JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, May 2013, Vol. 58, No. 3
doi: 10.1111/1556-4029.12072
Available online at: onlinelibrary.wiley.com

CASE REPORT

CRIMINALISTICS

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Forensic Identification of Indian Snakeroot (*Rauvolfia serpentina* Benth. ex Kurz) Using DNA Barcoding*

ABSTRACT: Indian snakeroot (*Rauvolfia serpentina*) is a valuable forest product, root extracts of which are used as an antihypertensive drug. Increasing demand led to overharvesting in the wild. Control of international trade is hampered by the inability to identify root samples to the species level. We therefore evaluated the potential of molecular identification by searching for species-specific DNA polymorphisms. We found two species-specific indels in the *rps*16 intron region for *R. serpentina*. Our DNA barcoding method was tested for its specificity, reproducibility, sensitivity and stability. We included samples of various tissues and ages, which had been treated differently for preservation. DNA extractions were tested in a range of amplification settings and dilutions. Species-specific *rps*16 intron sequences were obtained from 79 herbarium accessions and one confiscated root, encompassing 39 different species. Our results demonstrate that molecular analysis provides new perspectives for forensic identification of Indian snakeroot.

KEYWORDS: forensic science, DNA typing, Apocynaceae, CITES, medicinal plants, *Rauvolfia*, *rps*16 intron

Rauvolfia serpentina (L.) Benth. ex Kurz (Apocynaceae) is the principal source of Indian snakeroot, a valuable forest product that is used to produce antihypertensive drugs worldwide (1). The discovery of reserpine as the most active alkaloid of the root, responsible for lowering high blood pressure, aroused global interest in the species. The entire genus *Rauvolfia* L. currently comprises 73 species (2–4), ranging in size from small herbs (up to 15-cm tall) to large trees (over 30-m high). These occur both in tropical regions of Central and South America, Africa and Madagascar, as well as in (sub)tropical to temperate areas of India, China, and Japan (2,5–7).

The increasing demand for Indian snakeroot led to intensive harvesting of the living wild stock of *R. serpentina.* To ensure sufficiently high reserpine concentrations, roots are harvested when plants are 3- to 4-years old, which unfortunately leads to death of the whole plant. In 1993, it was estimated that 400–500 tons of *R. serpentina* roots were harvested annually in India, Pakistan, Sri Lanka, Burma, and Thailand (8). As a result, *R. serpentina* is now the only species of *Rauvolfia* listed on the CITES Appendix II list, as it is considered endangered by the International Union for Conservation of Nature (9). To ensure a legal and sustainable trade of Indian Snakeroot, control of international trade is needed. Currently, this control is hampered by the lack of an unambiguous identification tool for Indian snakeroot (8). Traditional identification keys in floras require flowers or fruits, and Indian snakeroot is mostly traded as sterile roots. Due to the difficulty of taxonomic identification of these roots, customs officials are able to capture only a small proportion of *R. serpentina* samples traded. Investigating the options for the development of species identification based on molecular characters is a major step forward to better control trade. Stimulated by the many ongoing DNA barcoding projects aiming to identify all living species and the huge drop in costs, molecular techniques have become more and more popular as an efficient instrument in applied sciences such as wildlife forensics. Identification by analyzing DNA of species illegally traded either as raw or processed material has recently been the aim of numerous investigations (10–15).

In this study, we aimed to identify sterile Indian snakeroot samples to the species level using DNA barcoding. To find DNA polymorphisms, we investigated the applicability of three fast-mutating chloroplast DNA regions (*trn*L-*trn*F intergenic spacer, *rpl*16 intron region, and *rps*16 intron region) to discriminate between *R. serpentina* and closely related species in the genus of *Rauvolfia*. Although, *rbcL*, *matK,* and *trnH-psbA* are proposed as the most informative markers for barcoding (16,17), we did not use these official plant Barcode of Life markers as they are too long to amplify DNA from confiscated dry root material using the standard primers. Furthermore, our aim was to distinguish *R. serpentina* from all other *Rauvolfia* species, regardless of the overall variation within the genus of *Rauvolfia*.

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*Funding provided by the Institute of Biology, Netherlands Centre for Biodiversity Naturalis and the Faculty of Archaeology.

Received 8 Dec. 2011; and in revised form 28 Feb. 2012; accepted 24 Mar. 2012.

In this particular case, the specific markers chosen have several advantages above the Barcode of Life markers. First of all, the *rps*16 intron region has proven to be a fast-mutating region within the Apocynaceae, holding sufficient informative characters for species identification (18–22). Second, with the sequence data of these markers already available in National Center for Biotechnology Information (NCBI) GenBank, we could use previously identified polymorphisms as a starting point for new primer design. Third, these markers are very short and therefore much more likely to amplify from highly degraded DNA extracted from traded root samples. We attempted to amplify our markers from the species of *Rauvolfia* most commonly traded (*R. caffra, R. serpentina, R. tetraphylla,* and *R. vomitoria*) (6,8) and the species considered most closely related to *R. serpentina* based on morphology and distribution (*R. cambodiana, R. beddomei, R. sumatrana,* and *R. verticillata*) (1,7). These species names are most likely to be used to disguise the presence of *R. serpentina* in traded material, and DNA sequences of these species are most likely to resemble *R. serpentina* DNA.

As the use of a DNA barcoding technique in forensic cases requires a validation study, we investigated the reliability and reproducibility of the results obtained according to the guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM).

Materials and Methods

Sample Collection and DNA Extraction

Leaves, and in a few cases roots, were collected from fertile and sterile herbarium specimens of different *Rauvolfia* species deposited at the Leiden and Wageningen branches of Netherlands Centre for Biodiversity Naturalis — National Herbarium of The Netherlands. All specimens were identified to the species level by Apocynaceae specialists Toon Leeuwenberg or Jan Wieringa. Several specimens (including types) of recently described new species were also sampled. In addition, a root sample supplied by the Dutch Customs Laboratory was analyzed (Table 1).

DNA extraction from leaves and roots was done using the Plant Mini Tissue Kit (Qiagen GmbH, Hilden, Germany) for herbarium dried-leaf material and a separate protocol using silica adsorption for the root material (23). Between 10 and 30 mg of leaf tissue was ground using a Retsch mill (Retsch GmbH, Haan, Germany). These samples were further processed according to the manufacturer's protocol of the Plant Mini Tissue Kit. About 20 mg of root tissue was ground to saw dust by firmly moving roots up and down against an iron grater. These samples were further processed using a separate protocol based on silica adsorption developed by Rohland and Hofreiter (23).

To avoid the risk of contamination with more concentrated herbarium-derived DNA, all extractions of roots were carried out in the ancient DNA facility of Leiden University following established protocols (24). As a control for possible contamination of the DNA extracted, part of the root samples was not only processed in Leiden, but also in the molecular laboratory of the Dutch Customs Laboratory in Amsterdam. In this way, replicate DNA sequences were obtained in physically separated laboratories from all root samples analyzed to confirm their authenticity.

PCR and DNA Sequencing

For primer design, initial DNA alignments were made using BioEdit Sequence Alignment Editor (version 7.0.9.0; Ibis Biosciences, An Abbott Company, Carlsbad, CA) using *Rauvolfia* sequences already available in NCBI GenBank. For the *trn*L-*trn*F intergenic spacer, published data of *R. serpentina* (AF214261 and AF214260), and *R. balansae* (AF214259) were used. For the *rpl*16 intron, published data of *R. sellowii* (DQ660796) and *R. vomitoria* (DQ660797) were used. For the *rps*16 intron, we used published data of *R. vomitoria* (DQ660607), *R. sellowii* (DQ660606), *R. verticillata* (AB364600), *R. sumatrana* (AB364599), and *R. serpentina* (AB 364598). Neither the *trn*F intergenic spacer nor the *rpL*16 intron region provided enough information to develop a marker for *R. serpenina*, therefore, these gene regions were not further investigated. In contrast, the *rps*16 alignment showed two indels that were unique for *R. serpentina*: one deletion of 13 base pairs (bp) starting at position 476 and one insertion of 8 bp starting at position 730 of *rps*16 NCBI GenBank accession AB364599 (*R. sumatrana)*. To investigate the usefulness of these indels for the development of a DNA marker for Indian snakeroot, we designed primers using Primer3 software (25) to amplify small fragments containing these mutations (Table 2). We tested these primers on an extended sampling of *Rauvolfia* species (Table 1) from both leaves and root samples. We examined samples of different age and different chemical treatments. Of several individuals from root and leaf, we also tested DNA dilution series and variable reaction mixes and reaction conditions (see further details below).

Standard Amplification Procedure

The standard polymerase chain reactions (PCRs) were carried out on a PTC 200 DNA engine (MJ Research, St. Bruno, Canada) in a 25-μL volume containing *c*. 5 ng of genomic DNA, 0.1 μM of each primer, 100 μM of each dNTP (Bioline, Londen, UK), Qiagen PCR buffer (50 mM KCl, 10 mM TRIS-HCl, pH 8.7, 1.5 mM $MgCl_2$), 1.5 mM $MgCl_2$ extra, 0.3 mg/mL BSA (Promega Corporation, Madison, WI), and 1 unit of Taq DNA polymerase (Qiagen). Positive and negative controls were included simultaneously in all the amplifications to check for contamination. The thermal cycling profile started with a 5-min denaturation step at 95°C, followed by 40 cycles of 20-sec denaturation at 94°C, 20-sec annealing at 51°C, and 20-sec elongation at 72°C, with a final extension step of 5 min at 72°C. The PCR products were purified using the Wizard SV and PCR Clean-up systems (Promega). DNA sequencing was done using a 96-capillary 3730xl DNA Analyzer automated sequencer 3730XL (Applied Biosystems, Inc., Foster City, CA) using standard dye-terminator chemistry (Macrogen Inc., Seoul, Korea).

Calculation of Genetic Similarity

Calculation of percentage of similarity of the *rps*16 intron DNA sequences retrieved was done by generating Kimura 2- parameter (K2P) distance matrices for both the del-13-bp and ins-8-bp regions as implemented in PAUP* version 4.0b10 (Sinauer Associates, Sunderland, MA).

Validation of DNA Barcoding Method

The specificity of the technique for barcoding Indian snakeroot was checked by including DNA samples of *R. serpentina* ($n = 6$) collected across a wide geographical range. Further

TABLE 1—*Information of herbarium specimens and confiscated root material analyzed. Vouchers are deposited at either the Leiden or Wageningen branch of the Netherlands Centre for Biodiversity Naturalis – National Herbarium of The Netherlands. Sequencing of accessions indicated with a dash (–) failed.*

Species/Sample	Voucher	Tissue Type	Collection Date	Geographic Origin	Barcode of Voucher	NCBI GenBank Acc. No.	
						del-13 bp	ins-8 bp
R. andina Markgr.	Quevedo S, R.C. 40	Leaf	03-03-1990	Bolivia	WAG0339506	HQ638298	HQ638222
R. andina Markgr.	Dillon, M.O. 4027	Leaf	16-10-1984	Peru	WAG0339505	HQ638299	HQ638223
R. aphlebia (Standl.) A.H. Gentry	Gómez, L.D. 22702	Leaf	11-03-1983	Costa Rica	WAG0339508	HQ638301	HQ638224
R. aphlebia (Standl.) A.H. Gentry	McPherson, G.D. 12301	Leaf	12-03-1988	Panama	WAG0339507	HQ638300	–
R. bahiensis A.DC.	Amorim, A.M. 1986	Leaf	14-08-1996	Brazil	WAG0339510	HQ638303	HQ638226
R. bahiensis A.DC.	Kallunki, J.A. 734	Leaf	23-04-1995	Brazil	WAG0339509	HQ638302	HQ638225
R. biauriculata Müll.Arg.	Stijfhoorn, E. 789	Leaf	19-05-1992	Dominica	WAG0339511	HQ638305	HQ638228
R. caffra Sond.	Lotsy, J.P.467	Leaf	15-01-1926	South Africa	L0285320	HQ638307	HQ638230
R. caffra Sond.	Sijde, H.A. v.d. 91	Leaf	04-11-1962	South Africa	L0285319	HQ638306	HQ638229
R. capixabae I.Koch & Kin.-Gouv.	Spada, J. 77/23	Leaf	18-11-1977	Brazil	WAG0339417	HQ638309	HQ638232
R. capuronii Markgr.	Capuron, R.P.R. SF27341	Leaf	10-12-1966	Madagascar	WAG0000199	HQ638310	HQ638233
R. gracilis I.Koch & Kin.-Gouv.	Vieira, M.G.G. 955	Leaf	06-11-1979	Brazil	WAG0248151	HQ638314	HQ638237
R. gracilis I.Koch & Kin.-Gouv.	Hallard, S. 14	Leaf	29-07-1976	Brazil	WAG0339440	HQ638313	HQ638236
R. gracilis I.Koch & Kin.-Gouv.	Cid Ferreira, C.A. 4464	Leaf	10-06-1984	Brazil	WAG0339419	HQ638315	HQ638238
R. gracilis I.Koch & Kin.-Gouv.	Hallard, S. 13	Leaf	03-12-2007	Brazil	WAG0339418	HQ638312	HQ638235
R. grandiflora Mart. ex A.DC.	Brito, H.S. 79	Leaf	12-08-1981	Brazil	WAG0339514	HQ638317	HQ638240
R. grandiflora Mart. ex A.DC.	Dias da Costa, A. 1	Leaf	05-05-1999	Brazil	WAG0339513	HQ638316	HQ638239
R. grandiflora Mart. ex A.DC.	Callejas Posada, R. 1611	Leaf	01-11-1983	Brazil	WAG0339437	HQ638318	HQ638241
R. hookeri S.R.Sriniv. & Chithra	Mohanan, N. 18708	Leaf	03-11-1993	India	WAG0339917	HQ638304	HQ638227
R. leptophylla A.S.Rao	Callejas Posada, R. 5487	Leaf	09-11-1987	Colombia	WAG0339515	HQ638320	HQ638243
R. letouzeyi Leeuwenb.	Wieringa, J.J. 3057	Leaf	05-11-1994	Gabon	WAG0179069	HQ638321	HQ638244
R. letouzeyi Leeuwenb.	Leeuwenberg, A.J.M. 12492	Leaf	10-11-1982	Gabon	WAG0000200	–	HQ638245
R. ligustrina Willd. ex Roem. & Schult.	Nee, M. 33724	Leaf	21-01-1987	Bolivia	WAG0339519	HQ638323	HQ638249
R. ligustrina Willd. ex Roem. & Schult.	Leeuwenberg, A.J.M. 14056	Leaf	29-05-1990	Cuba	WAG0339518	HQ638322	HQ638248
R. littoralis Rusby	Llatas Quiroz, S. 1724	Leaf	01-02-1986	Peru	WAG0339520	HQ638324	HQ638250
R. macrantha K.Schum. ex Markgr.	Jaramillo, N. 685	Leaf	18-07-1995	Peru	WAG0058689	–	HQ638251
R. mannii Stapf	Leeuwenberg A.J.M. 11540	Leaf	14-11-1977	Gabon	WAG0179290	HQ638327	HQ638276
R. mannii Stapf	Wilks C.M. 1245	Leaf	07-03-1986	Gabon	WAG0179280	HQ638326	HQ638277
R. mattfeldiana Markgr.	Cid Ferreira, C.A. 5092	Leaf	30-09-1984	Brazil	WAG0339421	HQ638329	HQ638254
R. mattfeldiana Markgr.	Jardim, J.G. 1242	Leaf	15-01-1998	Brazil	WAG0339523	HQ638328	HQ638253
R. media Pichon	Rakotomalaza, P.-J. 1785	Leaf	05-11-1998	Madagascar	WAG0131685	HQ638332	HQ638257
R. media	Pichon Pascal, O. 271	Leaf	07-12-1995	Mayotte	WAG0248361	HQ638330	HQ638255
R. media Pichon	Rabenantoandro, J. 1116	Leaf	02-11-2002	Madagascar	WAG0339524	HQ638331	HQ638256
R. mollis S. Moore	Nee, M. 34299	Leaf	01-03-1987	Bolivia	WAG0339919	HQ638334	HQ638247
R. mollis S. Moore	Schessl, M. 2413	Leaf	01-11-1991	Brazil	WAG0339918	HQ638333	HQ638246
R. mombasiana Stapf	Mhoro, E.B. 6173	Leaf	03-02-1989	Tanzania	WAG0339923	HQ638336	HQ638259
R. mombasiana Stapf	Setten, K. van 964	Leaf	22-06-1987	Kenya	WAG0339921	HQ638335	HQ638258
R. nana E.A.Bruce	Drummond, R.B. 7327	Leaf	25-03-1961	Zambia	WAG0249826	HQ638337	HQ638275
R. nitida Jacq.	Maas, P.J.M. 6419	Leaf	11-04-1985	Dominican Republic	WAG0248262	HQ638338	HQ638260
R. obtusiflora A.DC.	Leeuwenberg, A.J.M. 14748	Leaf	17-11-1996	Madagascar	WAG0248264	HQ638340	HQ638262
R. obtusiflora A.DC.	Birkinshaw, C.R. 200	Leaf	05-12-1992	Madagascar	WAG0058708	HQ638339	HQ638261
R. paraensis Ducke	Schunke Vigo, J. 14279	Leaf	15-03-1989	Peru	WAG0339436	HQ638341	HQ638263
R. polyphylla Benth.	Maguire, B. 35553	Leaf	13-04-1953	Venezuela	WAG0339439	–	HQ638265
R. polyphylla Benth.	Liesner, R.L. 9073	Leaf	05-02-1980	Colombia	WAG0339438	HQ638342	HQ638264
R. praecox K.Schum. ex Markgr.	Smith, D.N. 5886	Leaf	26-01-1984	Peru	WAG0339438	HQ638343	HQ638266
R. purpurascens Standl.	Hammel, B.E. 14122	Leaf	03-07-1985	Costa Rica	WAG0248148	HQ638344	HQ638267
R. sandwicensis A.DC.	Lau, J. 1890	Leaf	21-01-1986	USA, Hawaii	WAG0339442	HQ638345	HQ638268
R. sellowii Müll.Arg.	Folli, D.A. 1263	Leaf	10-01-1991	Brazil	WAG0339443	HQ638346	HQ638269
R. semperflorens (Müll.Arg.) Schltr.	Stauffer, H.U. 5788	Leaf	10-03-1964	New Caledonia	WAG0339445	HQ638347	HQ638270
R. serpentina (L.) Benth. ex Kurz	Condon, W. 19 (NCI Q6601916-T)	Leaf	17-06-1985	Nepal	L0285328	HQ638352	HQ638282
R. serpentina (L.) Benth. ex Kurz	Palee P. 56	Leaf	09-07-1992	Thailand	L0285327	HQ638351	HQ638281

TABLE 1—*Continued.*

Species/Sample	Voucher	Tissue Type	Collection Date	Geographic Origin	Barcode of Voucher	NCBI GenBank Acc. No. del-13 bp	NCBI GenBank Acc. No. ins-8 bp
R. serpentina (L.) Benth. ex Kurz	KIM 1118-169-1894	Root	1894	Java	WAG0144516	HQ638350	HQ638280
R. serpentina (L.) Benth. ex Kurz	KIM 1816-C7-1903	Root	1903	Indonesia	WAG0144515	HQ638349	HQ638279
R. serpentina (L.) Benth. ex Kurz	KIM 2646-73-1910	Root	1910	Java	WAG0144514	HQ638348	HQ638278
R. serpentina (L.) Benth. ex Kurz	PLA 017	Root	Unknown	Unknown	L0285331	HQ839863	HQ839863
R. sprucei Müll.Arg.	Maas, P.J.M. 8185	Leaf	21-10-1994	Peru	WAG0248152	HQ638357	HQ638294
R. sprucei Müll.Arg.	Oliveira, A.A. 2802	Leaf	31-07-1995	Brazil	WAG0339430	HQ638356	–
R. sprucei Müll.Arg.	Peters, C. 84/37	Leaf	06-11-1984	Peru	WAG0339429	HQ638358	HQ638295
R. sumatrana Jack	Schmutz E. 1544	Leaf	20-05-1967	Flores	L0285317	HQ638359	HQ638283
R. sumatrana Jack	Wilde, W.J.J.O. de 20832	Leaf	30-06-1985	Sumatra	L0285318	HQ638360	HQ638284
R. sumatrana Jack	Kostermans A.J.G.H.	Leaf	??-07-1970	Unknown	L0285332	HQ638361	HQ638285
R. sumatrana Jack	Jeswiet, J. 1080	Leaf	23-08-1925	Madura, Indonesia	WAG0339522	HQ638325	HQ638252
R. sumatrana Jack	Leeuwenberg, A.J.M. 13144	Leaf	02-04-1984	Indonesia	WAG0339428	HQ638355	HQ638272
R. tetraphylla L.	Bot. Garden Delft cult. s.n.	Leaf	15-09-1970	Unknown	L0285330	HQ638363	HQ638274
R. tetraphylla L.	Prezia s.n.	Leaf	02-11-1896	India	L0285329	HQ638362	HQ638273
R. verticillata (Lour.) Baill.	Larsen, K. 33425	Leaf	26-04-1974	Thailand	L0285324	HQ638364	HQ638286
R. verticillata (Lour.) Baill.	Larsen, K. 32832	Leaf	02-03-1974	Thailand	L0285325	HQ638365	HQ638287
R. verticillata (Lour.) Baill.	Sørensen, T. 4344	Leaf	22-07-1958	Thailand	L0285333	HQ638366	HQ638288
R. verticillata (Lour.) Baill.	Geesink, R. 6867	Leaf	23-05-1974	Thailand	L0285323	HQ638308	HQ638231
R. verticillata (Lour.) Baill.	Koster, H. 6	Leaf	15-01-1986	Sri Lanka	WAG0339512	HQ638311	HQ638234
R. verticillata (Lour.) Baill.	Setten, K. van 797	Leaf	05-08-1983	Unknown (culta NL)	WAG0339517	HQ638319	HQ638242
R. viridis Willd. ex Roem. & Schult.	Raynal-Roques, A.M. 15935	Leaf	01-06-1975	Guadeloupe	WAG0339432	HQ638368	HQ638290
R. viridis Willd. ex Roem. & Schult.	Groll-Meyer, J. van 203	Leaf	??-??-1905	Netherlands Antilles	WAG0339431	HQ638367	HQ638289
R. volkensii (K.Schum.) Stapf	Breyne, H. 6048	Leaf	10-07-1993	Burundi	WAG0179262	HQ638370	HQ638292
R. volkensii (K.Schum.) Stapf	Sigara 152	Leaf	09-01-1978	Tanzania	WAG0339433	HQ638369	HQ638291
R. vomitoria Afzel.	Rodenburg W.F. 88	Leaf	11-06-1974	Ghana	L0285321	HQ638371	HQ638293
R. weddelliana Müll.Arg.	Mori, S.A. 16716	Leaf	12-07-1984	Brazil	WAG0339435	HQ638372	HQ638296
R. weddelliana Müll.Arg.	Mori, S.A. 16795	Leaf	14-07-1984	Brazil	WAG0339434	HQ638373	HQ638297
R. weddelliana Müll.Arg.	Hatschbach, G. 33056	Leaf	11-11-1973	Brazil	WAG0339425	HQ638353	HQ638271
R. weddelliana Müll.Arg.	Hatschbach, G. 36001	Leaf	09-02-1975	Brazil	WAG0339424	HQ638354	–

TABLE 2—*Primer characteristics of two markers located in rps16 intron containing regions with species-specific mutations of* Rauvolfia serpentina*. Position based on NCBI GenBank accession AB364599.*

Name	Primer Sequence (5′–3′)	Position	Size Range (bp)	Ta (°C)
rps16(del-13 bp) F	AAACCCAATGATTTAAAACAAAGAT	397	137–160	51
rps16(del-13 bp) R	TTCATTTATTGAGTGGTCTTTACCC	549		
rps16-(ins-8 bp) F	TCMGGAACGAAGAAGAAAAA	612	159–177	51
rps16-(ins-8 bp) R	CCCCCTAGAAACGTATAGGAA	788		

cross-species amplification was checked with 38 other *Rauvolfia* species (Table 1). To check the tissue specificity of the technique, DNA in the validation study was extracted from both leaves and roots of *R. serpentina*. The sensitivity of the method was evaluated by performing PCR reactions using a range of DNA concentrations (from 0.1 to 50 ng/μL) of *R. serpentina*. Degraded DNA from dried root samples was also included in the validation study to investigate whether the technique could be

used for forensic purposes. The effect of chemicals on the used samples was included by extracting, amplifying, and sequencing DNA from herbarium samples, previously sprayed with mercuric chloride or methyl bromide to prevent damage by insect pests, or sprayed with ethanol prior to drying as a preservation method ("Schweinfurth"). Robustness of the PCR was tested by varying the $MgCl_2$ concentration (1.0–5.0 mM), annealing temperature (±3°C), and DNA Taq polymerases.

Results

Inter- and Intraspecific Variation

After sequence comparison of three genomic regions (*trn*L-*trn*F intergenic spacer, *rpl*16 intron, and *rps*16 intron) of a limited number of *Rauvolfia* species, it was discovered that the *rps*16 intron provided most variation at the species level. Therefore, we decided to develop a marker for species identification and sample for this genetic locus only. Clearest informative characters distinguishing *R. serpentina* from the other *Rauvolfia* species turned out to be two indels. The first one consisted of a deletion of 9 bp, that changed to 13 bp after the inclusion of more sequences in the alignment, and the second one was an insertion of 8 bp. Using newly designed primers, we amplified these two areas. For *R. serpentina,* they had the characteristic size of 137 bp and of 177 bp, respectively. Size for these fragments from the other samples ranged from 146 bp (*R. biauriculata, R. leptophylla, R. mombasiana, R. nana,* and *R. verticillata*) to 160 bp (*R. littoralis*) and from 156 bp (*R. mannii*) to 176 bp (*R. tetraphylla*), respectively. As compared with the full *rps16* intron, less than 1% of sequence divergence was omitted when samples were compared for the smaller del-13-bp and ins-8-bp fragments. Internal primers were then designed (Table 2), and PCR reactions were carried out with these on a total of 80 DNA extractions. Amplification and subsequent sequencing of only five of 160 (3%) reactions failed.

Degree of sequence divergence between the different *Rauvolfia* species analyzed was much smaller in the del-13-bp region as compared with the ins-8-bp region of the *rps16* intron sequenced and ranged up to 0.08 (*R. verticillata* and *R. polyphylla*). In both data sets, all *R. serpentina* individuals had identifiable sequences and could therefore be characterized by the described species-specific mutations. In contrast with *R. serpentina*, several other *Rauvolfia* species showed intraspecific variation ranging from 0.02 (*R. mollis* and *R. sprucei*) up to 0.03 (*R. gracilis*).

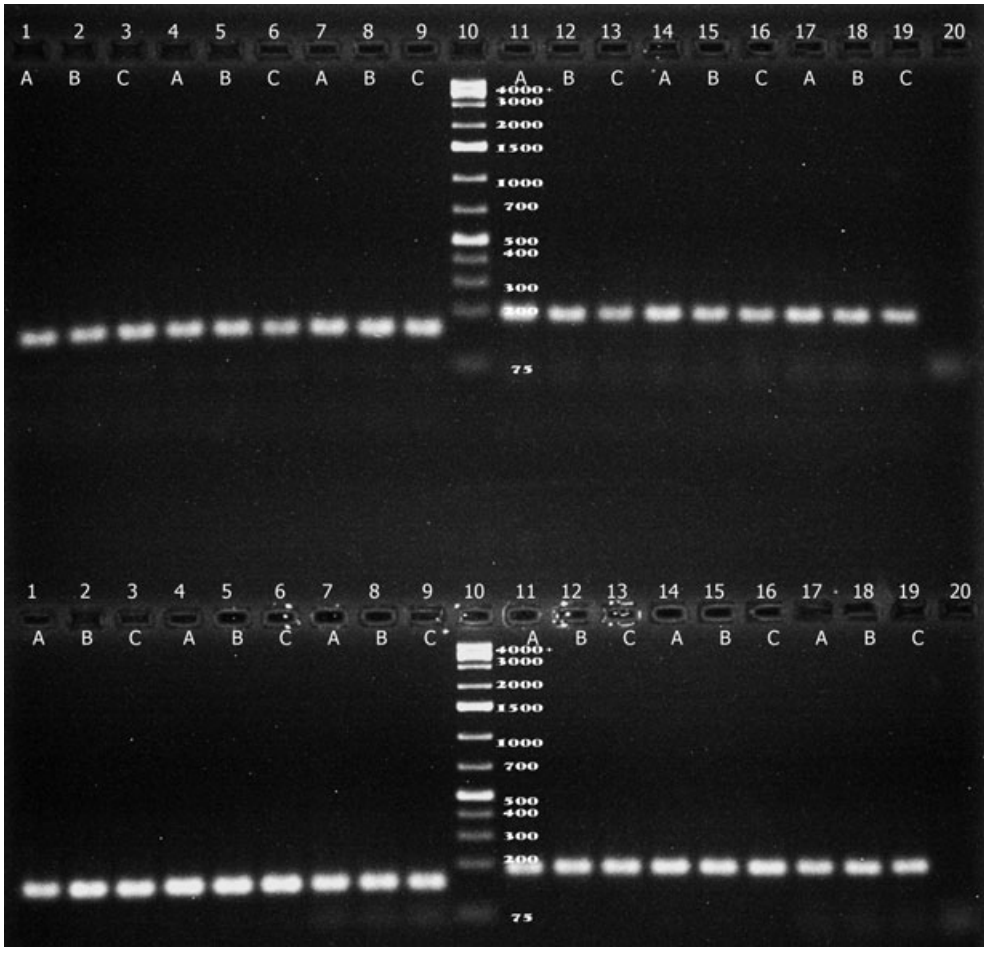

FIG. 1—*Agarose gel (2%) electrophoresis showing results for samples exposed to various annealing temperatures (first row) and $MgCl_2$ concentrations (second row) in the validation study. Both fragments of 137 and 177 bp from samples: (A)* Rauvolfia serpentina *(1985, L0285328), (B)* R. serpentina *(1896, WAG0144516), and (C)* R. serpentina *(confiscated, L0285331) are shown. Only the deviations from the standard protocol are mentioned. First row: Lanes 1–3 and 11–13: annealing temperature = 48°C; lanes 4–6 and 14–16: annealing temperature = 51°C; lanes 7–9 and 17–19: annealing temperature = 54°C. Second row: Lanes 1–3 and 11–13: 1 mM $MgCl_2$; lanes 4–6 and 14–16: 2 mM $MgCl_2$; lanes 7–9 and 17–19: 5 mM $MgCl_2$. Lane 10: molecular size marker: GeneRuler™1 kb Plus DNA Ladder (Thermo Fisher Scientific, Inc., Waltham, MA); lane 20: negative control.*

Validation of DNA Barcoding Method

Species specificity of the *rps*16 intron turned out to be high as unique sequences were obtained for all *R. serpentina* samples analyzed. Although DNA extracted from dried roots yielded more degraded DNA as compared with the DNA obtained from leaf samples, PCR amplification, and DNA sequencing was still successful, even after DNA samples were serially diluted. Even though samples treated with various chemicals yielded highly degraded DNA, nevertheless, DNA barcoding proved efficient even for these samples. According to SWGDAM, a validation study including a PCR-based procedure must demonstrate the effect of $MgCl_2$ and other thermocycling parameters. It was found that the PCR stayed specific in amplifications with 1.0 mM to 5.0 mM $MgCl_2$ concentrations. Specific amplifications were also obtained using annealing temperatures up to 3°C below and 3°C above the optimal annealing temperature of 51°C. There was no effect in changing the cycle number on the results obtained, and different DNA Taq polymerases produced similar results (data not shown). Altogether, the PCR reaction appeared to be very robust. All PCR products obtained in the validation study were sequenced and aligned with positive controls of *R. serpentina* and were 100% similar in the DNA sequence alignments.

Identification of Confiscated Sample

Of the 39 different *Rauvolfia* species analyzed, only the *R. serpentina* samples contained both a 13-bp deletion and an 8-bp insertion. In this data set, these mutations appeared to be specific for *R. serpentina* (Figs 1 and 2). The same mutations were also found in the DNA sequences derived from the confiscated Indian snakeroot sample and root samples taken from fertile herbarium samples that had been identified to species level by Dr. Toon Leeuwenberg, indicating that the confiscated root was indeed derived from *R. serpentina*. Completely identical sequences were found in these same samples processed in the molecular laboratory of the Dutch Customs Laboratory.

Discussion

All five root samples (four herbarium samples and one confiscated sample) tested and analyzed could be identified to species level by only sequencing parts of the *rps*16 intron region.

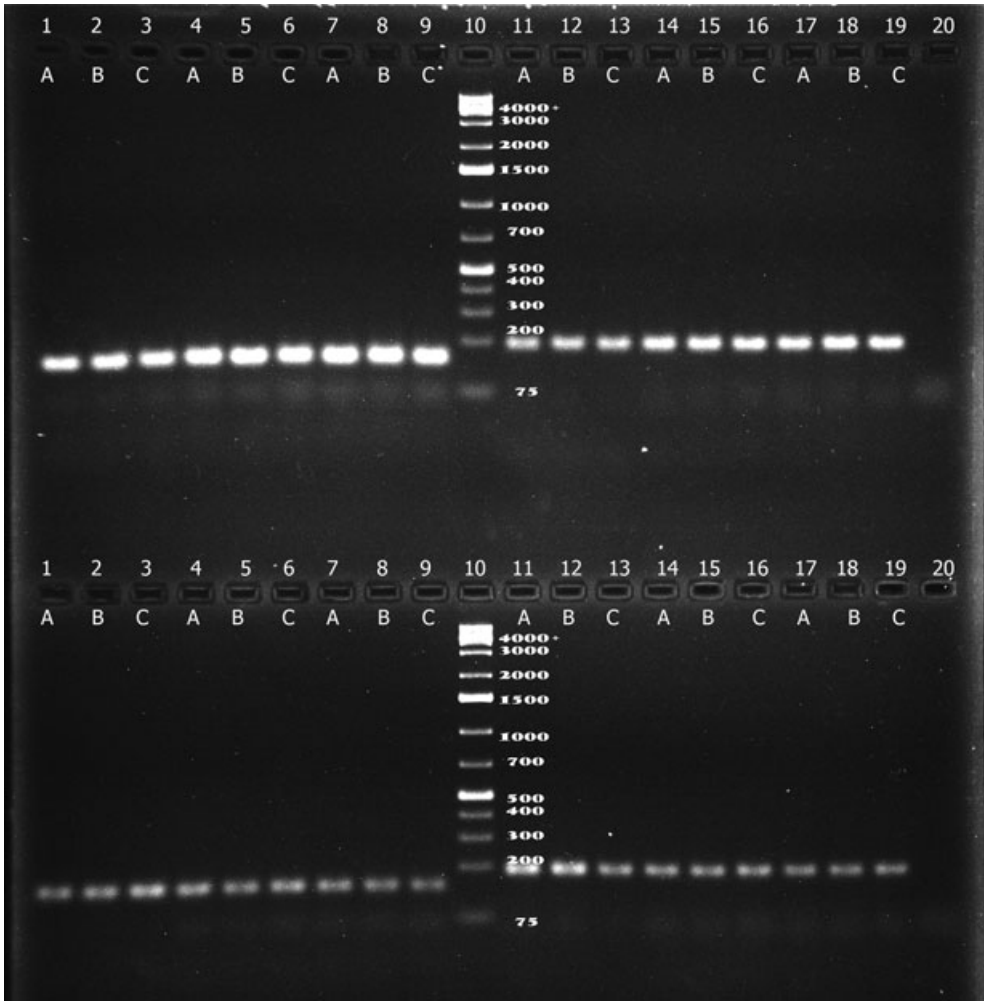

FIG. 2—*Agarose gel (2%) electrophoresis showing results for samples exposed to various number of cycles in the PCR (first row) and dilutions (second row) in the validation study. Both fragments of 137 and 177 bp from samples: (A)* Rauvolfia serpentina *(1985, L0285328), (B)* R. serpentina *(1896, WAG0144516), and (C)* R. serpentina *(confiscated, L0285331) are shown. Only the deviations from the standard protocol are mentioned. First row: Lanes 1–3 and 11–13: 35 cycles, lanes 4–6 and 14–16: 40 cycles, lanes 7–9 and 17–19: 45 cycles. Second row: Lanes 1–3 and 11–13: dilution factor 5, lanes 4–6 and 14–16: dilution factor 100, lanes 7–9 and 17–19: dilution factor 200. Lane 10: molecular size marker: GeneRuler™ 1 kb Plus DNA Ladder; lane 20: negative control.*

Although it is essential to add more *Rauvolfia* species to our *rps*16 sequence database, we have demonstrated by the inclusion of the most closely related species that the *rps*16 intron contains unique indels at the species level for *R. serpentina*. Woodson et al. (1) also suggest that *R. perakensis* King & Gamble and *R. confertiflora* Pichon are closely related to *R. serpentina*, but these species are nowadays considered synonyms of *R. verticillata* (Lour.) Baill and *R. media* Pichon, respectively (5), both of which have been included in this study. As none of the related species studied show the species-specific deletion or insertion, we consider it very likely that both indels are unique for *R. serpentina*. To exclude any possible misidentifications as much as possible, we advice the use of both *rps*16 regions in testing. We would also like to stress the importance of using well-identified specimens to produce a good reference data set.

The length of the *rps*16 intron fragments containing the putatively unique deletion and insertion amplified with our newly designed primers is relatively short: 137 and 177 bp, respectively.

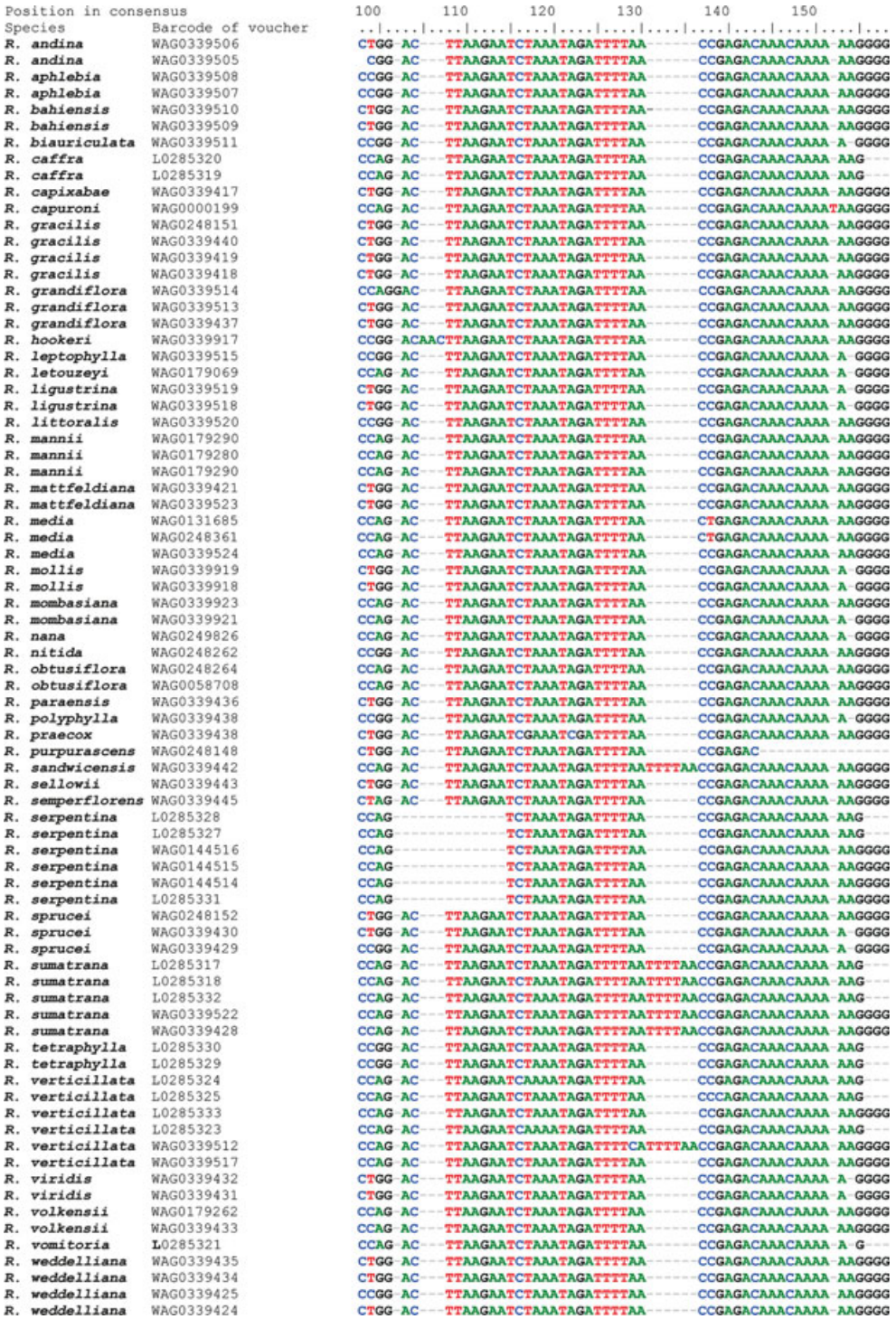

FIG. 3—*Alignment of part of the rps16 intron del-13-bp region.*

It should therefore be possible to amplify highly degraded DNA. Results obtained from our four root samples indicate the applicability of these markers even for material collected up to 115 years ago. Decreasing the fragment lengths even further by designing new standard primers seems feasible when the extracted DNA is still too degraded to amplify.

For unambiguous identification of Indian snakeroot, the specific indels for *R. serpentina* in the *rps*16 intron described in this study

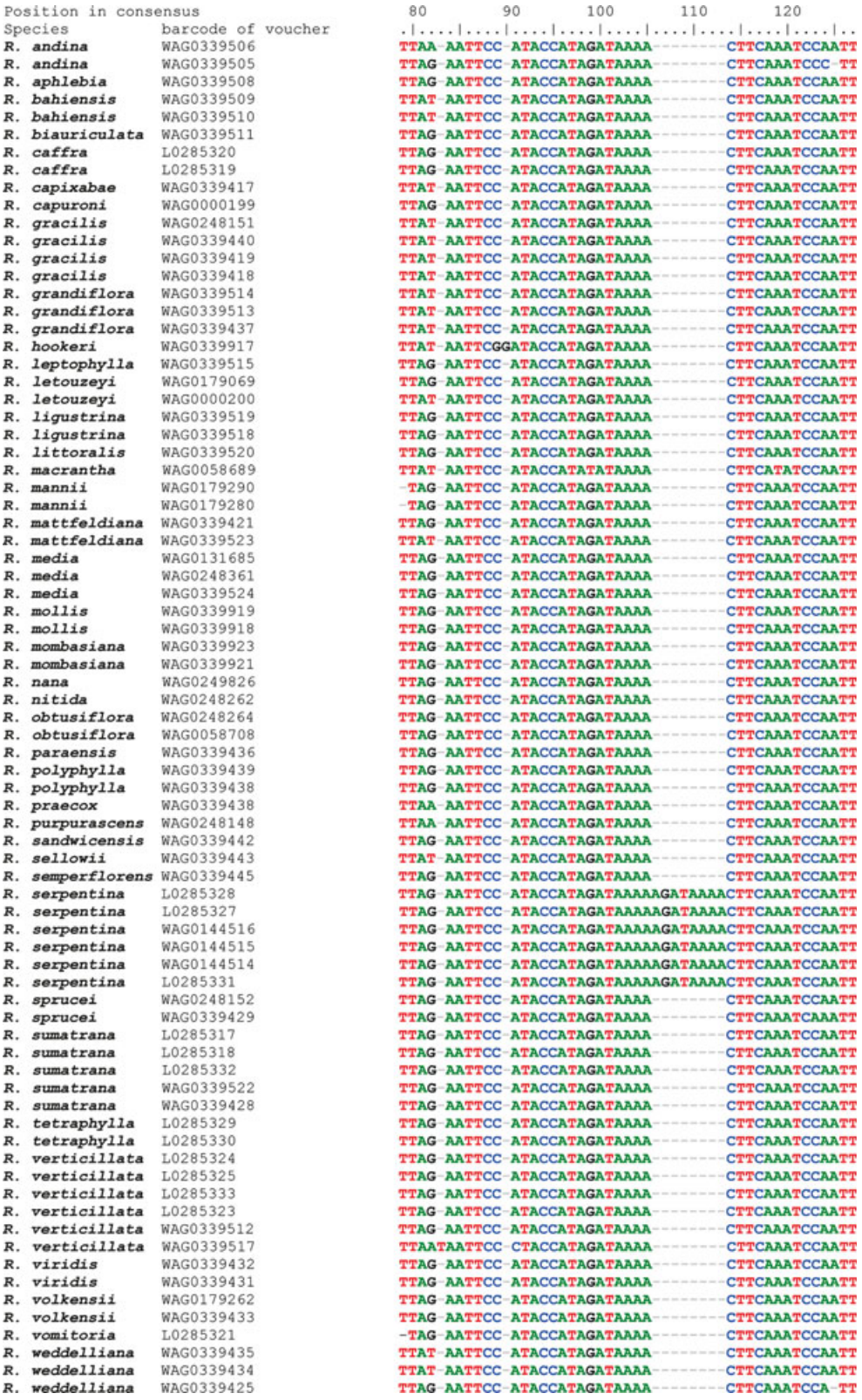

FIG. 4—*Alignment of a part of the rps16 intron ins-8-bp region.*

(Figs 3 and 4) appear to be both unique and interpretable in only a single way. In this perspective, DNA analysis is the most straightforward method to identify traded snakeroot samples.

According to Woodson et al. (1) anatomical characters such as broad rays and a very starchy xylem and phloem are highly specific for *R. serpentina*. As these authors screened 24 species, we think that wood anatomy could be an additional useful tool for species identification of traded roots as well. However, root anatomical variation within the genus remains underinvestigated and roots are only sporadically present in herbarium collections. In addition, wood anatomy requires an expertise that is probably lacking in an ordinary customs laboratory. According to Woodson et al. (1), chemical data of *Rauvolfia* species such as alkaloid composition also seem to be highly species specific. We therefore think that mass spectrometry could be considered for further investigation as an additional identification tool for traded *Rauvolfia* roots. On both techniques, wood anatomy and mass spectrometry, we performed a preliminary inquiry on several herbarium samples and the confiscated root sample. The results of these pilot studies support the findings of our molecular barcoding study (data not shown).

The validation study carried out shows that the primers developed here are effective for the amplification of forensic samples as they work with very low quantities of template DNA, amplify DNA of samples exposed to various chemicals and from highly degraded tissue such as dried roots. Finally, our DNA barcoding method succeeded to reveal the taxonomic identity of a sterile confiscated root sample. We therefore recommend applying this method in forensic identification of confiscated Indian snakeroot samples in law enforcement to improve conservation of this endangered plant species.

Acknowledgments

DNA extractions of the root samples were partly carried out in the Ancient DNA Facility of Leiden University. Lynne McIvor is kindly thanked for correcting our English grammar.

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