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NORWAY SPRUCE (*PICEA ABIES* [L.] KARST.) PROVENANCE VARIATION IN AUTUMN COLD HARDINESS: ADAPTATION OR ACCLIMATION?

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We tested autumn frost hardiness in three Norway spruce (*Picea abies* [L.] Karst.) provenances originating from different altitudes at two trial plots in Slovakia (Vel'ký Lom at 450 m a.s.l., Mútne-Zákamenné at 1,250 m a.s.l.) in a spinoff experiment of the IUFRO 1964/68 Inventory Provenance Experiment with Norway spruce. Two approaches were used to assess hardiness: the electrolyte-leakage method based on artificial freezing, and measurements of chlorophyll *a* fluorescence parameters. The frost injury index at -20°C differed between provenances, with a significant provenance-by-plot interaction. In material from the lower-altitude Vel'ký Lom trial plot, the high-elevation TANAP provenance exhibited much lower frost injury than the middle-elevation Habovka and low-elevation Beňuš provenances. In material from the high-altitude Mútne-Zákamenné trial plot, all three tested provenances showed approximately the same degree of frost injury. At -80°C no differences between provenances were observed, and the trees growing at the high-elevation site exhibited lower average frost injury than the trees at Vel'ký Lom. Most parameters of the kinetics of chlorophyll *a* fluorescence followed the same trends as frost injury, and differed significantly between plots. We suggest that the observed differences resulted from acclimation of trees to the conditions of the trial plots rather than from adaptation through natural selection.

Key words: *Picea abies*, Norway spruce, IUFRO 1964/68, cold hardiness, chlorophyll fluorescence, electrolyte leakage, provenance research.

INTRODUCTION

Frost hardiness is generally considered a genetically controlled trait exhibiting seasonal variation. The course of cold hardiness is driven by environmental factors. In most woody plants the period of autumn cold acclimation is divided into two phases (Weiser, 1970; Holliday et al., 2008). The slow phase, overlapping with growth cessation and dormancy induction, usually starts long before the first autumn frost occurs, as it is triggered by critical night length (Weiser, 1970; Cannell et al., 1990). During this phase, cold hardiness increases continuously. After the first subfreezing temperatures occur, the rapid phase begins, during which maximum cold hardiness is ultimately achieved (Weiser, 1970). Both photoperiod and temperature thus act as drivers of the hardening process, inducing changes in gene expression (Smallwood and Bowles, 2002; Joosen et al., 2006; Holliday et al., 2008). These changes engage a metabolic hardening mechanism which increases frost hardiness in autumn and decreases it in spring (Weiser, 1970; Repo, 1992). Although cold hardiness in different tissues seems to be generally controlled by the same genes, genetic control of autumn hardening is largely independent of spring dehardening (Aitken and Adams, 1996; Anekonda et al., 2000).

There is a trade-off between growth and hardening/dehardening phenology, as the timing of both

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TABLE 1. Characteristics of provenances and trial sites in the study

	No.	Longitude /Latitude	Altitude (m a.s.l.)	Soil	T^1 (°C)	P^2 (mm)
Provenance						
Beňuš	P1	19°53'/49°50'	700	Dystric Cambisol	5.7	852
Habovka 49b	P12	19°41'/49°15'	1,000	Dystric Cambisol	3.7	1,199
TANAP	P49	20°15'/49°11'	1,450	Haplic Podsol	2.9	1,154
Trial site						
Veľký Lom	T1	19°21'/49°20'	450	Eutric Cambisol	7.9	727
Mútne-Zákamenné	T5	19°17'/49°32'	1,250	Dystric Cambisol	3.1	1,336

[1]Mean annual temperature, [2]Mean annual precipitation

processes determines the length of the vegetation period. Therefore the timing of entry into dormancy frequently exhibits clinal variation along latitudinal or altitudinal gradients, as demonstrated by common-garden experiments with different provenances (Daehlen et al., 1995). Cold hardiness as such is heritable, although narrow-sense heritabilities are generally low (O'Neill et al., 2000; Andersson and Fedorkov, 2004; Bower and Aitken, 2006; Mahalovich et al., 2006). The extent to which geographical trends result from genetic adaptation in the Darwinian sense (i.e., changes of allelic structures at underlying structural loci) or from epigenetic modifications remains unclear. In conifers, daylength and temperature during zygotic embryogenesis and seed maturation affect spring flushing, autumn growth cessation, height growth as well as frost hardiness (Johnsen and Østreng, 1994; Johnsen et al., 2005; Kvaalen and Johnsen, 2008), and these effects last at least several years. Ultimately such memory effects lead to rapid changes in the phenological behavior of the offspring of transplanted provenances after one generation (Skrøppa et al., 2009). Finally, there is a strong environmental component in the variation of cold hardiness; the course of hardening/dehardening processes is strongly affected by ambient temperature, water availability, photoperiod and other exogenous factors (Kalberer et al., 2006; Garcia-Banuelos et al., 2008).

The aim of this study was to quantify the differences in autumn frost hardiness between contrasting provenances in a 45-year-old provenance experiment with Norway spruce (*Picea abies* Karst.). The results are discussed with respect to adaptation by natural selection and physiological acclimation as potential causes of such differences.

MATERIAL AND METHODS

The study is based on a spin-off of the IUFRO 1964/68 Inventory Provenance Experiment with Norway spruce (Krutzsch, 1974). Twelve Slovak provenances and selected foreign provenances represented in the experiment were planted in a series of 5 plots distributed along an altitudinal gradient from 450 to 1,250 m a.s.l. in 1968, in a randomized complete block design with 3 blocks and 49 plants per provenance and block (2 × 2 m spacing). No thinning has yet been performed.

For our study, three provenances originating from contrasting environments were selected in the lowermost and uppermost plots of the series (Tab. 1). Climbers collected branches (~80 cm) from the insolated upper part of the crown from 10 trees per provenance in 2009, in Vel'ký Lom on October 27 and in Mútne-Zákamenné on October 28. Those dates followed a cold weather episode with whole-day frosts, which occurred from October 12 to 15, 2009. Dominant trees distributed over all three blocks were chosen. Immediately after collection the branches were transported to the laboratory and kept in darkness at +4°C.

Two days after the collection, parameters of the rapid phase of chlorophyll *a* fluorescence yielding information on the efficiency of photosystem II (PSII) were measured in one-year-old needles using a Plant Efficiency Analyser fluorimeter (PEA, Hansatech Ltd., Kings Lynn, UK). The measurements were made after an adaptation period of 30 min at 50% intensity of saturation light (2,100 μmol m^{-2} s^{-1}), repeated and recorded at 1 s intervals. Two replicate measurements per sample were made. The following parameters were assessed: F_0 (basic fluorescence; fluorescence yield in the absence of photosynthetic light, when all reaction centers of PSII are open), F_m (maximum fluorescence), F_v (variable fluorescence, F_m-F_0), F_v/F_m (photochemical efficiency), T_m (time required for the increase of fluorescence from F_0 to F_m), and *Area* (area above the induction curve between basic and maximum fluorescence) (Maxwell and Johnson, 2000).

Cold hardiness was determined by the electrolyte-leakage method, which measures the ability of cell membranes to retain a high intracellular ion

TABLE 2. Analyses of variance of frost injury indices

Variable	Effect	DF	Mean square	*F*-test	
I_{-20}	Provenance	2	780.78	4.7399	*
	Trial site	1	2469.75	47.5333	***
	Trial site × Provenance	2	748.55	4.5442	*
	Block (Trial site)	4	46.18	0.2837	ns
	Provenance × Block (Trial site)	8	162.18	0.7341	ns
	Error	41	220.93		
I_{-80}	Provenance	2	346.78	3.9209	a
	Trial site	1	923.45	21.6079	**
	Trial site × Provenance	2	96.83	1.0948	ns
	Block (Trial site)	4	40.27	0.4600	ns
	Provenance × Block (Trial site)	8	87.25	0.7596	ns
	Error	41	114.86		

Significance levels: $^{***}p<0.001$; $^{**}0.001<p<0.01$; $^{*}0.01<p<0.05$; a – $0.05<p<0.1$; ns – $p>0.05$ nonsignificant

concentration. In the case of injuries to membranes, ions leak out of the cells into the apoplast. The extent of injury to cell membranes may be assessed by the conductivity of the incubation solution after incubation of needles in distilled water. The methodology followed Repo et al. (2000), with slight modifications.

Two days after harvesting, ~50 needles from each tree were prepared for each exposure temperature. The samples were placed in plastic bags with a little distilled water sprayed over them. Two freezing temperatures (-20°C, -80°C) and a control temperature (+4°C) were used. As we do not have controlled air-cooled chambers at our disposal we used a deep freezer and controlled the temperature decrease manually. The initial and end temperature of exposure was +4°C; temperature was decreased by 5°C per h until -20°C was reached in a commercial freezer, and then half of the samples were placed in a deep freezer (Jouan VX570F) where the temperature was decreased down to -80°C. The samples were kept at the target temperature for ~4 h, and warming was done at the same rate. To smooth random temperature fluctuations (namely during warming), the samples were kept in a polystyrene box during these procedures.

After the freezing treatment, ~30 needles of the shoots from each tree and each freezing temperature were selected for the electrolyte leakage test. Segments ~10 mm in length were cut from the middle of each needle, rinsed with distilled water and placed in test tubes (8 samples per tube) and weighed. Measurements were made in three replicates per tree. Distilled water (6 ml per 10 mg sample) was added to each test tube, which was then shaken at room temperature for 24 h before the first conductivity measurement (L_{1t}). Then the samples were heat-killed at 92°C for 20 min and shaken another 24 h before the second conductivity measurement (L_{2t}). The conductivity of the heat-killed sample gives a measure of the total amount of tissue electrolytes. The relative electrolyte leakage for a given freezing temperature t was then defined as

$$R_t = L_{1t}/L_{2t}$$

The index of frost injury at freezing temperature t was determined according to Flint et al. (1967):

$$I_t = 100 \times (R_t - R_0)/(1 - R_0)$$

where R_0 is relative electrolyte leakage without freezing (at +4°C).

The differences in photosynthetic parameters and frost injury indices between provenances and trial sites were tested by ANOVA using the GLM procedure (SAS, 1988). As the distributions of injury indices were left-skewed, they were square-root-transformed prior to statistical tests. Provenance and trial site were treated as fixed effects, and block as a random effect.

RESULTS AND DISCUSSION

Our analysis of variance of the frost injury indices indicated that the differences in cold tolerance were related mainly to trial plot conditions and only secondarily to provenance (Tab. 2). The material collected from the Vel'ký Lom trial plot situated at 450 m a.s.l. generally showed higher levels of damage from artificial freezing than the needles collected at the Mútne-Zákamenné plot situated at 1250 m a.s.l. (Fig. 1). There was a significant provenance × plot interaction for frost injury at -20°C, a temperature that occurs at least once per year everywhere in Slovakia except perhaps the lowlands. In the material from Vel'ký Lom, the high-elevation TANAP provenance exhibited much lower frost injury than the middle-elevation Habovka and low-elevation

TABLE 3. Analyses of variance of chlorophyll *a* fluorescence parameters

Variable	Effect	DF	Mean square	*F*-test	
F_0	Provenance	2	2.457E–04	0.3578	ns
	Trial site	1	0.490E–04	0.111	ns
	Trial site × Provenance	2	4.429E–04	0.6447	ns
	Block (Trial site)	4	4.350E–04	0.6322	ns
	Provenance × Block (Trial site)	8	6.886E–04	1.0636	ns
	Error	42	6.474E–04		
F_m	Provenance	2	2.919E–02	2.4865	ns
	Trial site	1	9.888E–02	15.092	*
	Trial site × Provenance	2	2.555E–02	2.1763	ns
	Block (Trial site)	4	0.597E–02	0.5271	ns
	Provenance × Block (Trial site)	8	1.113E–02	0.4347	ns
	Error	42	2.561E–02		
F_v	Provenance	2	3.235E–02	3.9583	a
	Trial site	1	8.403E–02	15.4004	*
	Trial site × Provenance	2	1.629E–02	1.9935	ns
	Block (Trial site)	4	0.504E–02	0.6424	ns
	Provenance × Block (Trial site)	8	0.769E–02	0.4024	ns
	Error	42	1.912E–02		
F_v/F_m	Provenance	2	9.123E–04	3.0226	ns
	Trial site	1	18.760E–04	14.2991	*
	Trial site × Provenance	2	1.066E–04	0.3532	ns
	Block (Trial site)	4	1.263E–04	0.4179	ns
	Provenance × Block (Trial site)	8	3.023E–04	1.038	ns
	Error	42	2.912E–04		
T_m	Provenance	2	0.436E–05	0.1263	ns
	Trial site	1	30.060E–05	7.4833	*
	Trial site × Provenance	2	1.053E–05	0.3051	ns
	Block (Trial site)	4	4.052E–05	1.1677	ns
	Provenance × Block (Trial site)	8	3.478E–05	1.2218	ns
	Error	42	2.847E–05		
Area	Provenance	2	0.162E–03	0.0848	ns
	Trial site	1	2.501E–03	3.6849	ns
	Trial site × Provenance	2	3.670E–03	1.9116	ns
	Block (Trial site)	4	0.615E–03	0.3248	ns
	Provenance × Block (Trial site)	8	1.884E–03	0.6863	ns
	Error	42	2.745E–03		

Significance levels as in Table 2

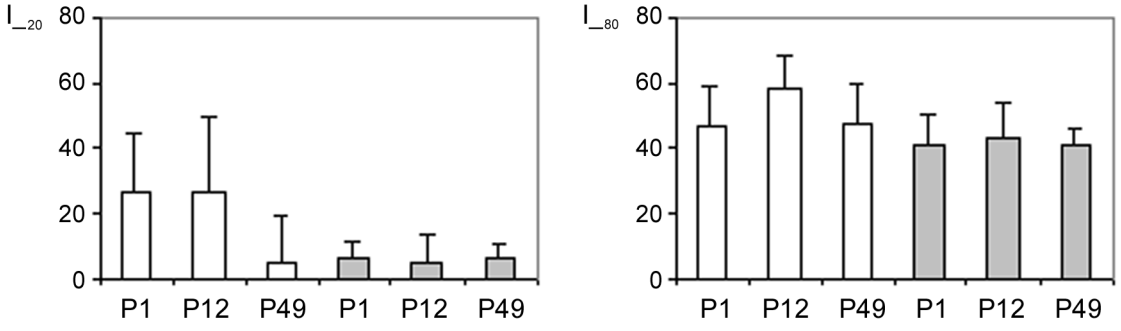

Fig. 1. Injury indices (I_{-20}, I_{-80}) for individual provenances (means ±SD). Unshaded columns – Veľký Lom trial site, shaded columns – Mútne-Zákamenné trial site.

Beňuš provenances (Fig. 1). In samples from the high-altitude Mútne-Zákamenné trial plot, all three tested provenances showed approximately the same low degree of frost injury. This finding is in accordance with the observation (Schrader and Graves, 2003) that plants growing in harsher conditions are more uniform in cold hardiness. The test temperature of -80°C is extremely low; spruce populations never encounter it in nature in Central Europe. This may be why the inter-provenance differences in frost injury are only marginally significant. Even at such low test temperature, however, the material from Mútne-Zákamenné exposed to colder climate exhibited slightly higher frost resistance than the material collected at Vel'ký Lom (Fig. 1).

As the material was collected shortly after a week of whole-day frosts and snowy weather, more severe in the north of Slovakia (Mútne-Zákamenné trial site), we expected this event to be reflected in photosynthetic capacity (cf. Repo et al., 2006) and that potential genetically based differences between provenances would be revealed. As mentioned above, fall cold hardiness develops in two stages: at temperatures between 10°C and 20°C, and subsequently at freezing temperatures (Kozlowski and Pallardy, 2002). The influx of cold air in mid-October occurred suddenly after a relatively warm beginning of autumn, so we suspected that the trees did not manage to develop hardiness and the temperature drop below zero exerted a severe stress, mainly on spruce provenances planted at Vel'ký Lom. However, the chlorophyll *a* fluorescence response of the material was modest. Again, it was affected primarily by trial site conditions (Tab. 3), but even between climatically contrasting plots the differences were generally small. No significant differences in fluorescence parameters between provenances were observed. The F_v/F_m ratio, that is, the ratio between variable and maximum fluorescence, is the parameter with the highest descriptive value, as it expresses the maximum quantum yield of the primary photochemical reactions of photosystem II. Values below 0.725 indicate physiological disturbances, but this was rarely found in our material; the values generally exceeded 0.8. Worth stressing are the extremely small differences we found: the coefficient of variation within the whole experiment was only 2.28%, meaning that even tree-level variation was negligible. Nevertheless, trees planted at the high-elevation Mútne-Zákamenné site had a slightly but significantly lower F_v/F_m ratio than those at Vel'ký Lom (Fig. 2, Tab. 3).

The parameter *Area* expresses the area above the induction curve between the basic (F_0) and maximum (F_m) fluorescence. It is proportional to the reserve of electrons on the reducing side of PSII. The area decreases when the fluency of reoxidation of the acceptor of electrons in the primary photochemical reactions is disturbed. In our case, *Area* varied randomly and there were no significant differences between trial sites or provenances (Tab. 3). Apparently the chlorophyll fluorescence parameters reflect the actual rather than accumulated stress levels. Our outcomes support Taulavuori et al.'s (2000) conclusion that chlorophyll fluorescence in vivo is not necessarily a proper indicator of cold hardiness.

Under the current experimental setup, climatic (long-term) effects could not be distinguished from weather (short-term) effects on the actual state of cold hardiness. Under lower temperatures, plants attain stronger cold tolerance and develop it more rapidly than in moderate conditions (Nielsen and Rasmussen, 2009), showing that tolerance to freezing responds quickly to the actual weather. This favors the hypothesis that the between-trial differences we observed are due to warmer weather at the low-elevation site at the time of collection. However, the trees planted at the two trial sites have undergone long-term exposure to contrasting climates, and this may also have contributed to a differential response (cf. Li et al., 2004). A reciprocal transplant experiment, feasible only with young plants and not subadult trees, is the only way answer this question.

Autumn cold hardiness is a heritable trait, although the reported heritability estimates in conifers are generally low to intermediate, ranging between 0.09 and 0.40 (Aitken et al., 1996; Anekonda et al., 2000; O'Neill et al., 2000; Bower and Aitken, 2006), and the genetic correlations between autumn and winter hardiness are also generally weak (Aitken and Adams, 1996). Even such low heritabilities potentially leave room for adaptation by natural selection when environmental contrasts are big enough to provoke divergence. Exploration of nucleotide variation at cold-hardiness-related genes in *Pseudotsuga menziesii* revealed signatures of selection at some loci (Eckert et al., 2009). At the phenotypic level, significant variation among different provenances and even clinal patterns along geographical or environmental gradients have been reported frequently, related mostly to latitude, which is associated not only with changes in precipitation and temperature regimes but also with photoperiod, known to play an important role in tree phenology (Beuker et al., 1998; Lu et al., 2003; Aldrete et al., 2008). Occasionally also clines or between-provenance contrasts associated with longitude or continentality (Thomas and Lester, 1992; Jensen and Deans, 2004; Eysteinsson et al., 2009) or altitude (Saenz-Romero and Tapia-Olivares, 2008) have been recorded.

As the natural range of Norway spruce in Slovakia is small, we could not have expected differences related to longitude or latitude; thus, climate differences associated with altitude remained the sole factor potentially triggering differentiation by selection. This

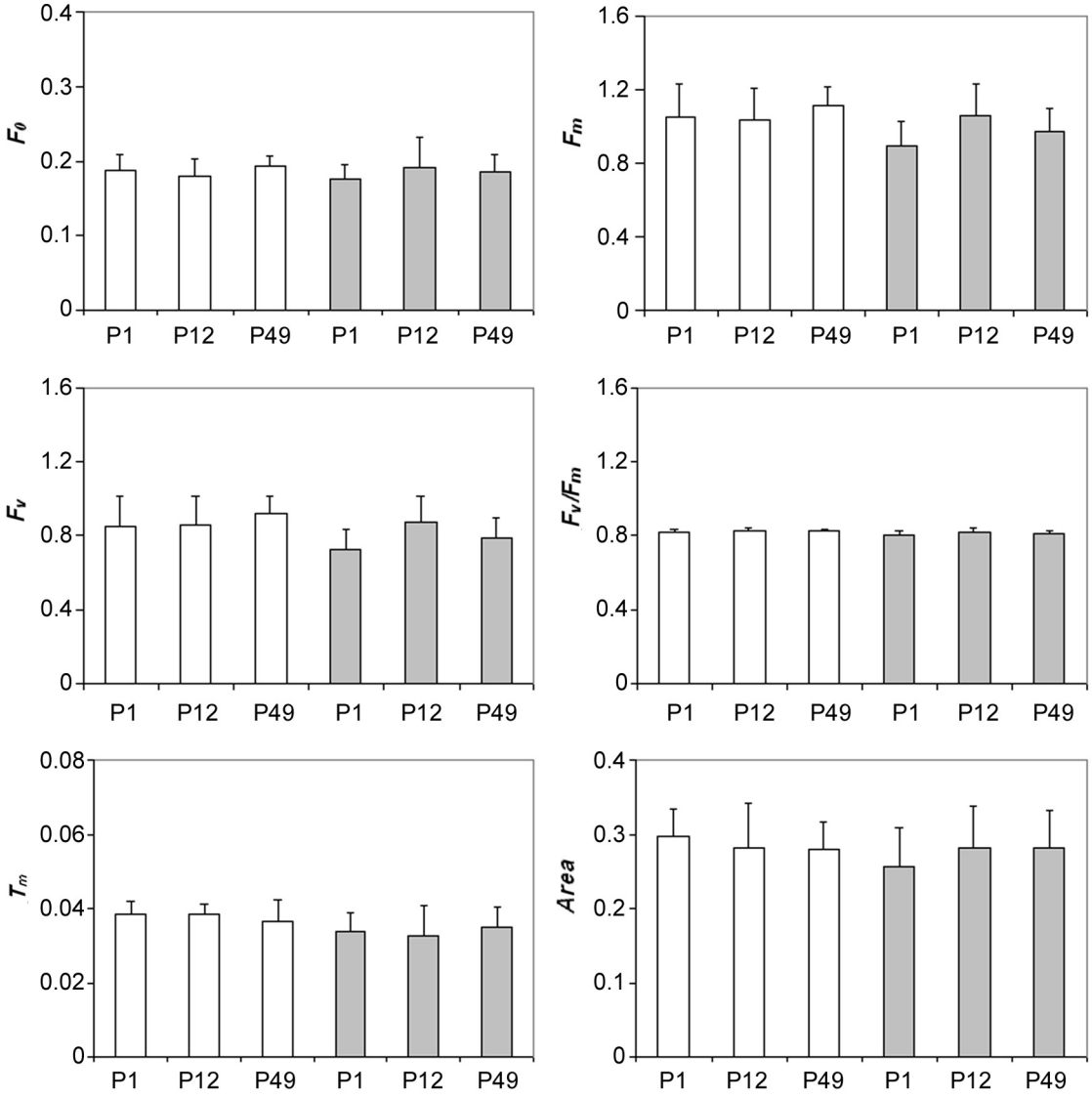

Fig. 2. Chlorophyll fluorescence parameters (F_0, F_v, F_m, F_v/F_m, T_m, *Area*) for individual provenances (means ±SD). Unshaded columns – Vel'ký Lom trial site, shaded columns – Mútne-Zákamenné trial site.

was confirmed only partially – only the subalpine TANAP spruce population exhibited higher cold hardiness, and only when grown in a mild climate. The lack of between-provenance differentiation may be associated with several factors. First, even when differential temperature regimes really trigger selection for cold hardiness in spruce, gene flow between altitudinal zones may hamper genetic divergence (Savolainen et al., 2007). Second, heritable changes of cold hardiness (including those observed in provenance experiments) may arise from epigenetic rather than genetic phenomena. Cold stress causes changes in plant DNA methylation patterns, such that expression of genes controlling physiological responses to cold can be modified without changing the nucleotide sequence (Sano, 2002; Steward et al., 2002). As shown by Daehlen et al. (1995), progenies of a spruce seed orchard produced in a cold year were hardier than those produced in a warm year.

In our material, environment seems to have played a much more important role in cold-hardiness development than heredity did. From a practical point of view, it means that cold hardiness need not be taken into account in considering the transfer of forest reproductive material of Norway spruce within such a small territory as Slovakia.

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