

1 **Sequence data from the internal and external transcribed spacers of nuclear**
2 **ribosomal DNA of *Cyclamen purpurascens* allow geographic mapping**

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9 Abstract

10 *Cyclamen purpurascens* (Alpine, European or purple cyclamen) is native to central
 11 Europe. Since decades it is discussed wether the occurrences of *C. purpurascens* north of
 12 the alps is native or if it was introduced. Here the nuclear ribosomal DNAs (rDNA) are
 13 sequenced in oder to obtain a phylogenetic geographic pattern. Phylogenetic analyses of
 14 ITS and NTS/ETS sequences distinguish three main clades coinciding with geographical
 15 distribution: Eastern alps (Austria), southern alps (Switzerland, Italy) and western Alps
 16 (France). The paper presents interspecific relationship of *C. purpurascens* based on
 17 geographic sequences of rDNA. The observed variations suggest that some plants were
 18 introduced via Benedictine gardens and the plants from Monastery gardens seem to origin
 19 from Lower Austria.

21 Introduction

22 *Cyclamen purpurascens* (Alpine, European or purple cyclamen) is a species in the genus
 23 *Cyclamen* of the family Myrsinaceae, formerly Primulaceae, and is native to central Europe
 24 from eastern France across the Alps to Slovakia and south to Croatia. North of the alps
 25 there are some occurrences of *C. purpurascens* like in southwest Germany (Mühlheim,
 26 Kisslegg, Salem, Brigachtal) and Switzerland (Schaffhausen) [1]. Welten & Sutter recorded
 27 the Jura to Oensingen (Mümliswil), Lake Lucerne and the Upper Lake Zurich as the next
 28 environs [2]. Since decades it is discussed wether the occurrences of *C. purpurascens*
 29 north of the alps is native or if it was introduced.

31 In 2011 Slovak *et al.* published an interesting paper about sequencing noncoding DNA

regions of *C. purpurascens* to distinguish geographic origins [3]. Unfortunately the polymorphisms in the sequenced regions were too low to generate an useful mapping. Nevertheless the idea of geographic mapping by sequencing of specific DNA regions seems to be challenging but also fascinating. Since chloroplast DNA sequences don't give enough data for phylogenetic analysis of *C. purpurascens* we investigated different approaches.

In an analysis of phylogenetic interrelationships of the genus *Cyclamen* L., Anderberg *et al.* sequenced the internal transcribed spacers of nuclear ribosomal DNA [4]. Ribosomal DNA (rDNA) consists of a tandem repeat of an operon, composed of non-transcribed spacer (NTS), external transcribed spacer (ETS), 18S, internal transcribed spacer 1 (ITS1), 5.8S, internal transcribed spacer 2 (ITS2) and 26S tracts (Fig 1). In preliminary studies we could find some variations not just in ITS1 and ITS2, but also upstream of 18S in the region of NTS/ETS in *C. purpurascens* from different geographic regions.

The aim of this study was to investigate the sequence of the NTS/ETS, ITS1 and ITS2 to do a geographic mapping of *C. purpurascens*. For this we collected and sequenced 97 leaves of alpine cyclamen of different geographical origin.

Fig 1. Schematic representation of ribosomal DNA (rDNA). *C. purpurascens* rDNA of NTS/ETS, 18S, ITS1, 5.8S and ITS2 is a part of a tandem repetitive cluster of 3155 basepairs. The rDNA contains NTS/ETS of ~600 basepairs, 18S of ~1800 basepairs, ITS1/5.8S/ITS2 of ~660 basepairs and 26S.

55 **Materials and Methods**

56 **Plant material**

57 A total of 97 leaves of *C. purpurascens* from different geographic origins were collected
58 and rDNA was sequenced (Fig 2; Table 1).

59 **DNA region**

60 DNA sequences from the ribosomal DNA were used for the present analysis. Ribosomal
61 DNA (rDNA) consists of a tandem repeat of an operon, composed of non-transcribed
62 spacer (NTS), external transcribed spacer (ETS), 18S, internal transcribed spacer 1
63 (ITS1), 5.8S, internal transcribed spacer 2 (ITS2) and 26S tracts. The spacers are regions
64 within the nuclear ribosomal DNA gene that separates the 18S, 5.8 S and 26S genes. The
65 ITS1 and ITS2 regions have been used in several phylogenetic reconstructions and
66 proved to be variable to a suitable degree for investigations [4]. Here we also analyzed the
67 NTS/ETS region (Fig 1).

68 **DNA extraction, PCR and sequencing**

69 50 mg of leave material was grinded in 1 ml buffer HS (10 mM Tris-HCl, pH 7.6, 10 mM
70 KCl, 10 mM MgCl₂, 400 mM NaCl, 2 mM EDTA, 1% SDS, 0,1 mM DTT, 20 µg Proteinase
71 K, 10 µg RNase) and incubated for 3 hours at 50°C. Debris was collected by centrifugation
72 and DNA was precipitated with isopropanol and washed with 70% EtOH. Genomic DNA
73 was also purified with columns from Roti Prep Genomic DNA kit from Roth.
74 PCR was performed with Polymerases (One Taq or Phusion) from New England BioLabs
75 and primers indicated in Table 2. Temperatures were calculated with Tm Calculator from

76 New England BioLabs. NTS/ETS was amplified with primer HM95/HM96. 18S was
 77 amplified with primer HM84/93, HM92/89 and HM94/93. ITS1/5.8S/ITS2 with primer
 78 HM102/HM82 or HM81/HM82. PCR products were gel purified with Monarch DNA Gel
 79 Extraction Kit from New England BioLabs and sequenced.
 80 The PCR product of NTS/ETS was sequenced with primers HM96, HM100 or HM 95. The
 81 PCR product of 18 S was sequenced with primers HM 84, HM93, HM92, HM89, HM94.
 82 The PCR product of ITS1/5.8S/ITS2 was sequenced with primers HM78, HM82 or HM79.
 83 Sequencing was performed by GATC Biotech (now Eurofins Genomics) in Cologne.
 84 Sequences were analyzed by Multiple Sequence Alignment by CLUSTALW, Kyoto
 85 Bioinformatics Center and SeqDoC by the Bioinformatics Facility of the ARC Special
 86 Research Centre for Functional and Applied Genomics.

87

88 **Fig 2. Map of central Europe.** Analyzed samples and their voucher numbers are indicated
 89 in red.

90

91 **Table 1. Plant materials.**

92 Plant materials used. Region, voucher information, localities and GenBank accession
 93 numbers are indicated.

Region/district	Voucher	Locality	GenBank accession numbers
Lower Austria	85-87	Horn, Waldviertel	MH908834, MH908835, MH908836
	20-22	Kaltenleutgeben	MH908845, MH908846, MH908847
	17-18	Breitenfurt	MH908848, MH908849
	26-28	Rax, Kesselgraben	MH908837, MH908838, MH908839
	23-24	Gerolding, Dunkelsteinerwald	MH908840, MH908841
	32-33	Kreuttal	MH908819, MH908820
	35-37	Leithagebirge	MH908816, MH908817, MH908818
	29-30	Bucklige Welt	MH908821, MH908822
Upper Austria	71-76	Roitham, Viecht, Fallholz	MH908793, MH908794, MH908795, MH908796, MH908797, MH908798,
Salzburg	14-16	Burglstein	MH908842, MH908843, MH908844
	48	Kapuzinerberg	MH908813
	51-52	Mönchsberg	MH908814, MH908815,
	54-60	Unken, Pinzgau	MH908806, H908807, MH908808, MH908809, MH908810, M908811, MH908812
Styria	63-69	Mixnitz	MH908799, MH908800, MH908801, MH908802, MH908803, MH908804, MH908805
	79-84	Saggau	MH908787, MH908788, MH908789, MH908790, MH908791, MH908792
	96-97	Karawanks, Bielschitz	MH908783, MH908784
Swiss	7-12	Zurich, botanical garden	MH908857, H908858, MH908859, MH908860, MH908861, MH908862
	13	Schaffhausen, Allerheiligen	MH908823
	38-40	Walensee, St. Gallen	MH908824, MH908825, MH908826
	46-47	Monte Caslano, Lake Lugano	MH908830, MH908831
	94-95	Schleitheim, Schaffhausen	MH908785, MH908786
	4	Orljavac, Benedictine Abbey of Saint Michael	MH908852
Croatia	41-43	Cittiglio, Lake Maggiore, Varese	MH908827, MH908828, MH908829
Italy	44	Arcumeggia, Varese	MH908833
	45	Monte Nudo, Varese	MH908832
France	5-6, 62	Saint-Vincent-de-Mercuze, Isère	MH908853, MH908854, MH908855
Germany	1-2	Brigachtal, Baden-Wurttemberg	MH908850, MH908851
	3	Mühlheim an der Donau, Baden-Wurttemberg	MH908856

94

95 **Table 2. Primers** Primers used for this study.

Primer	Sequence
HM75	CAAACGACCCGCGAACTT
HM76	GCTTAAACTCACGGGTAAT
HM77	TGCTTAAACTCACGGGTAATC
HM78	TCTCGCATCGATGAAGAAC
HM79	CACGGGGAGCCAATATC
HM80	GAACCTTATCGGTTTAGAGGA
HM81	ACGAATTCATGGTCCGGT
HM82	AGATTTTCACGCTGGGCA
HM84	AATCCGAACACTTCACCG
HM85	GTGGCAGAGTGGCCTTG
HM86	CCACTGAGATTCAGCCCT
HM87	TCGCACAATTGGTCATC
HM88	TCGATCACGGCAATTCCCC
HM89	GAATGATGCGTCGCCAG
HM90	CTTGGATGTGGTAGCCGTT
HM91	ATTGACACCCATCGAAC
HM92	CGACTTCTGGAAGGGAT
HM93	GGATACATTAGCATGGGA
HM94	GGTTCAGTGGACTTCTTG
HM95	AGGATCAACCAGGTAGCAT
HM96	TCTGTGCCTTGGCTATGT
HM99	GTTGCATTGTGCTGAGTC
HM100	AATCCATCCACCACTCAC
HM101	TGCCCTTTGTACACACC
HM102	AGTCATCAGCTCGCGTT

96

Results

Polygenetic analyses of *C. purpurascens* collected in different geographic regions

We sequenced 97 leaves of different geographical origin of *C. purpurascens* in the region of NTS/ETS and ITS1/5.8S/ITS2 (Table 1; Fig 1 and 2). Multiple sequence alignment was done with the 3155 basepair rDNA region (Fig 1). For a better overview some similar sequences were omitted. Also five too divergent sequences were omitted. This were one sequence from Unken (Pinzgau), one from Salzburg, two from Styria and one from Arcumeggia (Italy). Plants from Switzerland (Walensee, St. Gallen and Monte Caslano, Lake Lugano, Schaffhausen) are related to the plants collected in Italy (region Lake Maggiore). Another cluster could be found with three plants from France (St. Vincent de Mercuse, Isère, North of Grenoble). In Austria plants get more diverse in the analyzed rDNA region, the farer we collect samples from Lower Austria (Fig 3). So plants from Styria (Mixnitz) and also from Pinzgau (Unken) are highly diverse. Surprisingly leaves collected in Lower and Upper Austria are very much conserved in the region of NTS/ETS/18S/ITS1/5.8S/ITS2. This conserved sequence could also be found in some plants from Salzburg, in a sample from Schaffhausen (Switzerland), Mühlheim an der Donau (Germany) and also from Orlovac (Croatia). The conserved sequence could not be found in the plants from Brigachthal (Germany) and from Schleithen (Schaffhausen, Switzerland). The plants with the conserved sequence similar to the region of Vienna are all growing close to Benedictine gardens. So the leaf from Schaffhausen was collected in the garden of the former abby Allerheiligen, Mühlheim an der Donau is close to Beuron Archabbey and near Orlovac was a Benedictine abbey of Saint Michael.

120 **Fig 3. Polygenetic tree of *C. purpurascens* from different geographic origins.** Plants
 121 from Switzerland (Walensee, Monte Caslano, Schleithem) are related to the plants
 122 collected in Italy (Cittiglio, Caslano, Monte Nudo). Another cluster could be found with
 123 plants from France (St. Vincent de Mercuse). In Austria plants get more diverse in the
 124 analyzed rDNA region, the farer from Vienna. Plants from Styria (Mixnitz) and also from
 125 Pinzgau (Unken) are highly diverse. Leaves collected in Lower and Upper Austria are very
 126 much conserved in the region of ETS/18S/ITS1/5.8S/ITS2. This conserved sequence
 127 could also be found in some plants from Salzburg, in samples from Schaffhausen,
 128 Mühlheim and also from Orjavac. It could not be found in the plants from Brigachthal and
 129 Schleithem.

Sequence comparison of geographic regions

Sequence alignment comparison of 50 bases in the ETS of 14 plants from different origins show the difference of plants from Switzerland , Italy , France and Austria. So all analyzed plants from Austria and France have an additional G (TGGCCC) which is missing in plants from Italy and Switzerland (TGCCC). Nine bases downstream of this signal plants from France are having a unique signal of AAAA instead AAGA like the analyzed plants from Austria, Switzerland and Italy (Fig 4). Similar signals could be found in the ITS1 and ITS2 of the rDNA (data not shown).

Fig 4. Sequence alignment of nt 100-150 in the NTS/ETS of selected plants. Plants from Italy and Switzerland show the signal TTGCC and plants from Austria and France TTGGCC. The plants from France can be distinguished 9 bases downstream by AAAA instead of AAGA in Austria, Italy and Switzerland.

Sequence alignment of ETS from two plants of different origin

Downstream of this signals we could also find some single nucleotide differences in the region of ETS. Using two chromatograms, we aligned images of the two chromatograms. Here we compare nucleotide 100-500 of ETS of a leaf from Lower Austria to the corresponding sequence of a leaf from Cittiglio (Fig 5). The difference profile in the middle allows identification of base substitutions, insertions and deletions. As shown in Fig 5., at the 5' are two G in the plant from Lower Austria and just one G in the plant from Italy. Downstream of this signal are 5 additional substitutions in the ETF of the two analyzed plants.

Fig 5. Alignment of two chromatograms. Alignment of nucleotide 100-500 of ETS sequence from a plant from Lower Austria (upper lane) and one plant from Cittiglio, Italy (lower lane). The lane in the middle is showing the difference with one additional G in the sequence of the Lower Austria region and five C/T substitution.

Discussion

By sequencing the region of NTS/ETS/18S/ITS1/5.8S/ITS2 in the rDNA of *C. purpurascens* we could clearly distinguish the geographic region of the origin of the plant. So plants from Austria, Switzerland, Italy and France are having a unique signals in NTS/ETS, ITS1 and ITS2 (Fig 1, 3, 4, 5).

rDNA of plants from Austria get more diverse the farther we collect samples from Vienna. So plants from Styria and also from Pinzgau are having several substitutions in the analyzed region. Leaves collected in Lower and Upper Austria are very much conserved in the region of NTS/ETS/18S/ITS1/5.8S/ITS2. This conserved sequence could also be found in some plants from Salzburg, in a sample from Schaffhausen (Switzerland), Mühlheim an der Donau (Germany) and also from Orjavac (Croatia). Surprisingly it could not be found in the plants from Brigachthal (Germany) and Schleithelm (Schaffhausen, Switzerland).

The plants with the conserved sequence similar to the region of Vienna are all growing close to Benedictine gardens. So the leaf from Schaffhausen was collected in the garden of Allerheiligen, Mühlheim an der Donau is close to Beuron Archabbey and near Orjavac was a Benedictine abbey of Saint Michael.

The Benedictines had as early as in the 6th century medicinal herb gardens and distributed many plants across the Alps. Salzburg was and is the center of the Benedictines with the abbey of St. Peter. Alpine cyclamen were called "Wolfgang Erdäpferl" by the locals and was said to have a special effect on snakebite, migraine headaches and as a philter. The roots were sold to Pilgrims as salvation and fertility

181 symbol [5]. So we suggest that at some point *C. purpurascens* had to be replanted in the
 182 area of Salzburg. We assume that for this reintroduction people used plants from
 183 Benedictine gardens. The plants from Monastery gardens seem to origin from the forests
 184 of Vienna.

185

186 In the forests of Schleithem in the Canton of Schaffhausen is another occurrence of *C.*
 187 *purpurascens* which was described in 1989 [1]. The plants found in 1971 are very much
 188 related to plants in Switzerland. Unfortunately just beside this plants someone has planted
 189 some tubers from the Benedictine gardens. Although there are inflorescences it seems
 190 that no seeds germinated, not in Canton Schaffhausen and also not in Germany. That's
 191 why there are just small patches of alpine cyclamen and they did not spread.

192

193 Interestingly plants in canton Schaffhausen are not very close related to the *C.*
 194 *purpurascens* growing in Brigachthal which is just 30 km across the border. The plants in
 195 Brigachthal seem to relate very much to plants from Austria. But it is possible that alpine
 196 cyclamen are naive to limestone in Southern Black Forest / Schaffhausen region.
 197 Another very interesting region would be the Tatra Mountains in the Czech Republic which
 198 should be analyzed in further studies. Also molecular cytogenetic mapping of 5S and 35S
 199 rDNA loci was not addressed in this study and should be considered in future studies.

Conclusion

This is the first molecular analysis of the geographic origin of *C. purpurascens*. An evolutionary and geographically interpretation in a phylogenetic context has been possible. rDNA sequences are variable within the species and some regions show a specific pattern. With this information it is possible to determine the geographic origin of alpine cyclamen and to hypothesizing if it is native or was introduced at some point.

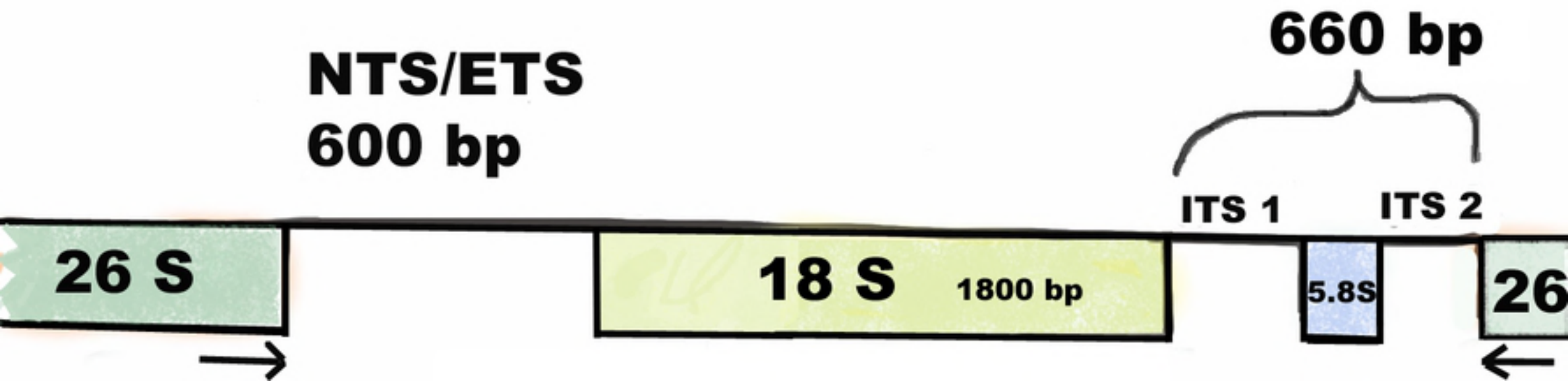
Acknowledgements

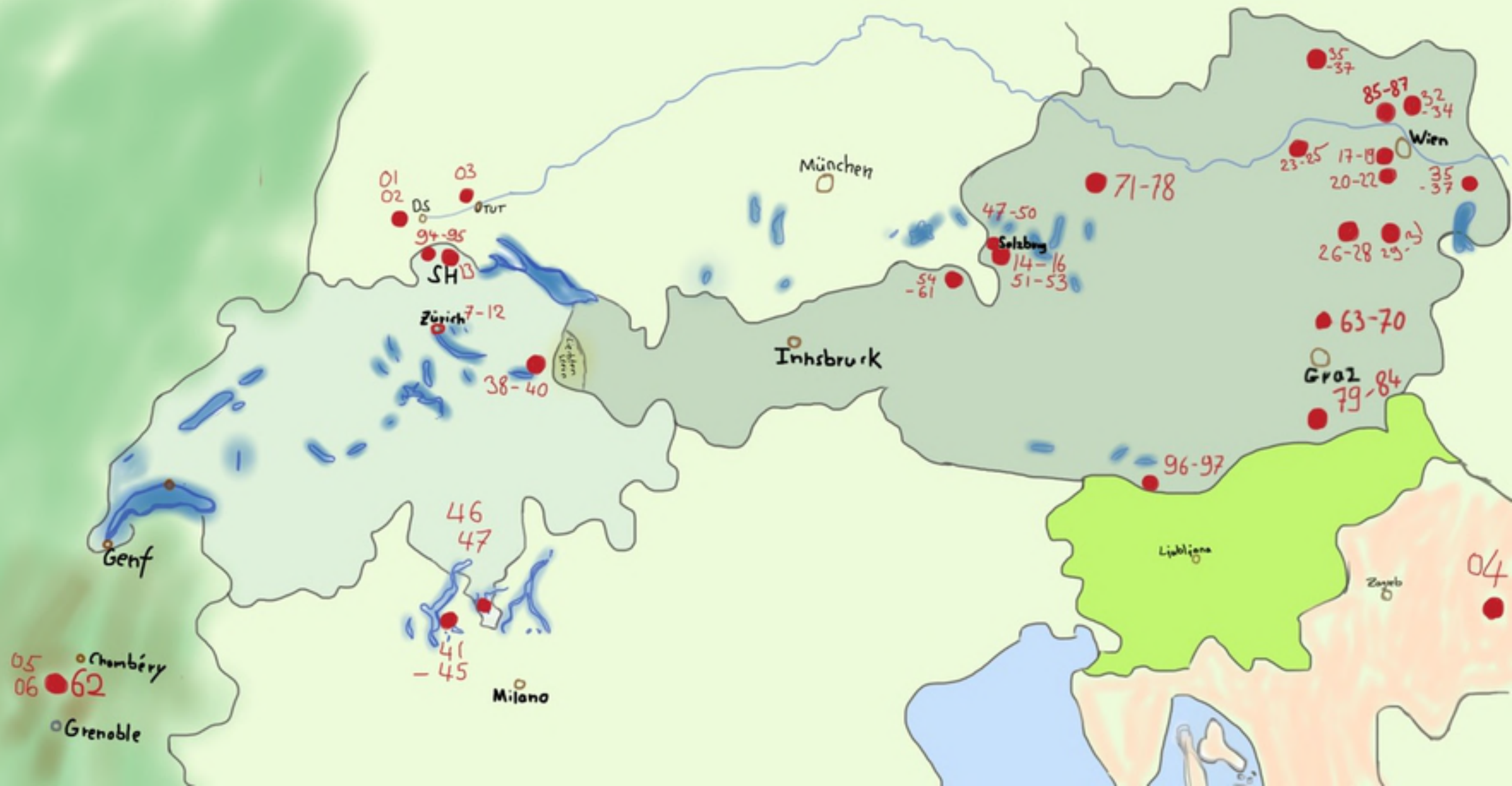
The author would like to thank the members of the Flora Austria (Verein zur Erforschung der Flora Österreichs) particularly Hermann Falkner, Stefan Lefnaer, Georg Pflugbeil, Markus Sabor, Michael Strudl and Maria Zacherl for sending leaves of *C. purpurascens* from all over Austria. Without their precious help this work would not have been possible. Many thanks to Petra Bachmann and Peter Braig of the Canton Schaffhausen and Peter Enz, garden manager at the Zurich Botanical Garden for their help. Thanks to Duca Jaag for the plant from Orjovac. This research was supported by Thomas Kring. The author received no specific funding for this work.

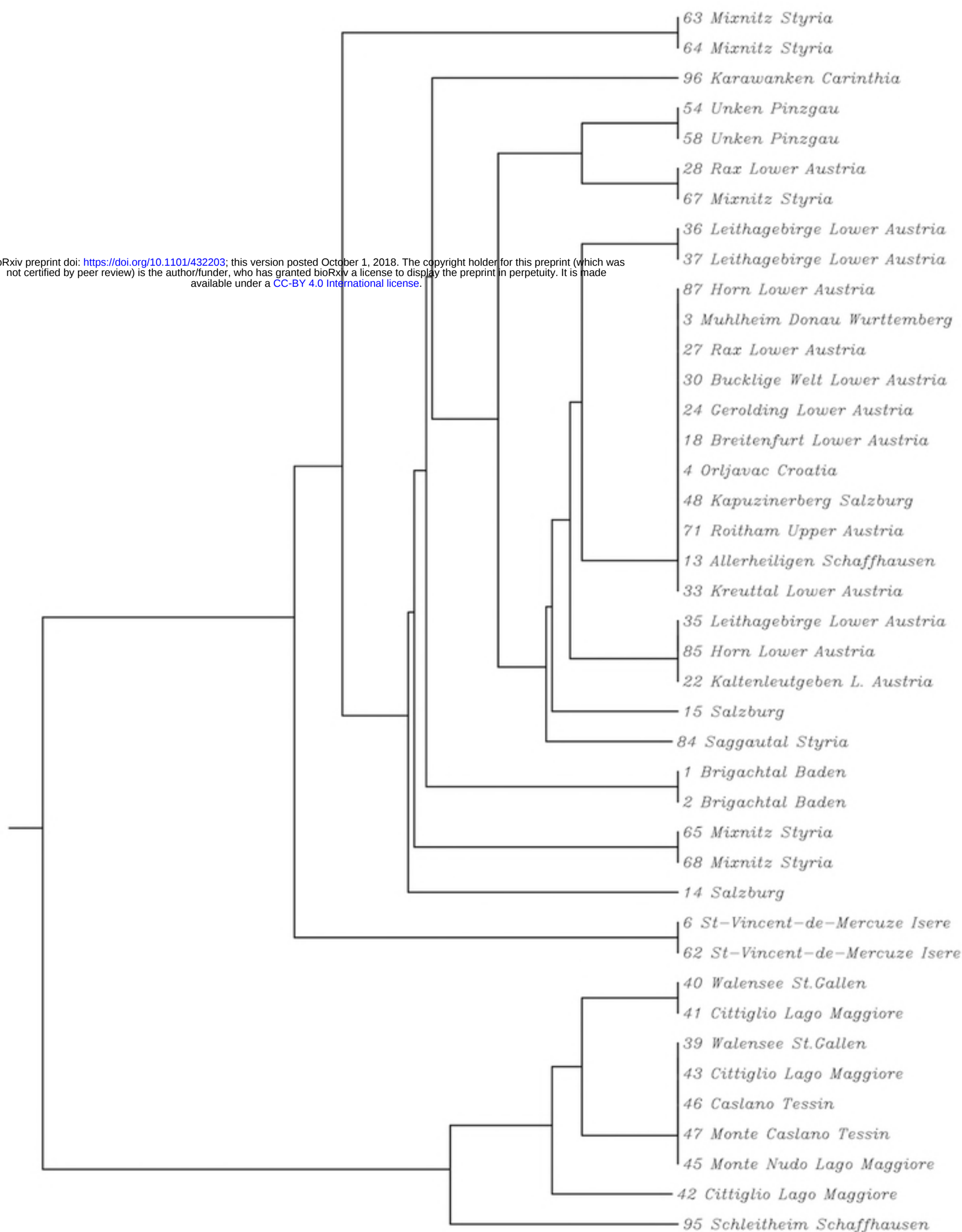
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06 | St-Vincent-de-Mercuze | Isere
62 | St-Vincent-de-Mercuze | Isere
63 | Mixnitz | Styria
54 | Unken | Pinzgau
18 | Breitenfurt | Lower.Austria
03 | Muhlheim.Donau | Wurttemberg
04 | Orljavac | Croatia
13 | Allerheiligen | Schaffhausen
71 | Roitham | Upper.Austria
40 | Walensee | St.Gallen
41 | Cittiglio | Lago.Maggiore
95 | Schleithem | Schaffhausen
46 | Caslano | Tessin
45 | Monte.Nudo | Lago.Maggiore

TCCCGTTGGTATTTTGGCCCGTTTCAAAATTGTCCCGAACGTGATTGGTT
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