MOLECULAR CHARACTERISTICS OF AN ANCIENT *PINUS SYLVESTRIS* (PINACEAE) ECOTYPE FROM THE GĄZWA PEAT BOG RESERVE (NE POLAND)

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Abstract. *Pinus sylvestris* f. *turfosa* is one of the most interesting morphotypes inhabiting mossy peat bogs. In the Gązwa Peat Bog Reserve (NE Poland), changes in hydrological cycles have allowed typical *P. sylvestris* to spread into it and have altered the community composition. The aim of this work was to find DNA markers distinguishing the *turfosa* form from typical *P. sylvestris* surrounding the Gązwa peat bog. This could confirm the genetic basis of its unique phenotype and help explain the origin of the *P. sylvestris* individuals that look like its typical form from that stand. DNA marker composition was analyzed in ten individuals of the *turfosa* form and in ten that looked like the typical *P. sylvestris* form growing in the Gązwa Reserve. Ten individuals of *P. sylvestris* surrounding the peat bog were the control. Four types of DNA markers (RAPD, ISJ, B-SAP, IS6110) were used. The data demonstrated that *Pinus sylvestris* f. *turfosa* from the Gązwa Reserve has a unique genotype differentiating this morphotype from typical *P. sylvestris* surrounding the peat bog. This suggests the ancient origin of the *turfosa* form. The intermediate DNA profiles of typical *Pinus sylvestris* from the peat bog suggest its hybrid origin. The gene pool of the *turfosa* form is endangered by its hybridization with the very abundant invasive *P. sylvestris* surrounding the peat bog.

Key words: *Pinus sylvestris* f. *turfosa*, relict ecotype, DNA markers, hybridization, Gązwa Reserve, northeastern Poland

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INTRODUCTION

Pinus sylvestris f. *turfosa* Woerl. is an endangered ecotype whose range is restricted to peat bogs, where it is a prominent component of *Sphagnetum magellanici pinetosum* communities (Jasnowski *et al*. 1968). This ecotype is easily distinguished from typical *P. sylvestris* growing on mineral soil by its short stature, curved trunk, lack of regular branch fascicles, and very characteristic umbrella-like crown (Fig. 1). In northeastern Poland the *turfosa* form used to be very common on mossy peat bogs. After the Second World War, widespread destruction of mossy peat bogs reduced these formerly abundant ecosystems to a dangerously low level, so that they occupied only 6% of the former peat bog areas in the region. As peat bogs disappeared, *P. sylvestris* f. *turfosa* populations declined, becoming isolated and endangered. Today, one of the most abundant populations of the *turfosa* form inhabits a mossy peat bog within the Gązwa Reserve, *ca* 60 km northeast of the town of Olsztyn. *P. sylvestris* f. *turfosa* was the only tree species growing there in the mid 20th century (Młynek & Polakowski 1962), as seen in old photographs. Unfortunately, indiscriminate drainage of nearby Jezioro Stama lake, which began in 1860 (Uggla 1968), initiated gradual and continuous alterations of the Gązwa peat bog associated with radical changes in hydrological cycles. It also triggered

Z. MIREK & A. NIKEL (eds) 2009. *Rare, relict and endangered plants and fungi in Poland.* W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków, pp. 561–567.

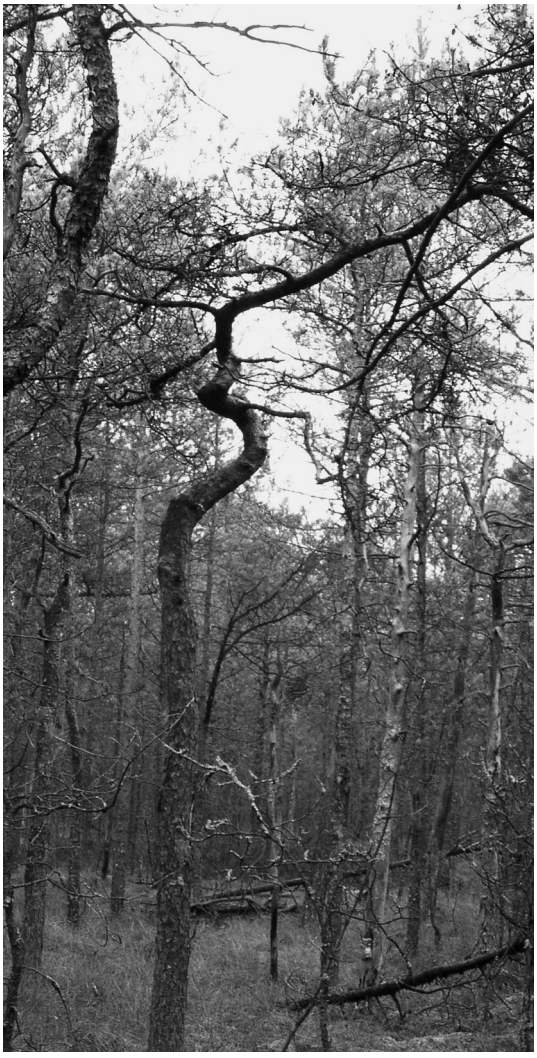

Fig. 1. *Pinus sylvestris* f. *turfosa* Woerl. from the Gązwa Reserve (photo K. Polok).

changes in the mossy peat bog species composition and created favorable conditions for invasions by typical *P. sylvestris* forms with straight stems and regular, well-developed nodes with preserved branch fascicles. Over the years, the offspring of typical *P. sylvestris* surrounding the peat bog have spread into it and altered the community composition.

Molecular analyses coupled with dendrometric and morphological data have confirmed the genetic basis of differences between the *turfosa* form and typical *P. sylvestris* growing on mineral soil (Polok *et al*. 2005a). Both phenotypes proved to be fixed. Middle-aged *turfosa* trees (30–85 years old) are about five times shorter than typical *P. sylvestris*, and their stem diameter is half that of the latter. The average height of middle-aged *turfosa* forms is 1.6 m, as against 8.6 m for typical *P. sylvestris*. The oldest trees (117–217 years old) of the *turfosa* form never exceed 10 m in height. With respect to stem diameter, the respective values are 4.2 cm and 9.2 cm for middle-aged trees, and 14.9 cm for the oldest *turfosa* trees. The low values for the *turfosa* form probably are due to very slow growth, as can be seen in its 0.5 mm average annual ring width, versus 1.2 mm in typical *P. sylvestris*. On the other hand, the needles are 20% longer in the *turfosa* form. Molecular analyses demonstrating very low polymorphism and specific DNA fingerprints confirmed the distinctiveness of the *turfosa* form (Polok *et al*. 2005a). On-site inspections showed that typical *P. sylvestris* performs very poorly in peat bogs and lives only up to 50–60 years, or two to four times less than the *turfosa* form. These morphological and molecular data as well as historical records suggest that the *turfosa* form reflects a unique and probably relict genotype, well adapted to peat bog conditions. Further morphological observations of the *P. sylvestris* populations inhabiting the Gązwa peat bog indicated that there are many intermediate forms in addition to the extreme phenotypes (*turfosa* forms and typical *P. sylvestris*). For example, trees that look like typical *P. sylvestris* at first glance have a twisted stem or perform much better than expected from their appearance. Studies of 16 anatomical traits of needles supported this suspicion and suggested the existence of different generations of hybrids between the *turfosa* form and typical *P. sylvestris* (Urbaniak *et al*. 2009). Notwithstanding the usefulness of such morphological data, the hybrid origin of intermediate phenotypes needed to be confirmed by molecular markers, which are not influenced by the environment. The intermediate forms might be thought to result from environmental variation. This work, then, aims to find specific DNA markers distinguishing the *turfosa* form from the typical *P. sylvestris* surrounding the Gązwa peat

bog, and then to use them to explain the origin of intermediate forms. We wanted to know whether they represent pure offspring of *P. sylvestris* surrounding the peat bog. If the DNA fingerprints indicate hybrid origin, are markers typical of both *turfosa* forms and typical *P. sylvestris* present in the intermediate phenotypes? If the intermediate forms are of hybrid origin, they may have several advantages over the pure *turfosa* form, such as rapid growth rate (Polok *et al*. 2005a). Genes introgressed from the *turfosa* form may enable them to survive on the mossy peat bog, giving them a selective advantage enabling them to invade the Gązwa peat bog and supplant *P. sylvestris* f. *turfosa*. In view of the low abundance of the *turfosa* population in the Gązwa Reserve and their hybridization with typical *P. sylvestris*, the gene pool of the former may be diluted, and consequently the oldest trees of the *turfosa* genotype could represent the last generation. The results of our work could have implications for conservation of the unique *turfosa* form, especially if the hybrid origin of typical pines from the Gązwa peat bog is documented.

Material and methods

The material consisted of *P. sylvestris* f. *turfosa* and typical pine growing in close proximity in the Gązwa Peat Bog Reserve. The control in these analyses was typical *P. sylvestris* growing on mineral soil in the vicinity of the peat bog.

Individuals of the *turfosa* form were randomly selected from the oldest ones of this category (117–217 years old). They possessed very characteristic phenotypes: height of 4–9.5 m, curved trunk, and umbrella-like crown. The second phenotypic category collected in the peat bog (also randomly) consisted of different *P. sylvestris* trees 20–35 years old and 7–11 m high, mostly resembling typical *P. sylvestris*, with a straight stem and a regular, conical crown. Although the visually determined morphological features suggested that this category represents typical *P. sylvestris*, their needles showed intermediate anatomical features (Urbaniak *et al*. 2009), so we refer to them as intermediate. The third category consists of randomly chosen 100–150-year-old typical *P. sylvestris* trees surrounding the peat bog. Ages in all categories were determined from cores taken with a Pressler borer (Polok *et al*. 2005a).

In the first step of analysis, DNA profiles were determined for the *turfosa* form and trees surrounding the peat bog in order to find diagnostic DNA markers separating the two phenotypes. In the second step, intermediate *P. sylvestris* was analyzed with the diagnostic DNA markers specific to the *turfosa* form and typical *P. sylvestris* in order to find any fingerprints of previous hybridization.

Ten individuals of each phenotypic category were taken for DNA isolation. Briefly, needles (2 g) were crushed in liquid nitrogen and DNA was isolated by a modified CTAB procedure (Polok *et al*. 2005a). Two categories of genome-scanning DNA markers, RAPD and semi-random ISJ markers, were employed (Table 1). A new type of marker developed from bacterial sequences was also applied: Bacteria-Specific Amplification Polymorphism, B-SAP (Polok 2007). Two such markers previously tested in a range of species were used, one developed from the *KatG* gene of *Mycobacterium tuberculosis* (katG markers) and the other developed from insertion element IS6110 of the same bacterium (IS6110 markers). PCR reactions revealing all marker categories (RAPD, ISJ, B-SAP) were performed according to Polok (2007). Nei's genetic similarity (I) was calculated based on shared PCR fragments (Clark & Lanigan 1993). The matrix of similarities was used in cluster analysis in STATISTICA 7.1. Euclidean distance and the average linkage method (UPGMA) were used to construct the dendrogram.

Results and discussion

Ninety DNA bands were detected in the study, including 27 RAPDs, 20 ISJs and 43 B-SAPs, including 27 developed from the *KatG* gene of *M. tuberculosis* (katG markers) and 16 developed from insertion element IS6110 of the same bacterium (IS6110 markers).

The unique character of the *turfosa* form was confirmed by the finding that it had half the number of polymorphic loci (17.8%) in comparison with

Table 1. Primers used for DNA amplification.

Type of marker	Abbreviation	Number of nucleotides	Primer sequence
RAPD	OPD-03	10	5'GTCGCCGTCA3'
	OPD-05	10	5'TGAGCGGACA3'
ISJ	ISJ2	18	5'ACTTACCTGAGGCGCCAC3'
	ISJ4	18	5'GTCGGCGGACAGGTAAGT3'
B-SAP (katG)	katG10	20	5'CAAAGTGTCCTTCGCCGACC3'
	katG11	20	5'TGCTCGACAAGGAGAACCTG3'
B-SAP (IS6110)	IS1	19	5'GGCTGAGGTCTCAGATCAG3'
	IS2	20	3'CAAGAACCTTTCCTACCCCA5'

the intermediate pines from the peat bog (37.8%) and the surrounding trees (37.8%) (Table 2). The largest differences between the *turfosa* form and the other two phenotypes were revealed by IS6110 (6.3% and 37.5%, respectively) followed by RAPD and ISJ (14–15% and 30–37%). Surprisingly, katG showed small differences in the number of polymorphic loci, probably because the B-SAP system based on the *KatG* gene is suited rather for species identification (Zieliński & Polok 2005; Polok 2007). Presumably the reduction of polymorphism in the *turfosa* form results from the bottleneck effect driven by strong selection pressure and by reduction of the size of the population growing in unfavorable peat bog conditions. This is a common phenomenon observed in many taxa, and is linked with environmental pressures or with the biological strategies of organisms: mating system, ploidy level, etc. (Nunney & Elam 1994; Avise 2004). Using 58 DNA amplification products, Polok *et al*. (2005a) also found extremely low genetic variation in *P. sylvestris* f. *turfosa*.

The results for *P. sylvestris* f. *turfosa* seem to contradict the widely held view of *Pinus sylvestris* as a species of high genetic variability. Analysis of seven European populations revealed as high as 71% ISJ polymorphic loci, 75% RAPD polymorphic loci, and 27% IS6110 polymorphic loci (Polok *et al.* 2005b). Even higher *P. sylvestris* variability was found by Goncharenko *et al*. (1994) at enzymatic loci: 90% polymorphic loci (P), 4.2 alleles per locus (A) and 0.226 mean heterogeneity (H). The values were about three times the values reported for flowering plants: P 34.2%, A 1.53, and H 0.113 (Hamrick & Godt 1990). Until now, *P. sylvestris* variability has been estimated mostly in populations of commercial value, and populations from peat bogs have received much less attention. The present results from the Gązwa Reserve demonstrate that the peat bog environment may seriously limit the accumulation of genetic variability.

The genetic distinctiveness of *P. sylvestris* f. *turfosa* was supported by the presence of diagnostic bands either in the *turfosa* form or in typical pine surrounding the Gązwa Reserve. A band generated by an ISJ primer was fixed in the *turfosa* form, while the other eight amplification products were observed only in material from the surrounding trees. The latter includes four bands generated by RAPD, one by katG, and three by IS6110 primers. The results correspond with the differences in needle morphology and anatomy found between *turfosa* forms and surrounding *P. sylvestris* (Urbaniak *et al*. 2009). The differences between the forms are statistically significant for 10 of 16 analyzed traits.

The intermediate DNA patterns of the intermediate *P. sylvestris* inhabiting the peat bog support its hybrid origin. Unexpectedly, several specific bands were not found in the *turfosa* form or the surrounding *P. sylvestris*. For example, IS6110 primers (Fig. 2) generated a complicated fingerprint of intermediate *P. sylvestris* from the peat bog. The bands indicated by arrows 1 and 2 in the figure were specific to the population sur-

Table 2. Proportion of polymorphic loci in three *Pinus sylvestris* groups from the Gązwa Reserve.

P. sylvestris group	*P. sylvestris* f. *turfosa* from the peat bog					Intermediate *P. sylvestris* from the peat bog					*P. sylvestris* surrounding the peat bog				
Marker	RAPD	ISJ	katG	IS6110	Total	RAPD	ISJ	katG	IS6110	Total	RAPD	ISJ	katG	IS6110	Total
No. of loci	27	20	27	16	90	27	20	27	16	90	27	20	27	16	90
No. of polymorphic loci	4	3	8	1	16	9	6	13	6	34	10	7	11	6	34
% of polymorphic loci	14.81	15	29.63	6.25	17.78	33.33	30	48.15	37.5	37.78	37.04	35	40.74	37.5	37.78
No. of diagnostic loci	0	1	0	0	1	3	0	1	0	4	4	0	1	3	8

rounding the peat bog but were also observed in all but one individual of the intermediate pines from the Gązwa Reserve. In the latter group of pines, in parallel with bands typical of the surrounding population, markers of *turfosa* origin were observed. Another band indicated by arrow 3 (IS6110/16) was exclusive to *P. sylvestris* surrounding the peat bog. No intermediate pines from the peat bog had band 3; nor did the *turfosa* form.

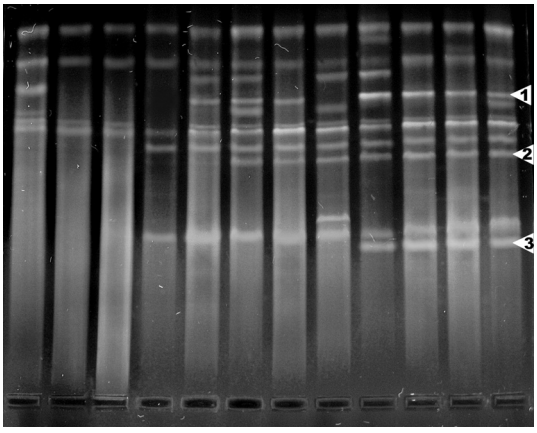

Fig. 2. Amplification patterns of three *Pinus sylvestris* groups from the Gązwa Reserve revealed by primers complementary to bacterial insertion element IS6110. Numbered arrows (1–3) indicate bands separating typical *P. sylvestis* from *P. sylvestris* f. *turfosa*; DNA profiles from left: 1–4 – *P. sylvestris* f. *turfosa*, 5–8 – intermediate *P. sylvestris* from the peat bog, 9–12 – typical *P. sylvestris* surrounding the peat bog (photo K. Polok).

Urbaniak *et al*. (2009) first documented the hybrid origin of *P. sylvestris* from the Gązwa Peat Bog Reserve, morphologically resembling typical pine. Similarly to young *turfosa* pine aged 30–85 years, this category of pines, aged of 20–55 years, presents a broad range of recombinant variation characteristic of the old *turfosa* form (117–217 years old) and typical *P. sylvestris* surrounding the peat bog. The present results and Urbaniak's findings suggest that pine of hybrid origin has a selective advantage in peat bog conditions.

At present the old *turfosa* form is very rarely represented in the Gązwa Peat Bog Reserve, and invasive 20–55-year-old pines of introgressive origin are the dominant element of the *P. sylvestris* population. Due to the demographic decline of the *turfosa* form and the open cross-fertilization taking place in the mixed population, pure *turfosa* forms may be virtually unrepresented in the next generations.

Identification of hybrids is much easier on the interspecific than the intraspecific level, because the genetic differences are greater between parental forms belonging to different species. There are only slight differences in genetic structure between *P. sylvestris* f. *turfosa* and the pines surrounding the peat bog, but they are consistent. It should be possible to correlate their molecular patterns with phenotype, and to detect intermediate forms.

Clustering confirmed the clear separation of *P. sylvestris* f. *turfosa* from the rest of the pine

Table 3. Nei's genetic similarities between three *Pinus sylvestris* groups from the Gązwa Reserve.

Group	DNA marker				
	RAPD	ISJ	katG	IS6110	Total
P. sylvestris f. *turfosa*, age 117–217 years, vs intermediate *P. sylvestris* from the peat bog, age 20–55 years	0.71	0.82	0.80	0.92	0.81
P. sylvestris f. *turfosa*, age 117–217 years, vs typical *P. sylvestris* surrounding the peat-bog, age 100–150 years	0.63	0.83	0.81	0.88	0.77
Intermediate *P. sylvestris* from the peat bog, age 20–55 years vs typical *P. sylvestris* surrounding the peat bog, age 100–150 years	0.94	0.84	0.82	0.91	0.90

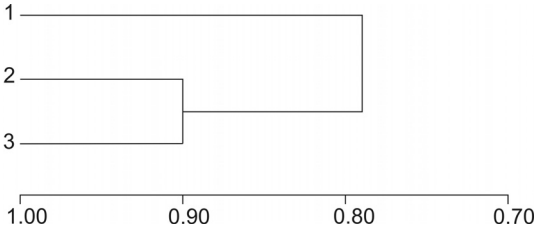

Fig. 3. UPGMA dendrogram based on Nei's genetic identity for four categories of DNA markers. 1 – *Pinus sylvestris* f. *turfosa*; 2 – intermediate *P. sylvestris* from the peat bog; 3 – typical *P. sylvestris* surrounding the peat bog.

phenotypes (Fig. 3). Intermediate *P. sylvestris* from the peat bog and surrounding trees joined at I = 0.90 and formed a single cluster in the dendrogram. The *turfosa* form joined them with the lowest genetic similarity (0.77). The intermediate value (I = 0.81) between *turfosa* forms and the intermediate pines from the peat bog again confirms the hybrid origin of the latter (Table 3, Fig. 3). RAPD markers gave the strongest discrimination of the analyzed phenotypes (Table 3).

CONCLUSIONS

1. *Pinus sylvestris* f. *turfosa* from the Gązwa Reserve has a unique genotype distinguishing this morphotype from typical *P. sylvestris* surrounding the peat bog. This suggests the ancient origin of the *turfosa* form.

2. The DNA profiles of intermediate forms of *Pinus sylvestris* from the peat bog suggest its hybrid origin between *Pinus sylvestris* f. *turfosa* and *P. sylvestris* surrounding the peat bog.

3. The genetic pool of the *turfosa* form is endangered by its hybridization with the very abundant invasive *P. sylvestris* surrounding the peat bog.

ACKNOWLEDGEMENTS. This work was supported by the European Community Project, Marie Curie Host Fellowships for the Transfer of Knowledge, GenCrop, Contract No MTKD-CT-2004-509834. We thank the anonymous reviewer for critically reading the manuscript.

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