

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.

20

## 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.

30

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

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

38

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

46

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

50

51 **Fig 1. Schematic representation of ribosomal DNA (rDNA).** *C. purpurascens* rDNA of  
52 NTS/ETS, 18S, ITS1, 5.8S and ITS2 is a part of a tandem repetitive cluster of 3155  
53 basepairs. The rDNA contains NTS/ETS of ~600 basepairs, 18S of ~1800 basepairs,  
54 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 MgCl<sub>2</sub>, 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
Carinthia	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
Croatia	4	Orljavac, Benedictine Abbey of Saint Michael	MH908852
Italy	41-43	Cittiglio, Lake Maggiore, Varese	MH908827, MH908828, MH908829
	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-Württemberg	MH908850, MH908851
	3	Mühlheim an der Donau, Baden-Württemberg	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	CTTGATGTGGTAGCCGTT
HM91	ATTGACACCCATCGAAC
HM92	CGACTTCTGGAAGGGAT
HM93	GGATACATTAGCATGGGA
HM94	GGTTCAGTGGACTTCTTG
HM95	AGGATCAACCAGGTAGCAT
HM96	TCTGTGCCTTGGCTATGT
HM99	GTTGCATTGTGCTGAGTC
HM100	AATCCATCCACCACTCAC
HM101	TGCCCTTTGTACACACC
HM102	AGTCATCAGCTCGCGTT

96

## 97 **Results**

### 98 **Polygenetic analyses of *C. purpurascens* collected in different** 99 **geographic regions**

100 We sequenced 97 leaves of different geographical origin of *C. purpurascens* in the region  
101 of NTS/ETS and ITS1/5.8S/ITS2 (Table 1; Fig 1 and 2).

102 Multiple sequence alignment was done with the 3155 basepair rDNA region (Fig 1). For a  
103 better overview some similar sequences were omitted. Also five too divergent sequences  
104 were omitted. This were one sequence from Unken (Pinzgau), one from Salzburg, two  
105 from Styria and one from Arcumeggia (Italy). Plants from Switzerland (Walensee, St.  
106 Gallen and Monte Caslano, Lake Lugano, Schaffhausen) are related to the plants  
107 collected in Italy (region Lake Maggiore). Another cluster could be found with three plants  
108 from France (St. Vincent de Mercuse, Isère, North of Grenoble). In Austria plants get more  
109 diverse in the analyzed rDNA region, the farer we collect samples from Lower Austria (Fig  
110 3). So plants from Styria (Mixnitz) and also from Pinzgau (Unken) are highly diverse.

111 Surprisingly leaves collected in Lower and Upper Austria are very much conserved in the  
112 region of NTS/ETS/18S/ITS1/5.8S/ITS2. This conserved sequence could also be found in  
113 some plants from Salzburg, in a sample from Schaffhausen (Switzerland), Mühlheim an  
114 der Donau (Germany) and also from Orljavac (Croatia). The conserved sequence could  
115 not be found in the plants from Brigachtal (Germany) and from Schleithem (Schaffhausen,  
116 Switzerland). The plants with the conserved sequence similar to the region of Vienna are  
117 all growing close to Benedictine gardens. So the leaf from Schaffhausen was collected in  
118 the garden of the former abby Allerheiligen, Mühlheim an der Donau is close to Beuron  
119 Archabbey and near Orljavac 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 Brigachtal and  
129 Schleithem.



## 130 **Sequence comparison of geographic regions**

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

138

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

## 143 **Sequence alignment of ETS from two plants of different origin**

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

152

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

## 157 Discussion

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

162  
163 rDNA of plants from Austria get more diverse the farther we collect samples from Vienna. So  
164 plants from Styria and also from Pinzgau are having several substitutions in the analyzed  
165 region. Leaves collected in Lower and Upper Austria are very much conserved in the  
166 region of NTS/ETS/18S/ITS1/5.8S/ITS2. This conserved sequence could also be found in  
167 some plants from Salzburg, in a sample from Schaffhausen (Switzerland), Mühlheim an  
168 der Donau (Germany) and also from Orjavac (Croatia). Surprisingly it could not be found  
169 in the plants from Brigachtal (Germany) and Schleithem (Schaffhausen, Switzerland).

170  
171 The plants with the conserved sequence similar to the region of Vienna are all growing  
172 close to Benedictine gardens. So the leaf from Schaffhausen was collected in the garden  
173 of Allerheiligen, Mühlheim an der Donau is close to Beuron Archabbey and near Orjavac  
174 was a Benedictine abbey of Saint Michael.

175  
176 The Benedictines had as early as in the 6th century medicinal herb gardens and  
177 distributed many plants across the Alps. Salzburg was and is the center of the  
178 Benedictines with the abbey of St. Peter. Alpine cyclamen were called "Wolfgang  
179 Erdäpferl" by the locals and was said to have a special effect on snakebite, migraine  
180 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 Schleitheim 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 Brigachtal which is just 30 km across the border. The plants in  
195 Brigachtal 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.

## 200 **Conclusion**

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

206

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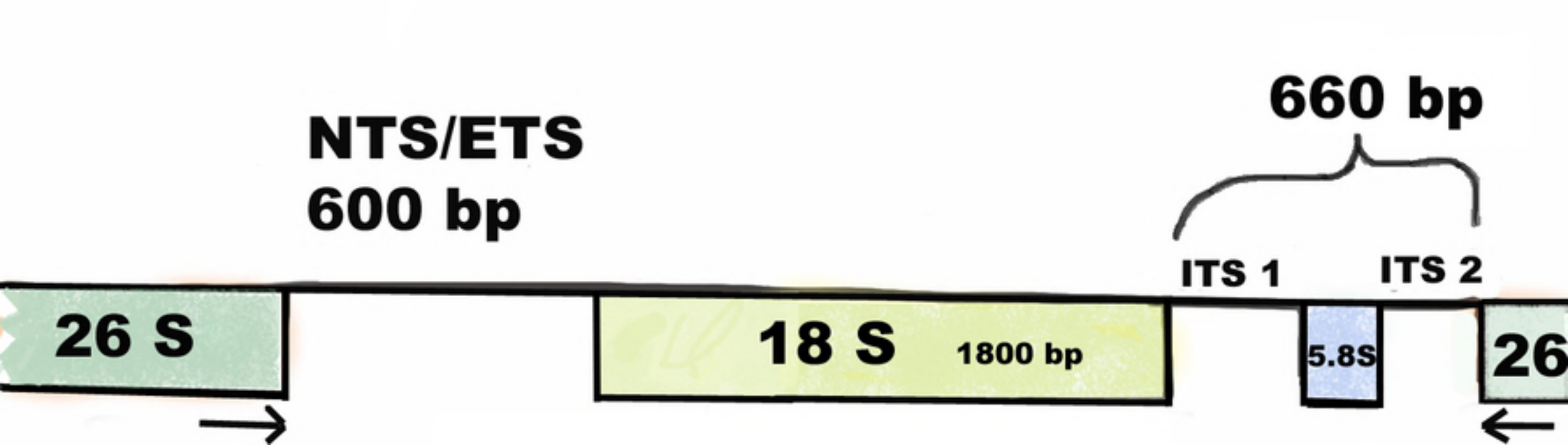
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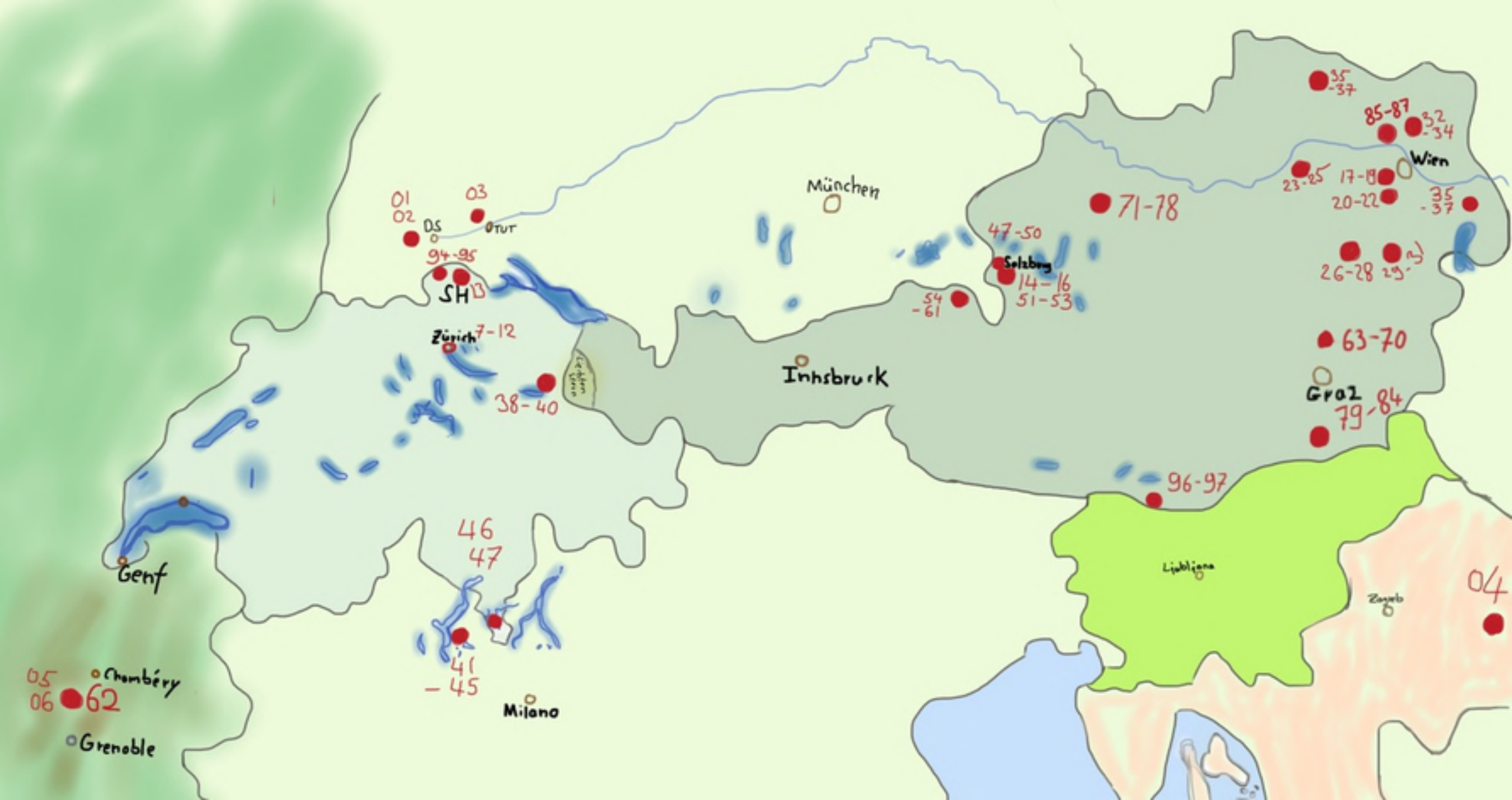
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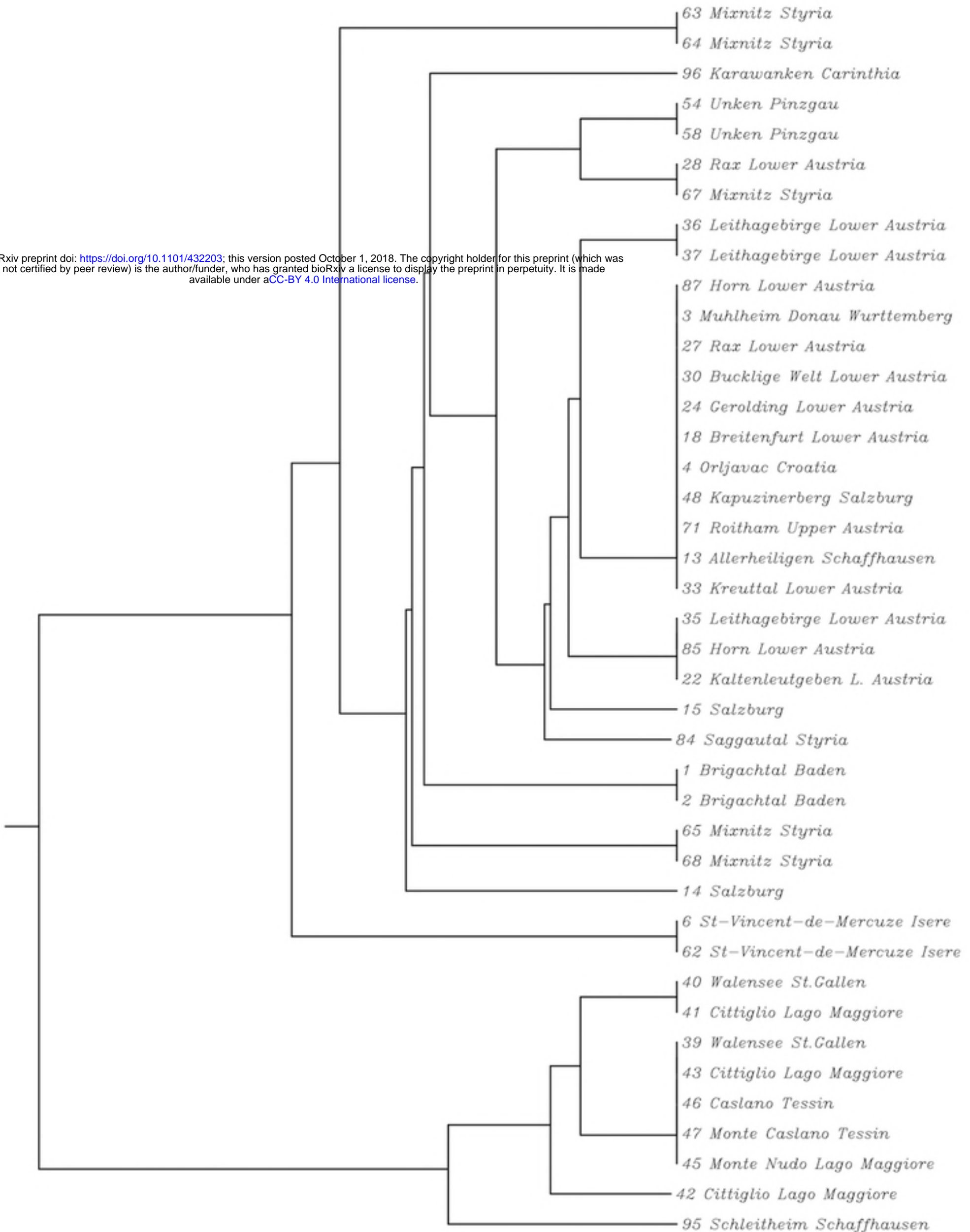
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06	St-Vincent-de-Mercuze	Isere	TCCCGTTGGTATTTTGGCCCGTTTCAAAATTGTCCCGAACGTGATTGGTT
62	St-Vincent-de-Mercuze	Isere	TCCCGTTGGTATTTTGGCCCGTTTCAAAATTGTCCCGAACGTGATTGGTT
63	Mixnitz	Styria	TCCCGTTGGTATTTTGGCCCGTTTCAAGATTGTCCCGAACGTGATTGGTT
54	Unken	Pinzgau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

