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Cyto- and chemotaxonomical studies on the sections *Officinalia* and *Coerulea* of the genus *Symphytum*

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Abstract

Th. W. J. Gadella, E. Kliphuis and H. J. Huizing 1983. Cyto- and chemotaxonomical studies on the sections *Officinalia* and *Coerulea* of the genus *Symphytum*. Bot. Helv. *93:* 169–192. Cytological and phytochemical studies of the *Symphytum officinale* polyploid complex, *S. asperum* and *S. peregrinum* clearly indicated that *S. peregrinum* is a distinct taxon. *S. peregrinum* differs cytologically, morphologically, chemically and in its distribution from *S. officinale* and *S. asperum*. Therefore it cannot be regarded as a hybrid between these species. *S.* × *uplandicum* (2n = 36, 40) differs from *S. peregrinum* (2n = 40) morphologically, chemically and in part cytologically.

The 2n = 40 cytotype of *S. officinale* differs both from the diploid (2n = 24) and tetraploid (2n = 48) cytotype in morphological aspect, but not in chemical respect. It contains the same pyrrolizidine alkaloids as *S. officinale* (2n = 24, 48). For that reason and also because this cytotype is interfertile with the 2n = 48 cytotype, it is regarded as conspecific with *S. officinale* (2n = 48). The lack of information of the exact identity of the Western 2n = 40 cytotype of *S. officinale* and the Eastern morphotype of *S. tanaicense* Stev. does, for the moment, not permit to give a taxonomic recognition and an assignment of the appropriate rank to these taxa.

Introduction

The genus *Symphytum* was divided into two species groups by Boissier (1897) and Kusnetsov (1910). They based their classification on the length of the corolla scales (included or exserted) and on the colour of the corolla, respectively. Bucknall (1913) did not accept this rather arbitrary and artificial classification. He based his own classification on the stem type (branched or simple) and on the root type (fusiform and branched or creeping and tuberous). In the group with branched stems and fusiform roots he

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distinguished five sections, two of which will be treated in this paper, with the sections *Officinalia* (with *S. officinale* L. and *S. uliginosum* Kern.) and the section *Coerulea* (with *S. asperum* Lepech. and *S. peregrinum* Ledeb., and two other species, left out of consideration within this paper).

Faegri (1931) regarded the relationship between the four species mentioned as so very close that he decided to unite them as subspecies in one collective species: *S. commune* Faegri. Their close relationship was confirmed by hybridisation experiments (Gadella 1972, Gadella & Kliphuis 1973, 1978). Especially the production of somewhat fertile hybrids is indicative of a close affinity.

In spite of the many studies devoted to these species, there is still much taxonomic confusion, which may follow from the compilation below:

1. *S. peregrinum* Lebed. was introduced from its native country, the province of Talysh (U.S.S.R.) into E. and W. Europe, where it became naturalised (Bucknall 1912, 1913). It crossed easily with the native *S. officinale*, giving rise to various hybrid types (Bucknall 1912). Wickens (1969) and Wickens in Davis (1978) did not see native specimens of *S. peregrinum* and took the description from Bucknall (1913).
2. *S.* × *uplandicum* Nym. is a synonym of *S. peregrinum* (Bucknall 1913).
3. *S. peregrinum* is intermediate in its morphology between *S. asperum* and *S. officinale* (Bucknall 1913).
4. *S. peregrinum* and *S.* × *uplandicum* are not synonymous, *S. peregrinum* is a good species, *S.* × *uplandicum* is a hybrid between *S. officinale* and *S. asperum* (Kusnetsov 1910, Schinz & Thellung 1913, Grossheim 1967, Dobrochaeva 1968b, Gviniashvili 1972). *S. peregrinum* differs from both *S. officinale* and *S. asperum*, morphologically, ecologically and in distribution. No comment is given on the status of *S. peregrinum* by Lindman (1911), Thellung (1915, 1919), Tutin (1956), Wade (1958), Basler (1972). These authors agree that *S.* × *uplandicum* is a hybrid between *S. asperum* and *S. officinale.*
5. There is a little, if any, difference between *S. peregrinum* and *S. asperum* (Schinz & Thellung 1913, Popov in Komarov 1953). In their opinion, *S. peregrinum* is probably best treated as a local race of *S. asperum.* Riedl (in Rechinger 1957) reports *S. asperum* from Talysh. According to Kusnetsov (l.c.) *S. peregrinum* is indigenous to Talysh and *S. asperum* is absent from that region. For that reason Riedl's material deserves a close re-examination. The possibility exists that Riedl confused *S. asperum* and *S. peregrinum.* Also Kurtto (1982) is in the opinion that the specimens from the Talysh province very probably belong to *S. peregrinum.*
6. The correct name for *S. uliginosum* Kern. is *S. tanaicense* Steven. (Degen 1930, Dobrochaeva 1968b, Murin & Majowsky 1982); *S. uliginosum* is either considered as a separate species (Kerner 1863) or as a subspecies of *S. officinale* (de Sóo 1926). The distribution area of *S. uliginosum* is insufficiently known, in particular westwards. The possibility cannot be excluded that the $2n = 40$ cytotype of *S. officinale*, occurring in the low lying peat lands of the Netherlands should be referred to this taxon (Gadella & Kliphuis 1967, 1973). Basler (1972), however, refers the $2n = 40$ cytotype of *S. officinale* to *S.* × *uplandicum.* This opinion was rejected on morphological evidence as well as on hybridisation experiments by Gadella & Kliphuis (1973) and on chemotaxonomic evidence by Huizing, Gadella & Kliphuis (1982).
   From this survey it is clear that the status of some of these taxa is questionable. This is particularly due to the absence of biosystematic information (Wickens 1969). This

applies especially to *S. peregrinum* and *S. tanaicense*. Therefore, cyto- and chemotaxonomical studies were carried out, supplemented by some hybridisation experiments, in order to make the obtained evidences conclusive for taxonomy and to gain a better insight in the evolutionary relationships within the polyploid complex.

Materials and Methods

Plant material

Plants of *Symphytum officinale* were collected from their natural habitats in various European countries. Seeds and living plants of *S. asperum* were provided by Mr. Hoog (Haarlem, The Netherlands). Other seed samples were received from Botanical Gardens. The hybrid *S.* × *uplandicum* was obtained from two different sources: they were either collected in nature or produced artificially. We received herbarium material of *S. peregrinum* Ledeb., collected by Dr. T. Gviniashvili(Thblisi) in "Caucasus Aserbaidshania", Lenkoran, in the village Girdani. This material was used by us for morphological studies as well as for chemotaxonomical comparison with other taxa. We are grateful to Dr. Gviniashvili for providing this interesting material. Voucher specimens of further plant material have been preserved in the Department of Biosystematics of the State University of Utrecht.

Cytological evaluation

We usually got satisfactory cytological results after fixation of the roottips in Karpechenko's fixative and staining according to Heidenhain's haematoxylin method. In most cases all chromosomes are clearly visible, their ends do not adhere to each other, thus making accurate chromosome counts possible. Microscopical slides have been preserved in the Department of Biosystematics of the State University of Utrecht.

Cytological studies of *S. peregrinum* were made by Dr. T. Gviniashvili. He counted $2n = 40$ in material from Azerbaijan (Lenkoran) in the village Aleksejeva. We are in the opinion that the material from Girdani (not studied cytologically) is conspecific with the material from Aleksejewa ($2n = 40$, counted by Gviniashvili).

Chemical methods

Extraction procedures

A part of the dry root which had been removed from the herbarium specimen of S. peregrinum which was kindly provided by Dr. T. Gviniashvili, was ground in a hammer mill and extracted exhaustively with n-pentane in an all-glass Soxhlet extractor (in order to remove lipids, phytosterols and naphthoquinones). After removal of the n-pentane at 50 °C using a vacuum rotary evaporator, the remaining semi-solid extract was adjusted with a calculated volume of n-pentane giving a similar strength as when the extract had been derived from an amount of 30 gram of dry root as mutually performed for our previous comparative chemotaxonomical investigations (Huizing, Gadella & Kliphuis 1982, Huizing, Malingré, Gadella & Kliphuis, submitted)

The dried remainder of the ground roots in the extraction apparatus was submitted to a second extraction with methanol. After removal of the methanol in vacuum at 50 °C in a rotary evaporator, the extract was taken up into 2N sulfuric acid and filtered through a fluted filterpaper. Possible pyrrolizidine alkaloid N-oxides present in the filtrate were reduced overnight by addition of zinc powder to the acidic extract. After removal of the excess of zinc by filtration, the filtrate was basified with ammonia till ph = 9.5 and extracted with three lots of 25 ml each of chloroform. The combined extracts were dried over anhydrous sodium sulfate. After removal of the chloroform at 50 °C using a vacuum rotary evaporator the remaining extract was adjusted with a calculated volume of chloroform giving a similar strength as when the extract had been derived from an amount of 30 gram of dry root. For comparative investigations dried roots from *S. officinale* ($2n = 24$), *S.* × *uplandicum* ($2n = 40$ and 36) and *S. asperum* ($2n = 32$) were treated in the same way.

Thin layer chromatography of extracts

For all comparisons of extracts employing thin layer chromatography, precoated silica gel G 1500 LS 254 plates (20 × 20 cm) from Schleicher & Schüll (Dassel, G.F.R.) were used. Aliquots of 5 µl of these extracts were supplied to plates by means of microcapillaries.

Phytosterols and triterpenoids were separated in a saturated chamber using hexane: diethyl-ether (30:60) as the eluent. Spots were visualised after spraying with a mixture of chlorosulfonic acid: acetic acid (1:2) followed by heating for 5 minutes at 130 °C (Stahl 1967).

Pyrrolizidine alkaloids were separated with a straight phase system (A) and an ion-pair system (B) in unsaturated chambers. Eluent A consisted of a mixture of chloroform: methanol: ammonia 25% (85:14:1) which has been described by Sharma et al. (1965) before. Prior to development by the ion-pair system (B), plates were impregnated by dipping into a solution of 0.1 M LiCl in methanol and subsequent drying for 15 minutes at 105 °C. Plates were stored in a vacuum desiccator at room temperature. The eluent consisted of a solution of 0.15 M LiCl in a mixture of chloroform: methanol (75:25) (Huizing et al. 1981).

Pyrrolizidine alkaloids were visualised by dipping of the dry, developed plates into a solution of 1% (w/v) chloranil in toluene. After dipping, the plates were heated at 105 °C for 15 minutes. Sulfuric acid (2N) was used as an intensifier spray (Huizing et al. 1980).

Gas chromatographical analysis of extracts

A 0.5 ml aliquot of the n-pentane extract was dried in a stream of nitrogen and 1 ml dimethoxy-propane, as a water scavenger, was added. After removal of the scavenger with a stream of nitrogen, 100 µl of a mixture of N, N-dimethyl-formamide: N,0- bis (trimethylsilyl) acetamide: trimethylchlorosilane (6:2:1) was added. Derivatisation was performed for 15 minutes at room temperature in capped Sovirel tubes. 1 µl of the reaction product was submitted to gaschromatography.

A 0.5 ml aliquot of the methanol extract was transferred to a Sovirel tube, dried and treated with dimethoxypropane as described above. After derivatisation with 100 µl TBT, consisting of a mixture of N-(trimethylsilyl) imidazole:N,0-bis(trimethylsilyl) acetamide:trimethylchlorosilane (3:3:2) for 2 hours at 60 °C, 1 µl of the reactionproduct was submitted to gaschromatography.

Separation of the trimethylsilyl (TMS) ethers was achieved in a Packard 429 gas chromatograph equipped with a flame-ionisation detector on a 25 m × 0.22 mm I.D. fused silica column coated with Cp Sil-5 (Chrompack, Middelburg, The Netherlands) under the following conditions:

- derivatised n-pentane extract: initial column temperature, 250 °C; final column temperature, 300 °C; temperature programming rate, 3 °C/min.
- derivatised methanol extract: initial column temperature, 220 °C; final column temperature, 270 °C; temperature programming rate, 3.5 °C/min.
- in both cases: injector block temperature, 250 °C; detector block temperature, 300 °C; nitrogen flowrate, 1.5 ml/min.

Combined gas chromatography - mass spectrometry (GCMS) of derivatised extracts

GCMS was performed on a Finnigan 3300 quadrupole mass spectrometer equipped with the standard chemical ionisation source and a 6110 data system. For electron impact MS an electron energy of 70 eV and for positive ion chemical ionisation MS an electron energy of 140 eV were employed. In the latter case ammonia was used as the reactant gas at 0.5 mbar source pressure. The calibrated ion source temperature was kept at 250 °C.

Compounds in the silylated pentane extract were separated on a 25 m × 0.25 mm I.D. fused silica Cp Sil-5 column. The column temperature was programmed from 250 °C to 310 °C at a rate of 4 °C/min. Mass spectra (EI) were taken with 1.4 sec cycle time.

Compounds in the silylated methanol extract were separated either on a wide-bore 25 m × 0.5 mm I.D. glass Cp Sil-5 column or a 25 m × 0.25 mm I.D. fused silica Cp Sil-5 column. In these cases the column temperature was programmed from 200 °C to 300 °C at rates of 6 °C/min (0.5 mm I.D. column) or 4 °C/min (0.25 mm I.D. column). Mass spectra (CI) were taken with 1.7 sec cycle time.

Results

Cytology

A list of chromosome numbers, together with some notes on the distribution and a provisional taxonomic assignment of the studied material is given in table I.

Table 1 Chromosome numbers, provisional taxanomic assignment and distribution of studied *Symphytum* taxa.

2n = 24:	*S. officinale* L.; some populations from England, G.D.R., France, G.F.R., Hungary, Italy, The Nederlands.
2n = 32:	*S. asperum* Lepech.; three populations from the Caucasus.
2n = 36:	*S.* × *uplandicum* Nym.; several populations from Belgium, Denmark, England, Ireland, Scotland, The Netherlands.
2n = 40:	*S. peregrinum* Ledeb.; chromosome number determined by Dr. T. Gviniashvili in material from Azerbaijan (Lenkoran, Aleksejeva).
2n = 40:	*S.* × *uplandicum* Nym.; several populations from Belgium, Denmark, G.F.R., England, Ireland, France, The Netherlands.
2n = 40:	*S. officinale* L.; several populations from low lying peat lands in the Netherlands, presumably also in Schleswig-Holstein (G.F.R.)
2n = 48:	*S. officinale* L.; many populations from Belgium, Bulgaria, Czechoslovakia, Austria, England, France, G.F.R., Hungary, Poland, Romania, Switzerland, Yugoslavia, The Netherlands.
2n = 56:	*S. officinale L.;* one population from Czechoslovakia (Tatra Mountains).

Morphology

1. The root
   In all material investigated, the roots were thick, vertical, fusiform and branched.

2. The height of the stem
   The height of the stems differs considerably. The plant with the shortest stems belong to *S. officinale* L. (2n = 40), 60–70 cm, and *S. peregrinum* (up to 70 cm). The diploids and tetraploids of *S. officinale* may reach a length of 120 cm, *S. asperum* up to 200 cm, *S.* × *uplandicum* (2n = 36) up to 130 cm, *S.* × *uplandicum* (2n = 40) up to 140 cm. In *S. officinale* (2n = 24, 48) the stems are distinctly winged, a characteristic not found in any of the other taxa.

3. The indument
   a. *The indument of the stems.* The diploid (2n = 24) and tetraploid (2n = 48) cytotype of *S. officinale* have a hispid stem. The stems are never scabrous and the indument renders them soft to the touch. The 2n = 40 cytotype has very prickly and asperous stems. The stems are provided with variable quantities of tubercular-based very stiff and scabrous setae, which render the plants very harsh to the touch. These scabrous hairs are deciduous in older stems and may be removed rather easily. The tubercular base of these straight or slightly curved setae is generally very conspicuous and much more pronounced than in any of the other taxa, which exception of the 2n = 36 cytotype of *S.* × *uplandicum.* In these plants it is also found, although to a much lesser extent. It is absent from the 2n = 40 cytotype of *S.* × *uplandicum.* This is understandable since the 2n = 36 cytotype arose from the cross between *S. asperum* and *S. officinale* 2n = 40. The stem of the 2n = 40 cytotype of *S.* × *uplandicum* is soft to the touch, the setae of the stem are neither prickly nor scabrid; if these setae have a tubercular base, this is always very small and deciduous, and never broad and

conspicuous as in *S. officinale* (2n = 40). The stem in *S. asperum* is very scabrid. The aculeate hairs are curved and subretrorse. They have a small tubercular base which is neither conspicuous nor broad or deciduous.
The stems of *S. peregrinum* have some scattered tubercular-based, more or less curved setae. Beside these, also large and short hairs without a tubercular base are found. The plants are not prickly and they are soft to the touch. In this respect they differ considerably from *S. asperum.*
b. The indument of the leaves. - The upper side of the leaves of the 2n = 24 and 2n = 48 cytotypes of *S. officinale* shows many short and long hairs which are never scabrid. These hairs sometimes have a small tubercular base, which is neither conspicuous nor deciduous. The under side of the leaves shows long appressed hairs along the veins and many much shorter hairs between the veins. The 2n = 40 cytotype of *S. officinale* has a lamina with a very scabrous upper side, which has many short tubercular-based prickly setae. Their tubercles are deciduous, especially in the older leaves. Between these hairs much shorter curved or uncinate hairs are present without tubercles. The under side shows many short, not appressed hairs without a tubercular base as well as some setae with a tubercular base.
The upper side of the leaves of the artificially produced *S.* × *uplandicum* 2n = 40 cytotype as well as that of the natural hybrid 2n = 40 has many appressed setae, the majority of which have a small tubercular base. These tubercular-based setae are not deciduous as in the 2n = 40 cytotype of *S. officinale.* The under side of the leaves shows many setae along the veins and many appressed hairs between them.
Some differences can be observed in the indument of the upper side of the leaves between the artificially produced *S.* × *uplandicum* 2n = 36 cytotype and the natural hybrid. In the first the picture agrees with that found in the 2n = 40 cytotype of *S. officinale,* in the second with that found in the 2n = 40 cytotype of *S.* × *uplandicum.* The under side of the leaves do not show a difference. They have some tubercular-based hairs along the veins and are otherwise glabrous. The upper side of the leaves of *S. asperum* is very scabrid. It is densely covered with short, more or less appressed hairs with a small tubercular base and beside these, smaller and much shorter hairs without a tubercular base are present. The veins of the under side of the leaves are provided with setae, up to 2.5 mm long. The surface is covered with much shorter, uncinate hairs, up to 0.2 mm long. In *S. peregrinum* some scattered tubercular-based setae are present next to many appressed setae without a tubercular base. This renders the leaves much softer to the touch than these of *S. asperum,* which are very scabrous. The under side of the leaves of *S. peregrinum* has many appressed setae and some smaller also appressed hairs.
c. *The indument of the calyx.* - The calyx lobes have long and rather stiff setae on their margins and in their dorso-median row. The pattern of the marginal setae is very regular in *S. officinale* (2n = 40), *S. peregrinum* and more or less also in *S.* × *uplandicum* (2n = 36). In these species the setae are almost evenly distributed along the margin. The distribution is more or less irregular in *S. officinale* (2n = 24, 48), *S. asperum* and *S.* × *uplandicum* (2n = 40). The dorso-median row of hairs consists of a few setae which are of the same type as those of the margin of the calyx lobes. Only in *S. officinale* (2n = 40) some of the marginal and dorso-median row of hairs are provided with a tubercular base.
In all taxa investigated the triangular area between the dorso-median and marginal row is densely covered with minute hairs.

4. The decurrence of the leaf base
The 2n = 24 and 2n = 48 cytotypes of *S. officinale* have decurrent leaf bases along the stem internodes, often from node to node. In the 2n = 40 cytotype this decurrence is usually less pronounced, at least at the lower and middle parts of the stems.
In *S. asperum* and *S. peregrinum* leaf decurrence along the stem is always absent. In the first the upper and uppermost leaves are sessile, or if petiolate, the base of the lamina is decurrent along the petiole or may show a tendency to it. In the second, decurrence along the petiole is always present. *S.* × *uplandicum* 2n = 36 lacks the leaf base decurrence. The picture agrees with that found in S. asperum. Leaf decurrence may be present in *S.* × *uplandicum* 2n = 40, usually it is not longer than 1 cm along the internodes.

5. The shape of the basal and stem leaves
The basal leaves of the three cytotypes of *S. officinale* are lanceolate or ovate, up to 60 cm long, acute at the apex, and acuminate and attenuate at the base. The lamina is 10–40 cm long and 2–12 cm wide. The petiole is 2–20 cm long. The middle and upper stem leaves are of the same type but they are much smaller.
The basal leaves of both cytotypes of *S.* × *uplandicum* are elliptic-lanceolate with an acuminate apex and a rounded more or less cordate base. The lamina is up to 30 cm long and up to 6 cm wide, the petiole up to 12 cm long. The stem leaves are smaller and often provided with a winged petiole. The uppermost leaves are nearly sessile with a cuneate base.
The basal leaves of *S. asperum* are ovate-elliptic, with an acuminate apex and a rounded or cordate base. The lamina is 15–19 cm long and 7–12 cm wide. The petiole may have a length up to 10 cm. The stem leaves are gradually smaller, 10–20 cm long, 4–10 cm wide, ovate or elliptic, acuminate at the apex and cuneate at the base.
The basal leaves of *S. peregrinum* are oblong, cuneate or somewhat rounded (but not cordate) at the base. They are much narrower than in *S. asperum*, 10–15 cm long and 3–6 cm wide. The petiole is up to 8 cm long. The middle and upper stem leaves are of the same type but they are much smaller.

6. The shape of the calyx
The calyx in S. officinale (2n = 24, 48) has an average length of 8 mm, in *S. officinale* (2n = 40) of 9 mm, in *S.* × *uplandicum* (2n = 36) of 5.5 mm, in *S.* × *uplandicum* (2n = 40) of 4 mm, in *S. asperum* of 3 mm and in *S. peregrinum* of 5 mm.
The calyx is divided to ¾ of its length in the three cytotypes of *S. officinale*, to ⅗ in both cytotypes of *S.* × *uplandicum*, to ⅔ in *S. asperum* and to ⅖ in *S. peregrinum*.
The calyx lobes are triangular-lanceolate and acute in the three cytotypes of *S. officinale*. They are linear-oblong and obtuse in flower, becoming triangular in fruit in *S. asperum* and triangular and obtuse in *S. peregrinum*. In *S.* × *uplandicum* (2n = 36) the calyx lobes are triangular-lanceolate and obtuse, in *S.* × *uplandicum* (2n = 40) triangular-lanceolate and sub-acute or acute.

7. The corolla
The length of the corolla varies from 9–19 mm. The differences are most conspicuous in *S. asperum*, where the length varies from 9–14 cm. In other taxa the length of the corolla is much more stable. In *S. officinale* (2n = 24, 48) it is 15–17 mm, in

S. officinale (2n = 40): 16–19 mm, in *S.* × *uplandicum* (2n = 36): 13–16 mm, in *S.* × *uplandicum* (2n = 40): 13–15 mm and in *S. peregrinum:* 14–16 mm.
The colour of the corolla is most variable in *S. officinale* (2n = 48): white or cream-coloured, light to dark purple or red and all possible colours intermediate between purple and red. The 2n = 24 cytotype of *S. officinale* always has white or cream-coloured corollas. The 2n = 40 cytotype of *S. officinale* has a light or dark purple corolla. The colour of the corolla in the two cytotypes of *S.* × *uplandicum* is different. It is either blue-purple in the 2n = 36 cytotype or pink-blue in the 2n = 40 cytotype. *S. asperum* has a sky-blue corolla and *S. peregrinum* a pink-coloured one. In *S. officinale* and *S. peregrinum* the colour of the buds is the same as that of the corolla after anthesis. In *S. asperum* they are deep red, in *S.* × *uplandicum* 2n = 36 cytotype dark purple and in the 2n = 40 cytotype pinkish. In the three cytotypes of *S. officinale* the corolla is urceolate; it is widely campanulate in *S. asperum* and narrowly campanulate in *S. peregrinum.* Both in the 2n = 36 and 2n = 40 cytotypes of *S.* × *uplandicum* the corolla is slightly campanulate.

8. The stamen
In *S. officinale* the stamens are longer than in the other species investigated: *S. officinale* (2n = 24, 48 and 40), up to 7 mm; *S.* × *uplandicum* (both cytotypes), 5–6 mm; and in *S. asperum* and *S. peregrinum,* 4–5 mm. In the 2n = 40 cytotype of S. officinale the anthers are somewhat longer than the corolla scales (squamae). In all other species and hybrids the scales tend to be longer. In all the material investigated the anthers turned out to be longer than their filaments.

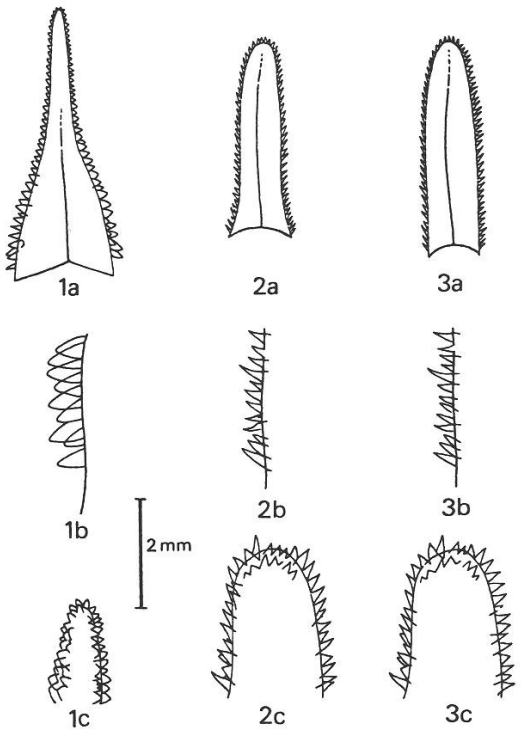


Figure 1. Corolla scales of all cytotypes of *Symphytum officinale* and *S.* × *uplandicum* (1a: shape; 1b: obtuse marginal papillae; 1c: apical papillae), *S. asperum* (2a: shape; 2b: acute marginal papillae; 2c: apical papillae) and *S. peregrinum* (3a: shape; 3b: acute marginal papillae; 3c: apical papillae).

In the three cytotypes of *S. officinale* the connective is projecting beyond the thecae meanwhile this is not the case in *S. asperum, S. peregrinum* and in both cytotypes of *S.* × *uplandicum.*

9. The squamae of the corolla
Pawlowsky (1961) described the shape of the corolla squamae. We follow his description (see also figure 1).
The squamae are triangular-lanceolate, 7–7.5 mm long and ca. 2 mm wide at the base with a more or less curved margin in the three cytotypes of *S. officinale* and in both cytotypes of *S.* × *uplandicum.* They are lingulate in *S. asperum* and *S. peregrinum* and have in the first a lenght of 6 mm and a width of 1 mm at the base and in the second a length of 6 mm and a width of 0.5–0.75 mm at the base. In both species the margin is straight. The apex in *S. officinale* (2n = 24, 48 and 40) and in *S.* × *uplandicum* (2n = 36) is very narrow and mucronate. It is broad and rotundate in *S. asperum, S. peregrinum* and *S.* × *uplandicum* 2n = 40. Many small papillae are present on the margins of the scales. Both in *S. asperum* and in *S. peregrinum* these papillae are longer, narrower, fewer in number and less densely crowded than in the three cytotypes of *S. officinale* and the two cytotypes of *S.* × *uplandicum.* They are regularly distributed along the margin in *S. asperum, S. peregrinum* and *S.* × *uplandicum* 2n = 40, more crowded at the tip of the scale margin in *S. officinale* (2n = 24, 48) and more densely crowded in the middle of the scale-margin in *S. officinale* (2n = 40) and *S.* × *uplandicum* (2n = 36). Only in *S. officinale* (2n = 24, 48 and 40) the papillae are obtuse, in all other species and hybrids they are acute.

10. The fruits
In the three cytotypes of *S. officinale* the nutlets are black and shining, they are 4–5 mm long and 2–2.5 mm wide. In *S. asperum, S. peregrinum* and in both cytotypes of *S.* × *uplandicum* the nutlets are brown and dull, 3–4 mm long and up to 3 mm wide at the base. In *S. asperum* the nutlets are areolate-granulate; in *S. peregrinum* venose-areolate and in *S.* × *uplandicum* (2n = 36 and 2n = 40) somewhat areolate-granulate.

Geographical distribution

S. officinale occurs in many parts of Europe with the exception of the extreme North. Eastwards it extends into W. Siberia and C. Asia. It is rare in the southernmost parts.

S. asperum is found in the Caucasus, North East Anatolia and adjacent Iran.

S. peregrinum is endemic to the Talysh Mountains (U.S.S.R.).

S. officinale and *S. asperum* are allopatric taxa, but there is a very small zone of overlap in the N.W. Caucasus (Kusnetsov 1910). Their hybrid is not mentioned from this sympatric zone, presumably because both species differ in their ecological requirements, *S. officinale* being a plant of lower elevations (usually much lower than 1000 m) whereas *S. asperum* prefers higher elevations (from foothills to the subalpine belt, up to 2000 m). *S. asperum* is especially common on banks of small rivers and streams, on edges of forests and in meadows in mountainous areas. The hybrids *S.* × *uplandicum* arose outside the Caucasus (Tutin 1956, Wade 1958). *S. asperum* is a valuable fodder plant for pigs and rabbits. It was introduced from the Caucasus into W. Europe (Tutin, l.c.). From these introductions the hybrid *S.* × *uplandicum* arose. From the studies by Gadella and Kliphuis (1969, 1973) it became clear that *S. asperum* hybridised with two

forms of *S. officinale*, i.c. the 2n = 40 and the 2n = 48 cytotype. It is neither known where these hybridisations occurred for the first time, nor how the present distribution of the hybrid can be explained. The hybrids were first described from the Swedish province of Uppland by Nyman (1854, 1855). At that location they are widely distributed and they occur near human settlements. They escaped from cultivation and hybridised with *S. officinale* and more rarely with *S. asperum*.

The 2n = 40 cytotype of *S. officinale* occurs in the Netherlands and N.W. Germany (as could be deduced from the photographs of some plants from Schleswig-Holstein in the paper from Basler 1972). In the Netherlands they occur in the low lying peat lands of Noord-Holland, Utrecht and Friesland, where they are locally very common. Populations with 2n = 40 may have a much wider distribution, since some of the characters of this cytotype closely match those of plants from Hungary and S. Russia. These plants are referred to as *S. uliginosum* Kern. by de Sóo (1926, 1931) and *S. tanaicense* Steven (which is the correct name of *S. uliginosum*) by Degen (1930) and Dobrochaeva (1968a). *S. uliginosum* is a lowland plant which is restricted to very moist and wet habitats, the plants are often flooded and occur along streams and in reed-beds.

S. peregrinum, finally, is typically a mountainous plant, which occurs in the lower and middle mountain zone at lower elevations than *S. asperum*.

In figure 2 the distribution areas are given for most of the taxa studied.

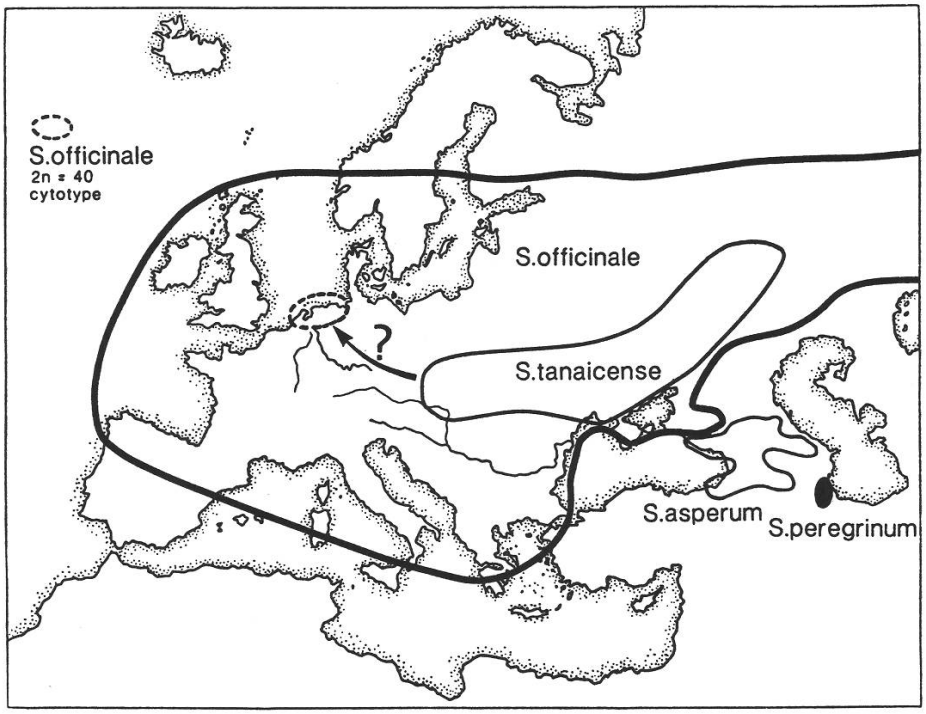


Figure 2. The distribution of the species *S. officinale* L., *S. tanaicense* Stev., *S. asperum* Lepech. and *S. peregrinum* Ledeb. in Europe. The W. European cytotype (2n = 40) of *S. officinale* and *S. tanaicense* are supposed to be very closely related or identical.

Chemotaxonomical studies

Fig. 3 shows the chromatographic patterns of n-pentane extracts of roots of *S. officinale* (2n = 24), *S.* × *uplandicum* (2n = 40), *S. asperum* and *S. peregrinum*, after development on a silica gel thin layer plate with an eluent consisting of a mixture of n-

hexane:diethylether (30:60). Spot 'a' which was detected in extracts from *S. officinale* and the hybrid between *S. officinale* (2n = 48) and S. asperum, i.e. *S.* × *uplandicum* (2n = 40), indicated the presence of the triterpenoid isobauerenol. In a comprehensive study of triterpenoid patterns derived from the former taxa, the occurence of this compound appeared to be general (Huizing, Malingré, Gadella & Kliphuis, submitted). On the other hand isobauerenol could not be demonstrated in *S. asperum* extracts. Spots designated with 'b' are indicative for the presence of steroids, e.g. cholesterol and phytosterols such as, amongst others, β-sitosterol.

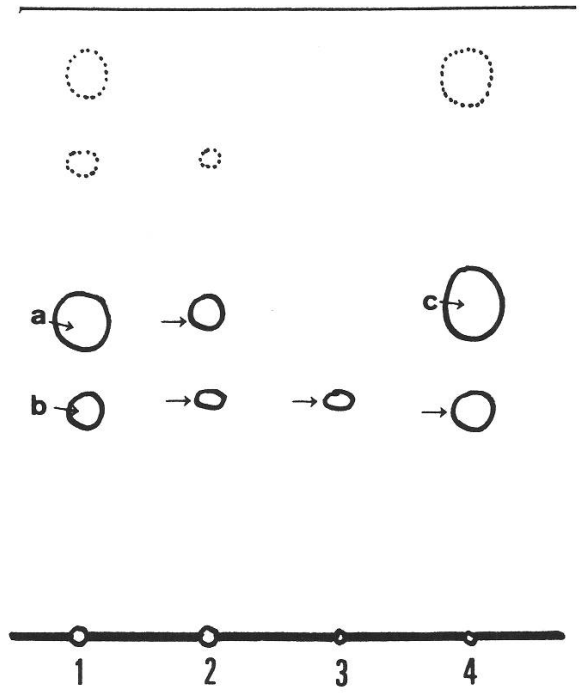


Figure 3. Thin layer chromatogram of phytosterols and triterpenoids from 1: *Symphytum officinale* (2n = 24), 2: *S.* × *uplandicum* (2n = 40), 3: *S. asperum* and 4: *S. peregrinum* Chromatograms were developed on pre-coated silica-gel (S&S) thin layer plates with an eluent consisting of a mixture of n-hexane:di-ethylether (30:60). Detection was performed by spraying with a mixture of chlorosulphonic acid: acetic acid (1:2) and subsequent heating for 5 minutes at 130 °C. a: isobauerenol; b: phytosterols and eventually steroids; c: unknown (not a!). Arrows indicate resembling spots.

The compound (or compounds) giving rise to spot 'c', which appeared in the pattern of the extract from *S. peregrinum* showed an almost identical migration as iso-bauerenol but a different colour response with the chlorosulfonic acid/acetic acid detection reagent.

Retention times of peaks eluting on application of capillary gas chromatography of a silylated n-pentane extract derived from *S. peregrinum* (fig. 4) as well as the evaluation of mass spectra of these eluted compounds by means of a gas chromatograph coupled with a mass spectrometer, did not reveal the presence of steroids or triterpenoids which had been formerly found in *S. officinale* (viz. brassicasterol, campesterol, β-sitosterol and isobauerenol), *S.* × *uplandicum* (viz. cholesterol in the 2n = 40 cytotype, campesterol, β-sitosterol and isobauerenol in both cytotypes, i.e. 2n = 40 and 36) and *S. asperum* (viz. campesterol and β-sitosterol) which also showed mutually higher retention times than the compounds observed in the chromatogram of *S. peregrinum*.

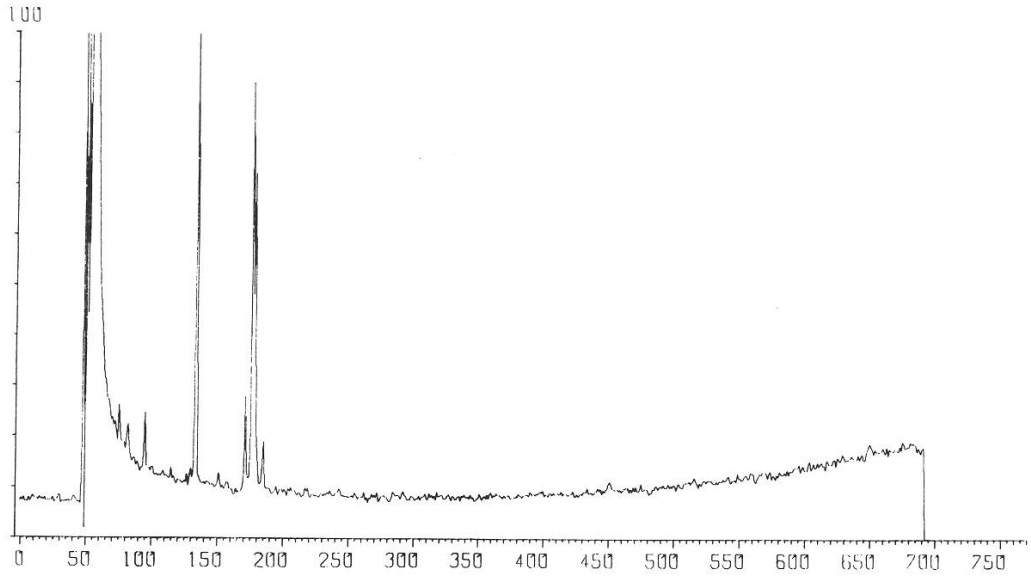


Figure 4. Capillary gas chromatography of a silylated n-pentane extract of *Symphytum peregrinum* roots. Separation was achieved on a fused silica Cp Sil-5 column (25 m × 0.25 mm I.D.) by means of linear temperature programming from 250 °C to 310 °C at a rate of 4 °C/min. Abscissa: scan numbers.

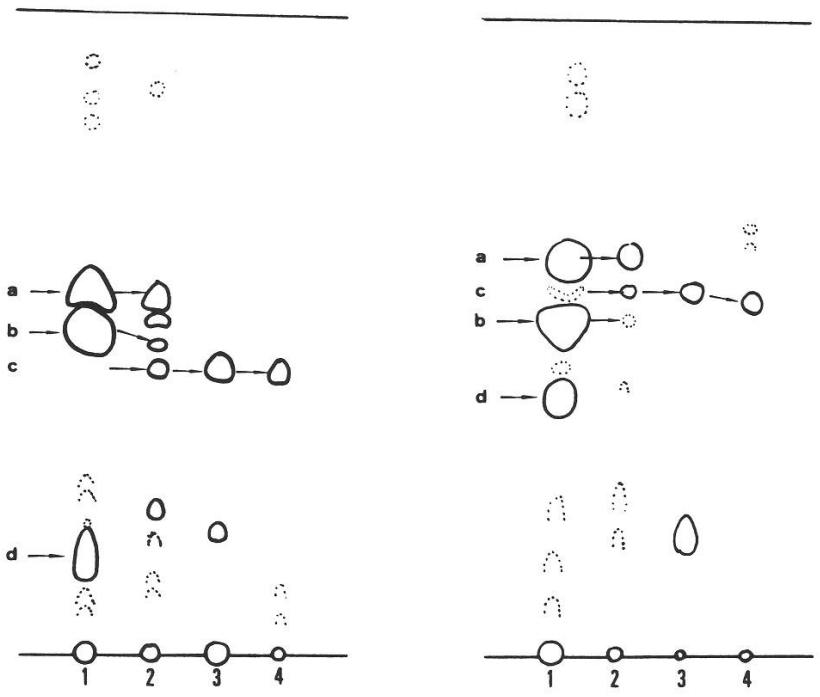


Figure 5. Thin layer chromatogram of pyrrolizidine alkaloids from 1: *Symphytum officinale* (2n = 24), 2: *S.* × *uplandicum* (2n = 40), 3: *S. asperum* and 4: *S. peregrinum.* Chromatograms were obtained on pre-coated silicagel thin layer plates (S&S) after development with the following eluents: left-hand figure: straight phase system consisting of a mixture of chloroform:methanol: ammonia (85:14:1).
right-hand figure: ion-pair system consisting of a 0.15 M solution of LiCl in a mixture of chloroform:methanol (75:25); plates were impregnated by dipping into a 0.1 M solution of LiCl in methanol and subsequently dried and stored in a vacuum desiccator.
Detection was performed with chloranil and sulfuric acid as an intensifier spray. a: symphytine; b: acetyl-lycopsamine; c: echimidine; d: lycopsamine. Of all designated compounds, stereoisomers might be present or even be predominant. Arrows indicate resembling spots.

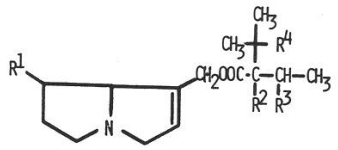
The absence of base peaks with m/z values of 129 in the mass spectra of the separated compounds in the silylated n-pentane extract of *S. peregrinum* roots as well as a direct comparison of the obtained spectra with spectra recorded from references, endorses the absence of steroids with a 5- en -3-01 moiety (cf. cholesterol and the formerly detected phytosterols) and triterpenoids (bauerenol and isobauerenol; Budzikiewicz 1972).

Figure 5 gives a comparison of the pyrrolizidine alkaloid patterns of extracts derived from *S. officinale* (2n = 24), *S. asperum* (2n = 32), one of their hybrids (*S.* × *uplandicum*, 2n = 40) and *S. peregrinum* (2n = 40) after development of the TLC-plates with a straight phase and an ion-pair system. Previous analyses of the detected main alkaloids in the extracts from the former three taxa (after their isolation by means of preparative TLC) and an extensive comparison of TLC patterns of the taxa which had been harvested at different sites and on different times, showed the general presence of lycopsamine, acetyl-lycopsamine and symphytine in all cytotypes of *S. officinale* (2n = 24, 40, 48). In all cases also stereoisomers of these alkaloids might be present or could be even predominant. Separation of diastereoisomeric forms generally can not be achieved by thin layer chromatography, albeit that lycopsamine and intermedine sometimes seem to separate by employment of the ion-pair system. In the extracts of *S.* × *uplandicum* also echimidine, being the main alkaloidal constituent in *S. asperum* extracts, generally was apparent (Huizing et al. 1982). From a comparison of the chromatographic pattern of *S. peregrinum* with those of the formerly described taxa, followed that echimidine might be the main alkaloid in this plant. However, in the ion-pair solvent system migration of the presumed echimidine was slightly retarded in comparison with echimidine from *S. asperum*.

Table 2
Expected quasi molecular ion $(M+)^+$ peak of fully silylated pyrrolizidine alkaloids and m/z value of the (eventually at C-7 esterified) amino alcohol moiety after C-9 cleavage by employment of positive ion chemical ionisation mass spectrometry.

Type of alkaloid (see figure 6)	quasi molecular ion peak at m/z	m/z value after C-9 cleavage
1-$(TMS)_3$	516	208/210
2-$(TMS)_2$	486	180/182
3-$(TMS)_2$	526	220/222
4-$(TMS)_3$	614	220/222
5-$(TMS)_3$	574	180/182

For a more reliable comparison of the identity of pyrrolizidine alkaloids in the extracts, gas chromatography in combination with positive ion chemical ionisation mass spectrometry of silylated alkaloids was performed. By employment of this technique quasi molecular ion peaks $(M+1)^+$ at a considerably high relative intensity become visible in the mass spectra. On the other hand, peaks formed after cleavage of the TMS ethers of the alkaloids at the C-9 position, gives direct evidence about the nature of the eventually also at C-7 esterified amino-alcohol. Indirectly the nature of the structure of the esterified necic acid at C-9 can be deduced from the difference of the m/z value of the quasi molecular ion and the m/z value of the fragment after C-9 cleavage (see table 2 and figure 6). A detailed description of interpretation of spectra and implication of these techniques will appear elsewhere.

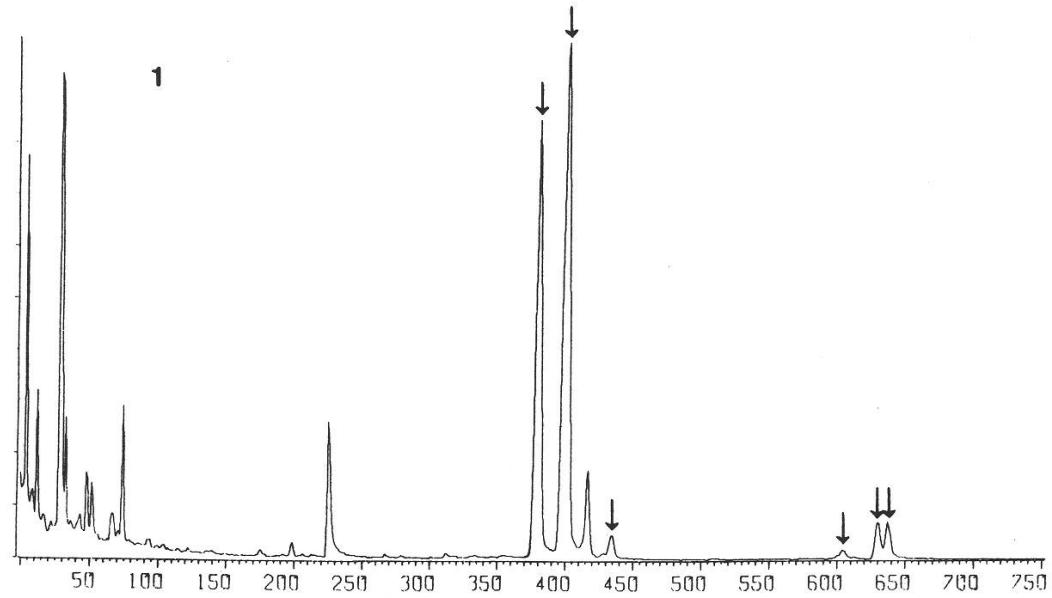


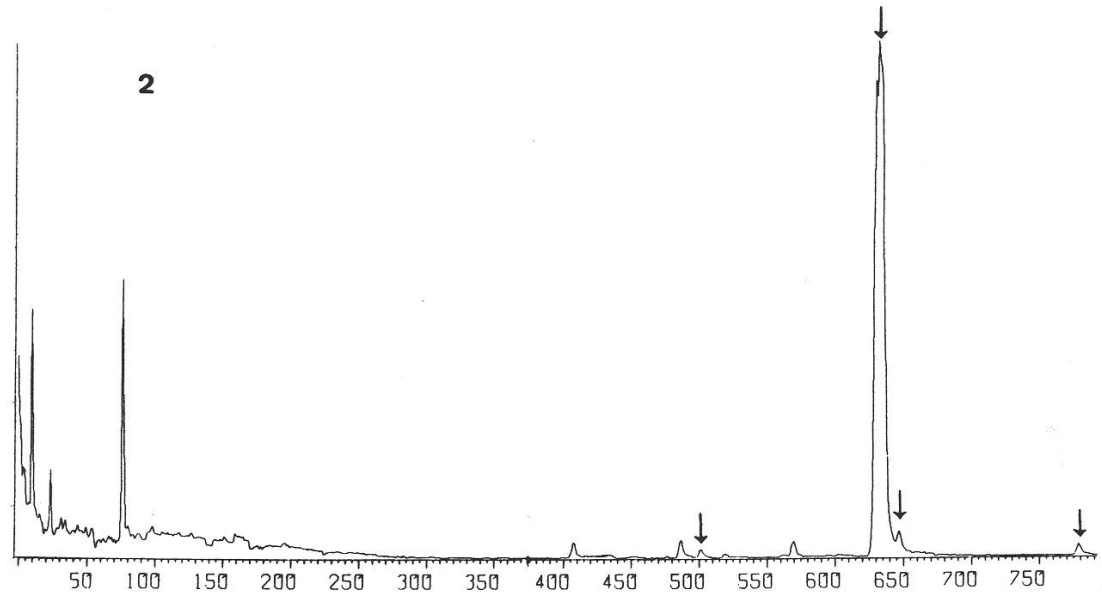
functional groups at:	*	trivial names:
R^1=R^2=R^3= OTMS; R^4= H	1	intermedine[2]/lycopsamine[2]
R^1= acetoxy; R^2=R^3= OTMS; R^4= H	2	acetyl-intermedine[2]/acetyl-lycopsamine[2]
R^1= angeloxy; R^2=R^3= OTMS; R^4= H	3	symphytine[1,2]/symlandine[2]
R^1= angeloxy; R^2=R^3=R^4= OTMS	4	echimidine[1,2]
R^1= acetoxy; R^2=R^3=R^4= OTMS	5	uplandicine[2]

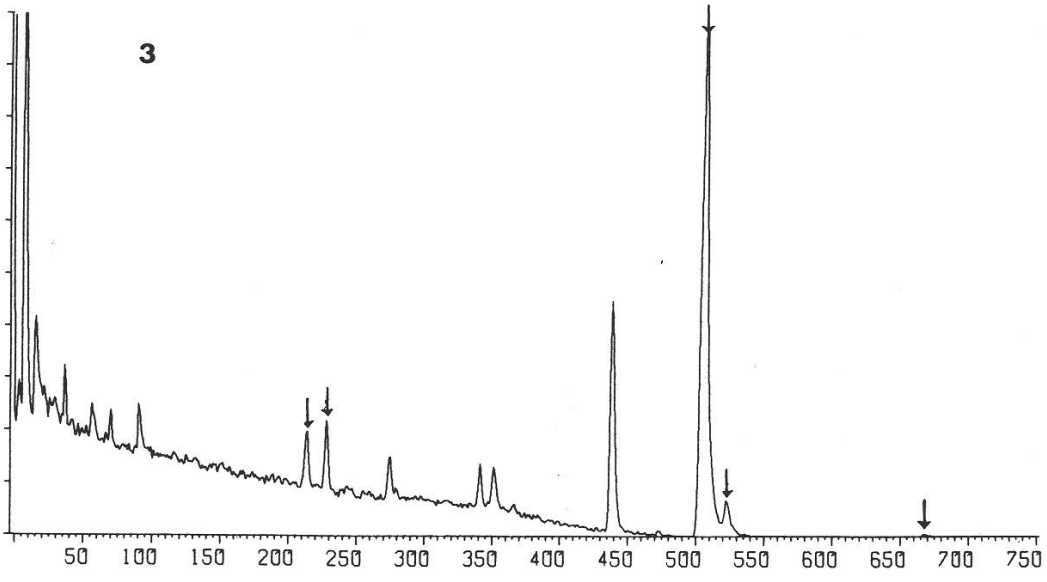
* see table 2: type of alkaloid

Figure 6. General structures of fully silylated pyrrolizidine alkaloids which might be expected in gaschromatograms of *Symphytum officinale* and *S.* × *uplandicum.* Although the esterifying acids may be present in some other isomeric form as indicated, e.g. as geometrical isomers (tiglic vs. angelic acid: trans- resp. cis-) or as diastereoisomers (trachelanthic vs. viridifloric acid: threo- resp. erythro-; echimidinic acids), only one of them has been designated. Specifications of the trivial names according to their structure are given in the following references: 1 = Furuya & Araki 1963 and Furuya & Hikichi 1971; 2 = Culvenor et al. 1980).

Fig. 7 shows gaschromatograms of the TMS derivatives of pyrrolizidine alkaloids, after silylation of extracts from roots of the studied *Symphytum* taxa. In the chromatogram of *S. officinale* (chromatogram 1 in fig. 7) the peak at scan number 28 (# 28) represents the trimethylsilyl derivative of trachechelanthic or viridifloric acid being for example the diastereoisomeric necic acids esterified to retronecine at the C-9 position for respectively intermedine and lycopsamine. These acids seem to be liberated upon storage of extracts or during the silylation procedure by partial hydrolysis. By analogy, echimidinic acid(s)-$(TMS)_3$ originating from echimidine, can be found in e.g. *S. asperum* extracts (# 77). Peak # 378 in the gaschromatogram of *S. officinale* points to the presence of lycopsamine-$(TMS)_3$. The proposed ion structures of the monitored fragments after chemical ionisation mass spectrometry of this compound are outlined in figure 8. The quasi molecular ion $(M + 1)^+$ peak at m/z 516 is explained by protonation of the tertiary nitrogen atom in the aliphatic amino-alcohol part of the pyrrolizidine alkaloid. m/z 210 and 120 can be rationalised by the successive elimination of viridifloric acid and trimethylsilyl alcohol. The ions at m/z 424, 208 and 118 contain an additional double bond. m/z 135 may be formed by a combination of substitution (Keough & Destefano 1981), elimination and dehydrogenation reactions. One double bond has apparently been reduced in the ion at m/z 212. As explained before, the m/z value of the quasi molecular ion and the m/z value derived after C-9 cleavage are diagnostic for recognition of the nature of the derivatised pyrrolizidines in the GCMS runs. Hence,


1
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750


2
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750


3
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750

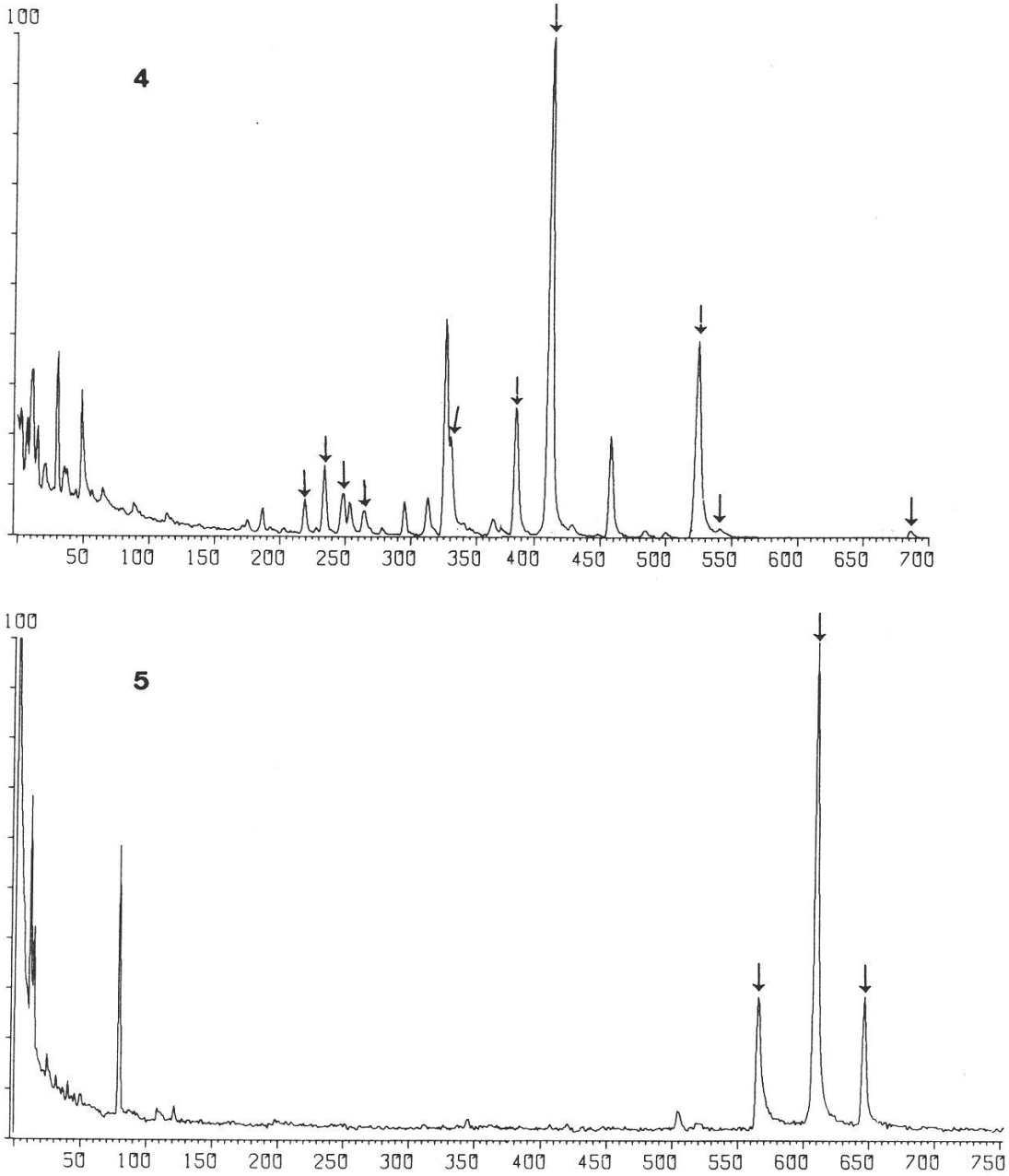


Figure 7. Capillary gas chromatograms of silylated methanol extracts with reduced pyrrolizidine alkaloids from *Symphytum officinale* (2n = 24; 1), *S. asperum* (2 and 3), *S.* × *uplandicum* (2n = 40; 4) and *S. peregrinum* (5). For experimental details see "materials and methods". Only retention times of chromatograms # 2 and # 5 may be compared. Arrows indicate silylated pyrrolizidine alkaloids which have been described in the text.

further examination of the spectra led to the identification of intermedine (# 398), acetyllycopsamine or more obviously acetyl-intermedine (# 435) and three symphytine isomers (# 605, 631, 638). (The nature of the compounds at # 226 and # 418 is unclear). The migration behaviour of the derivatised diastereoisomeric alkaloids lycopsamine and intermedine in GC-columns has been studied after isolation of the pure, not derivatised, compounds by means of affinity chromatography followed by TMS derivatisation and gas chromatographical separation of the products (Huizing 1981). This method made a later assignment of the nature of these diastereoisomeric

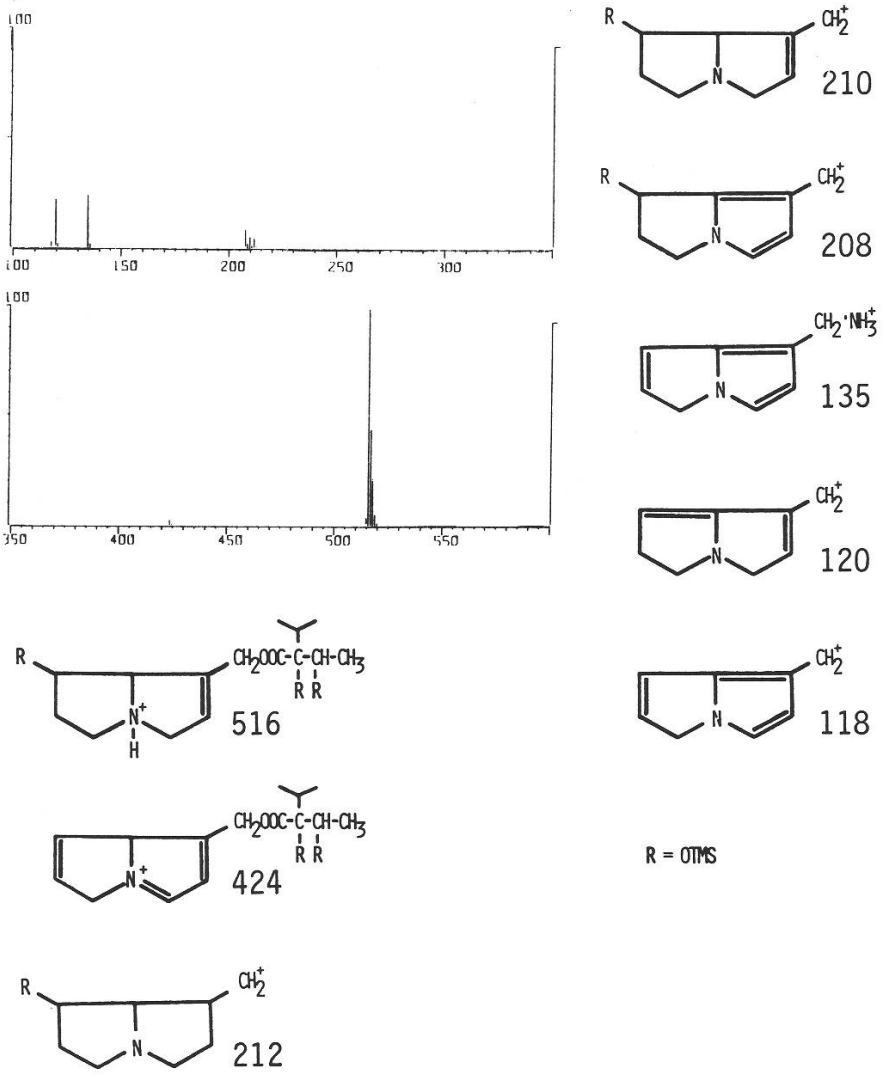


Figure 8. Proposed ion structures of fragments from lycopsamine-$(TMS)_3$ derived by employment of chemical ionisation mass spectrometry. Numbers given with the structures refer to the m/z values in the mass spectrum.

forms of the alkaloids in the gaschromatograms feasible, providing that both diastereoisomers are present. In *S. asperum* (chromatogram 2 in figure 7), besides echimidinic acid(s)-$(TMS)_4$ (# 76), symphytine or a stereoisomer (# 502), echimidine or isomers (# 632, # 648) and a small amount of presumably a higher homologue of echimidine (with tiglic or angelic acid esterified to the echimidinic acid moiety) was found. The mass spectrum of the latter compound showed a quasi molecular ion peak at m/z = 624 (57%) and fragments at m/z values 118 (100), 120 (70), 122 (25), 220 (2) and 222 (10). The spectrum of this compound was quite similar with that of echimidine - $(TMS)_3$ apart from the quasi molecular ion peak (see fig. 9). No clear structural assignments could be made for the other peaks in the chromatogram. In another extract of S. asperum (chromatogram 3 in fig. 6), lycopsamine (# 214), intermedine (# 229), echimidine or stereoisomers (# 508, # 523) and presumably the already mentioned tiglyl/angelyl-echimidine-$(TMS)_2$ were detected in the extracts. The unknown compound at # 440 was identical (by fragmentation pattern) with the compound at # 571 in chromatogram 2 (*S. asperum*, fig. 7) as were # 27 vs. # 408 and # 352 vs. # 487.

S. × *uplandicum* is the hybrid between *S. officinale* and *S. asperum* and shows an alkaloid pattern which is reflected as a summation of the patterns of the parent plants (Huizing, Gadella and Kliphuis 1982). In chromatogram 4 (fig. 7), lycopsamine (# 220), intermedine (# 235), acetyl-lycopsamine (# 249), acetyl-intermedine (# 265), symphytine isomers (# 331: mono-TMS; # 387 and # 413: di-TMS), echimidine isomers (# 526, # 541) and a small amount of the tiglyl/angelyl echimidine (# 687) can be recognised. A number of peaks could not be identified as pyrrolizidine alkaloids but a number seems to represent analogous series considering their MS fragmentation patterns, e.g. # 187 and # 296 with main peaks at m/z values of 118, 120 and 186 in common and further peaks respectively at 424 $(M+1)^+$ and 422, 512 $(M+1)^+$. A compound with the latter fragmentation pattern was also found in *S. asperum* (chromatogram 3, # 275). A further analogous series is present in *S.* × *uplandicum*, e.g. # 254, # 314, # 327 and # 459 which all have m/z values of 120, 136 and 151 in common. In *S. asperum* a similar compound (chromatogram 3, # 440) is present as in *S.* × *uplandicum* (chromatogram 4, # 459). In *S. officinale* none of these unknown compounds has been detected. *S. peregrinum* root extracts gave rise to a sound chromatogram (no. 5 in fig. 7) in which echimidinic acid(s) (# 80), an unknown compound (# 505) with peaks in its mass spectrum at m/z 118 (68%), 120 (48), 122 (18), 380 (4) and 524 $(M+1^+$; 100%), symphytine or an isomer (# 566), echimidine-$(TMS)_2$ or an isomer (# 610) and echimidine-$(TMS)_3$ or an isomer (# 647) were recognised. At higher scan numbers no

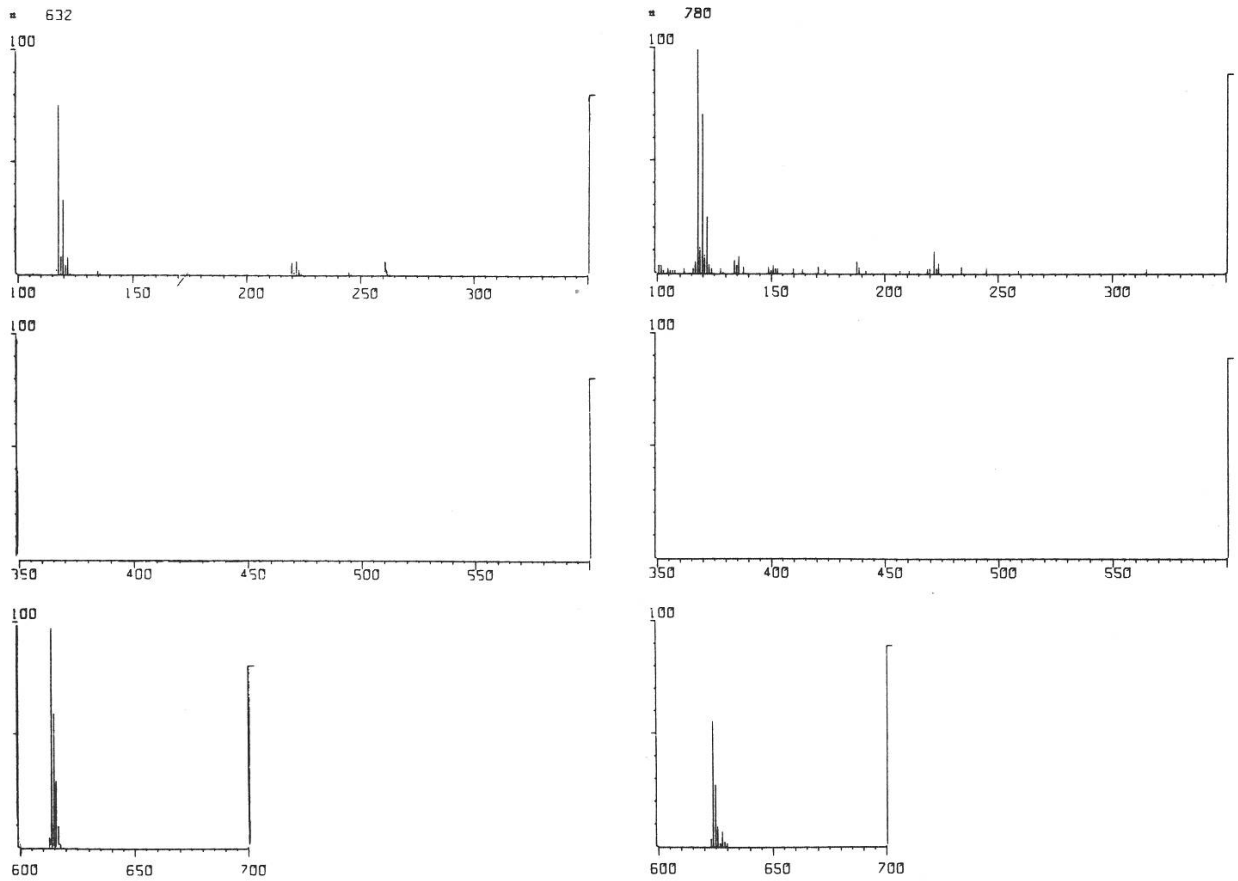


Figure 9. Comparison of mass spectra (chemical ionisation) of echimidine-$(TMS)_3$ or an isomer (scan number 632; see also chromatogram 2 in figure 7) and a presumably higher homologue of echimidine (scan number 780; chromatogram 2) both from *S. asperum* extracts.

compounds were detectable in the column effluent. The unknown compound (# 505) was not present in gaschromatograms of the other taxa.

Discussion

The analysis of morphological characters of the taxa studied makes clear that the artifically derived interspecific hybrids between *S. asperum* and *S. officinale* are intermediate in many aspects. This is also true for many hybrids collected in nature (Gadella & Kliphuis 1975), provided that no backcrossing to one of the parental species had occurred. Some methods for the analysis of populations suspected of hybridisation were devised by Anderson (1949). The simplest, albeit somewhat crude method, is the construction of a hybrid index. This is obtained by selecting a number of characters by which two species differ, assigning the score "2" to each of the attributes of the first, "0" to the second species and "1" to the intermediates. Within the *S. officinale/S. asperum/ S. peregrinum* complex 19 characters were selected. The score "2" has been assigned to each of the character attributes of *S. officinale* (2n = 24, 48), "0" to those of *S. asperum* and "1" to the intermediate plants. For a complete comparison of the character attributes within the former species complex, *S. peregrinum* has been added with the assumption that it might be a hybrid between *S. officinale* and *S. asperum* as suggested by Bucknall (1913). Results of this comparison are summarised in table 3. It shows that the artificially produced (and most natural) hybrids between *S. asperum* and *S. officinale* are exactly intermediate. The score of *S. peregrinum* differs considerably from

Character	*S. off.* 24/48	*S. off.* 40	*S. upl.* 36	*S. upl.* 40	*S. asp.*	*S. peregr.*
1 heigth of stem	2	2	1	1	0	2
2 stem winged	2	2	0	0	0	0
3 indument of stem	2	0	0	2	0	2
4 leaf decurrence	2	2	0	1	0	0
5 shape basal leaves	2	2	1	1	0	0
6 width basal leaves	2	2	2	2	0	0
7 indument leaves	2	0	0	2	0	2
8 length calyx	2	2	1	1	0	1
9 ratio calyx/corolla	2	2	1	1	0	1
10 indument calyx margin	2	0	2	0	0	2
11 connective of anthers	2	2	0	0	0	0
12 corolla shape	2	2	1	1	0	1
13 absence of blue in corolla	2	2	1	1	0	1
14 shape corolla scales	2	2	2	2	0	0
15 apex scales	2	2	2	0	0	0
16 shape scale papillae	2	2	2	2	0	0
17 pattern scale papillae	2	2	2	0	0	0
18 fruit colour	2	2	0	0	0	0
19 fruit surface	2	2	0	0	0	0
Total score	38	32	18	17	0	12

Table 3. A comparison of the characters of *S. officinale, S. asperum, S. peregrinum* and the two *S.* × *uplandicum* hybrids. In each case the score **2** is given to character attributes of *S. officinale* 2n = 24/48, **0** to *S. asperum* and **1** to intermediates. *S. peregrinum* has been added for comparison and was treated in the same way as the other taxa.

S. × *uplandicum* and from *S. asperum*. The 2n = 40 cytotype of *S. officinale* differs from the 2n = 24 and the 2n = 48 cytotype of *S. officinale*, but not to a large extent. The 2n = 40 cytotype of S. officinale differs considerably from the *S.* × *uplandicum* hybrids. The differences in chromosome number, morphology, geographical and altitudinal distribution, as well as the differences in steroid and alkaloid patterns of *S. peregrinum* and *S. asperum* are in favour to justify these taxa as being independent, albeit that a more definite assignment of the rank needs further investigations (hybridisation studies). There is not doubt that the two species are more closely related to each other than to *S. officinale* as can be deduced from table 3. This close relationship between the former two taxa has been suggested earlier by Popov (in Komarov 1953).

S. peregrinum and *S.* × *uplandicum* are not identical at all. They differ morphologically, partly cytologically, chemically and in their distribution. For that reason we completely disagree with Bucknall (1913) and with Wickens (1969), who took the description from Bucknall, without having seen native specimens of *S. peregrinum* (Wickens 1969, page 163).

S. × *uplandicum* did not arise in the Caucasus, because the parental species are sympatric in a very restricted area only. Additionally they are bound to different altitudinal zones with this area. Wade (1958) reported that *S. asperum* was introduced into the area of *S. officinale*, where hybridisation between and backcrossing of hybrids

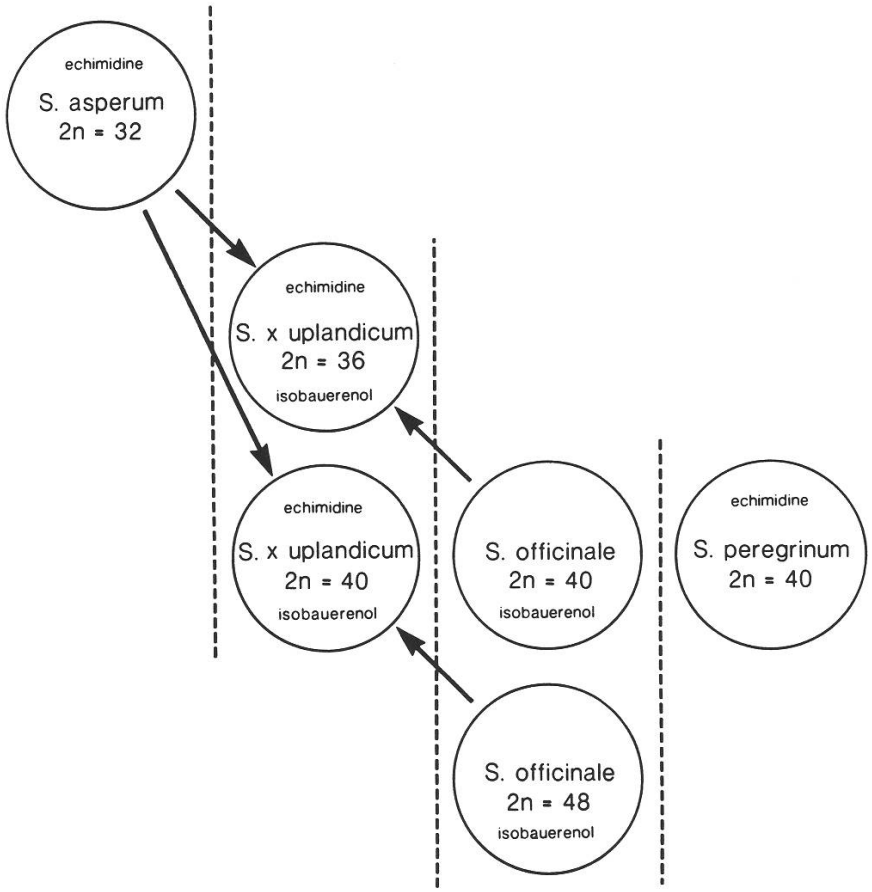


Figure 10. Diagram showing the ploidy level of the taxa *Symphytum asperum, S. officinale, S. peregrinum* and the *S.* × *uplandicum* hybrids. The occurrence of the diagnostic compounds isobauerenol and echimidine (or isomers) have been included in the diagram. *S. officinale* (2n = 24) shares the compounds of *S. officinale* (2n = 48).

with the parental species occurred as well. Usually backcrossing to the *S. officinale* parent took place. Two cytotypes of *S. officinale* (2n = 40, 48) were involved in this hybridisation process. The 2n = 48 cytotype is widely distributed in Europe, but the 2n = 40 cytotype occupies as far as is known up to now, a more restricted area (The Netherlands and NW Germany). However, it is highly unlikely that the *S.* × *uplandicum* 2n = 36 - hybrid originated from W. Europe, because the *S.* × *uplandicum* hybrids are supposed to have been introduced into W. Europe from Leningrad (Wade, l.c.). Therefore, we are in the opinion that plants with the morphological characters of the 2n = 40 cytotype most probably have a much wider distribution in Europe. The W. European plants share a number of characters with E. European plants of *S. tanaicense* Steven. However, the latter species has neither been studied cytologically nor chemically. If the material from the low-lying peat lands of The Netherlands is identical with Hungarian and S. Russian plants, they should be assigned taxonomically to *S. tanaicense.* However, the W. European 2n = 40 cytotype crosses readily with *S. officinale* (2n = 48), producing fertile hybrids (2n = 44). For that reason the authors express as their opinion that the crossability and the slight morphological differences indicate that the 2n = 40 and 2n = 48 cytotypes are conspecific. Possibly the differences in ecological requirements may justify a treatment at the level of subspecies, but before we can arrive at more definite conclusions a careful biosystematical comparison of the W. European 2n = 40 cytotype and *S. tanaicense* (syn. *S. uliginosum*) is clearly needed. Such studies should include a close morphological examination and crossing as well as phytochemical investigations. At any rate the 2n = 40 cytotype of *S. officinale* and *S.* × *uplandicum* (2n = 40) are not identical at all. They differ morphologically (Gadella & Kliphuis 1973) and chemically (Huizing, Gadella & Kliphuis 1982, Huizing, Malingré, Gadella & Kliphuis, submitted). The diagnostic cyto- and chemotaxonomical data of the taxa studied are summarised in the diagram in figure 10.

The 2n = 24 and 2n = 48 cytotypes of *S. officinale* are inseparable on morphological and chemical grounds as far as they have been studied. They do not hybridise in nature and with great difficulty in the experimental garden, giving rise to sterile triploid hybrids (2n = 36). Such hybrids could be produced only between white-flowered parents. The exact taxonomic position of the 2n = 56 cytotype is unclear for the moment. It needs further investigation.

Zusammenfassung

Zytologische und phytochemische Untersuchungen am *Symphytum officinale*-Komplex, *S. asperum* und *S. peregrinum,* zeigen, daß *S. peregrinum* eine gute Art ist, die *S. asperum* zwar nahe steht, aber doch deutlich von ihm verschieden ist. *S. peregrinum* unterscheidet sich in der Verbreitung und durch zytologische, morphologische und phytochemische Merkmale von *S. officinale* und *S. asperum.* Deshalb ist *S. peregrinum* bestimmt kein Bastard zwischen diesen Arten. Die beiden *S.* × *uplandicum*-Hybriden (2n = 36, 40) sind von *S. peregrinum* (2n = 40) in morphologischer, phytochemischer und zum Teil in zytologischer Hinsicht verschieden.

Diploide (2n = 34) und tetraploide (2n = 48) Pflanzen von *S. officinale* stimmen morphologisch nicht ganz mit *S. officinale* (2n = 40) überein, aber sie besitzen die gleichen Pyrrolizidine-Alkaloide. Weil sich außerdem gezeigt hat, daß *S. officinale*

$2n = 40$ und $2n = 48$ voll und fertil kreuzbar sind, werden die beiden Zytotypen als conspezifisch behandelt.

Weitere Arbeiten sind erforderlich, um die Pflanzen von *S. officinale* mit $2n = 40$ aus Westeuropa eingehend mit den osteuropäischen Pflanzen von *S. tanaicense* Stev. zu vergleichen und um eine endgültige taxonomische Entscheidung über die Identität dieses Zytotyps möglich zu machen.

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References

Anderson E. 1949. Introgressive hybridization. New York.

Basler A. 1972. Cytotaxonomische Untersuchungen an der Boraginaceen-Gattung *Symphytum* L. Untersuchungen an überwiegend norddeutschen Pflanzen der Arten *S. asperum* Lepech., *S. officinale* L. und *S.* × *uplandicum* Nym. Bot. Jahrb. Syst. *92:* 508–553.

Boissier E. 1897. Flora orientalis *4:* 171–177. Geneva.

Bucknall C. 1912. Some hybrids of the genus *Symphytum.* J. Bot. *50:* 332–337.

Bucknall C. 1913. A revision of the genus *Symphytum.* J. Linn. Soc. Bot. *41:* 491–556.

Budzikiewicz H. in G.R. Waller 1972. Biochemical applications of mass spectrometry. Wiley – Interscience, New York.

Culvenor C.C.J., J.A. Edgar, J.L. Frahn and L.W. Smith 1980. The alkaloids of *Symphytum* ×*uplandicum* (Russian comfrey). Aust. J. Chem. *33:* 1105–1113.

Degen A. von. 1930. Bemerkungen über einige orientalische Pflanzenarten LXXXXIX. Über *Symphytum uliginosum* Kern. Magyar Bot. Lapok *29:* 144–148.

Dobrochaeva D.N. 1968 a. On species independence of two species of the genus *Symphytum* L., *S. asperum* Lepech. and *S. peregrinum* Ledeb. (in Russian). Ukr. bot. Zhurn. *25:* 43–49.

Dobrochaeva D.N. 1968 b. On taxonomy of the genus *Symphytum.* 1. *Symphytum* section. (in Russian). Ukr. bot. Zhurn. *25:* 33–39.

Faegri K. 1931. Über in Skandinavien gefundenen *Symphytum*-Arten. Bergens Museum Årbok 1931: 1–47.

Furuya T. and K. Araki 1963. Studies on constituents of crude drugs. I. Alkaloids of *Symphytum officinale* L. Chem. Pharm. Bull. *16:* 2512–2516.

Furuya T. and M. Hikichi 1971. Alkaloids and triterpenoids of *Symphytum officinale* L. Phytochemistry *10:* 2217–2230.

Gadella Th.W.J. 1972. Cytological and hybridization studies in the genus *Symphytum.* Symp. Biol. Hung. *12:* 189–199.

Gadella Th.W.J. and E. Kliphuis 1967. Cytotaxonomic studies in the genus *Symphytum.* I. *Symphytum officinale* L. in the Netherlands. Proc. Kon. Ned. Acad. Wet. ser C *70:* 378–391.

Gadella Th.W.J. and E. Kliphuis 1969. idem. II. Crossing experiments between *Symphytum officinale* L. and *Symphytum asperum* Lepech. Acta Bot. Neerl. *18:* 544–549.

Gadella Th.W.J. and E. Kliphuis 1973. idem. V. Some notes on W. European plants with the chromosome number 2n = 40. Bot. Jahrb. *93:* 530–538.
Gadella Th.W.J. and E. Kliphuis 1975. idem. VII. Some hybrids between *S. asperum* Lepech. and *S. officinale* L. in Denmark. Proc. Kon. Ned. Acad. Wet. ser C *78:* 182–188.
Gadella Th.W.J. and E. Kliphuis 1978. idem. VIII. Chromosome numbers and classification of ten European species. Proc. Kon. Ned. Acad. Wet. ser C *81:* 162–172.
Grossheim A.A. 1967. Flora Kaukaza ed. 2. VII. Leningrad.
Gviniashvili Ts. 1972. Some data on the karyology of Caucasic species of *Symphytum* L. with respect to taxonomy (in Russian). Bot. Zhurn. *57:* 1120–1126.
Huizing H.J. 1981. Vortragsveranstaltung der Gesellschaft für Arzneipflanzenforschung. Planta med. *42:* 126.
Huizing H.J. and Th.M. Malingré 1981. Ion-pair adsorption chromatography of pyrrolizidine alkaloids. J. Chromatogr. *205:* 218–223.
Huizing H.J., F. de Boer and Th.M. Malingré 1980. Chloranil, a sensative detection reagent for pyrrolizidine alkaloids on thin-layer chromatograms. J. Chromatogr. *195:* 407–411.
Huizing H.J., Th.W.J. Gadella and E. Kliphuis 1982. Chemotaxonomical investigations of the *Symphytum officinale* polyploid complex and *S. asperum* (Boraginaceae). Part I: The pyrrolizidine alkaloids. Pl. Syst. Evol. *140:* 279–292.
Huizing H.J., Th.M. Malingré, Th.W.J. Gadella and E. Kliphuis 1983. idem. Part II. Phytosterols and triterpenoids. Pl. Syst. Evol. (submitted).
Keough T. and A.J. Destefano 1981. Factors affecting reactivity in ammonia chemical ionization mass spectrometry. Organic mass spectrometry *16:* 527–533.
Kerner A. 1863. Descriptiones plantarum novarum florae Hungaricae et Transsilvanicae. Österr. Bot. Z. *13:* 227–228.
Kurtto A. 1982. Taxonomy of the *Symphytum asperum* aggregate (Boraginaceae), especially in Turkey. Ann. Bot. Fenn. *19:* 177–192.
Kusnetsov N. 1910. Caucasian species of the genus *Symphytum* (Tourn.) L. (in Russian). Mém. Acad. Imp. Sci. St. Pétersbourg ser. 8. Phys. Math. *25:* 1–94.
Lindman C.A.M. 1911. Über *Symphytum orientale* L. und *Symphytum uplandicum* Nyman. Bot. Not. 1911: 71–77.
Murin A. and J. Majowsky 1982. Die Bedeutung der Polyploidie in der Entwicklung der in der Slowakei wachsenden Arten der Gattung *Symphytum* L. Acta Fac. Rer. Nat. Univ. Comenianae Bot. *29:* 1–25.
Nyman C.F. 1854, 1855. Sylloge Florae Europaea, Örebro.
Pawlowski B. 1961. Observationes ad genus *Symphytum* L. pertinentes. Fragm. Florist. Geobot. *7:* 327–356.
Popov M.G. in V.L. Komarov 1953. Flora U.S.S.R. *9:* 279–291. Moscow.
Riedl H. in K.H. Rechinger 1957. Boraginaceae in Flora Iranica *48/15:* 239–241.
Schinz H. and A. Thellung 1913. Beiträge zur Kenntnis der Schweizerflora XIV Mitt. Bot. Mus. Univ. Zürich *65:* 78–80.
Sharma R.K., G.S. Khajuria and C.K. Atal 1965. Thin layer chromatography of pyrrolizidine alkaloids. J. Chromatogr. *19:* 433–434.
Sóo R. de 1926. Diagnoses plantarum novarum et revisio formarum specierum nonnularum. Rep. Spec. nov. regn. veg. *27:* 316–322.
Sóo R. de 1931. Kritische Bemerkungen und neue Beiträge zur Kenntnis der ungarischen Flora. IV. (Zur Kenntnis von *Symphytum uliginosum/tanaicense*). Bot. Közlem. *38:* 125–133.
Stahl E. 1967. Dünnschicht-Chromatographie, 2. Aufl. Springer, Berlin.
Thellung A. 1915. Über das "Prickley Comfrey" der Engländer. Verh. Bot. Ver. Prov. Brandenburg *57:* 78.
Thellung A. 1919. Beiträge zur Adventivflora der Schweiz. Vierteljahrschr. Naturf. Ges. Zürich *64:* 779–780.
Tutin T.G. 1956. The genus *Symphytum* in Britain. Watsonia *3:* 280–281.
Wade A.E. 1958. The history of *Symphytum asperum* Lepech. and *S.* × *uplandicum* Nym. in Britain. Watsonia *4:* 117–118.

Wickens G.E. 1969. A revision of *Symphytum* in Turkey and adjacent areas. Notes Roy. Bot. Garden Edinburgh *24:* 157–180.
Wickens G.E. in P.H. Davis 1978. Flora of Turkey VI: 378–386. Edinburgh.

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