

ETS and plastid sequence data indicate a spontaneous origin of Scandinavian betony, *Betonica officinalis* L.

Original Article

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Arne Thell¹, Mats Hansson¹, Per-Erik Persson¹, Mark R. D. Seaward², Maik Veste^{3,4} and Mikael Hedrén¹

¹Department of Biology, Lund University, Lund, Sweden

²School of Archaeological Sciences, University of Bradford, Bradford, UK

³Institute of Botany (210), University of Hohenheim, Stuttgart, Germany

⁴Institute of Environmental Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Cottbus, Germany

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Background and aims: Betony (*Betonica officinalis* L.) is one of the rarest and most spectacular plants in the Scandinavian flora. A long-term question has been whether it is spontaneous or introduced, or whether it comprises both spontaneous and introduced populations. This study aimed to answer this question by analyzing sequence data from the nuclear external transcribed spacer (*ETS*) region and three regions of the plastid genome, the *trnT-trnL* intergenic spacer (*IGS*) region, *tRNA-Leu* (*trnL*) intron, and the *trnS-trnG* *IGS*. **Materials and methods:** Altogether 41 samples from 11 European countries were analyzed. A unique duplication in the *trnT-trnL* *IGS* was detected in material from Skåne (southern Sweden), the “Skåne-duplication.” Populations with this duplication are united on a moderately supported branch in the phylogeny based on plastid sequences. A distinct heath genotype from Yorkshire was discovered in the phylogeny based on plastid sequences and in a comparative cultivation. **Results:** Phylogeny based on *ETS* sequences does not support any Scandinavian group, whereas a principal coordinates analysis ordination based on variable *ETS* positions indicated a spontaneous origin for all Scandinavian populations, which comprise a genetically well-defined subgroup of the species, most closely related to other spontaneous populations from adjacent parts of continental parts of northern Europe. **Discussion:** Seven possible naturally occurring localities remain in Scandinavia, five in central Skåne, southernmost Sweden, and two on the southwestern part of the Danish island of Lolland.

INTRODUCTION

Betony (*Betonica officinalis* L.) has the longest documented history of all Scandinavian plant species. The history began almost 500 years ago, when the Danish humanist Christiern Pedersen (c. 1480–1554) in his book *Om Urte Vand* [On plant extracts] reported that “betony grows here in Skåne, in particular in Stodhage” [Stehag] (Pedersen, 1534, p. 21). His published note on betony was the first from the province Skåne, at that time a part of Denmark, and probably one of the earliest records in the world where a locality is connected with a plant species.

Betony may have been found locally frequent in western central Skåne in medieval times but was considered extinct from Skåne during the period 1770–1820 (Fries, 1823; Lilja, 1870, p. 403). In Stehag, the locality reported by Pedersen, it was not observed during two periods, 1740–1860 and 1957–1977 (Thell, 2016b). Leche (1744, p. 10) had found it “on meadows at Stehag” in 1740, and “on a meadow close to Lund and at Maglö farm” (Linnæus, 1745, p. 176, 1755, p. 201). The population in Maglö in Norra Mellby parish in northern central Skåne went extinct early (Lilja, 1838, p. 253; Thell, 2016a, 2016b). After Leche’s reports, it was not collected in Stehag until 1860 (Sandberg LD1157437). Twelve additional herbarium sheets from Stehag are stored in the Lund University botanical collections (LD). The most recent addition was collected in 1925; thereafter, betony became very rare. Forested meadows, the preferred habitat, were formerly frequent west of Stehag, but were transferred to settled areas or spruce

Author for correspondence:

Arne Thell

e-mail: arne.thell@biol.lu.se

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forests and earlier into agricultural land. Both Weverinck (1939, p. 49) and Rufelt (1949, p. 113) mention “a single, fenced population of betony just south of the railway station,” perhaps the place where the last plant was removed to a private garden in 1957 prior to the building process of the water supply station, finished in 1963. A small betony plant with few inflorescences was discovered in 1977 (Larsson, 1987, p. 111). The next observations were in 1980 (Karlsson, 1982, p. 81), again a single plant, and in 1985–1986 when 15 plants in 6 places were counted (Larsson, 1987, p. 111). In 1980, it was discovered in Kastberga in Västra Sallerup about 3 km southwest of Stehag (Larsson, 1987, p. 111).

At present, about 20 specimens of *B. officinalis* are growing in Stehag; however, about half of them may be the result of vegetative spreading. Two localities are positioned in Västra Sallerup, where about 25 individuals grow in Kastberga forest and 6 or 7 plants on Kastberga meadow around 500 m to the northwest. A population of 17–19 individuals grow on a dry hill in Trolleås parish at a distance of 500 m further to the northwest. The fifth locality of presumably naturally established betony is Kungsmarken in Södra Sandby just east of Lund, where a single individual was believed to constitute the entire Swedish population in the 1960s and 1970s. Consequently, a rescue project, described by Mattiasson (2010) and Thell (2016a, 2016b), was initiated in 1981. Only three seeds managed to germinate, but together with vegetatively propagated material from the single remaining specimen, cross pollination was successful. Sixty-three new plants (42 from seeds and 21 vegetatively reproduced plants) were set out in 1988 and Kungsmarken is currently the richest of the naturally occurring localities in Skåne with perhaps as many as 150 specimens. In the past, betony also used to grow in some neighboring parishes. Many localities were carefully listed by Lilja (1838, p. 253, 1870, p. 403), most of which, however, are extinct today. Some additional localities collected or reported rather recently may have been natural, one in Axelvold in Svalöv parish in western Skåne, where it was collected in 1994, and a second one in Mölle, Brunnby parish, where it grew on a meadow north of the railway station until recently, and on a vacant lot, now a private garden where it still grows (Thell, 2016b). Its occurrence in the north of Höör, between Stehag and the ancient Maglö locality, was first believed to be spontaneous (Tyler, 2015), but was later revealed to be planted. In Säby close to Landskrona, there is an introduced population, probably originating from central Skåne.

The first betony report from present-day Denmark appears in the study of Paulli (1648, III, p. 178), who states that “it grows wild in some places in the forests but since there are not very many of them, it is often introduced and planted in gardens.” The report is illustrated with a beautiful woodcut (Fig. 1). Kylling (1688) cites two places for betony, the now extinct occurrence in Rygaard (in Hellerup north of Copenhagen) and Lolland, where it managed to survive to this day. According to Müller in *Flora Danica* (1778, Vol. 13, Fig. 726), it was still common in the southwestern part of Lolland in the 18th century and was reported to occur in “large amounts”



Fig. 1. Woodcut of betony in Simon Paulli's *Flora Danica Det er: Dansk Urtebog* (1648)

along two parallel roads in Bjerremark (Andersen, 1942, p. 154). In 1999, the number of individuals was reduced to 11 (Helkjær, personal communication), and in 2016, only two flowering plants with two inflorescences each were observed (Fig. 2); in 2017, eight plants developed because the roadside vegetation was not cut so harshly and early as usual. The remaining plants were protected in an agreement with Lolland municipality. The second Danish locality, about 10 km to the northwest, in Tillitse parish in Lolland, is a forest population with too low sun irradiance; therefore, the seven remaining plants were rarely flower. Some, possibly naturally occurring populations, scattered in other parts of Denmark, are all extinct (Hornemann, 1821; Pedersen, 1969; Thell, 2016b).

Betony was considered a universal drug, particularly in medieval times, believed to cure a multitude of disorders and diseases in the fields of pain, fever, bad nerves, lung diseases, and in plague remedies (Allen & Hatfield, 2004; pp. 212, 213; Culpeper, 1790, pp. 31, 32; Pedersen, 1534, p. 21). Therefore, it was frequently introduced and cultivated (Ortus [Hortus] Sanitatis, 1517; Paulli, 1648, p. 178, plate 28), and it sometimes escaped from herb gardens. A long-debated question, hitherto not finally answered is whether betony in the flora of Skåne is spontaneous or artificially



Fig. 2. The roadside locality in Bjerremark on Lolland, where it was collected already in 1853, was the only locality with flowering plants in Denmark in 2016. Two plants with four inflorescences (arrow) were found. Roadsides are often the last retreat localities for meadow flowers in a modern landscape. One hundred individuals of local origin were planted there 2017–2018 (photo: A. Thell)

introduced by human. An attempt to answer this question was made by the Swedish botanist and geneticist Göte Turesson, who performed a comparative garden study of eight betony provenances (Turesson, 1930). He used material from two possibly naturally occurring localities in Skåne, Stehag, and Kungsmarken; two populations from Skåne believed to have a foreign origin, Malmö and Öved; and four samples, from Munich, Vienna, Zagreb, and

Moscow, representing the European continent (Turesson, 1930). Plants from Stehag and Kungsmarken flowered earlier and grew to a lower height than the other six provenances in the study. Turesson (1930) concluded that the spontaneous provenances had evolved in genetic isolation. Although the Scandinavian distribution is considered limited to Skåne and Denmark (Figs 3 and 4), an odd locality in Sollentuna near Stockholm exists, composed of several hundred individuals, marked as feral or ruderal by Hultén (1971), could also be spontaneous (Almgren, 1909; Almquist, 1909). It is geographically closer to Estonian localities than those in southern Sweden. Material from this locality is not investigated in this study.

Betony occurs on nutrient-rich, calcareous clay soils in rather dry, grass-rich meadows with bushes, often hazel and sloe, and open woods, particularly under oaks (Fig. 5). It is characteristically found in open habitats with the presence of high light and soil water fluctuations. The common meadow phenotype is spread in Europe (Fig. 6), whereas dwarf phenotypes occur in mountainous areas and in dry habitats where betony plants may be considerably smaller with relatively short and broad inflorescences. As observed in Yorkshire, England, where betony occurs in rather poor and dry meadows, even heathlands, it becomes “dwarf” in appearance (Stace, 2019, p. 657; Fig. 7). Subspecies and varieties are not generally accepted within *B. officinalis*; yet, one of our samples, sent from the Botanical Garden in Nancy, was labeled “*Betonica officinalis* var. *montana*,” originally from the French Alps. These “dwarf-forms” were included in the study.

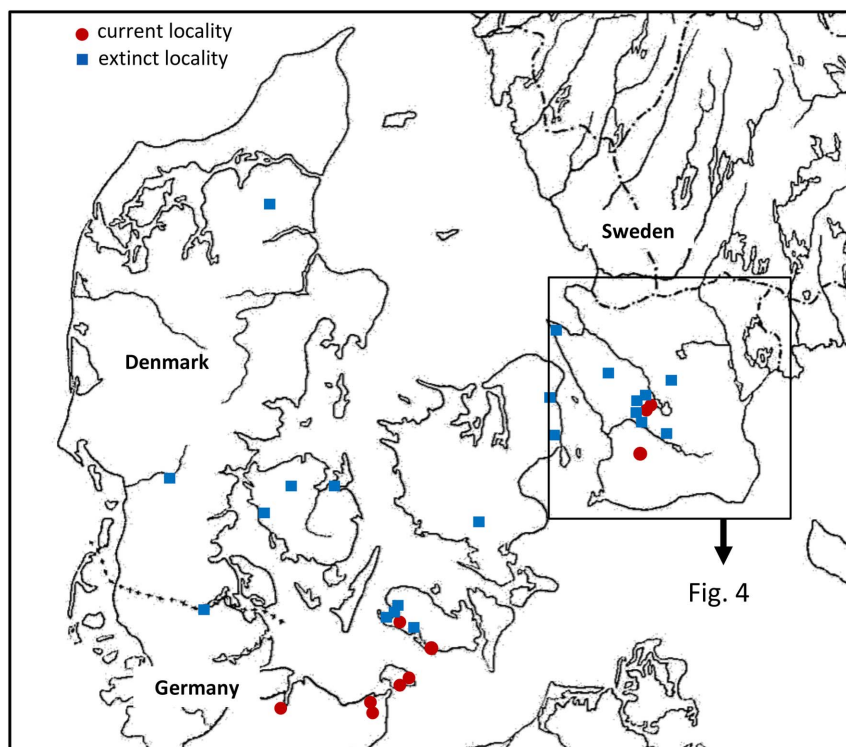


Fig. 3. Distribution of betony in Scandinavia, i.e., Skåne and Lolland, and the adjacent part of Schleswig-Holstein in Germany. Dots represent still existing localities. Note that the northernmost occurrence, in central Skåne, represents four nearby localities (see Fig. 4). Squares show possibly spontaneous but extinct localities. The map is based on Pedersen (1969), Thell (2016b), Simon Kellner, AG Geobotanik in Schleswig-Holstein, and Ulf Schiefelbein, Landesamt für Umwelt, Naturschutz und Geologie, Mecklenburg-Vorpommern

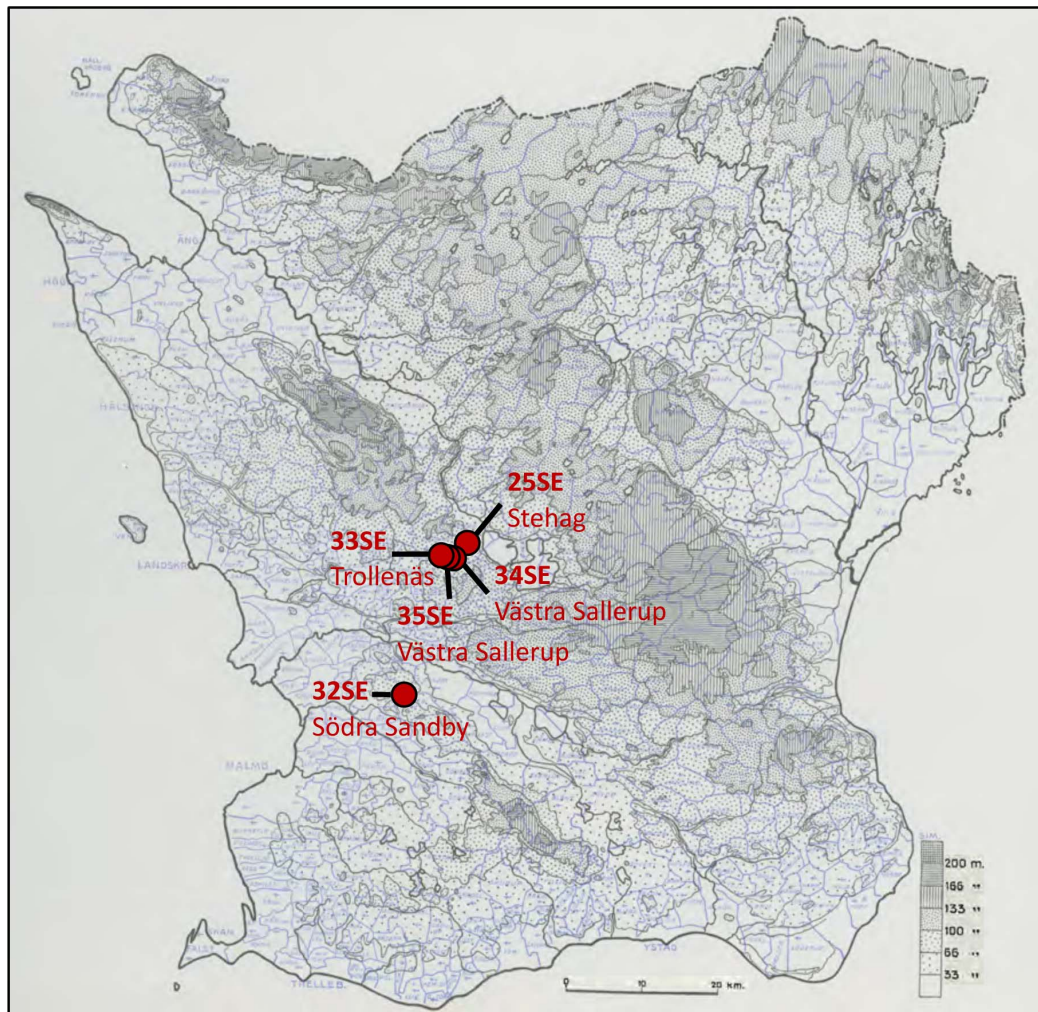


Fig. 4. The Swedish distribution in detail, concentrated to western central Skåne, southernmost Sweden, where five presumably spontaneous localities remain. The unique “Skåne-duplication” was found in plastid sequences from the three nearby localities 33–35



Fig. 5. A presumably spontaneous stand of betony in Stehag, July 6, 2017 (photo: N. Thell)

The Scandinavian betony populations occur in the north-westernmost outposts of its distribution range in Europe, western Asia, and northwestern Africa. Pedersen (1969) describes betony’s world distribution as central European–western Siberian. It occurs in most of Europe, but is rare in Ireland and Scotland, and avoids the Atlantic coast and the

steppe zone; in the northeast, it occurs in Estonia and via the lakes Ladoga and Onega to western Siberia and Caucasus, and in the south to Bulgaria, Greece, Italy, and northern Spain; it has also been found in Algeria. Betony was formerly very common throughout Germany (Geiger, 1839, p. 511). It is now almost absent in the northwest and listed in the IUCN Red List for Schleswig-Holstein (Rote Liste gefährdeten Arten; Mierwald & Romahn, 2006). It gradually becomes commoner toward the mountainous regions in the southeast (FloraWeb, 2018; Haeupler & Schönfelder, 1989; Wollert, 2005; Zacharias et al., 1988, p. 59; Zimmermann, 2014).

The seven current, possible natural populations from Scandinavia were included in this study, together with samples from extinct localities in Svalöv and Brunnby parishes, feral material from Säby, and cultivated material from Lund Botanical Garden and Odarslöv, where the latter are remnants of Tureson’s material.

The aim of this study is to test whether betony is spontaneous in Scandinavia by reconstructing its phylogeography by means of DNA analyses in a set of 41 betony samples from 11 European countries (Table 1), with emphasis on the remaining 7 putatively naturally occurring Scandinavian populations.



Fig. 6. Betony cultivated in Stehag July 6, 2017 with buff-tailed bumble bee, *Bombus terrestris* (photo: N. Thell)



Fig. 7. Fruiting "dwarfing" phenotype of betony to the left, growing among, for example, common heather, *Calluna vulgaris* (L.) Hull, on dry, nutrient-poor soil. United Kingdom, Yorkshire, Maltby, August 24, 2017 (38UK) (photo: A. Thell)

MATERIALS AND METHODS

Materials

Thirty-eight samples of *B. officinalis* were included in this study. As outgroups or reference materials, two specimens of *Betonica hirsuta* L. and one specimen of *Stachys alopecuroides* (L.) Benth. were selected (Figs 8 and 9). The samples were collected in 11 European countries (Table 1; Fig. 10). Most specimens used are stored in the botanical collections of the Biological Museum, Lund University (LD). Two samples were on loan from the herbarium in Oskarshamn (OHN), and two samples reached us through seed exchange with the Botanical Gardens of Nancy and Nantes in France. Four samples from Stehag in Skåne are preserved as DNA extracts only. Most specimens were collected by us and some were sent by colleagues. In Scandinavia, where betony is a rare and protected species, only fragments were collected for the DNA analyses. In such cases, we refer to old herbarium sheets from the same localities as vouchers (Table 1). All material, both old and new herbarium sheets, is registered in the public database *Sweden's Virtual Herbarium* (herbarium.emg.umu.se). All non-Swedish samples are assumed to be naturally established in their localities. For the province Skåne, on which the study is focused, the samples are either from the five presumably naturally occurring localities or from planted or escaped populations to confirm a presumed origin or, in one case, of completely unknown origin (Table 1).

DNA extraction

Total DNA used for PCR amplification and sequencing was mainly extracted with the DNeasy plant mini kit (Qiagen, Germany). A few DNA extracts were obtained by the REDExtract-N-Amp Plant PCR kit (Sigma, USA) but the produced DNA extracts were less suitable for PCR. Extractions were made from fresh or dried leaf material for all samples except sample 8 (Table 1), for which seeds were used.

Amplification and sequencing

Four gene regions were successfully amplified and sequenced (Tables 2–5), three plastid regions: the *trnT*–*trnL* intergenic spacer (*IGS*) region, the *tRNA-Leu* (*trnL*) intron, and the *trnS*–*trnG* *IGS*; partial sequence (Prince & Kress, 2006; Shaw et al., 2005); and one nuclear region, the external transcribed spacer (*ETS*) of the rDNA repeat region (Baldwin & Markos, 1998). However, additional regions were tested without success (Table 2). PCR reactions were performed with a Mastercycler pro (Eppendorf, Germany) using the following programs: *ETS*: 94 °C for 5 min (94 °C for 1 min, 58 °C 30 s, 72 °C for 1 min) × 30 cycles, 72 °C for 5 min; *trnT*–*trnL* spacer and *trnL* intron: 94 °C for 3 min (94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min) × 35 cycles, 72 °C for 5 min; *trnS*–*trnG* spacer: 94 °C for 4 min (94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min) × 26 cycles, 72 °C for 10 min. PCR products were visualized by agarose gel electrophoresis and those of good quality were directly purified with Illustra ExoProStar 1-Step (GE Healthcare, UK) and sent to Eurofins Genomics (Germany) for Sanger sequencing. In the case of an

Table 1. Material used in the study

Sample	Taxon	Country, province	District, locality	Year	Collector	Herbarium Acc. no	GenBank accession numbers			
							ETS	trnT-trnL	rRNA-Leu	trnS-trnG
1AT	<i>B. officinalis</i>	Austria, Niederösterreich	Kremsland, Willendorf NV	2013	Rühling	OHN222039	MG742548	MG742579	MG742619	MG742655
2AT	<i>B. officinalis</i>	Austria, Niederösterreich	Kremsland, Willendorf, Wetterkreutz	2014	Rühling	OHN238339	MG742549	MG742580	MG742620	MG742656
3BY	<i>B. officinalis</i>	Belarus, Homyelskaya	Gomel, Chenki forest	2015	Tsurykau	LD1769199	MG742550	MG742581	MG742621	MG742657
4BY	<i>B. officinalis</i>	Belarus, Homyelskaya	Gomel, Chenki forest [the same spot as number 3]	2017	Tsurykau	See 32	–	MG742582	–	MG742658
5DK	<i>B. officinalis</i>	Denmark, Lolland	Tillise, Rudbjerggård	1884 [2016]	Mortensen	LD1771056	MG742551	MG742583	MG742623	–
6DK	<i>B. officinalis</i>	Denmark, Lolland	Tågerup, Bjerremark	1853 [2017]	Mortensen	LD1757551	MG742552	MG742584	MG742622	MG742659
7FR	<i>B. officinalis</i>	France, Pays-de-la- Loire	Loire-Atlantique, Nantes	2017	Seed exchange in cult	LD1946483	–	MG742585	–	–
8FR	<i>B. officinalis</i> (var. <i>montana</i>)	France, Grand-Est	Meurthe-et-Moselle, Nancy	2017	Seed exchange	Extract only	MG742553	MG742586	MG742624	–
9DE	<i>B. officinalis</i>	Germany, Nordrhein- Westfalen	Bielefeld, Mühlenmarsch	2015	Breckle	LD1769455	MG742554	MG742587	MG742625	MG742660
10DE	<i>B. officinalis</i>	Germany, Sachsen	Görlitz, Schöna-Berzdorf	2015	Ritz and Wesche	LD1769327	MG742555	MG742588	MG742626	MG742661
11DE	<i>B. officinalis</i>	Germany, Sachsen	Görlitz, Schöna-Berzdorf	2015	Ritz and Wesche	LD1769391	MG742556	MG742589	MG742627	MG742662
12DE	<i>B. officinalis</i>	Germany, Schleswig-Holstein	Ostholstein, Dazendorf	2016	Thell and Hedrén	LD1895978	MG742557	MG742590	MG742628	MG742663
13DE	<i>B. officinalis</i>	Germany, Schleswig-Holstein	Ostholstein, Seegalendorf	2016	Thell and Hedrén	LD1899079	MG742558	MG742591	MG742629	MG742664
14HU	<i>B. officinalis</i>	Hungary, Pest	Pilis-Visegrád Mts, Pomáz, “Pankos-tető” at Kiskovácsi	2017	Farkas and Lökös	LD1909812	MG742559	MG742592	MG742630	MG742665
15HU	<i>B. officinalis</i>	Hungary, Pest	Pilis-Visegrád Mts, Pilisszentlászló, at meadow “Sikárosirét”	2017	Farkas and Lökös	LD1909876	–	MG742593	–	MG742666
16IT	<i>B. officinalis</i>	Italy, Trentino-Alto Adige	Trento, Carbonare	2004	S. and B. Snogerup	LD1892493	MG742560	MG742594	MG742631	MG742667
17LT	<i>B. officinalis</i>	Lithuania, Vilnius	Environs of Trīnapolis church and monastery	2017	Motiejūnaitė	LD1930737	MG742561	MG742595	–	MG742668
18PL	<i>B. officinalis</i>	Poland, Dolnoslaskie	Wzgórza dalkowskie, Szczyglice apud Głog’w	2004	Charytonowicz and Koziol	LD1891007	MG742562	MG742596	MG742632	MG742669
19PL	<i>B. officinalis</i>	Poland, Pomorskie	Kwidzyn [Marienwerder] Rezerwat przyrody Kwidzyńskie Ostnice	2017	Thell, Wszalek- Rożek, Jarońska, and Seaward	LD1909748	MG742563	MG742597	–	MG742670

(Continued)

Table 1. (Continued)

Sample	Taxon	Country, province	District, locality	Year	Collector	Herbarium Acc. no	GenBank accession numbers			
							ETS	trnT-trnL	rRNA-Leu	trnS-trnG
20SE	<i>B. officinalis</i>	Sweden, Skåne	Brunnby, 150 m NO of Mölle former railway station [extinct]	2001	Lindroth	LD1146758	MG742564	MG742598	MG742633	–
21SE	<i>B. officinalis</i>	Sweden, Skåne	Mölleråsvägen 15 Brunnby, Mölle,	2000	Lindroth	LD1769263	MG742565	MG742599	MG742634	MG742671
22SE	<i>B. officinalis</i>	Sweden, Skåne	Höör, Holma meadows [planted]	[2015]						
23SE	<i>B. officinalis</i>	Sweden, Skåne	Lund, Botanical Garden [old Swedish grown material]	2015	Tyler Thell and Veste	LD1801983 LD1769135	– MG742566	MG742600 MG742601	MG742635 MG742636	MG742672 MG742673
24SE	<i>B. officinalis</i>	Sweden, Skåne	Odarslöv, private garden (remains of the Turesson, 1930 material)	2015	R. Svensson	LD1770607	MG742567	MG742602	MG742637	–
25SE	<i>B. officinalis</i>	Sweden, Skåne	Stehag, Värilinge	1860 [2015]	Sandberg	LD1157437	–	MG742603	MG742638	MG742674
26SE	<i>B. officinalis</i>	Sweden, Skåne	Stehag, Värilinge	2017	Hedren	Extract only	MG742568	MG742604	MG742639	MG742675
27SE	<i>B. officinalis</i>	Sweden, Skåne	Stehag, Värilinge	2017	Hedren	Extract only	MG742569	MG742605	MG742640	MG742676
28SE	<i>B. officinalis</i>	Sweden, Skåne	Stehag, Värilinge	2017	Hedren	Extract only	MG742570	MG742606	MG742641	MG742677
29SE	<i>B. officinalis</i>	Sweden, Skåne	Stehag, Värilinge	2017	Hedren	Extract only	MG742571	MG742607	MG742642	MG742678
30SE	<i>B. officinalis</i>	Sweden, Skåne	Svalöv, Axelvold [extinct]	1994	Johansson	LD1151351	–	–	MG742643	–
31SE	<i>B. officinalis</i>	Sweden, Skåne	Säby, Säbyholm, in grassland close to road [escaped]	2007	Å. Svensson	LD1292510	MG742572	MG742608	MG742644	MG742679
32SE	<i>B. officinalis</i>	Sweden, Skåne	Södra Sandby, Kungsmarken	1906 [2015]	Pählman	LD1151831	MG742573	MG742609	MG742645	MG742680
33SE	<i>B. officinalis</i>	Sweden, Skåne	Trollenäs, Ulfstorp meadow	1924 [2015]	Lange	LD1152775	MG742574	MG742610	MG742646	MG742681
34SE	<i>B. officinalis</i>	Sweden, Skåne	Västra Sallerup, Kastberga skog	1947 [2015]	Lange	LD1157377	–	MG742611	MG742647	MG742682
35SE	<i>B. officinalis</i>	Sweden, Skåne	Västra Sallerup, Kastberga meadow	1940 [2015]	Nilsson	LD1551531	MG742575	MG742612	MG742648	MG742683
36UK	<i>B. officinalis</i>	United Kingdom, England	West Yorkshire, Wakefield, Moor Lane	2017	Persson, Seaward, and Thell	LD1908068	–	MG742613	MG742649	–
37UK	<i>B. officinalis</i>	United Kingdom, England	West Yorkshire, Wakefield, E part of Brockdale Nature Reserve	2017	Persson, Seaward and Thell	LD1908004	–	MG742614	MG742650	–
38UK	<i>B. officinalis</i>	United Kingdom, England	North Yorkshire, Maltby [dwarf phenotype]	2017	Persson, Seaward, and Thell	LD1933169	–	MG742615	MG742651	–
39IT	<i>B. hirsuta</i>	Italy, Trentino-Alto Adige	Trento, Tonale	2016	Hedren	LD1933876	MG742577	MG742617	MG742653	MG742684
40IT	<i>B. hirsuta</i>	Italy, Trentino-Alto Adige	Trento, Tonale	2016	Hedren	LD1899015	MG742578	MG742618	MG742654	MG742685
41IT	<i>Stachys alopecuroides</i>	Italy, Lazio	Rieti, Passo Terminillo	2016	Hedren	LD1934004	MG742576	MG742616	MG742652	–

Note. All specimens belong to *Betonica officinalis* L. except for numbers 39–41 which belong to the outgroups, *B. hirsuta* L., 39–40, and *Stachys alopecuroides* (L.) Benth. Year within square bracket indicates fresh collections at the old locality.



Fig. 8. *Betonica hirsuta* L., Trento, Italy, July 8, 2016 (39–40IT) (photo: M. Hedrén)



Fig. 9. *Stachys alopecuroides* (L.) Benth. Lazio, Italy, July 8, 2016 (41IT) (photo: M. Hedrén)

unspecific product, the desired band was excised from the gel for extraction and purification with a Nucleospin PCR clean-up and Gel extraction kit from Macherey-Nagel (Germany) before being sent to sequencing as above.

Phylogenetic methods

Nuclear and plastid sequences were analyzed both separately and merged. All sequences used in this study are obtained *de novo* (Table 1). No sequences of interest for completion were found in the GenBank. Sequences were processed in MEGA7 (Temple University, Philadelphia, USA; King Abdulaziz University, Jeddah, Saudi Arabia; and Tokyo Metropolitan University, Hachioji, Japan), aligned by Muscle and adjusted by visual inspection (Hall, 2013; Kumar et al., 2016). Phylogenetic trees were reconstructed in MEGA7 using two statistical methods, maximum parsimony (MP) using the

search method Tree Bisection Reconnection and maximum likelihood (ML) using the General Time Reversible Model. All sites, including gaps and missing characters were used. All gaps were recoded to letters and treated as a fifth character, except for a deletion in one of the outgroups, *B. hirsuta*, where only one site was recoded to letters. The “Skåne-duplication” was replaced as one character only. Finally, a bootstrap test was performed for both statistical methods with 1,000 replicates (Felsenstein, 1985). Separate analyses were performed for plastid sequences, *ETS* sequences, and a combined matrix of *ETS* and plastid sequences.

Phenetic analysis of the *ETS* sequence variation

Data for phenetic analysis of the *ETS* region were obtained by Sanger sequencing of genomic DNA. *ETS* is part of the nuclear rDNA repeat regions. rDNA genes are located in tandemly repeated units consisting of the three rDNA genes 18S, 5.8S, and 25S separated by the internal transcribed spacer regions *ITS 1* and *ITS 2*, respectively (Jorgensen & Cluster, 1988). The repeat units are separated by the *ETS* and the non-transcribed *IGS*. rDNA repeat units typically occur between 1,000 and 10,000 copies in any haploid genome, and may be distributed between one and several loci. Because of the large number of gene copies, only relative proportions of sequence variants could be estimated by inspection of the peak sizes in the electropherograms obtained from the sequencer. In addition, because sequence variants may occur within and between loci, the origin of different sequence variants could not be traced to any specific locus. Moreover, when more than one variable site was identified in a sequence, we had no information on how the variants at different positions along the *ETS* sequence were related to each other. To extract as much information as possible without adding bias, we therefore had to interpret each variable position as an independent character and perform a phenetic analysis of our combined data set (see Pillon et al., 2007 for an example of this methodology). Relative peak sizes recorded from the variable positions in *ETS* (Table 5; Fig. 11) were translated into allele frequency data and used in calculating pairwise Cavalli-Sforza Chord distances between accessions (Cavalli-Sforza & Edwards, 1967), as if allele frequencies were representing population averages. Due to the fact that allele frequencies were estimated from sequence data, linkage between positions could not be estimated and positions were essentially treated as unlinked loci. The matrix of pairwise genetic distances was rotated in a principal coordinates analysis (PCOA) to extract the maximum proportion of dispersion between samples along the first two principal coordinates. Cavalli-Sforza chord distances and the PCOA were calculated using NTSYSpc (Exeter Software, Setauket, NY, USA; Rohlf, 2005).

RESULTS

Identification of genetic variation

One hundred and thirty-eight sequences were produced for the phylogenetic analyses and submitted to GenBank. The entire matrix, after alignment, measured 2,375 bp, including



Fig. 10. Sites of the studied samples. Forty-one samples from 11 European countries were included in the study

the outgroups *B. hirsuta* and *S. alopecuroides*; 147 variations in the DNA (mostly SNPs) were found, of which 39 concerned the ingroup only (Table 3). The total matrix was composed of four molecular regions. The *ETS* matrix had a length of 435 bp. One deletion and 50 SNPs were found, of which 10 were of interest for the ingroup (Tables 3–5). The *trnT-trnL* IGS region in the chloroplast had a length of 712 bp, revealing 43 sequence variants of which 24 provided information of the ingroup. This variation also included a 19-bp-long duplication in some of the Swedish samples (the “Skåne-duplication”). Concerning the 499-bp intron region of the chloroplastic *trnL* gene, the alignment resulted in 11 SNPs of which only two were relevant for the ingroup. Finally, the partial *trnS-trnG* IGS in the chloroplast was 729-bp long, including 43 informative DNA variants. Because the major part of the variation depended on a 33-bp long deletion in *B. hirsuta*, only three SNPs provided information to the ingroup (Tables 3 and 4).

Phylogeography

Several branches with bootstrap support of more than 50% were revealed in the separate phylogenetic trees based on plastid and *ETS* sequences as well as in the combined tree based on the merged matrix (Figs 12–17). However, groups supported in phylogenetic analyses based on *ETS* sequences were not supported in the plastid phylogeny, and groups supported in phylogenetic analyses based on

plastid sequences were not supported in the *ETS* phylogeny because of lacking *ETS* sequences (Table 5). In addition, using different phylogeny methods, such as MP or ML, resulted in similar results but the ML method generally gave stronger support. Seven groups, such as I–VII, were supported by different matrices and methods (Table 5; Figs 12–17).

The phylogeny based on plastid sequences using MP resulted in five most parsimonious trees, tree length = 98, consistency index (CI) = 0.973333, retention index (RI) = 0.987013. Three groups with support were identified. A group of five samples wearing the “Skåne-duplication” (I) had maximum support in the bootstrap analysis (Table 5; Fig. 12). The strongest bootstrap support, 76%, was found for a group of two dwarf samples, 37–38UK (II), collected at dry places, dry meadow and heath in Yorkshire respectively (Table 5; Fig. 12). The second strongest supported branch, bootstrap support 69%, was composed of a sample from Moor Lane, 36UK, growing in a rather wet meadow in Yorkshire and one sample representing material introduced into Sweden, i.e., a population known to be planted in a meadow north of Höör, 22SE (III), in central Skåne (Table 5; Fig. 12). However, the method supported the same three groups with maximum support for taxa having the Skåne-duplication (Fig. 13). The ML method calculated a tree with the highest log likelihood: –3159.4764.

The *ETS* phylogeny using MP resulted in seven most parsimonious trees (tree length = 29, CI = 0.952381,

Table 2. Primers tried and used in the phylogenetic analyses

DNA-region	Primers and references	Primer sequences
ETS – intergenic spacer between the 18S and 26S rRNA genes, nuclear gene <i>trnT–trnL</i>, intergenic spacer region; plastid gene	18S-ETS (Baldwin & Markos, 1998, p. 450) and 5' ETS-B (Beardsley & Olmsted, 2002: 1094)	18S-ETS: 3'-ACTTACACATGCATGGCTTAATCT-5' ETS-B: 3'-ATAGAGCGCGTGAGTGGTG-3'
<i>trnT–trnL</i> – intergenic spacer region; plastid gene	Primers a and b Taberlet et al. (1991, p. 1106)	a: 5'-CATTACAAATGCGATGCTCT-3' b: 5'TCTACCGATTTTCGCCATATC-3'
<i>trnL</i>, intron; plastid gene	c and d in Taberlet et al. (1991, p. 1106)	c: 5'-CGAAATCGGTAGACGCTACG-3' d: 5'GGGGATAGAGGGACTTGAAC-3'
<i>trnL–trnF</i> – intergenic spacer region; plastid gene	e and f in Taberlet et al. (1991, p. 1106)	e: 5'-GGTTCAAGTCCCTCTATCCC-3' f: 5'-ATTTGAACTGGTGACACGAG-3'
<i>trnS–trnG</i> – intergenic spacer, partial sequence; plastid gene	<i>trnS</i> (GCU) and <i>trnG</i> (UCC) (Hamilton, 1999, p. 522)	5'-GCCGCTTTAGTCCACTCAGC-3' 5'-GAACGAATCACACTTTTACCAC-3'
5.8S rRNA gene and its flanking spacer regions (ITS); nuclear gene	ITS4 and ITS5 (White et al., 1991)	ITS4: 5'-TCCTCCGCTTATTGATATGC-3' ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3'
5.8S rRNA gene and its flanking spacer regions (ITS); nuclear gene	ITS1 and ITS4 (White et al., 1991)	ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' ITS4: 5'-TCCTCCGCTTATTGATATGC-3'
5.8S rRNA gene and its flanking spacer regions (ITS1 and ITS2); nuclear gene	ITS4 (White et al., 1991) and <i>leu1</i> (Urbatsch et al., 2000) as “ITS1”, later ITSleu1 (Bohs & Olmstead, 2001)	ITS4: 5'-TCCTCCGCTTATTGATATGC-3' ITSleu1: 5'-GTCCACTGAACCTTATCATTTAG-3'
5.8S rRNA gene and its flanking spacer regions (ITS1 and ITS2); nuclear gene	ITS5a and ITS241R (Prince & Kress, 2006)	ITS5a: 5'-TTATCATTTAGAGGAAGGAGAAGTC-3' ITS241R: 5'-CAGTGCCTCGTGGTGCGACA-3'
5.8S rRNA gene and its flanking spacer regions (ITS and ITS2); nuclear gene	ITS5a_alt (Stanford et al., 2000) and ITS241R (Prince & Kress, 2006)	ITS5a_alt: 5'-CCTTATCATTTAGAGGAAGGAG-3' ITS241R: 5'-CAGTGCCTCGTGGTGCGACA-3'
tRNA-His (GUG) to exon 1 of tRNA-Lys (UUU); plastid gene	<i>trnHt</i> and <i>trnK1A</i> (Demesure et al., 1995)	<i>trnHt</i> : 5'-ACGGAATTGAACCCGCGCA-3' <i>trnK1A</i> : 5'-CCGACTAGTTCGGGTTTCGA-3'
tRNA-Ser (UGA) to tRNA-fMet (CAU); plastid gene	<i>trnS2</i> and <i>trnfM</i> (Demesure et al., 1995)	<i>trnS2</i> : 5'-GAGAGAGAGGGATTGCAACC-3' <i>trnfM</i> : 5'-CATAACCTTGAGGTCACGGG-3'
tRNA-Ser (GGA) to tRNA-Thr (UGU); plastid gene	<i>trnS4</i> and <i>trnT3</i> (Demesure et al., 1995)	<i>trnS4</i> : 5'-CGAGGGTTCGAATCCCTCTC-3' <i>trnT3</i> : 5'-AGAGCATCGCATTTGTAATG-3'

Note. Those successfully employed are given in boldface.

Table 3. Number of sequences, total number characters, and parsimony informative characters used in the phylogenetic analyses

DNA region	Number of sequences	Number of characters	Parsimony informative characters	Parsimony informative characters restricted to the ingroup
<i>ETS</i>	31	435	51	10
<i>trnT–trnL</i> intergenic spacer region, chloroplast	40	712	43	24
<i>tRNA-Leu (trnL)</i> gene, intron, chloroplast	36	499	11	2
<i>trnS–trnG</i> intergenic spacer, partial sequence, chloroplast	31	729	43	3
Total	138	2375	147	39

RI=0.972973). Using the *ETS* matrix and MP resulted in one group with support: eight samples from Sweden, Belarus, Poland, Lithuania, and Austria (IVd) had a weak bootstrap support value of 66% (Table 5; Fig. 14). This group was not supported in the analysis based on the merged *ETS* and plastid matrices (Figs 16 and 17). Group IV had a support of 63% in the bootstrap analysis using the ML method. An additional group (V) was weakly supported,

54%, in the bootstrap analysis. One of the German samples, 11DE, formed a branch with the Italian sample, 16IT (Fig. 15). None of the groups supported in the plastid phylogeny were supported in the *ETS* phylogeny because of lacking data. Amplification of *ETS* sequences was for English samples and the “Skåne-duplication” is situated in one of the plastid sequences. The ML method resulted in a tree with the highest log likelihood: –762.8425.

Table 4. Informative plastid characters within *Betonica officinalis*

Region			<i>trnT-trnL</i>			<i>tRNA-Leu</i>			<i>trnS-trnG</i>		
Site	116	140	183< >184	237	250	425	147	183	184	329	529
1AT	G	T	_____	T	T	T	T	A	T	T	T
2AT	G	T	_____	T	T	T	T	A	T	T	T
3BY	G	T	_____	T	T	T	T	A	T	T	G
4BY	G	T	_____	T	T	N	N	N	T	T	G
5DK	G	T	_____	T	T	T	T	A	N	N	N
6DK	G	T	_____	T	T	T	T	A	T	T	T
7FR	G	T	_____	T	T	T	N	N	N	N	N
8FR	G	T	_____	T	T	T	–	C	N	N	N
9DE	G	T	_____	T	T	T	–	A	G	T	N
10DE	G	T	_____	T	T	T	T	A	T	T	T
11DE	G	T	_____	T	T	T	–	A	G	T	T
12DE	G	T	_____	T	T	T	T	A	T	T	T
13DE	G	T	_____	T	T	T	T	A	T	T	G
14HU	G	T	_____	T	T	T	T	A	T	T	T
15HU	G	T	_____	T	T	T	N	N	T	T	T
16IT	A	T	_____	–	T	T	–	A	G	T	T
17LT	G	T	_____	T	T	T	N	N	T	T	G
18PL	G	T	_____	T	T	T	T	A	T	T	T
19PL	G	T	_____	T	T	T	N	N	T	T	G
20SE	G	T	_____	T	T	T	–	A	N	N	N
21SE	G	T	_____	T	T	T	–	A	G	–	T
22SE	G	T	_____	T	T	C	–	A	G	T	T
23SE	G	G	_____	T	T	T	T	A	T	T	T
24SE	G	T	TCGAATATTATTCTATTCC	T	T	T	T	A	N	N	N
25SE	G	T	_____	T	T	T	T	A	T	T	G
26SE	G	T	_____	T	T	T	T	A	T	T	G
27SE	G	T	_____	T	T	T	T	A	T	T	G
28SE	G	T	_____	T	T	T	T	A	T	T	G
29SE	G	T	_____	T	T	T	T	A	T	T	G
30SE	N	N	NNNNNNNNNNNNNNNNNNNN	N	N	N	T	A	N	N	N
31SE	G	T	TCGAATATTATTCTATTCC	T	T	T	T	A	T	T	G
32SE	G	T	_____	T	T	T	T	A	T	T	G
33SE	G	T	TCGAATATTATTCTATTCC	T	T	T	T	A	T	T	G
34SE	G	T	TCGAATATTATTCTATTCC	T	T	T	T	A	T	T	G
35SE	G	T	TCGAATATTATTCTATTCC	T	T	T	T	A	T	T	G
36UK	G	T	_____	T	T	C	–	A	N	N	N
37UK	G	T	_____	T	G	T	–	A	N	N	N
38UK	G	T	_____	T	G	T	–	A	N	N	N

Note. The “Skåne-duplication” is positioned between characters 183 and 184 in the *trnT-trnL* sequence. N: missing character.

Table 5. Groups with bootstrap support in phylogenetic analyses using different matrices and methods

Group	Samples	Plastid sequences MP (Fig. 12)	Plastid sequences ML (Fig. 13)	ETS sequences MP (Fig. 14)	ETS sequences ML (Fig. 15)	ETS and plastid sequences MP (Fig. 16)	ETS and plastid sequences ML (Fig. 17)
I	The “Skåne-duplication”	91	100	–	–	92	92
II	The “dwarfy phenotype”	76	65	–	–	76	60
III	22SE + 36UK	69	54	–	–	80	0
IV	1AT, 3BY, 17LT, 19PL, 20–21SE, 29SE, 35SE	0	0	66	63	0	0
V	11DE + 16IT	0	0	<50	54	68	<50
VI	20–21SE	0	0	0	0	53	63
VII	17LT + 19PL	0	0	0	<50	0	55

Note. MP: maximum parsimony; ML: maximum likelihood.

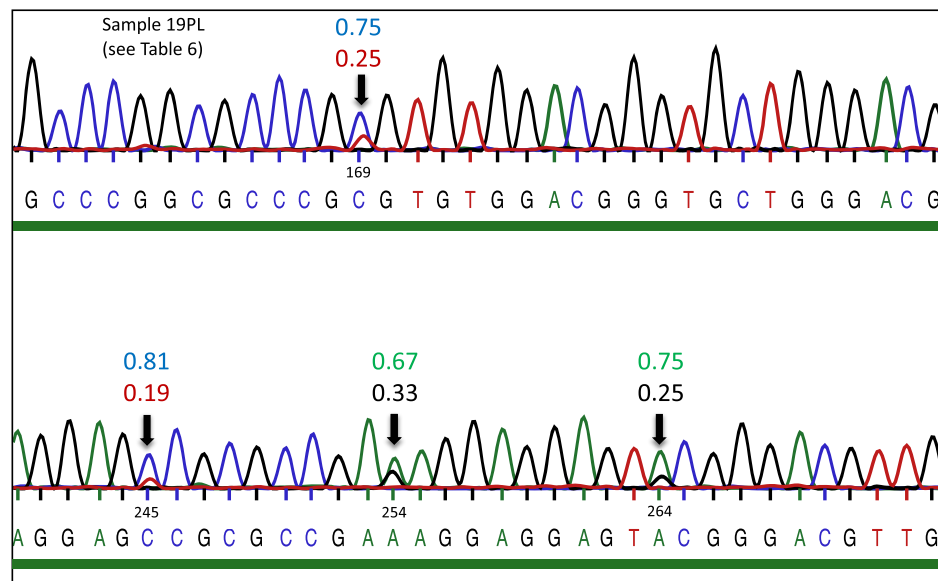


Fig. 11. Part of *ETS* sequence (sample 19PL, Table 1) showing four variable loci, 169, 245, 254, and 290, and their relative peak sizes (Table 6)

A total phylogeny of the merged *ETS* and plastid matrices using MP resulted in six most parsimonious trees (length = 157, CI = 0.940000, RI = 0.968586). This analysis confirmed three of the above five branches with moderate support. The dwarfy English samples, 37–38UK (II), having a support of 76%. The sample from Moor Lane in Yorkshire and the sample from Höör in Skåne (III), having a support of 80%. One of the German samples, 11DE and the Italian 16IT, form a branch, V, with bootstrap support 68%. Finally, with a weak bootstrap support of 53%, the two samples from Brunnby in Skåne, 20–21SE (VI), formed a branch in the total analysis (Table 5; Fig. 16). The samples wearing the “Skåne-duplication” was strongly supported in the bootstrap analysis, 92%. Using ML, the “Skåne-duplication” received the same strong support. In addition, the ML gives support, although weak (55%), to a group including the samples from northern Poland, 19PL, and Lithuania, 17LT (VII). The group composed of the German sample 11DE and the Italian 16IT has no support using ML (Table 5; Fig. 17). A tree with the highest log likelihood, −4238.5636, was calculated.

The variation detected at the 28 variable positions in the *ETS* sequences, including the 10 parsimony informative characters, provided a clearer picture of the geographic variation patterns than the phylogeny based on plastid sequences (Table 6). Representative samples from all seven putatively spontaneous localities in Skåne and Denmark, with or without the “Skåne-duplication,” clustered close together (Fig. 18). Furthermore, some cultivated or feral populations from Skåne, with origins we intended to verify, were positioned in the same group indicating local origins. Since six samples lack data from the variable *ETS* positions, their positions in the PCOA ordination were determined only or mainly by the 10 parsimony informative *ETS* characters and therefore have uncertain positions (Table 6; Fig. 18).

SNPs and autapomorphies

Unique SNPs in the *ETS* region were found exclusively in German material. One sample from Schleswig-Holstein had

three SNPs, whereas material from Sachsen had two SNPs. However, as many as 28 positions were variable within or between accessions (see below). Of the 31 *ETS* sequences, 26 were clear enough to estimate relative peak sizes directly from the chromatograms (Table 6; Fig. 11). Variable positions mostly involved two alternative bases, but two positions, 142 and 180, expressed three alternative bases, and all four bases were found at position 245 (Table 6). Individual samples were mostly variable with two alternative bases at any single position, but in sample 13DE from Germany, three different bases were expressed at position 142 (Table 6). The two outgroup species did not show any *ETS* variability and were excluded from the *ETS* analysis. Sequences where relative peak heights could not be estimated were coded entirely for the highest peak at each position, except for samples 10DE and 21SE, where variation could be estimated at a fraction of the variable positions, at one and two positions, respectively (Table 6).

Five of the samples from the province of Skåne had a unique 19 bases long duplication situated between positions 183 and 184 in the *trnT–trnL* sequence (Table 4). The sample from Italy had one autapomorphy and one unique gap. The sample from Lund Botanical Garden, referred to as “old established Swedish grown material” according to the former superintendent Lennart Engstrand, also had one autapomorphy in the *trnT–trnL* sequence. The collection from the French Alps labeled *B. officinalis* var. *montana* ined. [*Stachys officinalis* var. *montana* (Lej.) Dumort.] carried the single autapomorphy in the *trnL* intron sequence (Table 4), whereas no autapomorphies were observed in the *trnS–trnG* IGS.

DISCUSSION

Several primer pairs were tested in this explorative study, some of which failed (Table 2). A second nuclear rDNA region in addition to *ETS*, comprising the internal transcribed spacers (*ITS*), was also tested, but failed for

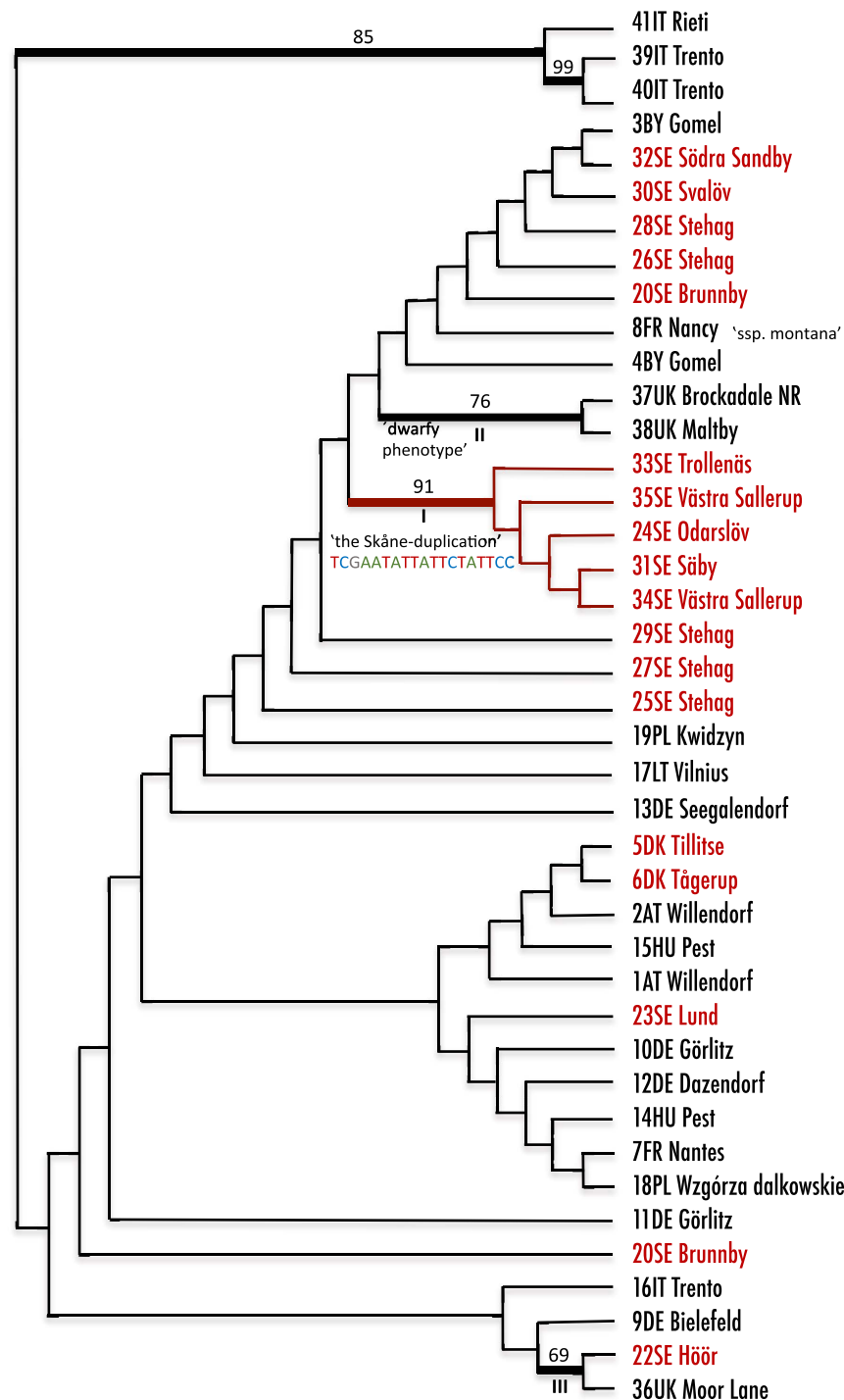


Fig. 12. Bootstrap consensus tree based on plastid sequences using the maximum parsimony (MP) method and the Tree-Bisection-Reconnection (TBR) model. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. Supported groups I–III, are indicated beneath thick branches

unknown reasons (Table 2), in spite of the fact that *ITS* has been successfully sequenced in an earlier study of *B. officinalis* published by Salmaki et al. (2012, 2013). *ITS* is also provided as an unpublished sequence JF330306 by Akcicek et al. in GenBank (www.ncbi.nlm.nih.gov), and has been sequenced in other members of the *Lamiaceae* (Silveira & Simpson, 2013) and in other families within the *Lamiales* (Beardsley & Olmstead, 2002). In the latter study, it was stated that *ETS* is relatively easy to amplify

compared to *ITS*, but without explaining or discussing possible reasons. Both *ETS* and *ITS* have been successfully used in resolving the phylogeny of not-too-distantly related species and genera of vascular plants. The *ETS* region is longer than *ITS* (*ITS1* + *ITS2*) and also offers more variation since it evolves faster (Baldwin & Markos, 1998; Beardsley & Olmstead, 2002); hence, it would probably add little useful information to our intraspecific study.

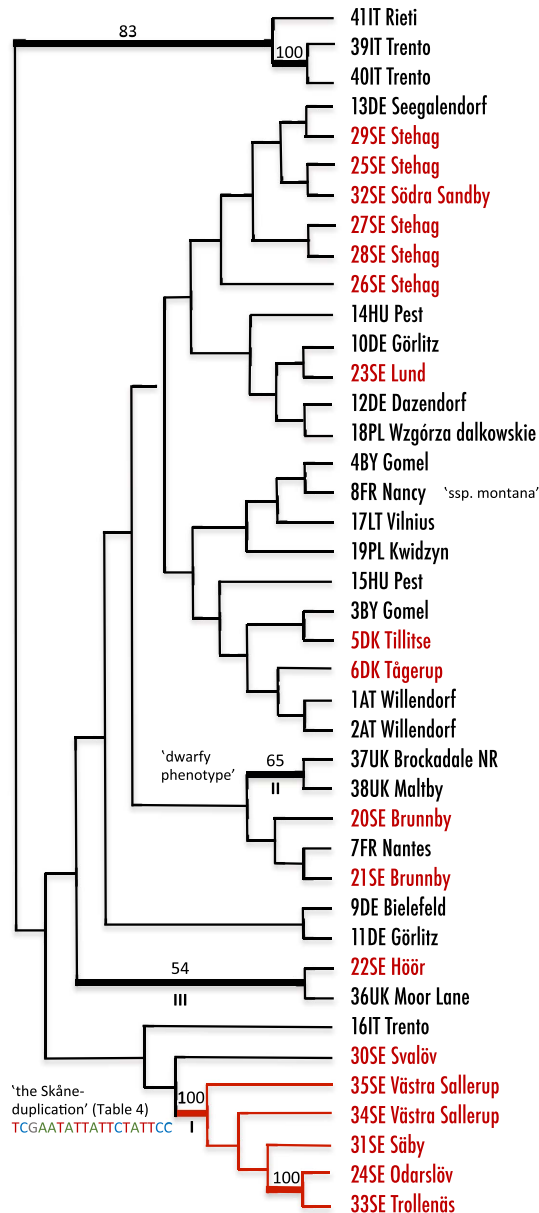


Fig. 13. Bootstrap consensus tree based on plastid sequences using the maximum likelihood (ML) method and the General Time Reversible (GTR) model. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. Supported groups I–III are indicated beneath thick branches

However, the phylogeography indicated a possible spontaneous origin for some of the Scandinavian populations, a pattern most apparent in the PCOA of the *ETS* data and for samples with complete sequences since they formed one group (Fig. 18). A spontaneous origin was indicated only for populations wearing the “Skåne-duplication” in the phylogenetic tree based on plastid sequences, which formed a branch with moderate support. Since the main focus of the study was on putatively spontaneous populations in Denmark and Skåne, the investigated localities or populations are described and discussed, including their history. In Denmark, there are two populations regarded as

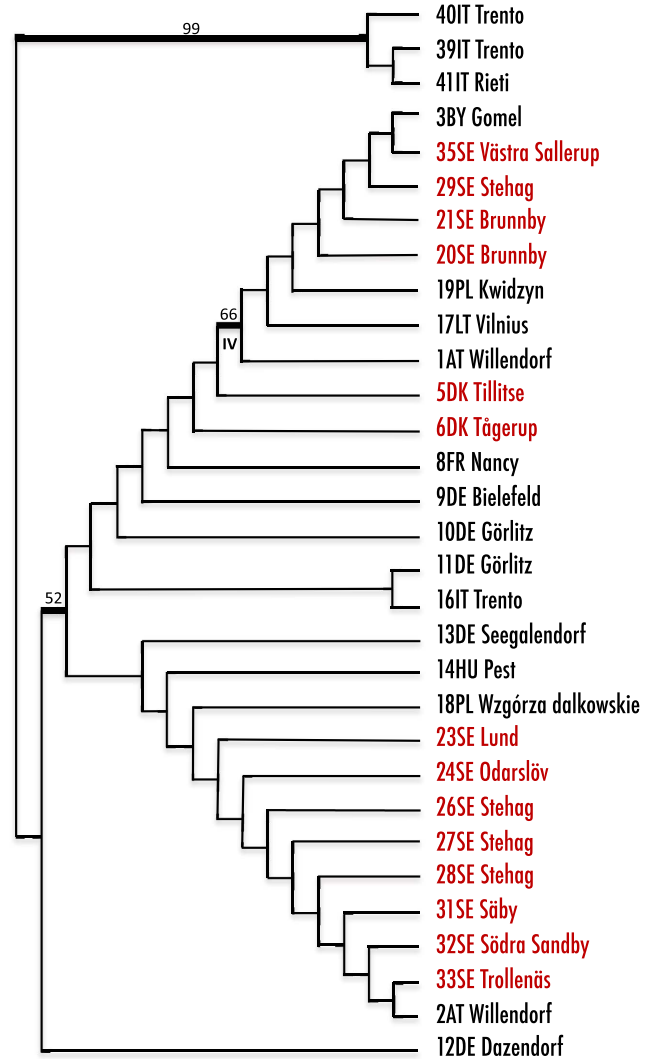


Fig. 14. Bootstrap consensus tree based on *ETS* sequences using the maximum parsimony (MP) method and the Tree-Bisection-Reconnection (TBR). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. The supported group IV, is indicated beneath the thick branch

spontaneous left, in Tillitse and Tågerup parishes. Stehag, Södra Sandby, Trollenäs, and Västra Sallerup are four parishes in Skåne that still hold betony localities of presumably spontaneous origin. Additional localities from Skåne, included in the analyses, have different background according to the results. Three populations from Skåne with unknown origins are positioned in the “Scandinavian group” in the *ETS* ordination, those from Brunnby parish, 20–21SE, and Lund Botanical Garden, 23SE. This result was not supported by the phylogenetic analyses. However, a branch composed exclusively of Swedish samples containing the “Skåne-duplication” was supported by the analysis based on plastid sequences, that is, material collected in the three neighboring localities in Trollenäs and Västra Sallerup parishes and, in addition, in cultivated material from

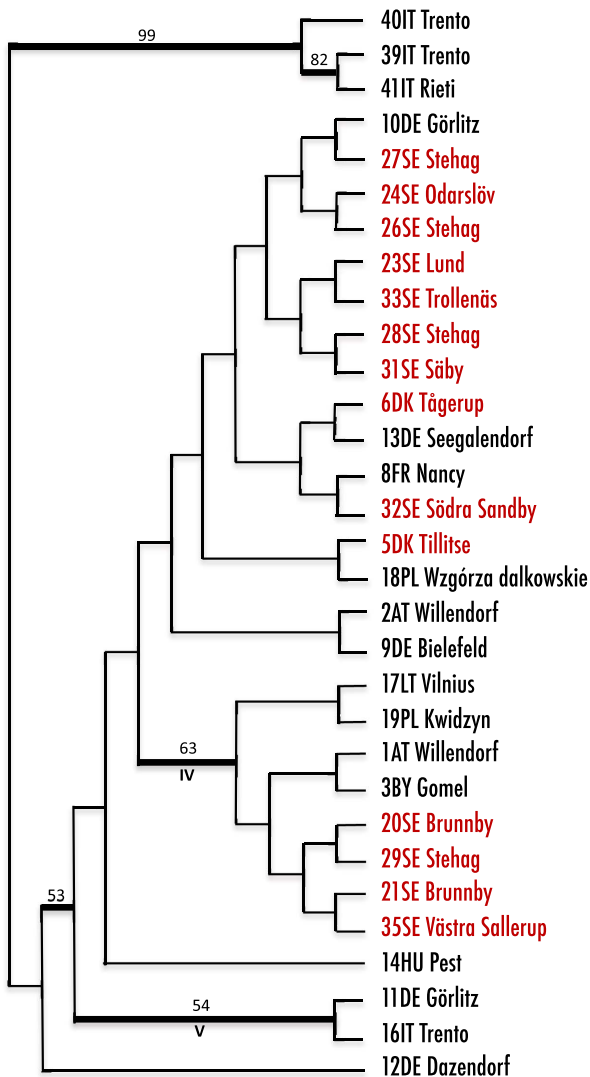


Fig. 15. Bootstrap consensus tree based on *ETS* sequences using the maximum likelihood (ML) method and the General Time Reversible (GTR) model. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. Supported groups IV and V, are indicated beneath thick branches

Odarslöv, 24SE, and in feral plants from Säby, 31SE. The cultivated material obtained from Odarslöv and Säby is believed to have an origin in central Skåne, whereas the population in the north of Höör has an origin in England, according to our results based on plastid sequences, 22SE and 36UK. We did not succeed to sequence *ETS* from these two samples.

Possibly spontaneous localities in Scandinavia

Denmark, Lolland: Tågerup and Tillitse parishes. The two still existing populations in Denmark, situated near Rudbjerggaard castle in Tillitse parish, 5DK and in Bjerremark in Tågerup parish, 6DK, are last remnants of a rather large distribution area in southwestern Lolland (Andersen, 1942; Müller, 1778). Both two populations were represented and

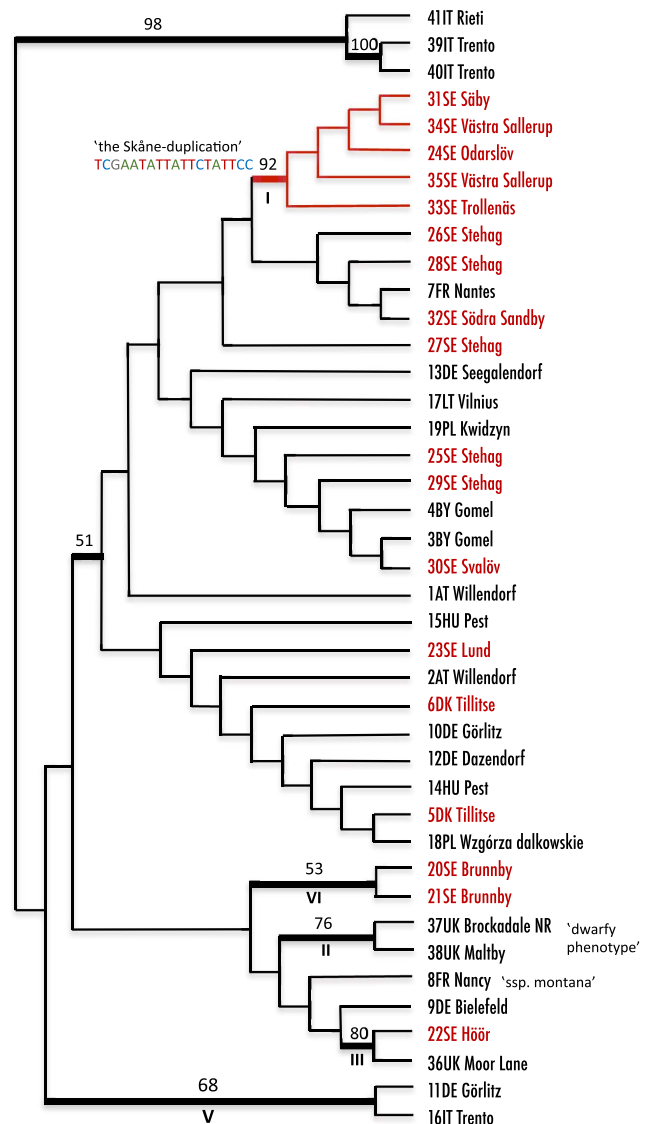


Fig. 16. Bootstrap consensus tree based on the merged plastid and *ETS* matrices using the maximum parsimony (MP) method and the Tree-Bisection-Reconnection (TBR). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. Supported groups II–VI, are indicated beneath thick branches

bore a relationship with the Swedish populations according to the PCOA ordination (Fig. 18).

Sweden, Skåne: Stehag parish. The historically interesting locality in Stehag was represented by several individuals in the analyses (25–29SE; Table 1). They were not located close together in the PCOA ordination, but were somewhat separated from each other within the Scandinavian group (Fig. 18).

Sweden, Skåne: Södra Sandby parish. The sample from Kungsmarken in Södra Sandby parish, 32SE, is closely related to the sample 26SE from Stehag, according to the PCOA ordination (Fig. 18).

Sweden, Skåne: Trollenäs and Västra Sallerup. Three localities, 33–35SE, are situated along 1 km, almost straight line from Trollenäs parish in the northwest to Kastberga meadow and forest in Västra Sallerup toward the southeast

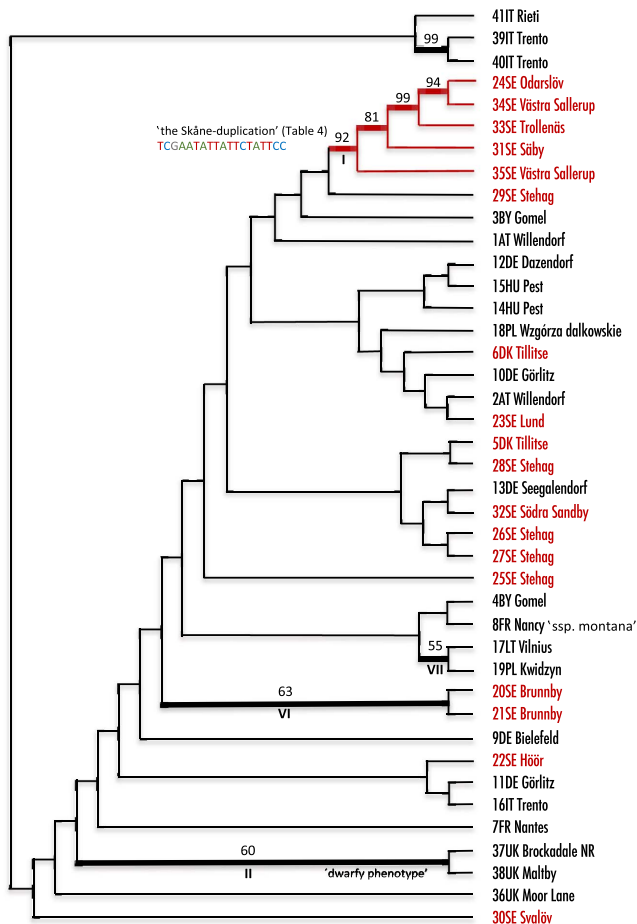


Fig. 17. Bootstrap consensus tree based on the merged plastid and ETS matrices using the Maximum Likelihood (ML) method and the Tree-Bisection-Reconnection (TBR). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown above thick branches. Supported groups I, II, VI, and VII, are indicated beneath thick branches

(Fig. 4). They constituted a core of the possibly spontaneous distribution in Skåne, geographically and genetically, since they received support both in the PCOA ordination and in the plastid phylogeny where they belonged to the branch of populations wearing the “Skåne-duplication” (Figs 12, 13, 16, 17).

Cultures with a possible origin in Scandinavia

Sweden, Skåne: Brunnby parish. One of the now extinct populations in Brunnby parish, had a natural appearance, growing on a meadow just northeast of the railway station in the township Mölle. However, since it was collected there remarkably rarely, only once, in 1904, before the early 2000s, it was perhaps introduced from material taken from central Skåne. The two samples from Brunnby form a weakly supported branch in the total analyses (Figs 16 and 17).

Sweden, Skåne: Lund. The sample collected in the spice garden in Lund Botanical Garden, “old established Swedish grown material” (Lennart Engstrand, *pers. comm.*),

probably originated from Skåne, perhaps from a mixed material, since other populations of betony also grow in the garden, including material originating from Kungsmarken in Skåne.

Sweden, Skåne: Odarslöv parish. Populations from Skåne, including this one from Odarslöv, with or without the “Skåne duplication” formed one group, in the PCOA ordination (Fig. 18). The sample from Odarslöv originates from the same material that Turesson (1930) used in his garden study, but unknown from which of his eight localities. However, it contains the “Skåne-duplication,” thus supported in the “Skåne-duplication” branch in the phylogenetic tree based on plastid sequences. Interestingly, the “Skåne-duplication” has not been found in material from the two other presumably naturally occurring localities in Skåne, i.e., Stehag and Kungsmarken in Södra Sandby, the two populations from Skåne represented in the study by Turesson (1930). Plants with the “Skåne-duplication” may have previously occurred in Stehag, or maybe still do, since the duplication was detected in samples collected only a few kilometers away in Västra Sallerup and Trolleås parishes, an area formerly connected by coherent meadow forests according to old maps.

Sweden, Skåne: Svalöv parish. The sample from Axelvold in Svalöv parish, 30SE, collected in 1994, has been searched at its original site at multiple occasions, but without success (Thell, 2016b). A fragment from the collection was included in the analysis, but only DNA from the *trnL* intron was successfully amplified, which was not enough for indicating its origin (Figs 12 and 13). The locality may have been spontaneous since it would fit rather well with the distribution area.

Sweden, Skåne: Säby parish. The population in Säby is believed to have escaped from the well-known botanist Arvid Nilsson’s culture at Säbyholm. This population was probably established from material collected in central Skåne, perhaps in Trolleås, the richest locality during Nilsson’s most active period (J. T. Johansson, personal communication). This presumption is supported by the presence of the “Skåne-duplication” (Table 4; Figs 12, 13, 16, and 17).

Scandinavian population with foreign origin

Höör parish. A foreign origin was supported only for the population from Höör, 22SE, among samples from Skåne, whose closest relative was found in Yorkshire (Figs 12, 13, 16).

Extrascandinavian populations

France and the United Kingdom – Different genotypes or taxa. Separated from the phylogeographic patterns, two distinct genotypes were distinguished in the plastid sequences, the “dwarfy” populations from France and the United Kingdom, one mountain genotype and one heath genotype, which may deserve taxonomic recognition (Figs 7, 12, 13, 16, 17). The mountain genotype has been described as *Stachys officinalis* var. *montana* (Lej.) Dumort., but this variety is rarely separated in modern literature. Meadow populations in France and the United

Table 6. The 28 variable *ETS* sites within *Betonica officinalis*, 10 of which are parsimony informative

Thell et al.

Note. Sequences 2, 6, 8, 9, and 10 were not sharp enough for measuring variation, for which only the 10 parsimony informative characters were included in the PCOA, marked in plane pace in Fig. 18. The outgroups, 39–41, were excluded from the PCOA ordination, since they were too deviant from *B. officinalis* sequences. Sequences with complete variation are in boldface.

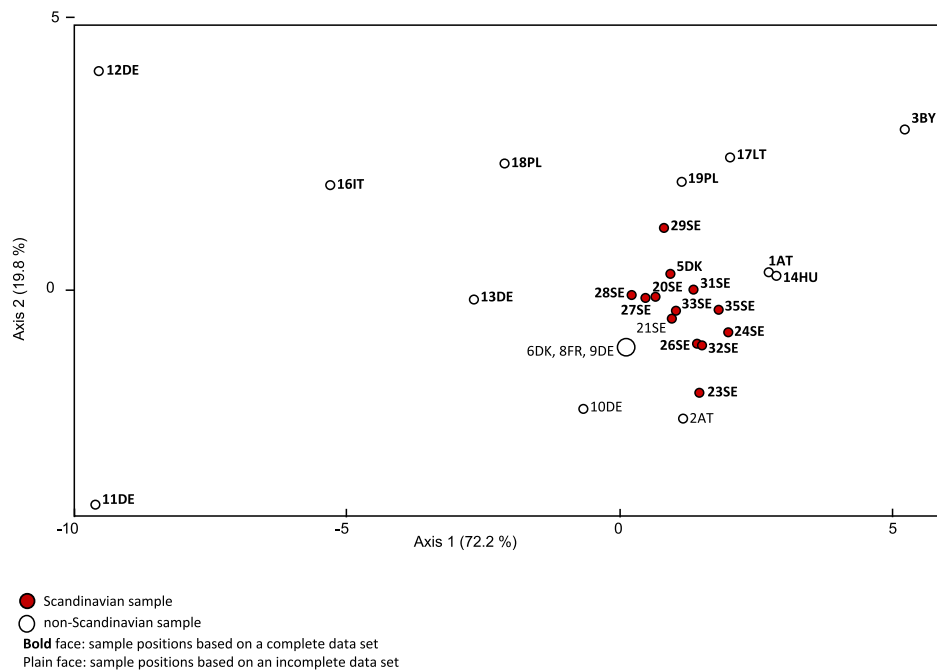


Fig. 18. PCOA ordination including representatives from all seven putatively spontaneous localities from Scandinavia, with or without the “Skåne-duplication,” clustered close together and is marked as filled dots. Six samples, 2, 6, 8–10, and 21, lack data from the variable loci, partly or entirely. Their positions were therefore uncertain and determined mainly by the ten parsimony informative *ETS* characters (Table 6). Samples with all variation determined are in bold face

Kingdom appeared to be more closely related to foreign meadow populations than with the local mountain and heath genotypes (Figs 12, 13, 16, 17). Betony is rare in western France and in Britain and Ireland. It is very rare in Ireland, where it occurs only in Cork and is also rare in Kerry counties (Parnell & Curtius, 2012), and also rare in Scotland and Wales according to Stace (2019, p. 657) who states that it may be “very dwarfy on grassy cliff tops,” but he does not recognize this form taxonomically. In Yorkshire, where betony was collected for this study, both standard and more or less dwarfy phenotypes were collected. Three samples were selected among the material for the study, where sample 36UK could be referred to as common meadow form, growing in a rather moist meadow, while sample 37UK is dwarfy and collected from a dry meadow on calcareous soil and sample 38UK grew on a moist heath (Table 1; Fig. 7). The two dwarfy samples formed a moderately supported branch in the phylogenetic tree, whereas 36UK differed genetically and formed a branch with the planted material from Höör, Sweden, except when using the merged matrix and the ML method (Figs 12, 13, and 16). We did not manage to sequence *ETS* from any of the Yorkshire samples. The dwarfy samples usually had comparatively short, rounded spikes, with fewer flowers (Figs 7 and 19), and often without an extra whorl of flowers a piece beneath the main cluster, characteristic for the common meadow form (Fig. 6). The different phenotypes collected in Yorkshire is currently being compared in a garden experiment to observe how they differ from each other and from other European samples when grown under the same conditions, and an early observation shows that the dwarfy phenotype remains dwarfy when grown in rich soil (Fig. 19). According to Stace (2019, p. 657), betony avoids heavy soils in Britain, as opposed to its preferences in Scandinavia.

Austria and Hungary. The Austrian samples, 1–2AT, are geographically closest to the Hungarian samples, 14HU and 15HU, and the population from Southern Poland, 18PL (Fig. 10). The two Austrian samples differed slightly from each other at *ETS* position 142, 169, and 262 (Table 6). The ordination based on *ETS* data indicated a relationship between 1AT and the Hungarian sample 14HU. The second Austrian sample, 2AT, is less reliable since it partly lacked data from the *ETS* positions variable in other samples from this area. Available sequences were identical for the two Hungarian samples and only one of them, 14HU, was included in the PCOA ordination.

Belarus, Lithuania and Poland. The two samples, 3BY and 4BY, belonging to the same population from Eastern Belarus had identical sequences, but only those from 3BY were complete. The position is rather isolated from other samples, but it appeared to be more closely related to the Lithuanian sample, 17LT, which in turn was most closely related to the sample from Pomerania in Northern Poland, 19PL, according to the PCOA ordination (Fig. 18), the positions of which were neither confirmed nor rejected by the parsimony analyses (Figs 11–13). The second Polish population, from Silesia in southwestern Poland, 18PL, had an isolated position in the PCOA ordination, but its position was not confirmed in the phylogenetic analyses, probably because it lacked the *trnL* sequence (Table 4; Figs 12 and 13).

Germany. The genetic variation of betony in Germany is the largest of the 11 countries included in the study. The German samples were not closely related to each other, as seen in the phylogenetic analyses as well as in the PCOA ordination (Figs 12–18), which cannot be explained by the fact that some of the German samples are geographically



Fig. 19. The dwarf phenotype from a heath in Yorkshire (38UK) remains dwarf when grown in rich soil, here seen in a comparative culture together with common meadow forms from Germany (13DE), Sweden (25-29SE), and Poland (19PL). June 8 and July 9, 2019 (photo: A. Thell)

distant, and in some cases are closer to populations in neighboring countries (Fig. 10). The variation is large even between the two samples from Schleswig-Holstein, collected only a few kilometers from each other. Here, three autapomorphies were found in the *ETS* sequence of the sample from Dazendorf, 12DE. The same amount of variation was observed for the two samples from Sachsen, both collected in the Görlitz district, where 11DE had three autapomorphies in the *ETS* region (Table 4). Although these German samples are geographically closer to the populations in Denmark and southwestern Poland, there was no support for a direct relationship with these populations (Figs 12–18).

Italy. Complete *ETS* and plastid DNA was obtained from a herbarium collection from 2003 from Northern Italy. This single Italian representative was characterized by one autapomorphy and a unique gap in the *trnT-trnL* region. There was no support for a close relationship with the geographically closest samples in France or Austria, neither in the phylogenetic trees nor in the PCOA ordination (Figs 12–18).



Fig. 20. Planting for the future. In addition to the supportive planting in Bjerremark, a backup locality for the rare Danish population was founded at Kristiansminde field station of Copenhagen University in Sorø. August 2018 (photo: N. Thell)

CONCLUSION FOR FUTURE BIOLOGY

Betony is decreasing in most parts of Europe due to the loss of habitats. The species is listed as endangered in several countries, among them Sweden and Denmark (Tyler et al., 2007, p. 471; Hartvig, 2015, pp. 613, 614). Except Kungsmarken in Södra Sandby parish in Skåne, where a successful rescue project has been undertaken, the number of individuals has slowly declined at the naturally occurring localities in Scandinavia. The decrease began a long time ago with the loss of open woods and meadows. The main threats today are intense forestry, inappropriate mowing, overgrowth, grazing by deer (mainly the flower stalks), and foraging by wild boars, which destroy the roots; the latter threat is probably the most severe one, at least in Scandinavia. However, the Kungsmarken locality project encouraged us to undertake new rescue projects to save the historically interesting populations, first and foremost in Stehag, where betony is documented since 1534, and at the roadside locality in Bjerremark on Lolland, where it was collected in 1853, according to a herbarium sheet kept in Lund (Thell, 2016b). Since 2017, local material is planted both at the localities and at backup localities followed by careful documentation. The conservation project started in Denmark where betony was most critically endangered and the decline had been most evident. Seeds were taken from the two remaining flowering

plants in 2016 (Fig. 2). During the next 2 years, 100 seedlings were placed along the roadside in Bjerremark, of which most seem to survive much depending on a management plan that was established with the municipality of Lolland. Roadsides are the last retreat places in the modern farmland for many meadow plants and furthermore the roadside flora often suffers from too intensive care.

The results of this study, including the discovery of the “Skåne-duplication,” support the contention by Turesson (1930) that “spontaneous” betony populations probably exist in Scandinavia. These are reasons enough for protecting the remaining localities for the future and for providing backup localities through cultivation. Such localities are established both on municipal land in Stehag and in Denmark where a backup locality was founded at Kristiansminde field station of Copenhagen University in Sorø (Fig. 20).

A comparative cultivation of betony from various countries is constantly being expanded, where we use the same materials as in the genetic studies (Fig. 19). Flowering time and plant size will be compared, following Turesson (1930), but hopefully additional characters that offer infraspecific variation will be detected.

Next step in the genetic study is to examine a further improved set of samples using Next Generation Sequencing, namely, Restriction site Associated DNA sequencing, which we believe will provide an even more nuanced picture of the infraspecific variation of this beautiful species.

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