

# *Plantago schrenkii* is *P. maritima*: morphological and molecular evidence

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Received 19 July 2014, final version received 5 Sep. 2014, accepted 22 Oct. 2014

Shipunov, A. 2015: *Plantago schrenkii* is *P. maritima*: morphological and molecular evidence. — *Ann. Bot. Fennici* 52: 33–37.

*Plantago schrenkii* (dwarf plantain) from the Russian Arctic is frequently segregated at species level from *P. maritima* (sea plantain). Using simultaneous morphometric and molecular analyses, I came to the conclusion that *P. schrenkii* is just an ecological form of sea plantain and should not be recognized at any taxonomical level.

## Introduction

*Plantago maritima* (sea plantain) is a highly variable species with a very large geographic distribution. The variability led to the segregation of multiple “species” such as *P. juncooides*, *P. decipiens*, *P. borealis*, *P. groenlandica* and others (Pilger 1937), which were subsequently included in *P. maritima* (Rahn 1996).

Arctic forms of *P. maritima* were often regarded as separate species. One example is *P. schrenkii* (dwarf plantain), first collected in 1837 from Waigatsch island (Russian Arctic). It was believed to have clear morphological differences from typical *P. maritima*, especially the small size of leaves and inflorescences.

Shipunov (2000), in the review of *Plantago* from European Russia, accepted *P. schrenkii* based on results of the statistical analysis first published in my dissertation (Shipunov 1998). However, that conclusion did not seem to be robust, and I always felt that the problem of *P. schrenkii* must be re-assessed for several reasons. First, characters which discriminate *P. schrenkii* are highly polymorphic and are often seen in *P.*

*maritima* plants growing in stressful ecological conditions (for example, on poor substrates such as seaside rocks). In addition, it is well-known from the classic works of Gregor (1938, 1950) that typical *P. maritima* populations often include some dwarfish plants. In my dissertation (Shipunov 1998), I used morphometric data from fewer than 40 *P. schrenkii* plants, and the separation between them and typical *P. maritima* was not absolutely clear. Last, there was no possibility for a molecular analysis. Therefore, I decided to review the problem based on better sampling and analysis of relevant molecular markers.

## Material and methods

All material was collected from the White Sea coast, near the Arctic circle. This area is well-known for the co-occurrence of *P. maritima* and *P. schrenkii* (Andreev 1930, Sergienko 1977). Typically, the latter could be found in the locations where rocky habitats are prevalent. A total of 394 plants were measured in order to obtain morphometric data. I collected 15 silica gel dried

plant tissue samples from one measured plant per locality (28 localities in total) in order to extract and sequence the DNA.

The main characters measured were (1) length of lower photosynthetic leaf (abbreviation: LOWER), (2) length of leaf closest to the stalk (UPPER), (3) length of maximal stalk (STALK), (4) length of spike on the maximal stalk (SPIKE), and (5) total number of flowers on the measured spike (FLOWER). All these characters were believed to distinguish *P. maritima* from *P. schrenkii* (Shipunov 2000). In addition, distance to the nearest plantain, substrate type (reflecting the “hardiness” of substrate) and the distance from seashore were registered as “ecological variables”. All metric measurements were in millimeters.

Statistical methods included principal component analysis (PCA) with ANOSIM testing on the cluster differences (Oksanen *et al.* 2013) and Spearman’s correlation. For all statistic calculations, the R package (R Core Team 2014) was used.

The “logical discrimination” was applied to the collected data. Dichotomous identification keys allow for the step-by-step determination based on the series of negations and confirmations; this method was made algorithmic in R. For example, *P. schrenkii* characters were encoded as “(FLOWER < 10) & (SPIKE < 140) & (UPPER < 90)”. I used this approach for the discrimination of samples in both morphometric and molecular parts of the study.

The DNA was extracted using a MO BIO PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). Dry plant leaf material (~0.1 g) was ground using a sterile mortar and pestle and then processed in accordance with the supplied protocol. We sequenced the ITS2 region of the ribosomal intergeneric spacer, which has proven to be best for revealing species-level phylogenetic relationships among *Plantago* (Ronsted *et al.* 2002, Hoggard *et al.* 2003) using the primers ITS2\_S2F (Chen *et al.* 2010) and ITS4 (White *et al.* 1990). PCR was carried out as follows: the reaction mixture in a total volume of 20  $\mu$ l contained 5.2  $\mu$ l of PCR Master Mix (components from QIAGEN Corporation, Germantown, Maryland), 1  $\mu$ l of 10  $\mu$ M forward and reverse primers, 1  $\mu$ l of DNA solu-

tion from the extraction above and 11.8  $\mu$ l purified water. Samples were incubated in a thermal cycler: 94° for 5 min, then 35 cycles of 94° for 1 min; 51° for 1 min, 72° for 2 min, and finally 72° for 10 min. Single band PCR products were sent for purification and sequencing to Functional Biosciences, Inc. (Madison, Wyoming) and sequenced there in accordance with standard protocol. Sequences were then assembled and edited using Sequencher™ 4.5 (Genes Codes Corporation, Ann Arbor, Michigan, USA) and then aligned with ClustalX (Thompson *et al.* 1997) using gap opening cost 9, gap extension cost 0.05 and IUB weight matrix.

Part of the DNA was sequenced with help of the Barcoding of Life Consortium, as a part of “SAPNA” (“*Plantago* of North America”) project. Other barcoding fragments (for example, *rbcL* and *matK*) were also sequenced within this project but ITS2 provided the best resolution.

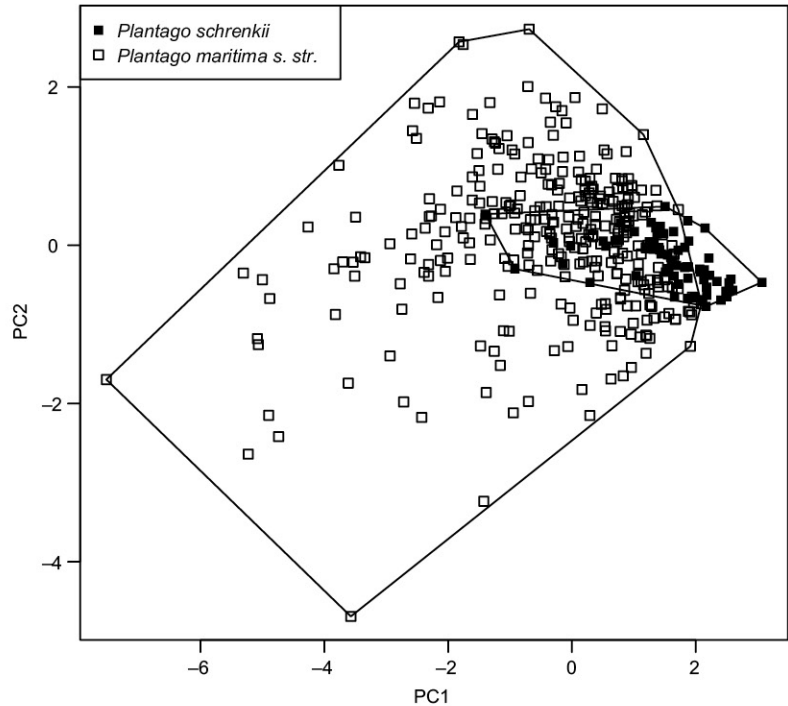
## Results

### Morphological analysis

Logical discrimination resulted in two groups: putative *P. schrenkii* and more typical *P. maritima*. Among the DNA samples, five appeared to belong to the first group. Among all samples, 68 (17%) were determined as *P. schrenkii*.

The difference between these two groups was assessed using principal component analysis of morphological characters. The two first principal components combined for 85% of variation. However, those components were unable to separate *P. schrenkii* (Fig. 1). Analysis of (dis)similarity between clusters showed low values: ANOSIM  $R = 0.05284$ ,  $p = 0.06$ .

Correlation between morphological characters and ecological variables was especially high for the UPPER and FLOWER: they correlated with the type of substrate (Spearman’s  $\rho = -0.4765257$ ,  $p < 0.0001$ ) and distance from seashore (Spearman’s  $\rho = 0.7350455$ ,  $p < 0.0001$ ), respectively. In other words, on more stressful substrates the leaves become shorter, and the number of flowers is higher on the plants growing farther from seashore.



**Fig. 1.** Principal component analysis of morphological characters. Ordination hulls emphasize the intersection between groups.

## Molecular analysis

Sequences of the whole ITS2 region had a length of 299–320 base pairs. Their alignment started from the 413 position of the full ITS1/5.8S/ITS2 region (*P. maritima*, GenBank AY101879) reflecting the absence of significant differences between two groups (see Appendix).

## Discussion

Several authors have accepted *P. schrenkii* as a separate species (Andreev 1930, Sergienko 1977, Tzvelev 1979, Shipunov 2000). However, there is a clear trend in publications toward a broader concept of *P. maritima*, which now includes numerous previously distinguished forms (Kuiper & Boss 1992, Rahn 1996). The present study on *P. schrenkii* supports the broader concept. The dwarf Arctic plants should not be recognized at any taxonomical level, but represent just ecological modifications or forms.

The more is known about the genetic and ecological polymorphism in *Plantago*, the more specialists shift to wider taxonomic concepts at

species level, not only in the *P. maritima* group but also in other widely distributed, frequently invasive plants such as *P. major*, *P. media*, *P. lanceolata* and *P. coronopus* (Rahn 1996, Shipunov 2000). It is likely that many of those taxa have not yet reached the stage of stabilization (Grant 1981) and “are caught” in the intermediate stage of the speciation process.

## Acknowledgements

I am grateful to all participants and heads of the Moscow South-West High School White Sea Expedition for their support, and especially to P. Borisova, D. Zakharova, K. Aquilar and M. Kuzmina for their invaluable help. Many thanks also to the anonymous reviewer for the constructive comments.

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Appendix. Alignment of ITS2 fragment sequences. Plants determined as *Plantago schrenkii* marked with “s” postfix. First three sequences are from GenBank: AY101879 and HQ593837 were obtained from cultivated plants, geographic origin of AJ548986 is “North America” (Hoggard et al. 2003).

AY101879_P_maritima	507	TGCAGAAATCCCGTGAACATCGAATCTTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	500
AJ548986_P_maritima	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	504
HQ593837_P_maritima	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	505
p01_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	506
p02_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	507
p03_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	508
p04_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	509
p05_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	510
p06_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	511
p08	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	512
p09	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	513
p10	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	514
p11	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	515
p12_s	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	516
p13	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	517
p14	95	TGCAGAAATCCCGTGAACATCGAATCTTTGAACGGAAGTTGGCCGACGACCCCTTGGGCTGAGGCAACCCCTCGCTGGGGGTACCGCATCCGGTC	518
AY101879_P_maritima	602	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	609
AJ548986_P_maritima	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	600
HQ593837_P_maritima	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	601
p01_s	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	602
p02_s	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	603
p03_s	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	604
p04_s	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	605
p05_s	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	606
p08	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	607
p09	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	608
p10	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	609
p11	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	610
p12_s	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	611
p13	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	612
p14	190	GCCCGTCCGAGTCCCTTCGGATAGCTCCGCTGAGCTCGCTGGCCGAAAATGGGATCCGCTCATCCGACGGATGTC	613
AY101879_P_maritima	697	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	698
AJ548986_P_maritima	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	699
HQ593837_P_maritima	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	700
p01_s	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	701
p02_s	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	702
p03_s	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	703
p05	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	704
p06_s	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	705
p08	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	706
p09	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	707
p10	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	708
p11	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	709
p12_s	285	ACAACAGTGGTGAAGAATCATTTGTGCTTGTGCTTCAACCTGTCGCTGATCGCTGCGGACCAACCGGCTGTCAATGGCCCTT	710
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