MORPHOLOGICAL AND GENETIC RELATIONSHIPS OF *MYOSOTIS LAXA* SSP. *BALTICA* AND SSP. *CAESPITOSA*, AND TYPIFICATION OF *M. LAXA* SSP. *BALTICA*

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ABSTRACT

Intraspecific taxonomy of *Myosotis laxa* has been unclear for a long time. *M. laxa* ssp. *baltica* has been treated as a microendemic taxon of the Baltic Sea region, which has evolved in the Åland Islands and has spread northwards; the spread to southeast has been declared doubtful. Morphologically intermediate individuals between *M. l.* ssp. *caespitosa* and *M. l.* ssp. *baltica* exist; these have sometimes been classified as *M. l.* ssp. *laxa*. The aim of this paper is to clarify phylogenetic relationships between subspecies of *M. laxa s.l.* Here, *M. laxa* ssp. *baltica* is lectotypified. We proved that typical *M. l.* ssp. *baltica* does occur in the south-eastern Baltic region, namely in Estonia, using herbarium and freshly collected material. A group of plants were identified as typical *M. l.* ssp. *baltica*, but many specimens showed intermediate characters between *M. laxa* ssp. *baltica* and ssp. *caespitosa*. The two subspecies could be clearly differentiated neither by morphological characteristics nor by ITS sequences. *M. laxa s. l.* appeared to be monophyletic according to the ITS phylogeny. We propose that *Myosotis laxa* ssp. *baltica* is a coastal ecotype of *Myosotis laxa,* which has adapted to the fluctuating conditions of coastal habitats. Genetically, it has not yet evolved into a separate species and therefore it would be reasonable to assign it a rank of variety. However, further investigation including wider taxon and geographical sampling is needed to finally clarify the position of all subspecies.

KEY WORDS: Baltic Sea region, phylogeny, rDNA ITS, microendemism, taxonomy.

INTRODUCTION

Genus Myosotis

The genus *Myosotis* (family Boraginaceae) contains about 100 species and is spread in temperate zones of northern and southern hemisphere. Molecular data support the hypothesis that the genus originates from the northern hemisphere (Judd and al. 2002; Winkworth and al. 2002). European species of the genus have been studied systematically using morphological and caryological characters (Schuster 1967; Grau and Schwab 1982; Przyvara 1986/1987; Apelgren 1990a, b, 1991). Still, some problematic taxa remain in the genus, for example, the *M. laxa* complex.

The M. laxa Lehm. complex

The complex contains three subspecies: *M. laxa* ssp. *laxa*, *M. laxa* ssp. *caespitosa* (Schultz) Hyl. ex Nordh., and *M. laxa* ssp. *baltica* (Sam.) Hyl. ex Nordh. *M. laxa* was described by Lehmann in 1818, being the oldest name in this complex and is therefore used as the species name. It was described as an annual with weak branching and spreading stems, sparse inflorescences, small flowers, and calyces as well as pedicels much elongating after flowering (Lehmann 1818). Lehmann did not designate a type specimen, he only said that he has seen a dried specimen from North-America (Lehmann 1818), the present location of which is not known.

From Europe, *M. caespitosa* was described a year later by Schultz (1819). It differs from *M. laxa* by a more erect growth habit, lesser branching, denser inflorescences, shorter calyces and pedicels (which do not elongate considerably after flowering). The type material of *M. caespitosa* is indicated in type description as "ad pagum Ballin hinter dem Hofgarten, et prope Neobrandenb. am kleinen Jhlpol." (Schultz 1819), but has not been found subsequently (Schuster 1967) and it has not been possible to detect its present location in herbaria, either. Probably the same taxon was already mentioned by Lehmann (1818) as *M. lin-*

gulata, a taxon with no description (*nomen nudum*). *M. l.* ssp. *caespitosa* is considered to be distributed in the entire northern Eurasia.

Lindberg (1915) first noticed a special coastal taxon of *Myosotis* in Åland and he identified it as *M. laxa* Lehm. Later, Samuelsson (1926) described probably the same taxon as a new species, *M. baltica* Sam. His species description was rather short, in Swedish and largely resembling that of *M. laxa* by Lehmann (1818); at the same time Samuelsson declared that this *M. baltica* is neither *M. laxa* sensu Lehmann nor sensu Lindberg (Samuelsson 1926). Samuelsson did not typify the name. Later, Lindberg (1934) stressed that *M. baltica* Sam. is the same taxon he had earlier mentioned as a coastal *M. laxa* and it corresponds to the Lehmann's description of *M. laxa* and should be treated under latter name (Lindberg 1934). Specimens from Exsiccatae Fennicae! and their photos (Lindberg 1915) confirm this. However, Lindberg (1934) admits that *M. baltica* differs from the American taxon generally regarded as *M. laxa* Lehm. (Britton and Brown 1898), and may therefore be treated as a separate species.

There has also been some confusion in the citation. *M. baltica* Sam. ex Lindm. (Schuster 1967) is wrong – *M. baltica* Sam. in Lindm. is correct, as the description was published in Lindman's flora (Samuelsson 1926), but with Samuelsson's name clearly indicated. However, the "in"-phrase is mostly omitted nowadays. *M. baltica* Sam. ex H. Lindb. (The International Plant Names Index 2004. http://www.ipni.org, accessed 19.11.2007). is also not correct, as the publication of the species name by Samuelsson (1926) was completely valid considering that it was published before 1935. The clarification by Lindberg (1934) that this is the same species recognised by him as *M. laxa* does not change this. Thus the simplest form *M. baltica* Sam. is the best.

Currently prevalent understanding that *caespitosa* and *baltica* are subspecies of *M. laxa* was published in Norsk Flora (Nordhagen 1940). *M. laxa* ssp. *laxa* is a third, North American subspecies, as mostly interpreted. Apelgren (1991b) found that all three taxa are mutually continuous and lowered their taxonomic range to that of variety. She considered that *M. l.* ssp. *laxa* is amphiatlantic and all three subspecies or varieties also occur in Europe (Apelgren 1991b). The same view is now accepted in Sweden (Krok and Almquist 2004). However, some authors consider *M. laxa* ssp. *laxa* to include *M. l.* ssp. *baltica* (Hegi 1927; Schuster 1967; Lid and Lid 1994). This view is supported by finding *baltica-* or *laxa-*like plants in Kamchatka (Schuster 1967) and Japan (Saiki and Osegawa 1972).

Contrarily, some authors still consider the members of this complex as separate species (Nikiforova 2001; Cepurfte 2003). Earlier, the taxa have also been treated as subspecies of *M. scorpioides* L. (syn. *M. palustris* (L.) Nath.): *M. s.* ssp. *laxa* (Lehm.) Hegi and *M. s.* ssp. *caespitosa* (C. Schultz) Herm. (Hegi 1927).

M. laxa ssp. baltica

The taxon is restricted to the Baltic Sea region with rare findings from western Sweden and Norway (Hultén and Fries 1986). It is considered to be neoendemic and to originate from the Åland Islands in the Baltic Sea (Jonsell 1988; Apelgren 1991). According to Jonsell (1988), *M. laxa* ssp. *baltica* belongs to a group of coastal microendemics. This author considers Baltic microendemics as morphologically distinct taxa with restricted range in Scandinavia and other northern countries. Though the postglacial period in Scandinavia and Baltic countries is considered to be too short for speciation to take place (Ingelög et al. 1993), environmental conditions of the coastal zone of the Baltic Sea, namely land lifting, seasonal changes of water level and salinity as well as erosion, may accelerate the differentiation of populations (Jonsell 1988). The characteristic coastal microendemics are annual, early flowering, with shorter life cycle and autogamous, as opposed to the sister taxa occurring in the same region (Jonsell 1988).

M. laxa ssp. *baltica* is most common in south-western Finland and Åland (Ulvinen 1998) with decreasing occurrence northwards (Palmgren 1961; Apelgren 1990a). Åland has been proposed as a putative taxon formation centre (Palmgren 1961; Apelgren 1990a). It has been hypothesised that *M. laxa* ssp. *baltica* spread from Åland to the north of the Baltic Sea by prevailing streams (Apelgren 1990b). Palmgren (1927) claimed that this species does not occur in Estonia, referring to Kupffer (1925). However, *M. baltica* was described in 1926 (Samuelson 1926) and Kupffer could not have been aware of it at the time. Still, Apelgren (1990b, 1991) also expresses doubts about the presence of typical *M. baltica* in Estonia. She does not refer to the material from the Estonian herbaria and has seen only some, probably not typical specimens collected from Estonia, not indicating the source of this material. She also relies on the theory that the taxon has developed in Åland and on the fact that there are no streams from there to south (the prevailing streams in the Baltic Sea go counter-clockwise; Alhonen 1966). On the contrary, *M. laxa* ssp. *baltica* is stated to occur in Estonia in the atlas of European Flora (Hultén and Fries 1986) and in several keys and floras (Viljasoo 1969; Lazdauskaite et al. 1996; Reier 1999; Cepurfte 2003). The taxon has been found from western Estonia and the islands (Kukk and Kull 2005; Fig 1), though it is relatively rare, occurring more abundantly on small islets (Viljasoo 1969; Vissak 1991; Ploompuu 1995; Rebassoo 1960, 1997).

M. laxa ssp. *baltica* grows on the seashore, where moisture and salinity fluctuate considerably during the vegetation period, and there is a danger of erosion or of getting buried (Ericson and Wallentinus 1979). Characteristic features of the taxon are actually adaptations to such changing conditions. Larger seeds/fruits enable faster sprouting and growth as well as promoting growth in unfavourable conditions, for example, in the dark under the sand or seaweed layer (Harper 1977; Weller 1985; Baskin and Baskin 1998). As seed size is one of the least plastic characters, this should be regarded as an adaptation (Harper 1977). *M. laxa* ssp. *baltica* flowers earlier than other subspecies, during the period when the water level is at its lowest (Ericson and Wallentinus 1979; Apelgren 1991). As the flowering starts soon after sprouting, the flowers are formed on the lower part of the stem; this is why there are leaves in the inflorescence. In cultivation, *M. laxa* ssp. *baltica* also started flowering two weeks earlier than *M. laxa* ssp. *caespitosa* (Apelgren 1986, 1991). Most of the diagnostic features persisted in cultivation, thus they should be genetically determined (Apelgren 1990b, 1991).
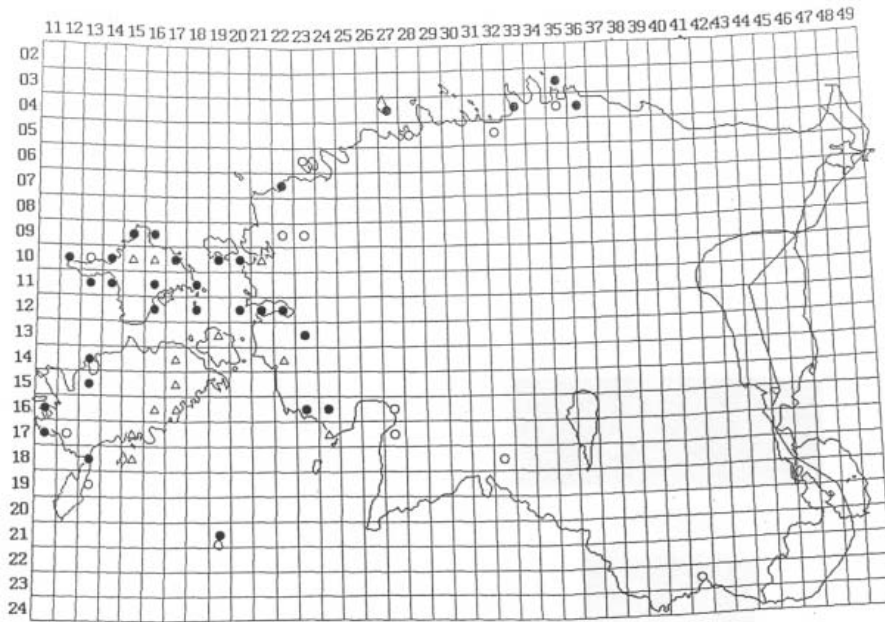
Fig. 1. Distribution of *M. laxa* ssp. *baltica* in Estonia (Kukk and Kull 2005).
● – findings after 1970; ○ – 1920-1970; Δ – before 1920 (herbarium specimens that have been identified later)

Distinctive characters

Most commonly used characters for distinguishing *M. laxa* ssp. *caespitosa* and *M. laxa* ssp. *baltica* are duration of life cycle, diameter of corolla, lengths of pedicel and calyx in fruiting stage, length of stem and presence of rosette leaves at the flowering stage (Table 1). 'Typical' individuals, i.e. those most similar to the original taxon descriptions, can be easily separated based on these characters, but many intermediate individuals are commonly found (Apelgren 1990b). Later, Apelgren (1991) treats these intermediate ones as *M. laxa* var. *laxa,* which in her opinion is a heterogeneous taxon. According to Apelgren (1991) the latter is mostly perennial; calyces and pedicels elongate after flowering, but the length of calyx does not exceed 5 and pedicel 7 mm; branching is extensive and inflorescence is sparse with at least one leaf within it; flowers are of various size and colour.

M. scorpioides

This was compared to *M. laxa* as one of the putatively closest taxa, as the subspecies of the latter have been some-times treated as subspecies of *M. scorpioides* (see above) and it belongs to the same series *Palustres*. The hybridisation of the subspecies of *M. laxa* between themselves and with *M. scorpioides* has also been hypothesised (Schuster 1967; Przyvara 1986/1987). It is widespread in the northern hemisphere.

Internal transcribed spacer region (ITS) of the nuclear rRNA gene complex

ITS region is probably the most widely used DNA marker in the angiosperm molecular systematics, particularly at the genus and lower taxonomic levels. Some authors declare that ITS was not the most appropriate marker for systematics, as ITS can be extremely variable within populations and even within individuals because of its multicopy nature (Alvarez and Wendel 2003; Bailey et al. 2003). However, concerted evolution has generally homogenized sequence variation among the numerous ribosomal DNA copies within an individual (Kay et al. 2006). Thus, it still remains the most efficient locus for generating species-le-

TABLE 1. Distinctive characters of *M. scorpioides, M. laxa* ssp. *baltica* and *M. laxa* ssp. *caespitosa* according to Samuelsson (1926), Schuster (1967), Viljasoo (1969), Grau and Merxmüller (1972), Lazdauskaite et al. (1996) and Ulvinen (1998).

Character	*M. laxa* ssp. *baltica*	*M. laxa* ssp. *caespitosa*	*M. scorpioides*
Duration of life cycle	Annual	Annual, biennial or perennial	Annual, biennial or (usually) perennial
Branching	Rare; occurs at lower part	Strong; occurs at upper part	Branches all over
Presence of leaves in inflorescence	Usually present	Usually not present	Usually not present
Diameter of corolla	2-5 mm	4-5 (6) mm	6-10 mm
Incisions of calyx	Up to 1/2	Up to 1/2	Up to 1/3
Shape of sepals	Oblong-triangular	Oblong-triangular	Triangular
Relative length of style	Not longer than calyx	Not longer than calyx	Longer than calyx
Length of calyx in fruiting stage	Up to 8 mm, elongates a lot after flowering	Up to 5 mm, elongates less	Up to 5 mm, elongates less
Length of pedicel in fruiting stage	Up to 25 (35) mm, much longer than calyx	Up to 10 mm, not much longer than calyx	Up to 20 mm, longer than calyx
Length of fruit	Up to 2.5×1.4 mm	Up to 1.5×1.0 mm	Up to 2.0×1.0 mm
Strength of stem	Weak	Strong	Strong
Length of stem	Up to 30 cm	Up to 50 cm	Up to 50 cm
Presence of runners	Missing	Missing	Present
Condition of the rosette leaves at flowering stage	Usually dead	Usually alive	Usually alive
Flowering time	May to July	May to September	May to September

vel phylogenetic inferences in most plant groups (Kay et al. 2006) and the results have been mostly congruent with those obtained using other nuclear genes, cDNA or morphology (Judd et al. 2002; Winkworth et al. 2002; Yuan et al. 2004).

ITS sequences have been used for solving phylogenetic relationships in the genus *Myosotis* and family Boraginaceae (Långström 2002; Winkworth et al. 2002; Hilger et al. 2004). The study of the evolution of the genus *Myosotis* in the northern and southern hemispheres (Winkworth et al. 2002) included also *M. laxa* ssp. *caespitosa,* which appeared in the joint clade with *M. rehsteineri* Wartm. and *M. debilis* Pomel, both originating from Europe (bootstrap support value 96%). *M. laxa* ssp. *baltica* and ssp. *laxa* were not included in that study. As the ITS region is quite variable in the genus, it should also be suitable for separating subspecies.

Aims

The aim of this paper is to clarify phylogenetic relationships of *M. laxa* ssp. *caespitosa* and *M. laxa* ssp. *baltica* and to propose appropriate taxonomic ranks for them. More specifically, we ask:

1) are the two subspecies of *M. laxa* separate taxa on the basis of the ITS sequences and morphology?;

2) which is the diagnostic value of the morphological characters for distinction of *M. laxa* ssp. *caespitosa* and *M. laxa* ssp. *baltica*?;

3) does the morphologically typical *M. laxa* ssp. *baltica* occur in Estonia?

MATERIALS AND METHODS

Materials

The majority of the herbarium specimens identified as *M. laxa* ssp. *baltica* and several identified as *M. laxa* or *M. l.* ssp. *caespitosa* were examined from the Estonian (TAA, TU) and Scandinavian herbaria (S, H), paying special attention to morphologically typical specimens of *M. laxa* ssp. *baltica*. Loans were asked from B and K containing *M. laxa* collected from Nordic countries. As mentioned above, type specimen(s) of *M. laxa* ssp. *baltica* were not chosen by Samuelsson (1926), but material collected and identified by him was examined (from S and B), and the type description was followed (Samuelsson 1926). The material collected by Lehmann from the Herbarium of the Swedish Museum of Natural History (S) was also examined, but the collections did not contain material identified as *M. laxa*. The material used by Lindberg and identified as *M. laxa*, including specimens from Exsiccatae Fennicae, was examined at the Herbarium of the University of Helsinki (H).

Fresh specimens were collected by E. Kook from Estonia during the years 2002-2003 as follows: 67 specimens of *M. laxa* ssp. *caespitosa*, 62 specimens of *M. laxa* ssp. *baltica*, 20 specimens of *M. scorpioides* and 10 specimens of *M. arvensis* (L) Hill. (mainly from the Island of Hiiumaa and from Western Estonia, Fig. 2). *M. arvensis* (L.) Hill was included to serve as outgroup in the subsequent analyses. The plants were identified to subspecies *caespitosa* or subspecies *baltica* (ssp. *laxa* was not used), 16 morphological characters (see below) were recorded and leaf samples were collected for DNA extraction. The latter were frozen at -20°C and lyophilised with cooling trap Hetotrap CT60 at -60°C (in 2002) or dried with silica gel (in 2003). If available, fruits were also collected: 72 fruits from 13 individuals of *M. laxa* ssp. *caespitosa* and 78 fruits from 17 individuals of *M. laxa* ssp. *baltica* and measured.

Morphological characters

Morphological characters were chosen from amongst those used in most descriptions and keys or considered to differ between taxa by several authors (Samuelsson 1926; Lindberg 1933; Schuster 1967; Viljasoo 1969; Lazdauskaite et al. 1996; Ulvinen 1998). 14 morphological characters were measured directly (Table 2), two calculated. Fruit length and width were measured using binocular microscope MBS-2 (40×).

Molecular analyses

DNA was extracted from approximately 0.1 g of dried leaf tissue using slightly modified (without liquid nitrogen)
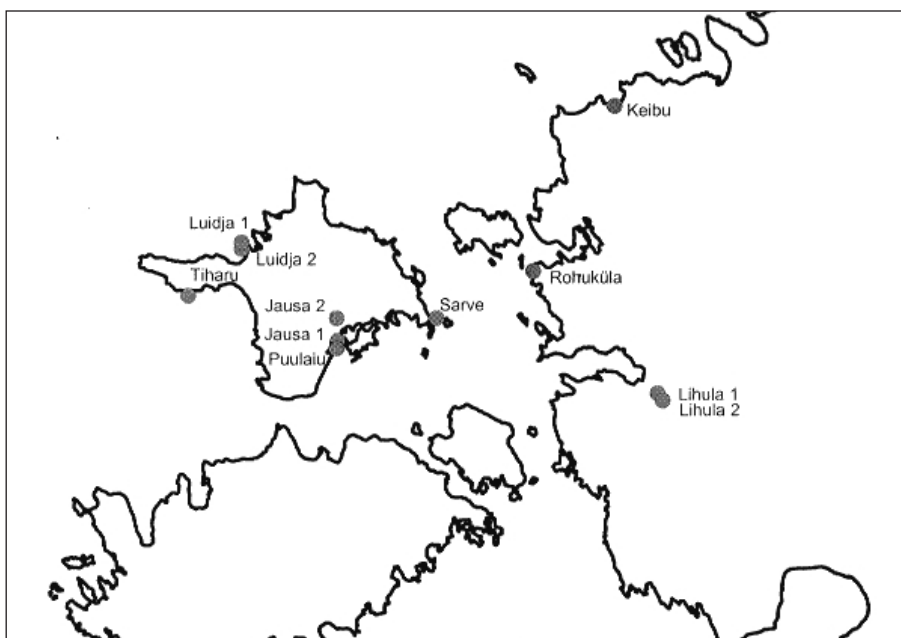

Fig. 2. Map showing the locations in Estonia from where *M. laxa* ssp. *caespitosa*, *M. laxa* ssp. *baltica*, *M. scorpioides* and *M. arvensis* were collected in years 2002-2003.

TABLE 2. Measured morphological characters and their states in parsimony analysis.

Character	Character states
1. Number of leaves in inflorescence	0-6
2. Presence of leaves in inflorescence	Calculated: no (0), yes (1); not used in most of the analysis types
3. Inflorescence type	Sparse (0), dense (1)
4. Length of the longest pedicel at fruiting stage (mm)	0-5 mm (1); 6-10 mm (2); 11-15 mm (3); over 15 mm (4)
5. Length of calyx of the flower with the longest pedicel at fruiting stage (mm)	4-5 mm (1); 6-7 mm (2); 8-9 mm (3)
6. Branching intensity	Not branched (0); branched in upper part (1); branched all over (2); branched in lower part (3)
7. Diameter of stem at base (mm)	Up to 1 mm (1); 1-2 mm (2); 3-4 mm (3)
8. Diameter of stem just below inflorescence (mm)	Up to 1 mm (1); 1-2 mm (2); over 2 mm (3)
9. Color of the lower part of stem	Reddish (1); not reddish (green) (0)
10. Incisions of calyx	Up to 1/3 (1); more (2)
11. Condition of the rosette leaves at flowering stage	Fresh (1); dried (2)
12. Average diameter of opened flower (of 2 flowers; mm)	1-3 mm (1); 4-6 mm (2); 7-10 mm (3)
13. Average length of leaf (of 2 leaves, mm)	mm, not used in most of the analysis types
14. Average width of leaf (of 2 leaves, mm)	mm, not used in most of the analysis types
15. Ratio of length and width of leaf	Calculated; 1.5-2.9 (1); 3.0-3.9 (2); 4.0-4.9 (3); 5.0-5.9 (4); 6.0-6.9 (5)
16. Presence of runners	Present (1); missing (0)

protocol of Doyle and Doyle (1987). PCR was carried out in a total volume of 25 µl containing 250 mM of each dNTP, 1× PCR buffer with $MgSO_4$, 1U Pfu polymerase (MBI Fermentas), 15 pmol of primers ITS4 (5' TCCTCCGCTTATTGATATGC 3') and ITS5 (5'GGA-AGTAAAAGTCGTAACAAGG 3'; White et al. 1990), 1 µl (1-10 ng) of template DNA, 0.001 mg BSA (bovine serum albumin). Thermocycling was carried out in Mastercycler® Personal (Eppendorf AG) using the following reaction conditions: 94°C 2 min, 35 cycles of 94°C 1 min, 48°C 1 min, 72°C 1 min, and finally 72°C 10 min. PCR products were purified with nuclease and phosphatase mix Exo-Sap-IT™ (USB Corporation, USA) or UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories Inc., USA) following the manufacturers' instructions. Sequencing reactions were carried out using Thermo Sequenase™ Primer Cycle Sequencing Kit (Amersham Biosciences) on sequencer ALFexpress II (Amersham Biosciences). Well readable sequences of the length of 630-684 bp were obtained from 20 specimens (1 *M. arvensis*, 1 *M. scorpioides*, 7 identified as *M.*

TABLE 3. Voucher specimens. All were collected in Estonia by Ene Kook in 2002 or 2003 and preserved at the Herbarium of the University of Tartu (TU).

Notation	(Sub)species	Locality	Number in herbarium	Genbank accession number
Ja12	*M. laxa* ssp. *caespitosa*	Jausa, Hiiumaa island	TU250127	EU594642
Ja19	*M. laxa* ssp. *caespitosa*		TU250121	EU594643
Ja110	*M. laxa* ssp. *baltica*		TU250126	EU594644
Ja113	*M. laxa* ssp. *baltica*		TU250116	EU594645
Ja222	*M. laxa* ssp. *caespitosa*		TU250085	EU594646
Ja226	*M. laxa* ssp. *caespitosa*		TU250123	EU594643
Lu192	*M. laxa* ssp. *baltica*	Luidja, Hiiumaa island	TU250128	EU594647
Lu196	*M. laxa* ssp. *baltica*		TU250131	EU594648
Li134	*M. arvensis*	Lihula, Lääne district	TU250132	EU594649
Li144	*M. laxa* ssp. *baltica*		TU250129	EU594650
Li145	*M. laxa* ssp. *baltica*		TU250120	EU594651
Ro50	*M. laxa* ssp. *baltica*	Rohuküla, Lääne district	TU250125	EU594652
Sa157	*M. laxa* ssp. *baltica*	Sarve, Hiiumaa island	TU250130/3	EU594653
Sa158	*M. laxa* ssp. *baltica*		TU250130/4	EU594653
Sa167	*M. laxa* ssp. *caespitosa*		TU250140	EU594659
Sa169	*M. laxa* ssp. *caespitosa*		TU250124	EU594654
Ti69	*M. scorpioides*	Tiharu, Hiiumaa island	TU250240	EU594655
Ti72	*M. laxa* ssp. *baltica*		TU250087	EU594656
Ti73	*M. laxa* ssp. *baltica*		TU250122	EU594658
Ti82	*M. laxa* ssp. *caespitosa*		TU250084	EU594657

laxa ssp. *caespitosa,* 11 identified as *M. laxa* ssp. *baltica*). The voucher specimens with notations and GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed) accession numbers for their sequences are listed in Table 3.

Data analysis

Characters not having normal distribution were log-transformed; number of leaves in inflorescence was standardized. Morphological data were analysed using principal components analysis, stepwise discriminant analysis, and analysis of variance (ANOVA) defining subspecies as a factor implemented in package STATISTICA 6.0 (Stat Soft Inc. 2001). For comparison of means, Student's t-test and Tukey test were used. For characters 2, 3, 9, 10, 11 and 16 (Tables 2 and 3) nonparametric Kruskal-Wallis ANOVA was performed. For cluster analysis, the average values of morphological characters by subspecies in populations were used and UPGMA with Manhattan distance performed with the same program package. *M. arvensis* was not included in these analyses.

Fruit data were available for only small number of individuals, because in most of the populations fruits were not ripe at the time of specimen collection. Therefore fruit data were analysed separately and t-test carried out.

DNA sequences were processed and complementary strands aligned using ALFwin™ Sequence Analyser 2.00 (Amersham BioSciences). These together with earlier sequenced representatives from GenBank were aligned using ClustalW and corrected manually in BioEdit (Hall 1999). We included *Myosotis rehsteineri* Wartm. ex Reut. as the closest sister species of *M. laxa* ssp. *caespitosa* according to Winkworth et al. (2002), other related sequenced taxa from Europe and one species from every major branch of the phylogenetic tree of *Myosotis. Myosotis* taxa most distantly related to *M. laxa* ssp. *caespitosa* (*M. australis, M. cadmea, M. discolor, M. personii,* and *M. verna*) were used as outgroup. Gaps in the beginning and/or end of some sequences were treated as missing data. Phylogenetic analyses were implemented in PAUP 4.0b10 (Swofford 2002) as follows: 1) parsimony analysis with heuristic search, tree-bisection-reconnection (TBR) and multiple parsimony

(MULPARS) options; and 2) neighbour joining analysis with the Hasegawa, Kishino and Yano (1985) model (HKY85), which estimates a transition-transversion ratio, takes into account base frequencies and thus generalizes the other models. The relative support for the clades was determined by bootstrap analysis employing 100 replicates (Felsenstein 1985).

Genetic differentiation between subspecies and populations of *M. laxa* was estimated using hierarchical AMOVA (analysis of molecular variance) implemented in Arlequin 2.000 (Schneider et al. 2000). Significance of differentiation was assessed using permutation tests. For estimating pairwise genetic distances, F_{ST}, an estimate of differentiation based on allele frequencies, was calculated. This parameter can be used as distance between populations (Schneider et al. 2000).

RESULTS

M. laxa ssp. baltica in herbaria and typification of the name Myosotis baltica Sam

Five specimens seen from the collection of Samuelsson collected from Sweden (1925-1932) stored in the herbarium of the Swedish Museum of Natural History (S), appeared to match the original species description, i.e. were typical. Seven specimens from Kew herbarium (K) and two specimens from Berlin herbarium (B), collected from Sweden, belonged also to the ssp. *baltica* according to the type description. One of the specimens from the collection of Samuelsson kept in B (collected in 1929, B 10 0151942!) has been designated by Dickoré in 1988 as possible type material with question mark (note on the herbarium specimen), but was not officially typified and is not suitable for lectotype. The specimens mentioned by Lindberg (1915) and classified as *M. laxa,* including the specimens from Exsiccatae Fennicae also corresponded to the type description of Samuelsson (1926) as well as to the description of *M. laxa* by Lehmann (1818). The only specimens from Samuelsson's original material suitable for lectotypification are from S! collected in 1925 from Uppland, Sweden. All Samuelsson's other specimens (S!,B!) were collected at
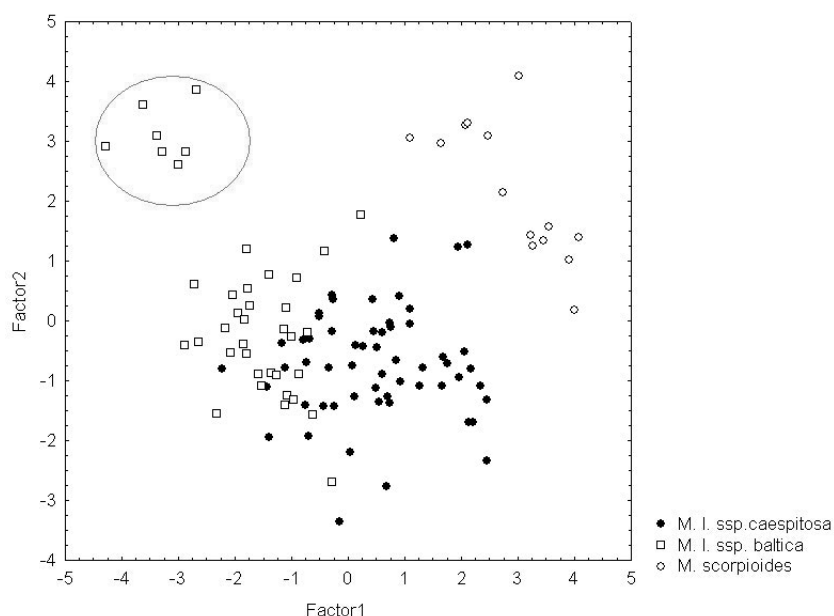

Fig. 3. Principal components analysis of morphological characters of *M. laxa* ssp. *caespitosa*, *M. laxa* ssp. *baltica* and *M. scorpioides*. Factors 1 and 2 describe 26.8% and 18.0% of the variability (eigenvalues 3.2 and 2.2), respectively. The typical *M. l.* ssp. *baltica* specimens are encircled.

a later date. The typification and synonymy can be thus summarized:

M. laxa **Lehm.** ssp. **baltica** (Sam.) Hyl. ex Nordh., Norsk Fl. 529 (1940)

≡ *M. baltica* Sam. (in Lindm.), Sv. Fan. Fl. 458 (1926)

– Lectotype (designated here): Uppland, Rådmansö Hamnskär i Lygne skärgård, 12/7 1925 G. Samuelsson S HS-6990! Isolectotype: Uppland, Rådmansö Hamnskär i Lygne skärgård, 12/7 1925 G. Samuelsson S HS-6991!

≡ *M. laxa* var. *baltica* (Sam.) Apelgren, Acta Univ. Ups. 306: 21 (1991)

= *M. laxa* Lehm., Pl. Asperif. I: 83 (1818) sensu auct. (Lindberg 1915, 1934; Schuster 1967)

= *M. laxa* Lehm. ssp. *laxa* s.l., sensu auct. (Lid and Lid 1994)

= *M. scorpioides* L. em. Hill. ssp. *laxa* (Lehm.) Hegi, Ill. Fl. Mitteleur. 2165 (1927)

Occurrence of morphologically typical M. laxa ssp. baltica in Estonia

20 morphologically typical specimens of *M. laxa* ssp. *baltica* collected from Estonia were found from the local herbaria (TU, TAA). The oldest specimen was collected in 1932 from northern Estonia. The specimens found in Estonia were very similar to those collected and identified by Samuelsson. 21 typical individuals of *M. laxa* ssp. *baltica* were collected during the field works from four populations in Estonia (Rohuküla, Sarve, Puulaiu, and Tiharu see Figure 3). The habitats of these were also typical, i.e. on the seashore close to the water.

Morphological characters

Principal component analysis of morphological characters of *M. scorpioides, M. laxa* ssp *baltica* and *M. laxa* ssp. *caespitosa* showed that *M. scorpioides* can be well discriminated from *M. laxa* s.l. (Fig 3). A group of typical *M. laxa* ssp. *baltica* separates very clearly from *M. laxa* ssp. *caespitosa*, but most of the specimens of the two subspecies are not distinguishable.

A similar result was obtained in UPGMA analysis that revealed some well-defined clusters: cluster 2 (Fig. 4) comprises both *M scorpioides* populations; clusters 1 and 3 contain morphologically more or less typical *M. laxa* ssp. *baltica* populations; the largest cluster 4 contains both subspecies of *M. laxa*, mostly *M. laxa* ssp. *caespitosa*.

M. scorpioides was 95% correctly classified according to the discriminant analysis, but subspecies of *M. laxa* were not well discriminated (Table 4).

Among the 16 analysed morphological characters, 14 proved usable for differentiation of the three studied taxa; only length and width of leaves were not informative (Table 5). Nine characters were statistically significantly different between *M. laxa* ssp. *baltica* and *M. laxa* ssp. *caespitosa* and thus can be used for taxon distinction: length of pedicel, length of calyx, colour of stem, inflorescence type (dense or sparse), branching, presence and number of leaves in inflorescence, rosette leaves fresh or dried at flowering stage and diameter of stem at base (Fig. 5). The means of the character values are presented in Table 5. When the analysis was performed by populations, the variation of several characters overlapped largely for different taxa from some populations, while in other populations the differences were clear (data not shown).
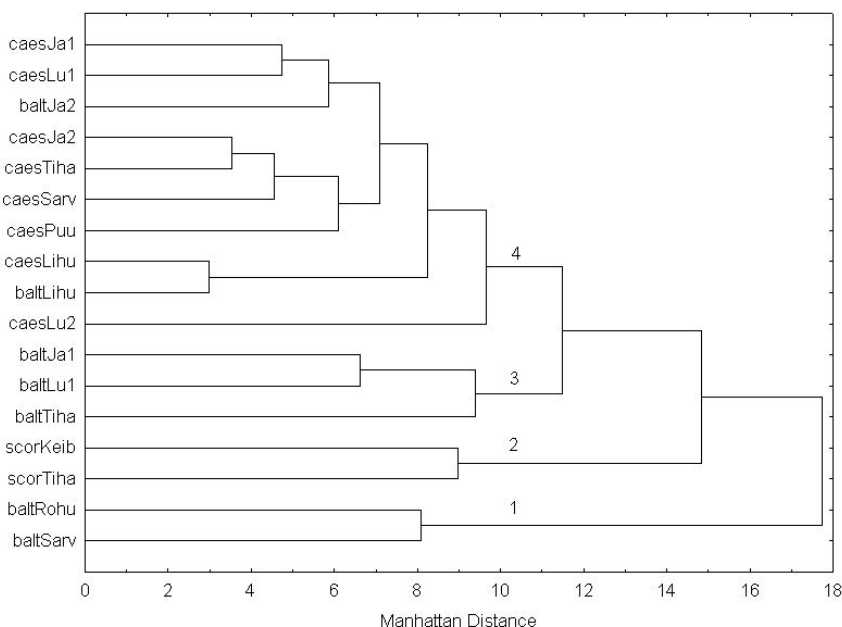

Fig. 4. UPGMA analysis of morphological characters by populations (Manhattan distance). The populations are coded as follows: taxon (caes-*M. laxa* ssp. *caespitosa,* balt-*M. laxa* ssp. *baltica* and scor-*M. scorpioides*); site (Tiha-Tiharu, Sarv-Sarve, Lu-Luidja, Lihu-Lihula, Puu-Puulaiu, Ja-Jausa, Rohu-Rohuküla, Keib-Keibu, see Figure 2); population number (if several in neighbourhood).

TABLE 4. The classification matrix for *M. scorpioides*, *M. laxa* ssp. *baltica* and ssp. *caespitosa* obtained by discriminant analysis. Observed identification is presented in rows, classification predicted by the analysis is given in columns.

Taxon	Percent correct	*M. l.* ssp. *caespitosa*	*M. l.* ssp. *baltica*	*M. scorpioides*
M. l. ssp. *caespitosa*	85.1	57	10	0
M. l. ssp. *baltica*	95.2	3	60	0
M. scorpioides	95.0	1	0	19

TABLE 5. Results of ANOVA or Kruskal-Wallis tests by species and average values of morphological characters.

Character	Transfor-mation	F or H	p	Average values of taxa			
				M. laxa ssp. *caespitosa*	*M. laxa* ssp. *baltica*	*M. scor-pioides*	Total
Number of specimens				67	63	20	150
1. **Leaves in inflorescence (count)**	standard	26.3	<0.001	0.82	2.17	0.95	1.41
Std deviation				0.69	1.45	**0.94**	1.28
2. **Leaves in inflorescence (0, 1)**	–	59.8	<0.001	0.68	0.95	0.04	0.71
Std deviation				0.48	0.25	0.22	0.45
3. **Inflorescence type (1-2)**	–	54.1	<0.001	1.36	1.05	1.90	1.30
Std deviation				0.48	0.21	**0.31**	0.46
4. **Length of pedicel (mm)**	log	100.4	<0.001	8.10	14.97	6.55	10.78
Std deviation				1.83	5.05	**1.79**	5.06
5. **Length of calyx (mm)**	log	24.0	<0.001	5.21	6.14	4.85	5.55
Std deviation				0.84	1.01	**0.67**	1.03
6. **Branching intensity (0-3)**	log	17.9	<0.001	1.31	2.17	1.90	1.75
Std deviation				0.50	1.04	**1.02**	0.93
7. **Diameter of stem at base (mm)**	log	6.0	0.003	2.66	2.25	2.25	2.43
Std deviation				0.77	0.95	**0.39**	0.84
8. **Diameter of stem near inflorescence (mm)**	–	9.8	<0.001	1.91	1.83	1.30	1.79
Std deviation				0.48	0.64	**0.38**	0.58
9. **Color of stem (0, 1)**	–	60.7	<0.001	0.83	0.08	0.45	0.47
Std deviation				0.49	0.28	**0.51**	0.54
10. **Incisions of calyx (1-2)**	–	133.4	<0.001	1.99	1.98	1.00	1.85
Std deviation				0.12	0.13	**0.00**	0.35
11. **Presence of rosette leaves (0, 1)**	–	12.9	0.002	0.25	0.05	0.05	0.14
Std deviation				0.44	0.21	**0.22**	0.35
12. **Diameter of corolla (mm)**	–	74.9	<0.001	4.74	4.40	7.95	5.02
Std deviation				0.92	1.08	**1.10**	1.50
13. **Length of leaf (mm)**	–	0.4	0.67	32.90	32.43	35.25	33.02
Std deviation				12.29	13.76	**6.84**	12.35
14. **Width of leaf (mm)**	–	1.8	0.17	8.07	7.94	6.83	7.85
Std deviation				2.61	2.87	**1.58**	2.63
15. **Ratio of length and width of leaf**	log	14.7	<0.001	4.13	4.07	5.25	4.25
Std deviation				0.88	0.76	**0.83**	0.91
16. **Presence of runners (0, 1)**	–	10.2	0.006	0.34	0.26	0.65	0.35
Std deviation				0.48	0.44	0.49	0.48

Fruits of *M. laxa* ssp. *baltica* were significantly longer than these of ssp. *caespitosa* (Fig. 6, Table 6). The difference in width was not statistically significant. However, if analysed by populations, fruits of the morphologically typical ssp. *baltica* from Rohuküla population were significantly wider than these of specimens of the same subspecies from other populations (t=-4.24; p=0.0007; data not shown). In general, fruits of *M. laxa* ssp. *baltica* were slightly smaller than expected based on previous data from literature (see Tables 1 and 6).

Analysis of the ITS sequences

Heuristic analysis of parsimony found 269 shortest trees, using 621 characters (nucleotides), of which 504 were con-

stant, 45 non-informative and 72 informative. The shortest trees after iterative weighting of characters had 132 steps, the indices were CI=0.86, HI=0.14 and RI=0.90. The topology of the neighbour joining tree was similar to that of parsimony analysis and only the NJ phylogram is presented. *M. laxa s.l.* formed a monophyletic group that included *M. rehsteineri* and *M. scorpioides* (Fig. 7) with bootstrap support of 94%, or 55% if excluding *M. scorpioides*. Within that group three subclades can be recognised: one containing a single specimen of *M. scorpioides,* other specimens of both subspecies of *M. laxa* from Sarve population and the largest one all other specimens, including *M. rehsteineri*. Morphologically typical specimens of *M. laxa* ssp. *baltica* (e.g., Ro50 and Sa157) were grouped into different
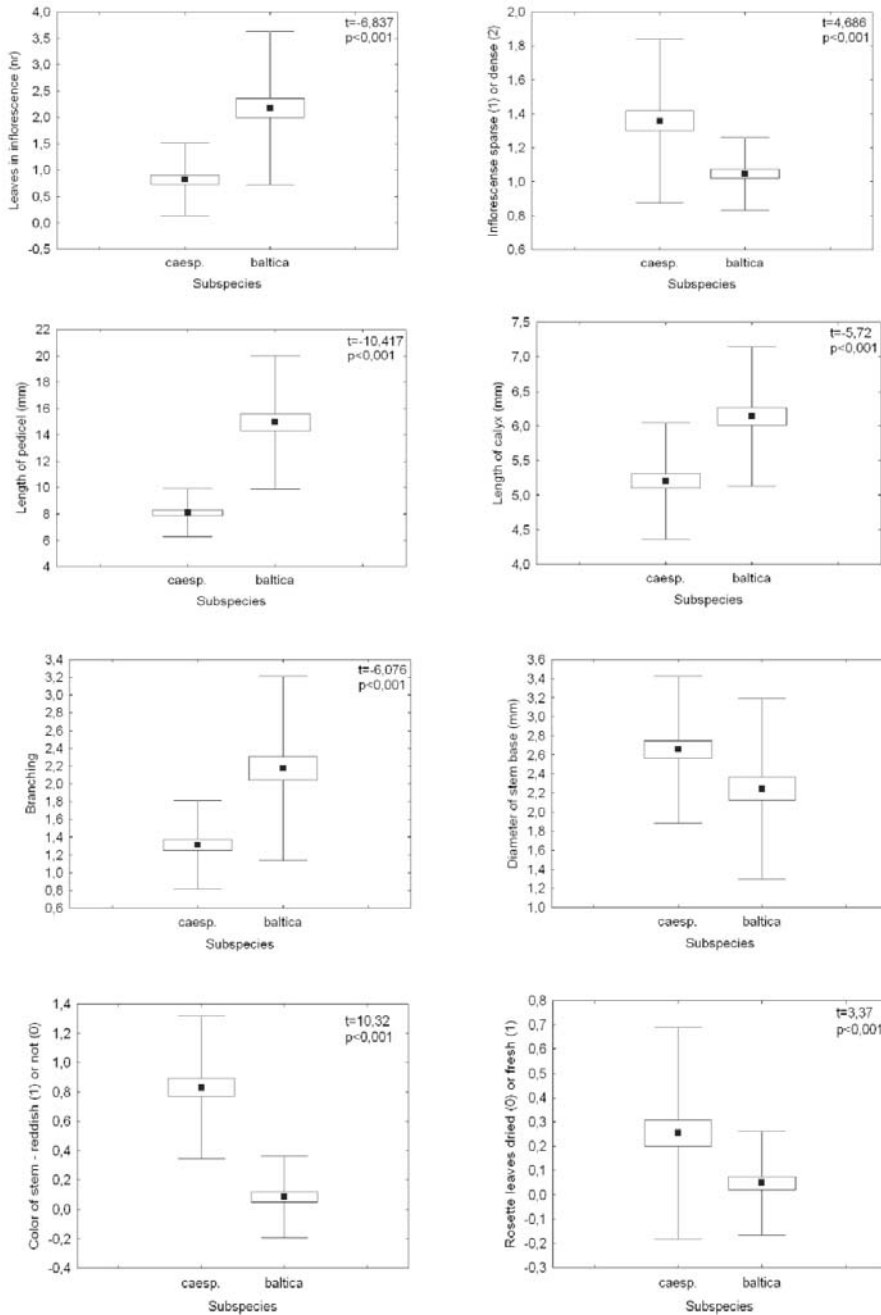
Fig. 5. Means ■, standard errors □ and standard deviations ⊥ of morphological characters which show statistically significant difference between *M. laxa* ssp. *caespitosa* and *M. laxa* ssp. *baltica*. Presence of leaves in inflorescence is not shown because it is highly correlated with the number of leaves in inflorescence.

TABLE 6. Average values of fruit length and width of *M. laxa* ssp. *baltica* and *M. laxa* ssp. *caespitosa*.

Subspecies	Specimens	Fruits	Average length (mm)	Standard deviation	Average width (mm)	Standard deviation
M. laxa ssp. *baltica*	17	78	**1.57**	0.11	**1.13**	0.12
M. laxa ssp. *caespitosa*	13	72	**1.43**	0.15	**1.05**	0.09
Total	30	150	**1.50**	0.15	**1.09**	0.12

clades. No clear grouping based on populations can be observed, except a clade containing specimens from the Sarve population with bootstrap support of 58%. *M. arvensis* sequenced by us (aLi134) grouped together with *M. arvensis* sequenced by Winkworth et al. (2002) in a well supported cluster close to the outgroup (bootstrap support 100%).

AMOVA yielded negative variance components at the hierarchical level of species (Table 7). The AMOVA methodology relies on estimates of relationships between alleles in the same population or group of populations relative to alleles of different populations or groups of populations (Weir 1996). Negative variance components can result from very small, but positive estimates of genetic structure indices from data (Weir 1996). This implies that alleles are more related between than within species and most of the variability occurs within species, between and within populations. Pairwise genetic distances between the subspecies (F_{ST} = -0.04; p=0.64) and populations (data not shown) were not significant.
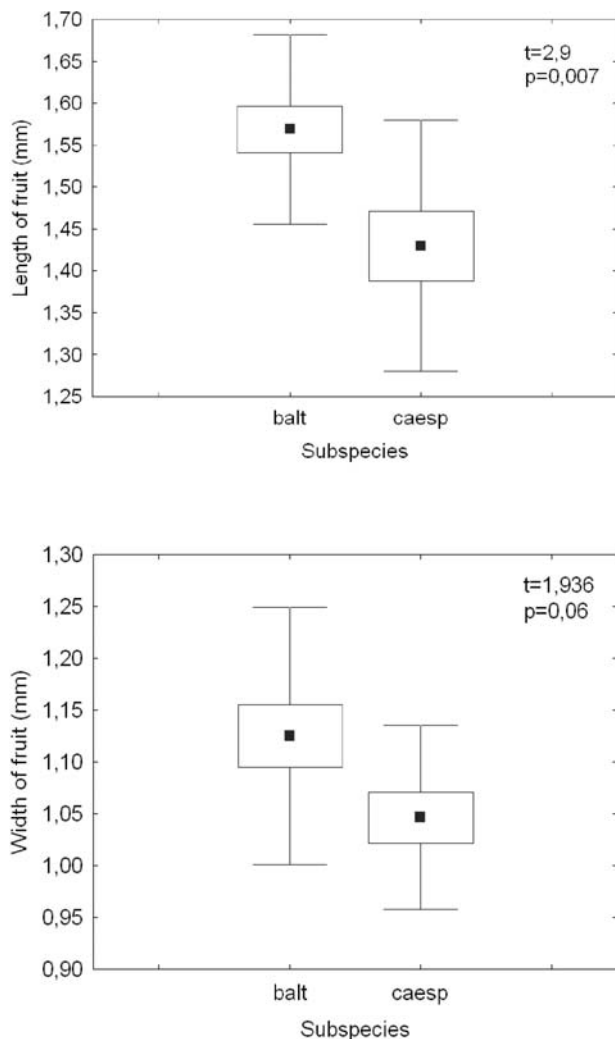
all from the characterization of coastal *M. laxa* by Lindberg (1915) and is very similar to the description of *M. laxa* by Lehmann (1818). Lindberg (1934) considered that this could be lapsus calami by Samuelsson. The separation of *M. laxa* ssp. *caespitosa,* at least at the level of variety or subspecies, seems reasonable, because typical subspecies *caespitosa* is morphologically well distinct from subspecies *baltica,* as also confirmed by our data. However, there are specimens showing a range of variation, which cannot be clearly classified into either of the two above-mentioned subspecies. The range of variation can be seen particularly clearly if both subspecies occur at the same site. The only clearly distinguishing character is the length of pedicel at the fruiting stage.

Apelgren (1991) found a solution in declaring that the third subspecies, *M. laxa* ssp. *laxa,* considered occurring only in America by most floras and identification keys, includes the intermediate specimens and grows also in Eurasia. It is somehow understandable, as there are some records of *baltica-* or *laxa*-like plants from several parts of Eurasia (Schuster 1967; Saiki and Osegawa 1972). We have seen such *baltica*-like herbarium specimens (TU) from Kiev (Ukraine), Tula and Kamchatka (Russia). However, the separation of the subspecies *baltica* and *laxa* is almost impossible, as there are no types, ssp. *laxa* is morphologically very variable and its description largely overlaps with that of ssp. *baltica.* Although Apelgren (1991) lowers the taxonomic rank of all three taxa to variety, there still seem to be too many units within the species.

Our data, both morphological and molecular, did not support clear distinction of subspecific taxa, neither two nor three. ITS-sequence analysis showed that *M. laxa s. l.* together with *M. rehsteineri* is monophyletic with bootstrap support of 94%, but neither *M. laxa* ssp. *caespitosa* nor *M. laxa* ssp. *baltica* is monophyletic. It is possible, that *M. rehsteineri* also belongs to the complex. The latter has been treated as a variety *M. caespitosa* var. *grandiflora* Gaudin (Schuster 1967), and was recently positioned as a sister taxon of *M. laxa* ssp. *caespitosa* (Winkworth et al. 2002). This species should also be included in further research.

Although phylogenetic analysis performed with morphological characters supported the monophyly of ssp. *baltica,* the ITS-sequence data did not support this. Phylogenetic relationships within *M. laxa s. l.* are largely unresolved. According to AMOVA the genetic variability within subspecies was much more extensive than between them and genetic distance between species was not significant, thus the subspecies cannot be distinguished by ITS-sequences.

Comparison of the herbarium specimens and freshly collected material from Estonia with authentic material of Samuelsson, as well as with the type description, provides evidence that the morphologically typical *M. laxa* ssp. *bal-*

Fig. 6. Means ■, standard errors □ and standard deviations ⊥ of width and length of fruit of the subspecies of *M. laxa*.

DISCUSSION

The status of the three subspecies of *M. laxa* is unclear and the history of the intraspecific taxa is very confusing. None of the taxa has been typified before. Therefore, having seen the original material of *M. laxa* ssp. *baltica* by Samuelsson, we typified that taxon. However, in our opinion, Lindberg (1915, 1933) was right that the description of *M. laxa* (Lehmann 1818), though described from North America, corresponds rather well with that of *M. laxa* ssp. *baltica*. It is not well understandable why Samuelsson (1926), describing *M. baltica* as a separate species, declared that this new species is neither *M. laxa* sensu Lehmann nor sensu Lindberg, while his description does not differ at

TABLE 7. AMOVA analysis results for subspecies of *M. laxa* (F_{st}=0.32; p=0.002), based on pairwise differences. F_{st} significance test based on 1023 permutations.

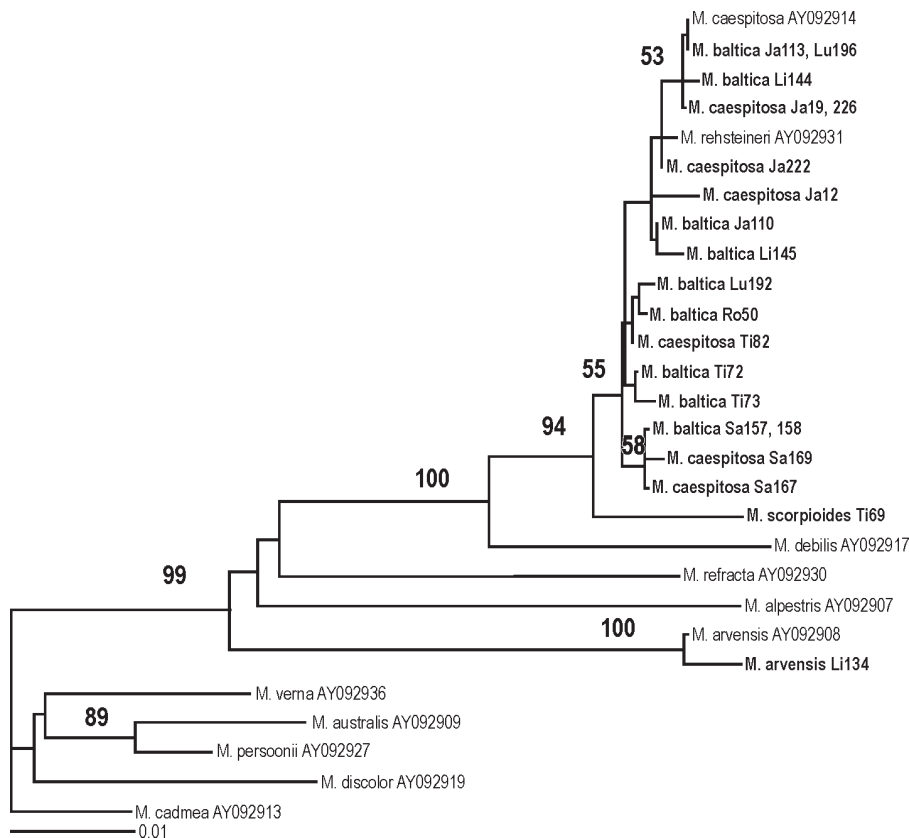| Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Between subspecies	1	0.88	-0.16	-13.24
Within subspecies (between populations)	7	13.12	0.56	45.36
Within populations	9	7.50	0.83	67.88
Total	17	21.50	1.23	100.0

Fig. 7. Neighbour joining analysis with HKY85 distance of nuclear ribosomal ITS-sequences of study specimens (bold) and related GenBank accessions. Bootstrap values >50% are shown above the branches. The accession numbers, habitat codes and voucher specimens of the analysed specimens are shown in Table 3. The identical sequences were analysed together as one, the code then consists of several accession numbers. Sequences from GenBank are denoted with their GenBank codes. *M. australis*, *M. cadmea*, *M. discolor*, *M. personii,* and *M. verna* were used as an outgroup.

tica does occur in Estonia, as well as in Sweden and Finland. Our results of the analyses of morphological characters are similar to the earlier results (Apelgren 1990b) in sense that there are both typical specimens and intermediate ones, often present at the same locality. Consequently the wide range of morphological variability of *M. laxa s. l.* including typical ssp. *baltica* occurs in Estonia as well as in Finland and Sweden. Analysis of morphological characters revealed that specimens classified as *M. laxa* ssp. *baltica* are more variable than these identified as *M. laxa* ssp. *caespitosa*. We hypothesise that there is one circumpolar species *M. laxa* with ecotypes or varieties, but the intraspecific taxa are not distinct. Thus, treating them as subspecies is not recommended and as species is not acceptable. As generally thought, subspecies should differ morphologically and/or genetically and have at least slightly separate distribution ranges. However, trees of individual genes may differ from the tree of taxa, especially in the case of reticulate evolution. Thus further investigation including all putative taxa of *M. laxa* as well as *M. rehsteineri,* wider geographical sampling together with different molecular markers, including chloroplast DNA is needed to clarify completely the position of these taxa.

Furthermore, we question the theory that *M. laxa* ssp. *baltica* is an endemic that originates from Åland and has spread from there around the Baltic Sea by streams (Apelgren 1990b). Firstly, the streams in the Baltic Sea are dependent on winds, temperature, air pressure, water level, inflow from rivers, vertical circulation and other factors; therefore the flow at any point and at any time can be from a variety of directions (Astok and Mardiste 1995). Thus, we cannot say that the seeds can travel only counter-clockwise in the Baltic Sea. Secondly, the theory would imply that *M. laxa* ssp. *caespitosa* is 'native' in Estonia and *M.*

laxa ssp. *baltica* arrived later from Sweden. The latter should therefore be monophyletic and genetically distinct from the former. As it is not so, we should consider *M. laxa* ssp. *baltica* as an ecotype adapted to coastal habitats with fluctuating conditions. Such adaptation has probably appeared several times in evolution in different regions of the Baltic Sea and may well do so in the future. The fact that it is less common in other regions compared to Åland could also be determined by the environmental conditions: other regions do not have so extensive land lifting as Åland and the coast of the Gulf of Bothnia (Ericson and Wallentinus 1979). As *M. laxa* ssp. *baltica* can be separated from *M. laxa* ssp. *caespitosa* neither by ITS-sequences nor by morphological characters, might its taxonomic rank even be lowered to variety as already proposed by Apelgren (1991).

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