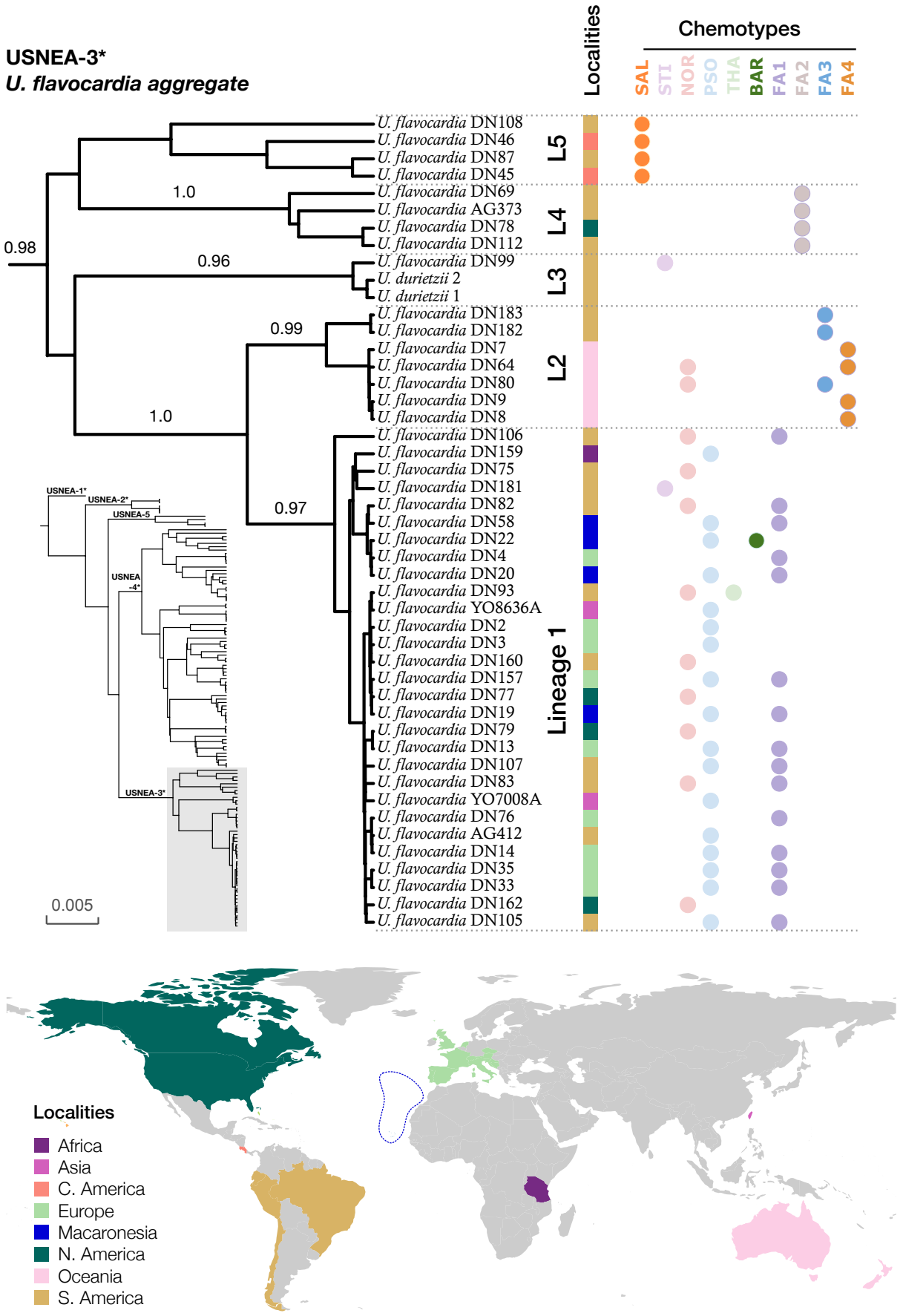

Figure 1. Species tree from STACEY analysis of the *Usnea flavocardia* aggregate nested into USNEA-3 as described by Truong et al. (2013a) with specimen localities colored according to the map on the lower side and chemotypes on the right side (SAL: salazinic acid STI: stictic acid; NOR: norstictic acid; PSO: psoromic acid; THA: thamnolic acid; BAR: barbatic acid; FA: fatty acids). The whole phylogeny is inserted on the left with the USNEA-3 clade highlighted in grey. Only posterior probabilities (PP) higher than 0.9 are shown above branches.

Table 1. List of specimens used with their species affiliation, lab number, country of origin, and chemical compounds, the number of genes sequenced and their GenBank number. When sequences were retrieved from GenBank, the reference article is given. Chemical compound abbreviations are the following. SAL: salazinic acid; CST: constictic acid; STI: stictic acid; NOR: norstistic acid; GAL: galbinic acid; PSO: psoromic acid; PRO: protocetraric acid; LOB: lobaric acid; THA: thamnolic acid; SQU: squamatic acid; BAR: barbatic acid; DIF: diffractaic acid; ALE: alectorialic acid; TER: terpenoids; FA: fatty acids.

Species	Lab number	Origin	Voucher/Code herbier	Chemotype	Done by	Available loci (nb)	GenBank/ENA accession numbers		
							ITS	*mcm7*	*rpb1*
Usnea aspera	AG140	Brazil	Gerlach & Penati-P36 (G)	SAL, NOR	Gerlach et al. 2019	3	MF669805	MT439122	MW231995
Usnea brasiliensis	AG13	Brazil	Gerlach-P52 (G)	PRO, PSO	Gerlach et al. 2019	3	MF669812	MT439123	MW231996
Usnea brasiliensis	AG15	Brazil	Gerlach & Penati P1-3 (G)	PRO, PSO	Gerlach et al. 2019	3	MF669806	MT439124	MW231997
Usnea brasiliensis	AG16	Brazil	Gerlach-P54 (G)	PRO, PSO	Gerlach et al. 2019	3	MF669807	MT439125	MW231998
Usnea brasiliensis	AG204	Brazil	Gerlach-P3 (G)	PRO, PSO	Gerlach et al. 2019	3	MF669808	MT439126	MW231999
Usnea ceratina	AG143	Brazil	Gerlach & Penati-1974-P20 (G)	BAR, DIF	Gerlach et al. 2019	3	MF669813	MT439127	MW232001
Usnea ceratina	AG200	Brazil	Gerlach & Penati 2016/P1-8 (G)	DIF	Gerlach et al. 2019	3	MF669814	MW273715	MW273442
Usnea ceratina	CER_02	UK	Törra, 06.10.2016 (TU66736)	BAR, DIF	Mark et al. 2016	3	KU352618	KU352178	KU352024
Usnea ceratina	CER_05	Portugal	Törra, 30.09.2008 (TU66737)	BAR, DIF	Mark et al. 2016	3	KU352619	KU352179	KU352025
Usnea chilensis	AG4869	Brazil	Rosiak 06 (G)	GAL	Gerlach et al. 2019	3	MF669815	MT439128	MW232002
Usnea chilensis	AG4949	Brazil	Alves s.n. (G)	GAL	Gerlach et al. 2019	3	MF669816	MW273716	MW273443
Usnea cirrosa	AG4906	Brazil	Gumboski 5020 (ICN)	SAL	Gerlach et al. 2017	3	KY021903	KY204413	KY204435
Usnea cladocarpa	AG5242	Costa Rica	PC2015/664 (G)	PRO	Gerlach et al. 2017	3	KY021904	KY204415	KY204437
Usnea cornuta s.str.	AG17	Brazil	Gerlach & Penati P-54 (G)	SAL	Gerlach et al. 2019	3	MF669859	MT439130	MW232005
Usnea cornuta s.str.	AG18	Brazil	Gerlach-P41 (G)	SAL	Gerlach et al. 2019	3	MF669860	MT439134	MW232008
Usnea cornuta s.str.	AG19	Brazil	Gerlach & Penati P44 (G)	SAL	Gerlach et al. 2019	3	MF669861	MT439135	MW232009
Usnea cornuta s.str.	AG30	Brazil	Gerlach & Penati P-52 (G)	SAL	Gerlach et al. 2019	3	MF669828	MT439140	MW232013
Usnea cornuta s.str.	AG113	Brazil	Gerlach-P7 (G)	SAL, CST	Gerlach et al. 2019	3	MF669835	MT439129	MW232003
Usnea cornuta s.str.	AG171	Brazil	Gerlach-P47 (G)	CST	Gerlach et al. 2019	3	MF669869	MT439132	MW232006
Usnea cornuta s.str	AG176	Brazil	Gerlach & Penati P46 (G)	SAL	Gerlach et al. 2019	3	MF669847	MT439133	MW232007
Usnea cornuta s.str.	AG198	Brazil	Gerlach-P18 (G)	CST	Gerlach et al. 2019	3	MF669872	MT439137	MW232010
Usnea cornuta s.str.	AG233	Spain	Aptroot 75462 (G)	SAL	Gerlach et al. 2019	3	MF669862	MT439138	MW232011
Usnea durietzii	1	Peru	HTL 19364j-290 (F)	n/a	Wirtz et al. 2006	1	DQ235504	n/a	n/a
Usnea durietzii	2	Peru	HTL 19364j-290 (F)	n/a	Wirtz et al. 2006	1	DQ235503	n/a	n/a
Usnea dasaea	DN121	Portugal	G00599772	SAL, GAL	**This study**	3	**PX105448**	**PX095167**	**PX103806**
Usnea dasaea s.lat.	AG35	Brazil	Gerlach & Penati 2016/P1(G)	GAL	Gerlach et al. 2019	3	MF669878	MW273708	MW273451
Usnea dasaea s.lat.	AG34	Brazil	Gerlach & Penati P18 (G)	GAL	Gerlach et al. 2019	3	MF669831	MT439209	MW232075
Usnea dasaea s.lat.	AG37	Brazil	Gerlach-P41 (G)	GAL	Gerlach et al. 2019	3	MF669880	MT439210	MW232076
Usnea dasaea s.lat.	AG128	Brazil	Gerlach & Penati P27 (G)	GAL	Gerlach et al. 2019	3	MF669885	MT329208	MW232074
Usnea densirostra	AG137	Brazil	Gerlach & Penati 2016/P33 (G)	SAL, NOR	Gerlach et al. 2019	3	MF669804	MW273717	MW273441
Usnea densirostra	AG4935	Brazil	Gerlach et al. 1494 (ICN)	NOR	Gerlach et al. 2019	3	KY021906	KY204417	KY204438
Usnea dodgei	AG54	Brazil	Gerlach & Penati P18 (G)	SAL, NOR	Gerlach et al. 2019	3	MF669886	MT439173	MW232042
Usnea dodgei	AG133	Brazil	Gerlach 2016/P28 (G)	NOR	Gerlach et al. 2019	3	MF669923	MW273718	MW273481
Usnea dodgei	AG173	Brazil	Gerlach 2016/P50 (G)	SAL,NOR,TER	Gerlach et al. 2019	3	MF669887	MW273719	MW273454
Usnea erinacea s.lat.	AG78	Brazil	Gerlach & Penati (G)	PRO	Gerlach et al. 2019 **& this study**	3	MF669888	MT439174	**PX103807**

Usnea erinacea s.lat.	AG79	Brazil	Gerlach & Penati (G)	PRO	Gerlach et al. 2019 **& this study**	3	MF669889	MW273710	**PX103808**
Usnea erinacea s.lat.	AG4894	Brazil	Gerlach 1498(ICN)	PRO	Gerlach et al. 2019	3	KY021910	KY204419	KY204440
Usnea esperantiana	DN100	Chile	G00598617	SAL	**This study**	3	**PX105447**	**PX095197**	**PX103805**
Usnea eulychniae	180	Chili	G00599346	THA	**This study**	3	**PX105449**	**PX095168**	**n/a**
Usnea flammea	AG230	Spain	Aptroot 75512 (G)	STI, LOB	Gerlach et al. 2019	3	MF669890	MT439176	MW232045
Usnea flavocardia s.str.	YO7008A	Taiwan	Ohmura 7008 (TNS)	PSO	**This study**	3	**PX105470**	**PX095189**	**PX103828**
Usnea flavocardia s.str.	YO8636A	Taiwan	Ohmura 8636A (TNS)	PSO	**This study**	2	**PX105476**	**PX095196**	**n/a**
Usnea flavocardia s.lat.	AG373	Brazil	Gerlach (G)	FA	**This study**	3	**PX105483**	**PX095203**	**PX103839**
Usnea flavocardia s.lat.	AG412	Brazil	Gerlach (G)	PSO	**This study**	3	**PX105467**	**PX095186**	**PX103825**
Usnea flavocardia s.str.	DN2	Portugal	G00563681	PSO	**This study**	3	**PX105463**	**PX095182**	**PX103821**
Usnea flavocardia s.str.	DN3	Portugal	G00563681	PSO	**This study**	3	**PX105466**	**PX095185**	**PX103824**
Usnea flavocardia s.str.	DN4	Portugal	G00563682	FA	**This study**	3	**PX105468**	**PX095187**	**PX103826**
Usnea flavocardia s.lat.	DN7	Australia	G00584343	FA	**This study**	3	**PX105492**	**PX095212**	**PX103846**
Usnea flavocardia s.lat.	DN8	Australia	G00584344	FA	**This study**	3	**PX105493**	**PX095214**	**PX103848**
Usnea flavocardia s.lat.	DN9	Australia	G00584342	FA	**This study**	3	**PX105494**	**PX095215**	**PX103849**
Usnea flavocardia s.str.	DN13	Switzerland	G00563771	PSO	**This study**	3	**PX105453**	**PX095172**	**PX103812**
Usnea flavocardia s.str.	DN14	Switzerland	G00563771	PSO	**This study**	3	**PX105454**	**PX095173**	**PX103813**
Usnea flavocardia s.str.	DN19	Macaronesia	G00563740	PSO	**This study**	3	**PX105460**	**PX095179**	**PX103818**
Usnea flavocardia s.str.	DN20	Macaronesia	G00563735	PSO	**This study**	3	**PX105461**	**PX095180**	**PX103819**
Usnea flavocardia s.str.	DN22	Macaronesia	G00563776	PSO, BAR	**This study**	3	**PX105462**	**PX095181**	**PX103820**
Usnea flavocardia s.str.	DN33	France	G00598047	PSO	**This study**	3	**PX105464**	**PX095183**	**PX103822**
Usnea flavocardia s.str.	DN35	France	G00598048	PSO	**This study**	3	**PX105465**	**PX095184**	**PX103823**
Usnea flavocardia s.lat.	DN45	Costa Rica	G00560467	SAL	**This study**	2	**PX105496**	**PX095218**	**n/a**
Usnea flavocardia s.lat.	DN46	Costa Rica	G00560469	SAL	**This study**	2	**PX105497**	**PX095219**	**n/a**
Usnea flavocardia s.str.	DN58	Macaronesia	G00598036	PSO	**This study**	3	**PX105469**	**PX095188**	**PX103827**
Usnea flavocardia s.lat.	DN64	New-Zealand	OTA 071024	NOR, FA	**This study**	3	**PX105491**	**PX095211**	**PX103845**
Usnea flavocardia s.lat.	DN69	Chile	G00598134	FA	**This study**	3	**PX105484**	**PX095204**	**PX103840**
Usnea flavocardia s.str.	DN75	Chile	G00598315	NOR	**This study**	3	**PX105471**	**PX095190**	**PX103829**
Usnea flavocardia s.str.	DN76	Czech Republ.	Šoun & Strelbová 2019 (MBH)	FA	**This study**	3	**PX105472**	**PX095191**	**PX103830**
Usnea flavocardia s.str.	DN77	United States	G00563950	NOR	**This study**	3	**PX105473**	**PX095192**	**PX103831**
Usnea flavocardia s.lat.	DN78	United States	G00563950	FA	**This study**	3	**PX105485**	**PX095205**	**PX103841**
Usnea flavocardia s.str.	DN79	United States	G00563949	NOR	**This study**	2	**n/a**	**PX095193**	**PX103832**
Usnea flavocardia s.lat.	DN80	New-Zealand	OTA 071316	NOR	**This study**	2	**n/a**	**PX095213**	**PX103847**
Usnea flavocardia s.str.	DN82	Chile	G00598131	NOR	**This study**	3	**PX105474**	**PX095194**	**PX103833**
Usnea flavocardia s.str.	DN83	Chile	G00598129	NOR	**This study**	3	**PX105475**	**PX095195**	**PX103834**
Usnea flavocardia s.lat.	DN87	Chile	G00598813	SAL	**This study**	2	**PX105498**	**PX095220**	**n/a**
Usnea flavocardia s.str.	DN93	Chile	G00563944	THA	**This study**	3	**PX105477**	**PX095197**	**PX103835**
Usnea flavocardia s.lat.	DN99	Chile	G00599002	STI	**This study**	3	**PX105478**	**PX095198**	**n/a**
Usnea flavocardia s.str.	DN105	Chile	G00598308	PSO	**This study**	3	**PX105450**	**PX095169**	**PX103809**
Usnea flavocardia s.str.	DN106	Chile	G00598308	NOR	**This study**	3	**PX105451**	**PX095170**	**PX103810**

Table 1. Continued.

Species	Lab number	Origin	Voucher/Code herbier	Chemotype	Done by	Available loci (nb)	GenBank/ENA accession numbers		
							ITS	*mcm7*	*rpb1*
Usnea flavocardia s.str.	DN107	Chile	G00599020	PSO	**This study**	3	**PX105452**	**PX095171**	**PX103811**
Usnea flavocardia s.lat.	DN108	Chile	G00598997	SAL	**This study**	2	**PX105495**	**PX095217**	**n/a**
Usnea flavocardia s.lat.	DN112	Brazil	G00562768	FA	**This study**	2	**PX105482**	**PX095202**	**n/a**
Usnea flavocardia s.str.	DN157	Netherlands	v. d. Pluijm n°3372	PSO	**This study**	3	**PX105455**	**PX095174**	**PX103814**
Usnea flavocardia s.str.	DN159	Tanzania	SGT 326/b (G)	PSO	**This study**	3	**PX105456**	**PX095175**	**PX103815**
Usnea flavocardia s.str.	DN160	Chile	G00563944	NOR	**This study**	3	**PX105457**	**PX095176**	**PX103816**
Usnea flavocardia s.str.	DN162	Canada	G00584075	NOR	**This study**	3	**PX105458**	**PX095177**	**PX103817**
Usnea flavocardia s.str.	DN181	Chile	G00598257	NOR, STI	**This study**	2	**PX105459**	**PX095178**	**n/a**
Usnea flavocardia s.lat.	DN182	Chile	G00598781	FA	**This study**	2	**PX105489**	**PX095209**	**n/a**
Usnea flavocardia s.lat.	DN183	Chile	G00598781	FA	**This study**	2	**PX105490**	**PX095210**	**n/a**
Usnea gaudichaudii	117	Chile	G00599378	NOR, DIF	**This study**	3	**PX105479**	**PX095199**	**PX103836**
Usnea gaudichaudii	118	Chile	G00599372	DIF	**This study**	3	**PX105480**	**PX095200**	**PX103837**
Usnea gaudichaudii	119	Chile	G00599371	DIF	**This study**	3	**PX105481**	**PX095201**	**PX103838**
Usnea geissleriana	AG238	Spain	Aptroot 75487 (G)	SAL, NOR	Gerlach et al. 2019	3	MF669934	MT439182	MW232049
Usnea lusitanica ad int.	DN123	Portugal	G00599762	STI	**This study**	3	**PX105486**	**PX095206**	**PX103842**
Usnea lusitanica ad int.	DN124	Portugal	G00599763	STI	**This study**	3	**PX105487**	**PX095207**	**PX103843**
Usnea lusitanica ad int.	DN128	Portugal	G00599768	STI	**This study**	3	**PX105488**	**PX095208**	**PX103844**
Usnea moreliana	AG84	Brazil	Gerlach (G)	TER	Gerlach et al. 2019	3	MF669893	MW273721	MW273458
Usnea moreliana	AG100	Brazil	Gerlach (G)	TER	Gerlach et al. 2019	3	MF669896	MW273723	MW273461
Usnea oreophila	AG7	Brazil	Gerlach & Penati 2016/P33 (G)	SQU	Gerlach et al. 2019	3	MF669913	MK636593	MK636600
Usnea oreophila	AG8	Brazil	Gerlach & Penati 2016/P33 (G)	SQU, BAR, THA	Gerlach et al. 2019	3	MF669914	MK636594	MK636601
Usnea oreophila	AG141	Brazil	Gerlach & Penati 2016/P33 (G)	SQU	Gerlach et al. 2019	3	MF669915	MK636595	MK636602
Usnea oreophila	AG142	Brazil	Gerlach & Penati 2016/P36 (G)	SQU	Gerlach et al. 2019	3	MF669916	MK636596	MK636606
Usnea perhispidella	AG41	Brazil	Gerlach & Penati (G)	STI	Gerlach et al. 2019	3	MF669883	MT439211	MW232077
Usnea perhispidella	AG82	Brazil	Gerlach P55 (G)	STI	Gerlach et al. 2019	3	MF669898	MT439212	MW232078
Usnea perhispidella	AG155	Brazil	Gerlach & Penati 2016/P58 (G)	STI	Gerlach et al. 2019	3	MF669899	MW273727	MW273463
Usnea perhispidella	AG159	Brazil	Gerlach & Penati 2016/P1-13(G)	STI	Gerlach et al. 2019	3	MF669900	MW273728	MW273464
Usnea perhispidella	AG165	Brazil	Gerlach & Penati 2016/P1-17 (G)	STI	Gerlach et al. 2019	3	MF669902	MW273729	MW273465
Usnea perhispidella	AG166	Brazil	Gerlach & Penati 2016/P1-17 (G)	STI	Gerlach et al. 2019	3	MF669903	MW273730	MW273466
Usnea perhispidella	AG178	Brazil	Gerlach & Penati 2016/P1-4 (G)	STI	Gerlach et al. 2019	3	MF669904	MW273731	MW273467
Usnea perhispidella	AG179	Brazil	Gerlach & Penati 2016P1-5 (G)	STI	Gerlach et al. 2019	3	MF669905	MW273732	MW273468
Usnea rubicunda	AG60	Brazil	Gerlach & Penati 2016/P19 (G)	STI	Gerlach et al. 2019	3	MF669908	MW2737333	MW273470
Usnea rubicunda	AG61	Brazil	Gerlach 2016/P30 (G)	STI	Gerlach et al. 2019	3	MF669909	MW273734	MW273471
Usnea rubicunda	AG207	Brazil	Gerlach & Penati 2016/P1-3 (G)	TER	Gerlach et al. 2019 **& this study**	3	MF669910	**PX095216**	**PX103850**
Usnea rubicunda	AG235	Spain	Aptroot 75478 (G)	STI	Gerlach et al. 2019	3	MF669911	MT439213	MW232079
Usnea rubicunda	AG4890	Brazil	Alves s.n. (ICN)	SAL	Gerlach et al. 2019	3	KY021923	KY204428	KY204445
Usnea rubicunda s.lat.	AG184	Brazil	Gerlach 2016/P30 (G)	STI	Gerlach et al. 2019	3	MF669912	MW273735	MW273472

Usnea rubicunda s.lat.	AG4807	Brazil	Gerlach et al. 1499 (ICN)	STI	Gerlach et al. 2019	3	KY021925	KY204430	KY204447
Usnea rubicunda s.lat.	AG4891	Brazil	Gerlach et al. 1497 (ICN)	STI	Gerlach et al. 2019	3	KY021924	KY204429	KY204446
Usnea steineri	AG97	Brazil	Gerlach (G)	TER	Gerlach et al. 2019	3	MF669930	MW273749	MW273484
Usnea subaranea	CT123	Ecuador	Truong 313 (G)	USN	Gerlach et al. 2019	3	JQ837292	JQ837337	JQ837416
Usnea subdasaea	AG40	Brazil	Gerlach P1 (G)	GAL	Gerlach et al. 2019	3	MF669882	MT439221	MW232086
Usnea subdasaea	AG4806	Brazil	Magnago 1099 (G)	GAL	Gerlach et al. 2019	3	MF669931	MT439222	MW232087
Usnea subflammea s.lat.	AG65	Brazil	Gerlach & Penati P1-2 (G)	STI	Gerlach et al. 2019	3	MF669932	MT439224	MW232089
Usnea subflammea s.lat.	AG66	Brazil	Gerlach 2016/P39 (G)	STI	Gerlach et al. 2019	3	MF669933	MW273750	MW273485
Usnea subflammea s.lat.	AG239	Spain	Aptroot 75474 (G)	STI	Gerlach et al. 2019	3	MF669935	MT439223	MW232088
Usnea subscabrosa	AG67	Brazil	Gerlach & Penati 2016/P19 (G)	PRO	Gerlach et al. 2019	3	MF669936	MW273751	MW273486
Usnea subscabrosa	AG212	Brazil	Fazolino (G)	PRO	Gerlach et al. 2019	3	MF669937	MT439225	MW232090
Usnea viktoriana	DIP_15	Switzerland	Tõrra, 05.02.2011 (TU)	ALE	Mark et al. 2016	3	KU352628	KU352188	KU352034
Usnea viktoriana	SUB 60	Italy	Tõrra, 09.06.2010 (TU)	ALE	Mark et al. 2016	3	KU352663	KU352244	KU352089
Usnea viktoriana	SUB 53	Switzerland	Tõrra, 17.05.2011 (TU)	ALE	Mark et al. 2016	3	KU352661	KU352242	KU352087
Usnea wasmuthii	wasm 05	UK	Tõrra, 07.10.2006 (TU)	BAR, SAL	Saag et al. 2011	3	JN086334	KU352260	KU352104
Usnea wasmuthii	wasm 09	UK	Tõrra, 07.10.2006 (TU)	BAR	Saag et al. 2011	3	JN086337	KU352107	KU352107
Total							126 (98.4%)	126 (98.4%)	115 (89.8%)

Chemical analyses

In *Usnea*, thin-layer chromatography (TLC) is a major tool for species identification. Secondary metabolites of all specimens studied and cited in this paper were determined by TLC based on standard procedures using solvent systems A, B and C (Culberson & Ammann 1979), with solvent B modified following Culberson & Johnson (1982).

DNA extraction and amplification and alignments

DNA was extracted from a total of 54 specimens: *Usnea flavocardia* (45 specimens), *U. gaudichaudii* and *U. lusitanica* ined. (3 specimens each), *U. eulychniae*, *U. esperantiana* and *U. dasaea* (1 specimen each). In each case, an entire branch of the specimen (algal and fungal tissues) was used for DNA extraction. We applied a modified protocol from Zolan & Pukkila (1986), i.e., an SDS-Phenol-Chloroform protocol with 2% sodium dodecyl phosphate (SDS) to break the cell walls and a Phenol:Chloroform:IAA mix (15:24:1) to remove proteins. We also used 3 DNA extracts from *U. erinacea* (2 specimens) and *U. rubicunda* (1 specimen; Table 1), previously obtained by Gerlach et al. (2019) for which only the Internal Transcribed Spacer (ITS) was sequenced.

For the molecular studies we used three markers that already showed good efficacy in separating the different clades within the subgenus *Usnea* s.str.: ITS, a protein-coding gene (*mcm7*) and the largest subunit of RNA polymerase II (*rpb1*) (Truong et al. 2013a; Mark et al. 2016; Truong & Clerc 2016; Gerlach et al. 2017, 2019; Table S1). For ITS, 54 PCR amplifications were performed using Us*ITS*4-R and Us*ITS*3-F primers (Truong et al. 2013a). A total of 57 PCR amplifications were processed for each of *mcm7* using Us*MCM7*-R and Us*MCM7*-F primers (Gerlach et al. 2017) and *rpb1* using primers Us*RPB1*-R/Us*RPB1*-F (Gerlach et al. 2017) and primers Rpf-Usn 2R/Rpf-Usn 3F (Mark et al. 2016). Amplified products were checked on a 1.5 or 2% agarose gel, stained with SYBER Green I (Thermo Fisher Scientific) and with a migration time ranging between 30 min. and 1 hour, depending on the fragment length. The PCR products were then purified using NucleoFast© plates (Macherey-Nagel) before being sent to Macrogene Europe for Sanger sequencing. PCR parameters were the same as in Gerlach et al. (2019) for all primer pairs, except for Rpf-Usn 2R/Rpf-Usn 3F for which we followed Mark et al. (2016) recommendations. All the amplification conditions are given in Table S1.

The DNA sequences were assembled and corrected in Sequencher software (Nishimura 2000). The alignment of DNA sequences was next generated using the *ClustalW Multiple Alignment* with the default settings, as implemented in BioEdit (Hall 1999), then manually corrected. The corrected ITS alignment was quality-checked using the GUIDANCE online server (Penn et al. 2010) which issued a good score and validated the alignment.

Species delimitation analysis

To estimate the potential number of species, we used STACEY version 1.2.5 (Jones et al. 2015; Jones 2017),

a multispecies coalescent approach (MSC, Yang & Rannala 2010), as implemented in BEAST 2.6.2 (Bouckaert et al. 2014). We used *U. subaranea* Truong & P. Clerc to root the tree according to Truong et al. (2013a) since it belongs to the basal clade of the subgenus *Usnea* s.str. (Clade I). Compared to other MSC approaches, STACEY considers each specimen as a putative species, thus avoiding improper assignment of individual sequences to species that could seriously bias the analysis outcome. This is a great advantage when the studied group is taxonomically poorly understood, as in lichens for which STACEY has been successfully used in different studies (e.g., Kanz et al. 2015; Mark et al. 2016; Gerlach et al. 2019; Boluda et al. 2019, 2021). STACEY analysis eventually builds MSC clusters (MSCC) on pairwise probabilities that two individuals fall within the same cluster. The xml input file was prepared with the STACEY template supplied by BEAUTI2 (Bouckaert et al. 2014). Substitution models were determined using MEGA X v10.0.5 (Kumar et al. 2018) as TN93 for ITS and JC69 for *mcm7* and *rpb1*, respectively. We assumed a *relaxed exponential molecular clock* with a *clock rate* of 1.0, an offset of 0.0, and lower and upper bounds of 0.0 and 5.0, respectively. The species tree priors were set as follows: birth-death model with *collapse height* (ε) of 1.10^{-4} and a *relative death rate* of 0.5, as recommended by Jones (2016). For the growth rate (bdcGrowthRte.t:Species), we chose a log-normal distribution with a mean of 1 and a standard deviation of 1.25. The initial value for node collapse weight (ω) was set at 0.5 according to a beta distribution [0.0, 1.0], with alpha and beta parameters set at 1.0. Population size was set to [0.0, 2000], under popPriorScale, adding a Gamma distribution with parameters alpha = 2 and beta = 259. All the other parameters were set as default.

The input xml file was run for 200 million generations on three parallel chains, by sampling every 10,000 log and 100,000 trees. Chain convergence was checked using Tracer v1.7.1 (Rambaut et al. 2018). Species and genes trees were combined using *Log Combiner* and summarized using *Tree Annotator* v2.6.0 using a 10% burn-in. The trees were viewed with Figtree v1.4.3 (Rambaut 2017). The resulting species tree file was run with *Species Delimitation Analyser* (SDA; Jones et al. 2015; Jones 2016) to calculate the probability that pairs of specimens belong to the same MSC cluster. We defined 10% of burn-in, 1.10^{-4} of collapse height and 1.0 similarity cut off. The output file was uploaded on RStudio v1.1.456 (R Team 2016) using the script provided by Jones et al. (2015) and modified by Simon Crameri (https://github.com/scrameri/smtools/tree/master/SpeciesDelimitation) to get the similarity matrix.

Results

Sequence data

In this study, we generated 153 new sequences for the genus *Usnea* from 57 specimens. A total of 13 sequences failed (two and eleven for ITS and *rpb1*, respectively). Among those, 123 were obtained from the 45 specimens of *U. flavocardia* (73.3% of which were sequenced for three loci and 22.2% for two loci). Twenty-six (26) sequences were obtained from *U. gaudichaudii*, *U. lusitanica*, *U. dasaea U. eulychniae* and *U. esperantiana* (three, three, one, one and one specimen, respectively) with only one *rpb1* failed sequence for *U. eulychniae*. The remaining 4 sequences were obtained from the DNA extracts of Gerlach et al. (2019) and helped to provide sequences for three loci for most specimens (Table 1). We obtained at least two sequences for 98.4% of our specimens, except for the South American taxon *U. durietzii* since only the ITS sequence was available on GenBank for these. Overall, 88.3% of the specimens have three markers (113 specimens), 10.2% display two markers (13 specimens) and 1.5% display only one marker (2 specimens). Among the 128 specimens analyzed, 1.6% are missing ITS and *mcm7*, and 10.2% *rpb1* (Table 1). All sequences were deposited in GenBank repository under accession numbers PX105447-PX105498, PX095166-PX095220 and PX103805-PX103850 for ITS, *mcm7* and *rpb1*, respectively. The data matrix consists of 505 nucleotide characters for ITS, 439 for *mcm7* and 533 for *rpb1*, respectively.

Gene trees, species trees and supported lineages

In our study, we discovered a new highly supported clade (Posterior Probabilities (PP) = 1), made up of two species, *Usnea gaudichaudii* and *U. eulychniae*, endemic to Chile, here named as USNEA-5 (Fig. 1 and Fig. S1) distinct from USNEA-1, USNEA-2, USNEA-3 and USNEA-4 of Truong et al. (2013a).

Our study additionally shows that all specimens identified as *U. flavocardia*, as well as both specimens of *U. durietzii*, fall within USNEA-3 according to Truong et al. (2013a).

Within *U. flavocardia*, at least four different lineages were detected with high posterior probabilities (PP ≥ 0.96; lineages 1, 2, 3 and 4) (Fig. 1). Lineages 1 and 2 form a monophyletic clade (PP = 1) with which the relationship with lineages 3 and 4 is not resolved. One additional clade, lineage 5, is not supported (PP < 0.8). The topologies of individual gene trees were generally in agreement with each other and with that of the species tree with the notable exception of lineage 5 (Figs S2–S4). This lineage is only supported for *mcm7* (PP = 0.97). The specimens with psoromic acid (*U. wirthii*) and with norstictic acid (*U. flavocardia* s.str.) were found together within lineage 1.

Species delimitation

The similarity matrix (Fig. 2) gives the posterior probability that two individuals belong to the same Multi-Species Coalescent cluster (MSCC) according to the species tree of Fig. 1. The five lineages highlighted above are confirmed here. All of the samples of lineage 1 display high probabilities to belong to lineage 1, except for two of them (*U. flavocardia*_DN181 and *U. flavocardia*_DN106) for which the probabilities are lower. Still, they were not attributed to any other MSCC. In lineage 2, 4 and 5, two or more sub-clusters were evidenced, whereas

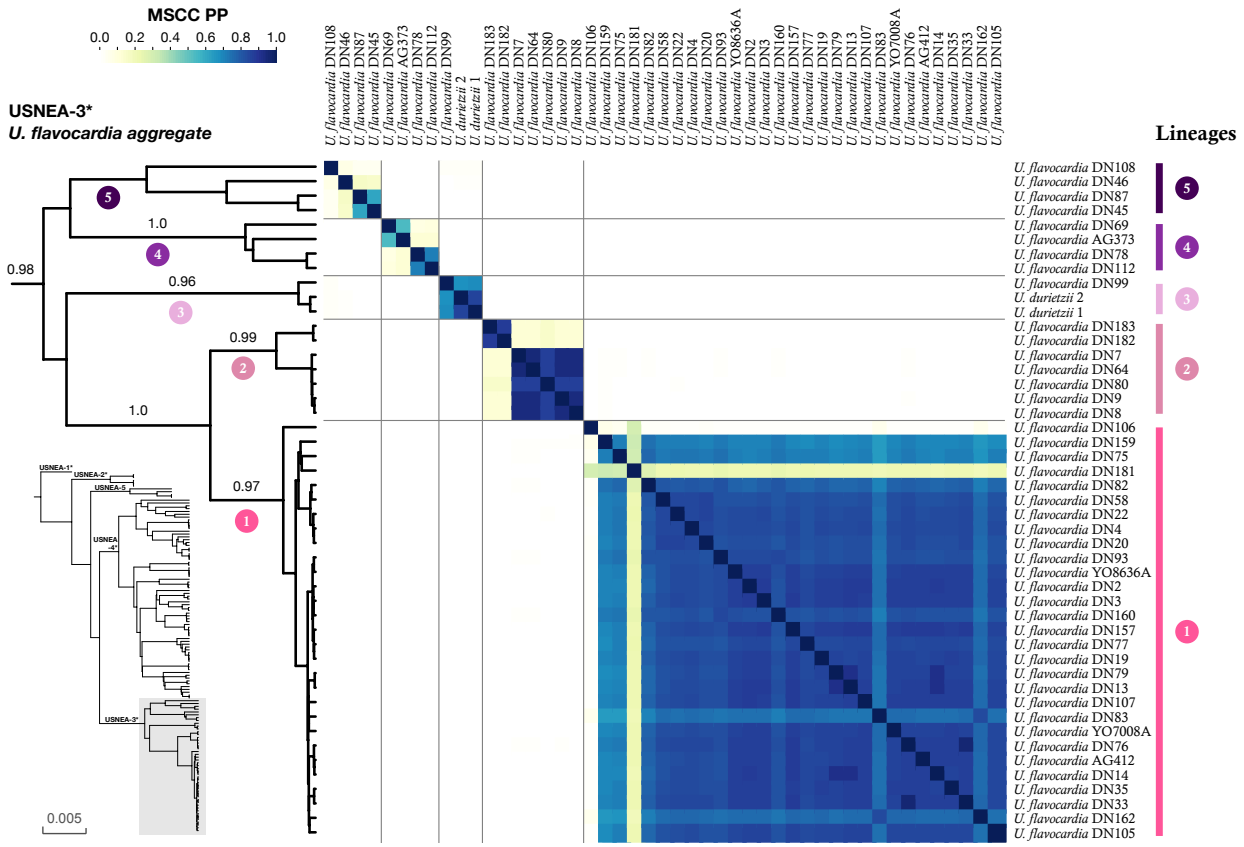

Figure 2. Similarity matrix from STACEY analysis for the *Usnea flavocardia* aggregate with 47 specimens. The squares represent posterior probabilities (white = 0, dark blue = 1) that two individuals belong to the same Multi-Species Coalescent cluster (MSCC) according to species tree of Figure 1 also represented on the left. Lineages are numbered from 1 to 5 according to the text. The whole phylogeny is inserted on the lower left side with the clade names after Truong et al. (2013a) and the USNEA-3 clade highlighted in grey.

lineage 3 corresponds to a MSCC to which all individuals are strongly attributed.

A putative correlation between lineages and specific chemotypes

Usnea flavocardia is shown to be highly diverse from the chemical point of view. The major secondary metabolites found in this aggregate belong to depsidones (salazinic and norstictic acids) and β-orcinol depsidones (psoromic acid). Other secondary compounds such as depsides (barbatic acid) and β-orcinol depsides (thamnolic acid,) are present in some samples. The latter two are new chemotypes for *U. flavocardia*, as well as a set of four different new and unknown fatty acids: FA1 (Rf classes values: A:5, B:3-4, C:5), FA2 (A:5-6, B:6-7, C:5-6), FA3 (A:6, B:5, C:5-6) and FA4 (A:1-2, B:2, C:2).

We also found a putative correspondence between the different lineages and their chemistry pattern (Fig. 1). According to our sampling, each lineage is characterized by a specific chemistry except lineage 1 (29 specimens), which may include at least six different chemotypes, namely the psoromic acid chemotype (PSO ± FA1; 16 specimens; 55%), the norstictic acid chemotype (NOR ± FA1; 8 specimens; 27.5%), the thamnolic-norstictic acid chemotype (THA-NOR; 1 specimen; 3.5%), the stictic acid chemotype (STI; 1 specimen; 3.5%), the psoromic-barbatic acids chemotype (PSO-BAR, 1 specimen; 3.5%) and a fatty acid chemotype (FA1; 2 specimens; 7%).

Lineage 2 (7 specimens) includes specimens with two different fatty acids (FA3 and FA4) which can be accompanied by norstictic acid (NOR). Lineage 3 (3 individuals) contains one individual with stictic acid and the remaining two belonging to *U. durietzii* whose chemotypes were not available as the sequences were retrieved from GenBank. Lineage 4 (4 specimens) displays a unique fatty acid chemotype profile (FA2). The phylogenetically weakly supported lineage 5 (4 specimens) includes specimens with salazinic acid (SAL) only.

In our study, norstictic acid is present only in specimens from South America (60% of the 10 specimens collected in South America) and from North America (100% of the 3 specimens collected in North America). The psoromic acid chemotype is predominantly present in specimens from Europe (78% of the 9 specimens collected in Europe), Macaronesia (100% of the 4 specimens collected in Macaronesia), Asia (100% of the 2 specimens collected in Asia) and Africa (one specimen collected), but also occurs in South American specimens (30% of the 10 specimens collected in South America). The stictic acid chemotype, new to *U. flavocardia*, seems to be rare in this aggregate since it was only found in two specimens from South America. The fatty acid FA1 was found either alone (2 specimens from Europe) or occurring together with psoromic acid (3 specimens from Macaronesia and 1 from South America) or with norstictic acid (1 specimen from South America). Barbatic and

thamnolic acids were newly found in *U. flavocardia*, associated with psoromic acid (1 specimen) in Macaronesia, and with norstictic acid in South America (Chile; 1 specimen), respectively.

Discussion

As for the *Usnea cornuta* group (Gerlach et al. 2019), the use of molecular sequences has revealed an unexpected genetic diversity within a group previously thought to be a single species, namely *U. flavocardia*. This genetic diversity was first suggested by Lücking et al. (2020) in their *Usnea* phylogeny, using published ITS data from specimens collected worldwide, showing a main *Usnea flavocardia* clade with European and Macaronesian specimens (lineage 1 in this study) sister of a clade containing a specimen from New Zealand (probably lineage 2 in this study). With our sampling, *U. flavocardia* now includes five different lineages, four of which are highly supported. Henceforth, we will refer to it as the *U. flavocardia* aggregate. As with the *U. cornuta* group, chemotypes correlate well with the different lineages, at least in our sampling, and can be suggested as diagnostic characters that should further be confirmed. Once again, this result confirms that, specifically in difficult groups such as the genus *Usnea*, phylogenetics is a necessary tool to delineate species, together with morphological, anatomical, chemical and ecological data.

An unexpected new clade within *Usnea*

The two species *Usnea gaudichaudii* and *U. eulychniae*, morphologically very different from *U. flavocardia*, were sequenced for the first time in this study to determine their placement within *Usnea*. The species tree (Fig. S1) shows that they belong to a new strongly supported clade, here named USNEA-5, adding to the four clades already known from the molecular phylogeny of the genus *Usnea* (Truong et al. 2013a), albeit with unresolved relationship to the other clades. *Usnea gaudichaudii* and *U. eulychniae* were originally described from specimens collected in the Atacama Desert along the coasts of northern Chile (Motyka 1936–1938; Follmann 1967). Although *U. gaudichaudii* is mentioned as having been found in Venezuela and Brazil (Motyka 1936–1938; Consortium of Lichen Herbaria 2025), these reports were most likely based on false determinations. Both species are endemic to the Atacama Desert and live in one of the driest and probably oldest deserts on Earth (Jung et al. 2019). *Usnea gaudichaudii* is a short, shrubby species with numerous apothecia and branches that are purple red pigmented in their terminal parts, a unique color in the genus. It produces diffractaic and barbatic acids in the medulla. *Usnea eulychniae* is a subpendulous to pendulous taxon with capillaceous terminal parts, small punctiform soralia and thamnolic acid in the medulla. Both species grow among a rich biological community, mostly on candelabriform or arborescent cacti of the genus *Eulychnia* living in a narrow coastal desert strip strongly influenced by frequent maritime orographic fogs, called the Desert Fog zone (Rundel 1978). This peculiar endemism, linked to a very specific habitat, most probably resulted from strong genetic isolation leading to the differentiation of this highly supported fifth clade in the genus. Another example of such isolation due to similar extreme conditions in South America is given with the genus *Cenozosia*, a fruticose genus belonging to the *Ramalinaceae*, whose species are endemic to the Atacama Desert Fog zone and furthermore associated with a *Trebouxia* photobiont that was recently demonstrated to also constitute a newly identified cluster within the Trebouxioid algae (Jung et al. 2023).

Usnea flavocardia Räsänen vs *U. wirthii* P. Clerc

One of our objectives in this work was to determine whether the molecular data confirms the synonymy of *Usnea wirthii* (chemotype psoromic acid) under *U. flavocardia* s.str. (chemotype norstictic acid) (Clerc 2004). The species tree (Fig. 1) and the similarity matrix (Fig. 2) both show that no significant difference exists between the two taxa since they form a highly supported clade (PP = 0.97 for the species tree) coined as lineage 1. We were not able to sequence the type specimens of *U. flavocardia* and *U. wirthii*, but morphological and anatomical studies, not presented here, as well as chemical evidence strongly suggest that lineage 1 is conspecific with *U. flavocardia* Räsänen s.str. (Clerc 1984, 1997, 2004; Clerc & Otte 2018; Clerc & Kissling 2019). Lineage 1, indeed, contains specimens DN33 and DN35 with psoromic acid collected in South France previously identified as *U. wirthii* (*U. wirthii* could not be found in the vicinity of its type locality) and specimens DN82, 83 with norstictic acid collected in Corral (Chile), the type locality of *U. flavocardia* s.str. Therefore, we consider that lineage 1 corresponds to *U. flavocardia* s.str. in the following and confirm the genetic proximity of *U. wirthii* with the former species.

The *Usnea flavocardia* aggregate contains five lineages that are well characterized by their chemistry

All of the specimens of the *U. flavocardia* aggregate used in this study fall within a single highly supported clade that also contains the saxicolous taxon *U. durietzii* which was not initially thought to belong to this aggregate. The *U. flavocardia* aggregate belongs to the USNEA-3 clade as defined by Truong et al. (2013a). The relationships between the five different lineages are poorly resolved as shown in the species tree (Fig. 1). This may be due to various factors such as the lack of information within the sequenced markers, incongruent histories among loci, and/or a recent diversification of this aggregate, leading to ILS. Whereas, the first cause could be leveraged using more genes, the two others (Naciri & Linder 2015) might be intimately related to the diversification process itself and its rate. In that latter case, it is expected that adding more genes would not necessarily help resolve the deeper relationships between the different lineages, as it is often recorded in radiating lineages (Naciri & Linder 2020).

Usnea flavocardia Räsänen has often been noted for its chemical diversity with norstictic, galbinic, psoromic, salazinic and stictic acids (Clerc 2004; Truong et al. 2011;

Ohmura 2014). Our results confirm this chemical variability and furthermore show that a good correspondence can be found between the lineages and particular chemotypes. Such a correlation between chemistry and genetics has already been described by Gerlach et al. (2019) in the *U. cornuta* group.

Lineage 1 (*Usnea flavocardia* s.str.)

Our sampling was biased toward Chile and Europe since the aim of this study was to compare *U. flavocardia* described in Chile with *U. wirthii* described in Europe. Lineage 1 predominates as it contains, by far, most of the *U. flavocardia* aggr. specimens analyzed in this study (29 specimens; 64% of the sequenced specimens; Fig. 1). The species tree (Fig. 1) and the similarity matrix (Fig. 2) show that this lineage is quite homogeneous, with all the specimens displaying high probabilities of falling within the same cluster, except for two Chilean specimens, DN106 containing norstictic acid and FA1, and DN181 containing stictic acid (Fig. 2). Lineage 1 contains the highest chemical and geographic diversities among the *U. flavocardia* aggr. with six chemotypes associated with an almost worldwide distribution (Brazil, Chile, USA, Canada, Canary Islands, Portugal, France, the Netherlands, Switzerland, Taiwan and Tanzania).

Three out of these six chemotypes have previously been reported from *Usnea flavocardia* s.str. (Syn. *U. wirthii*): 1. psoromic acid ± FA1 (Clerc 1984), 2. norstictic ± FA1 (Clerc 1984, 2004; Clerc & Diederich 1991; Ohmura 2014) and 3. stictic acid, a chemotype that was first reported from South America by Truong et al. (2011). Although the probability of its belonging to lineage 1 is low according to the STACEY matrix (Fig. 2), the specimen DN106 (norstictic acid, FA1) has no obvious anatomical, morphological or chemical character differentiating it from other specimens of lineage 1. Specimen DN181 collected in central Chile (Maule), also with an unusual chemistry for lineage 1 (stictic acid), likewise shows no morphological or anatomical differences from *U. flavocardia* s.str. Further studies using additional Chilean specimens would therefore be necessary to unravel the status of this stictic acid chemotype and possibly gain more information with further specimens sharing the same genetics as DN106.

Three further chemotypes are found in lineage 1 that have not been reported so far in *U. flavocardia* s.str.: 1. psoromic and barbatic acids, 2. psoromic and thamnolic acids and 3. the fatty acid FA1. Barbatic and thamnolic acids are new substances in *U. flavocardia* s.str. Barbatic acid was found together with psoromic acid in a specimen collected on the Canary Islands (specimen DN22). Complementary chemical analyses (P. Clerc, unpublished results) show that this chemotype is frequent and restricted to Macaronesia. Thamnolic acid was found only in one locality situated in the Atacama Desert (Chile) in the fog oasis "Las Lomitas" within the National Park "Pan de Azúcar" (specimen DN93). Finally, psoromic acid and the fatty acid FA1 are found exclusively in lineage 1 and seem to be diagnostic of this lineage.

Lineage 1 occurs on all continents apart from Oceania (Fig. 1). As previously noted, it contains specimens collected in Central and South America (10 specimens), Europe (9), Macaronesia (4), North America (3), Asia (2) and Africa (1). The number of specimens studied is not sufficient to draw any definitive conclusions, however some geographical trends can already be highlighted within lineage 1 with the norstictic acid chemotype present only in South and North America, while the psoromic acid chemotype is predominantly present in Europe, but also occurring in South America, Macaronesia, Asia and Africa. This chemical pattern is not reflected in the genetic analysis after discarding DN181 and DN106. As a matter of fact, STACEY analysis showed a fairly homogenous MSC cluster. This could be due to ILS, a recurrent problem with species delimitation (Naciri & Linder 2015). This happens when the speciation is very recent and when the effective population sizes are important (Naciri & Linder 2015). Rosenberg (2003) suggested that 5.3 N_e generations are required for a lineage to acquire the monophyly of 99% of its loci. Given the worldwide distribution of Lineage 1, we might hypothesize that the effective population size of lineage 1 is high resulting in putative strong ILS within the lineage and in weakly supported relationships among specimens (Naciri & Linder 2015). The lack of geographical or chemical patterns within Lineage 1 could therefore be due to a recent diversification associated with high effective population sizes mediated by transoceanic long-distance dispersal (LDD) of vegetative diaspores (isidiomorphs, soredia, thallus fragments) via winds or birds as it has been described for other lichen species (Galloway & Aptroot 1995; Muñoz et al. 2004; Geml et al. 2010; Lewis et al. 2014). There is indeed evidence in the *Parmeliaceae* that transoceanic LDD events occur and that they have played a major role in species diversification within this family (Amo de Paz et al. 2011; Fernández-Mendoza et al. 2011). Alternatively, the used loci might lack resolution at the scale of this rapid diversification or might not be the most appropriate ones to highlight such a signal, if it exists, failing at showing a correlation between chemotypes and genetics.

In summary, Lineage 1 conforms well with *U. flavocardia* s.str. with both the psoromic and norstictic acid chemotypes. Moreover, the placement of *U. wirthii* within *U. flavocardia* (Clerc 2004) is here confirmed. Using other markers such as RAD sequences, for instance (Grewe et al. 2018), would be necessary to further characterize this lineage and find putative signs of differentiation.

Lineage 2

Seven specimens (16%) of the *U. flavocardia* aggr. collected in Australasia and South America are included in lineage 2. This clade is strongly supported and appears sister to lineage 1 (Fig. 1). It is characterized by the presence of fatty acids FA3 and FA4. Neither FA3 or FA4 occur in another lineage of this aggregate. In two specimens (29%), norstictic acid co-occurs with FA3 or FA4. Lineage 2 has an intercontinental distribution, although restricted to the southern hemisphere where the two subclades (Figs 1, 2) show a clear geographic separation between Chile (2 specimens) and Oceania (5). A specimen collected by H.U. Stauffer in South Africa (G261564) was

identified as belonging to this lineage, based on anatomical, morphological and chemical characters as it contains the fatty acid FA4, but it couldn't be sequenced since it is an old specimen. Here again, long-distance dispersal events between Chile, South Africa and Oceania seem to be a straightforward hypothesis to explain this disjunction. According to Muñoz et al. (2004), winds are, for pteridophytes, bryophytes and lichens, the major long-distance dispersal vector in the southern hemisphere.

Our results together with preliminary morphological and anatomical studies strongly suggest that lineage 2 corresponds to a still undescribed new species, that inhabits both South America and Australia, and most probably South Africa, the two subclades identified so far showing no obvious morphological differences.

Lineage 3

This well-supported lineage contains two specimens of *Usnea durietzii* Motyka from Peru and a specimen of the *Usnea flavocardia* aggr. (DN99) from Chile displaying the stictic acid chemotype (Fig. 1). Its relationship to the other 4 lineages is not resolved. It was a surprise to find *Usnea durietzii* nested within the *U. flavocardia* aggr. *Usnea durietzii* occurs on rocks, mainly in South America throughout the Andean Cordillera from northern Venezuela to the Magellan Straits and Tierra del Fuego (Walter 1985). It usually contains salazinic and norstictic acids, rarely one or the other alone. Walter (1985) considered this species to belong to the subgenus *Neuropogon*, probably because the branch extremities were somewhat blackened. However, Rodriguez et al. (2011) questioned this placement since they showed unresolved relationships between *U. durietzii* and the *Neuropogon* clade. Our study seems to confirm that *U. durietzii* does not belong to the subgenus *Neuropogon*, but to the subgenus *Usnea* s.str. Morphologically, the presence of red cortical dots close to the basal part of its thallus, even though sometimes inconspicuous, as well as to the yellow pigmentation around its central axis (although very rare: 4 out of 90 specimens present in the fungarium G), is consistent with the inclusion of *U. durietzii* in the *U. flavocardia* aggr. Specimen DN99 with stictic acid and sister to *U. durietzii* is typical of the *U. flavocardia* aggr. with numerous red cortical dots over the whole thallus and a yellow-pigmented medulla around the central axis. This specimen, however, has a different type of soralia than the one found in *U. flavocardia* s.str. We collected several specimens in Chile with the same morphology and chemistry, suggesting that the singleton DN99 corresponds to a second putative undescribed new species that falls sister to *U. durietzii*.

Lineage 4

Four specimens (9%) of the *U. flavocardia* aggr. are included in the strongly supported lineage 4 (Fig. 1). This lineage contains specimens from Brazil (2), Chile (1) and USA (1), therefore showing a distribution restricted to the Americas. This lineage, whose relationship to the other lineages is not resolved, is characterized by the fatty acid FA2 not found in any other lineage within the aggregate. The similarity matrix (Fig. 2) shows a weak genetic differentiation into two groups (Fig. 2); however, these do not show geographic patterns. Preliminary morphological and anatomical studies show that these specimens are different from those in the other lineages. We collected several other specimens with the same morphology and chemistry, strongly suggesting that this fourth lineage corresponds to another undescribed new species.

Lineage 5

Lineage 5 is not supported in the species tree, although a high PP (0.97) is found on the *mcm7* gene tree. It comprises a group of specimens whose distribution is restricted to Central and South America: Chile (2) and Costa Rica (2). The presence of salazinic acid in this lineage is unique in this aggregate. The morphological and anatomical characters displayed by the specimens of lineage 5 are diverse and need a thorough morphological and anatomical investigation, as well as more sequence data to understand its circumscription.

Conclusion

According to our results, the *Usnea flavocardia* aggregate includes at least five different lineages, four of which are phylogenetically well supported, and one is weakly supported. Miralles & Vences (2013) suggested using at least five specimens per putative lineage for an efficient species delimitation analysis, a minimum threshold not reached by some of these putative species. It was indeed highly unexpected to find such a high diversity within the *U. flavocardia* aggregate; the sampling was only aimed at checking the synonymy of *U. flavocardia* and *U. wirthii*, while trying to encompass as much geographical diversity as possible. Lineage 1 and 2, each with more than five specimens, are both highly supported, as is lineage 4 with only four specimens. Only lineage 5 with four specimens is not supported and, together with the singleton within lineage 3, would need additional samples to reach a good species delimitation. Despite this drawback, it is still possible to identify several geographic trends. According to our sampling, lineage 1 is found worldwide, while lineages 3, 4 and 5 are restricted to the Americas and lineage 2 is found only in the southern hemisphere. Although the former statements should be confirmed with additional data, our results suggest an important intercontinental distribution in two of our five lineages that can be explained by long-distance dispersal by winds and birds.

These results show that the diversity of this group has been largely underestimated, both at the genetic and chemical levels. We confirm the hypothesis of Clerc (1998) that chemistry is a good predictor of species delimitation in *Usnea* when correlated with other characters, as also shown by Gerlach et al. (2019) in the *U. cornuta* group.

Author Contributions

Conceptualization, P.C; fieldwork, D.R., P.C., I.P., T.G, R.H; formal analysis, D.R., P.C. and Y.N.; writing: original

draft preparation: D.R.; figures, sequence submission to ENA, C.P.; writing, review and editing, Y.N., P.C, C.P. and TG; lab work, D.R., P.C., S.J; provided sequences, Y.O., A.G.; funding acquisition, D.R., P.C. and Y.N. All authors have read and agreed to the published version of the manuscript.

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Supplementary electronic materials

Figure S1. Species tree from STACEY analysis of 128 *Usnea* specimens with specimen localities colored according to the map on the lower left side and chemotypes on the right side (SAL: salazinic acid; CST: constictic acid; STI: stictic acid; NOR: norstictic acid; GAL: galbinic acid; PSO: psoromic acid; PRO: protocetraric acid; LOB: lobaric acid; THA: thamnolic acid; SQU: squamatic acid; BAR: barbatic acid; DIF: diffractaic acid; ALE: alectorialic acid; TER: terpenoids; FA: fatty acids). The clades are named after Truong et al. (2013). Only posterior probabilities (PP) higher than 0.9 are shown above branches. Download file

Figure S2. ITS individual gene tree STACEY analysis of 128 *Usnea* specimens. Download file

Figure S3. *mcm7* individual gene tree STACEY analysis of 128 *Usnea* specimens. Download file

Figure S4. *rpb1* individual gene tree STACEY analysis of 128 *Usnea* specimens. Download file

Table S1. List of the sequenced loci with indication of the primers used, their sequence, the PCR conditions per locus and the associated literature. Download file

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