

# Origin and evolution of invasive naturalized material of *Rhododendron ponticum* L. in the British Isles

RICHARD I. MILNE and RICHARD J. ABBOTT

Division of Environmental & Evolutionary Biology, School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK

## Abstract

Information concerning the area of origin, genetic diversity and possible acquisition of germplasm through hybridization is fundamental to understanding the evolution, ecology and possible control measures for an introduced invasive plant species. *Rhododendron ponticum* is extensively naturalized in the British Isles, but it is not known whether native material in Turkey, Spain or Portugal gave rise to the naturalized material, or to what extent introgression has affected this material. Chloroplast (cp) and nuclear ribosomal DNA (rDNA) restriction fragment length polymorphisms (RFLPs) were sought which could distinguish between native material of *R. ponticum*, and between 15 other *Rhododendron* species including *R. ponticum*'s closest relatives. Thereafter, a total of 260 naturalized accessions of *R. ponticum* from throughout the British Isles was examined with respect to informative polymorphisms. It was found that 89% of these accessions possessed a cpDNA haplotype that occurred in native material of *R. ponticum* derived almost entirely from Spain, while 10% of accessions had a haplotype unique to Portuguese material. These results therefore indicated an Iberian origin for British material. rDNA or cpDNA evidence of introgression from *R. catawbiense* was found in 27 British accessions of *R. ponticum*, and such accessions were significantly more abundant in Britain's coldest region, eastern Scotland, than elsewhere. This could indicate that introgression from *R. catawbiense* confers improved cold tolerance. Introgression from *R. maximum* and an unidentified species was also detected.

*Keywords:* adaptive introgression, cold tolerance, hybridization, invasions, RFLPs, *Rhododendron ponticum*

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## Introduction

Introduced plant species now occur in virtually every inhabited part of the world. A small minority of such plants can become aggressively invasive and displace native species from their habitats (Kelly 1981; Pysek & Prach 1995; Coghlan 1997). The study of these species is critical to the conservation of plant communities subject to invasion, and can provide information on how invasive species respond to new habitats.

Introduced taxa will often contain low levels of genetic diversity due to the founder effect and genetic drift experienced during and immediately following successful colonization and establishment (Barrett & Husband 1989; Novak & Mack 1993). Low genetic diversity within introduced material could constrain evolutionary divergence

and possibly limit range expansion. However, should introduced material hybridize with native taxa or other introduced taxa, it might acquire through introgression appropriate genetic variation for adaptation to new environments. This could aid its spread in the area colonized. Examples exist of introduced plants contributing germplasm to native relatives (Rieseberg *et al.* 1990a; Abbott 1992; Abbott *et al.* 1992a,b; Strefeler *et al.* 1996), and also of introductions gaining germplasm from native species which might improve adaptation to local conditions (Heiser 1951; Harlan & deWet 1963; Rieseberg *et al.* 1990a; Rieseberg & Wendel 1993; Strefeler *et al.* 1996). Occasionally, hybrids between introduced and native species have been shown to displace native species from their natural habitat (Wojcicki & Marhold 1993; Daehler & Strong 1997; Vila & D'Antonio 1998a,b), while hybrid derivatives of two introduced species are known that exhibit enhanced reproductive capability (Hollingsworth *et al.* 1998) and/

Correspondence: R. I. Milne. Fax: +44-1334463366; E-mail: rim@st-andrews.ac.uk

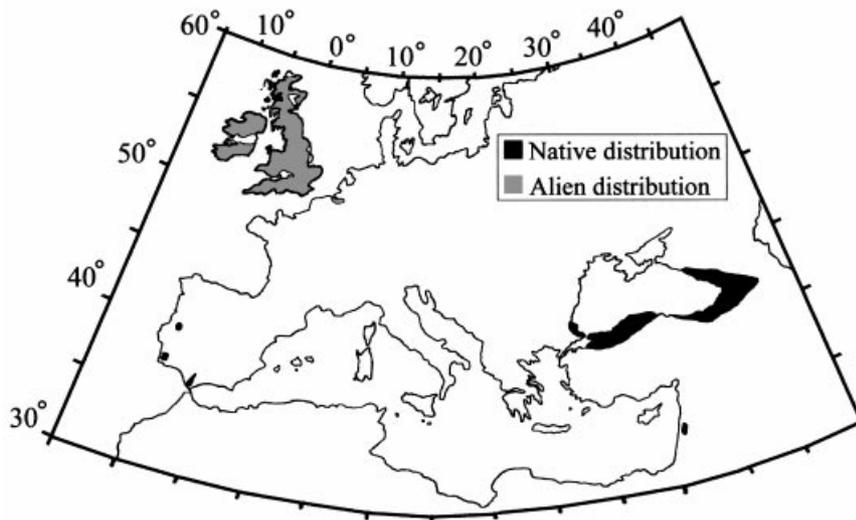


Fig. 1 Native and alien distribution of *Rhododendron ponticum* (after Cross (1975)). The species also occurs as an alien at isolated sites in France and Norway (Popova 1972).

or greater ecological range than either parent (Roche & Roche 1991).

To determine if an introduced species has undergone genetic change during colonization and establishment, due to genetic bottlenecking and/or introgression of genes from native or other introduced taxa, it is necessary to: (1) locate the native population(s) which served as the source of introduced material; (2) determine whether the introduced material contains only a sample of the genetic variability present in the source population(s); and (3) determine whether hybridization with other species has resulted in the acquisition of genes from these taxa. Here we report an investigation aimed at obtaining this information for an introduced and highly invasive species in the British Isles, *Rhododendron ponticum*.

*R. ponticum* is extensively naturalized in the British Isles (Fig. 1), where it has very few natural enemies (Judd & Rotherham 1992). It is currently recognized as a threat to native communities (e.g. in Killarney, southwest Ireland; Turner & Watt 1939; Cross 1981; Kelly 1981), and is a pest of forestry (Brown 1953, 1954; Simons 1988). It is an example of a species whose ecology in the British Isles previously could not be meaningfully compared with that of material from which it was derived, as it was uncertain from which area it was introduced, and also to what extent naturalized material had been altered by introgression. The species is a native of the area to the south of the Black Sea (i.e. the Caucasus, northern Turkey and the southeast corner of Bulgaria) and, disjunctly, Lebanon and three small areas in the Iberian Peninsula, i.e. in southwest Spain, and southern and central Portugal (Jessen *et al.* 1959; Cross 1975; Fig. 1). The Black Sea and Iberian populations were formerly considered to be specifically or subspecifically distinct, but there are no consistent morphological characters which distinguish

these populations (Chamberlain 1982; Davidian 1992; Sales 1997). The first introduction of *R. ponticum* to the British Isles appears to have been in 1763, from southwest Spain (Curtis 1803; Coats 1963), but subsequent introductions have certainly occurred, of which some were from the Black Sea area (Curtis 1803; Loudon 1838), and some authors consider naturalized British material to be of Black Sea area origin (Cross 1975; Stace 1991). Thus it cannot be determined from historical or morphological information from which area naturalized British material originates.

It seems likely from the morphology of some naturalized material that the species has been subject to introgression from other introduced and cultivated *Rhododendron* species since its introduction. Native material of *R. ponticum* has glabrous ovaries and calyx lobes up to 1 mm long (Popova 1972; Chamberlain 1982; Davidian 1992), whereas naturalized British material frequently has hairy ovaries and longer calyx lobes, and displays a greater range of variation in corolla colour and morphology (Michalak 1976; Milne 1997). This indicates that introgression, most probably from the American species *R. catawbiense* and/or *R. maximum*, has occurred (Cox & Hutchinson 1963; Michalak 1976; Clapham *et al.* 1987; Davidian 1992; Stace 1997). *R. ponticum* was allowed to hybridize freely in cultivation with both of these species, and to a lesser extent *R. arboreum*, in the late 18th and early 19th centuries (Coats 1963; Bean 1976; Michalak 1976). As hybridization is common in *Rhododendron*, the possibility also exists that introgression from some of the 500 other *Rhododendron* species cultivated in Britain (Bean 1976) has occurred through accidental or deliberate crossing in cultivation. This makes it difficult to determine with certainty which species might have contributed any morphological characteristic noted in a naturalized individual of *R. ponticum*.

Introgression may have altered the ecology of *R. ponticum* in the British Isles compared with native material. The climate of the British Isles is colder than that of native localities – the mean January temperatures are between 5 and 10 °C lower (depending on region; Brown *et al.* 1992; de Blij & Muller 1993) – and if increased tolerance to lower temperatures was conferred by introgression this could be expected to have a significant effect on the ecology and invasive capability of *R. ponticum* in the British Isles. Following the exceptionally severe winter of 1894–5 it was noted that ‘Catawba’ (i.e. involving *R. catawbiense*) hybrids survived intact whereas large numbers of individuals of *R. ponticum* were killed or damaged (Field 1895). *R. catawbiense* and *R. maximum* are tolerant of far lower temperatures than *R. ponticum* (respectively, –50, –50 and –15 °C; Davidian 1982; Nilsen 1991). Therefore the possibility exists that introgression from these species has increased the cold tolerance of *R. ponticum*, and therefore its fitness, in the British Isles.

To determine the location(s) of source material of native *R. ponticum* from which naturalized British material is derived, a survey of chloroplast (cp) DNA variation was conducted on naturalized material sampled from throughout the British Isles and also native material sampled from northern Turkey and the Iberian Peninsula. This approach assumed that there is sufficient cpDNA variation within native material to determine from which part of the species’ native range naturalized British material is derived. Differences in cpDNA haplotype(s) have been reported between populations, or races, of other species separated since the last glaciation, or longer (Sewell *et al.* 1996; Soltis *et al.* 1997; Comes & Kadereit 1998). Fossil evidence (Jessen 1948; Jessen *et al.* 1959) indicates that Black Sea and Iberian plants of *R. ponticum* have been isolated from each other since an interglacial period between 300 000 and 400 000 years ago, at a time when the climate of Europe was wetter and warmer than in the current interglacial, and which was possibly the last time that the species was distributed across central Europe between the Black Sea and the Iberian Peninsula (Jessen *et al.* 1959). Thus, it was considered feasible that Black Sea and Iberian material would differ in cpDNA haplotype, allowing a survey of cpDNA variation to determine whether *R. ponticum* naturalized in the British Isles is derived from populations from one or both of these two regions.

To investigate the possibility that naturalized British material of *R. ponticum* is introgressed with genes from related species, both cpDNA and ribosomal (r) DNA variation was surveyed in naturalized and native material of *R. ponticum*, and also in material of the close relatives of this species that are cultivated in the British Isles. rDNA variants are useful markers of the nuclear genome and have been used in previous studies of the effects of

hybridization in plants, either alone (e.g. Rieseberg *et al.* 1990b; Rieseberg 1991; Brubacker & Wendel 1994), or in combination with cpDNA variants (e.g. Rieseberg *et al.* 1990a; 1990b; Harris & Ingram 1992).

## Materials and methods

### Plant collection

Native material of *Rhododendron ponticum* was collected from the three areas of the Iberian Peninsula where it occurs, i.e. southwest Spain (26 accessions from six sites), southern Portugal (33 accessions from 13 sites) and central Portugal (10 accessions from two sites), and also from three regions of Turkey near to the Black Sea, i.e. northeast Turkey around Artvin, northern Turkey near Bolu, and the Istranca Daglari, northwest of Istanbul (Table 1). Material was also sampled from all other species of *Rhododendron* subsection Pontica, i.e. *R. catawbiense* (five accessions), *R. maximum* (two accessions), *R. macrophyllum* (three accessions), *R. brachycarpum* (one accession each of ssp. *brachycarpum* and ssp. *faureii*), *R. degrobianum* (= *R. japonicum*), one accession each of ssp. *degrobianum*, ssp. *heptamerum*, ssp. *heptamerum* var. *hondoense* and ssp. *yakushmanum*, *R. makinoi*, *R. caucasicum*, *R. smirnovii*, *R. ungerii*, *R. aureum*, and *R. hyperythrum* (single accessions). In addition, material was sampled from single accessions of four species outside subsection Pontica, i.e. *R. arboreum*, *R. adenopodum*, *R. argyrophyllum*, and *R. fortunei*. All samples were obtained from plants grown at the Royal Botanic Garden, Edinburgh, except for samples from one accession of each of *R. catawbiense* and *R. maximum* collected from the wild by M. Strate, and from one accession of *R. catawbiense* supplied from cultivation by Dr J. Balfour.

In addition to the material described above, 260 accessions of naturalized British *R. ponticum* were sampled. This material was collected in two ways. First, 177 accessions were collected as single individuals, randomly selected from populations distributed throughout the British Isles. A complete list of localities of populations sampled is given in Milne (1997). For convenience, these localities have been grouped into the following 13 areas: (1) western Scotland, i.e. Scottish mainland west of the 238 km eastern national grid line, and north of the 610 km northern grid line but excluding Kintyre Peninsula (28 accessions); (2) Scottish Islands, i.e. the Islands of Arran, Raasay and Skye, western Scotland (six accessions); (3) Kintyre, i.e. the Kintyre Peninsula of western Scotland, south of the 710 km northern grid line (eight accessions); (4) eastern Scotland, i.e. Scotland north of the Tay and east of the 238 km national grid line (24 accessions); (5) Fife and Lowlands, i.e. the Scottish counties of Fife and Stirlingshire, west to Stirling plus Edinburgh city and the northern bank of the river Tay (13 accessions); (6) northern England,

**Table 1** Localities of native material of *Rhododendron ponticum* surveyed, and the number of accessions examined with different enzyme:probe combinations

Region	No. of accessions examined with different enzyme:probe combinations (A–D)*				Brief description of locality	Northing and easting	Date of collection†
	A	B	C	D			
Northeast Turkey	6	4	1	7	Hill ≈ 10 km westsouthwest of Artvin. Woodland and grassland above treeline	41°07'N, 41°44'E	21 June 1994
Northeast Turkey	28	7	1	17	Slopes below Tiryal Daga mountains near Damar, 4 km southeast of Murgul	41°14'N, 41°36'E	22 June 1994
Northeast Turkey	2	1	0	2	Woodland below Gul Dagi, 10 km along road running southwest from Murgul	≈ 41°11'N, 41°32'E	23 June 1994
Northeast Turkey	1	1	1	1	By the road just south of Hopa; shady undergrowth at roadside	41°24'N, 41°29'E	21 June 1994
Northeast Turkey	2	2	0	2	Roadside below Camlihemsin	41°01'N, 41°03'E	21 June 1994
Northeast Turkey	4	5	1	5	Very steep stream valley above Camlihemsin	41°01'N, 41°03'E	21 June 1994
Northeast Turkey	3	3	3	2	By main road (83) above Ikisdere; scrub between the river and the road	40°46'N, 40°35'E	27 June 1994
Northern Turkey	5	2	5	1	12 km from Bolu, on main road (E5) to Duzce. Woods beside the road	40°43'N, 31°30'E	2 July 1994
Northwest Turkey	5	4	5	2	By road from Demirkoy northwest to Golyaka, understorey in woodland	41°50'N, 27°41'E	29 June 1994
Northwest Turkey	4	2	4	1	Just above Kiyikoy, near coast; understorey in woodland	41°38'N, 28°06'E	29 June 1994
Total (Turkey)	60	31	21	40			
Southwest Spain	5	5	1	5	Parque natural Los Canutos, 2 km north of main road from Algamasilla to Pelayo	36°06'N, 5°32'W	16 December 1994
Southwest Spain	6	6	1	5	Near El Tinadero, ≈ 3 km north of the road	≈ 36°11'N, 5°37'W	17 December 1994
Southwest Spain	7	7	2	6	Hill slope northwest of Algeciras, near Chorroquina, along the stream Mogeia Comady in La Miel valley	≈ 36°08'N, 5°32'W	18 December 1994
Southwest Spain	4	0	0	0	Cadiz Prov., Alcalá de los Gazules, el Picacho El Medio Gorge, mountain stream (water all year) over sandstone	36°32.5'N, 5°38'W	November 1998 (JA)
Southwest Spain	4	0	0	0	Cadiz Prov., Alcalá de los Gazules, el Picacho, El Algibe Gorge, mountain stream (water all year) over sandstone	36°32.5'N, 5°38'W	November 1998 (JA)
Total (southwest Spain)	26	18	4	16			

Central Portugal	8	8	2	8	Near Campia — Oliviera de Frades, Mata do Cambarinho	40°40'N, 8°12'E	November 1993 (IH)
Central Portugal	2	2	2	2	Esbarreja, Riviera das Amdonihás, Ponte de Mimhoteina	≈ 40°45'N, 8°25'E	15 November 1994 (IH)
Total (central Portugal)	10	10	4	10			
Southern Portugal	2	2	0	2	Just west of the road between Monchique and Nave Redondo near the hill Cerro do Rochal	37°23'N, 8°29'W	19 December 1994
Southern Portugal	3	2	1	3	Near San Teotónio, Pedreneiras, Ribeira de Agua de Peixe	37°29'N, 8°41'W	19 December 1994
Southern Portugal	5	5	1	5	Near relva Grande, close to Barranco do Salvador, along Ribeira do Lameino and smaller streams	37°25'N, 8°40'W	19 December 1994
Southern Portugal	1	1	0	1	Roadside between relva Grande and Monchique, near Foz do Farelo	37°21'N, 8°37'W	19 December 1994
Southern Portugal	2	1	0	1	Near Barranco de Maceina and the road between Monchique and Nave Redondo, by Barranco de Mantiones	37°22'N, 8°33'W	20 December 1994
Southern Portugal	3	3	1	3	Near Barranco de Maceina, tributary to Ribeira de Perna de Negra	37°22'N, 8°33'W	20 December 1994
Southern Portugal	1	1	0	1	By Barranco do Congo, near Ribeira de Perna de Negra	37°23'N, 8°34'W	20 December 1994
Southern Portugal	1	0	0	1	Roadside, by Ribeira de Perna de Negra	≈ 37°23'N, 8°34'W	20 December 1994
Southern Portugal	2	2	0	2	Roadside between Monchique and Foia	37°18'N, 8°34'W	20 December 1994
Southern Portugal	4	4	1	4	South-facing slope just below hill-top at Foia, Bicas	37°18'N, 8°35.5'W	20 December 1994
Southern Portugal	3	3	1	3	North slope of Picota Hill	37°18'N, 8°31'W	20 December 1994
Southern Portugal	3	3	0	2	Roadside between Monchique and Nave Redondo	≈ 37°23'N, 8°29'W	20 December 1994
Southern Portugal	3	3	1	3	Roadside between Monchique and Nave Redondo, under eucalyptus	≈ 37°23'N, 8°28'W	20 December 1994
Total (southern Portugal)	33	30	6	31			
Iberian total (Spain + Portugal)	69	58	16	57			

\*The letters represent the following combinations: A, *HpaII*/pLsC7–10; B, *BclI*/pLsC6; C, *BglIII*/pLsC4 and *BglIII*/pLsC6; D, *DraI*/pTa71 for all material, plus *SstI*/pTa71 for material from Spain and Portugal only.

†All material collected by R. Milne except (IH) collected by Ian Hedge *et al.* and (JA) collected by Juan Arroyo.

i.e. between the 200 and 610 km northern grid lines, excluding the Lake District, East Anglia and Wales (q.v.) (11 accessions); (7) Lake District, i.e. England north of the 360 km northern grid line and west of the 360 km eastern grid line (11 accessions); (8) Wales (14 accessions); (9) East Anglia, i.e. England north of the 200 km northern grid line and east of the 550 km eastern grid line (seven accessions); (10) southern England, i.e. south and west of the 200 km northern and 480 km eastern grid lines (eight accessions); (11) southeast England, i.e. south and east of the 200 km northern and 480 km eastern grid lines (18 accessions); (12) Killarney National Park, southwest Ireland (13 accessions); and (13) Connemara, western Ireland (16 accessions).

Second, groups of plants were collected from four populations from each of eastern and western Scotland. These populations were located at: (1) Killin, Perthshire, NN574321 (five accessions); (2) Craigtoun, east Fife, NO467148 (six accessions); (3) Loch Katrine, east-central Scotland, NN497071 (eight accessions); (4) Killiekrankie, near Pitlochry, Perthshire, NN896611 (nine accessions); (5) Gaerloch, Wester Ross, NG839720 (12 accessions); (6) Lochranza, Arran, NR923505 (14 accessions); (7) Knapdale, northern part of the Kintyre Peninsula, NR865582, NR859721, and NR9492 (14 accessions from three close populations treated as one); (8) Torridon, Wester Ross, NG876573 (15 accessions). From these accessions certain morphological characters were noted, i.e. calyx lobe length, degree of ovary hairiness, corolla colour, corolla marking colour, corolla length and corolla lobe width.

#### *Sample preservation, DNA extraction and restriction fragment length polymorphism (RFLP) analysis*

From each sampled accession about five young leaves were torn into 1 cm squares and sealed in bags with 25 g of coarse silica gel, that was replaced each day until the material was fully desiccated, before storing at  $-20^{\circ}\text{C}$ . DNA extraction from dried leaf material and RFLP analysis of cpDNA and rDNA was performed according to the protocols summarized in Milne *et al.* (1999). For RFLP analysis of cpDNA, the pLsC cpDNA fragments cloned from *Lactuca sativa* (Jansen & Palmer 1987) were used as heterologous probes. All the pLsC probes employed fall within the large single copy (LSC) region of the cpDNA molecule except pLsC6 and part of pLsC4 which fall within the inverted repeat (IR) region. For rDNA analysis, the pTa71 rDNA fragment cloned from *Triticum aestivum* (Gerlach & Bedbrook 1979) was employed as the probe.

#### *Detection and characterization of cpDNA and rDNA RFLPs*

The cpDNA RFLP profiles of material from different

areas of the native distribution of *R. ponticum* were initially compared by examining single accessions from each area with a large set of enzyme:probe combinations. Single Turkish and Iberian accessions were compared using 22 restriction enzymes (*Bam*HI, *Bcl*II, *Bgl*III, *Cfo*I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*II, *Kho*I, *Kpn*I, *Mlu*I, *Nru*I, *Pst*I, *Sal*I, *Sca*I, *Sma*I, *Sst*I, *Stu*I, *Xba*I and *Xho*I) in combination with the following pLsC probes or sets thereof: pLsC4, pLsC8, pLsC2 + 6, pLsC1 + 16, and the combined pLsC probes 7, 9, 14 and 10 (7–10), which are arranged contiguously in the cpDNA molecule (Jansen & Palmer 1987). Thus, the screen involved a total of 110 enzyme:probe combinations. A comparison of single accessions from the three Iberian regions where *R. ponticum* occurs (southwest Spain, and southern and central Portugal) was then conducted using the same set of enzymes and probes, except that enzymes *Kho*I, *Mlu*I, *Nru*I and *Xba*I and the probe pLsC8 were excluded from the analysis, while seven enzymes not used previously were included, i.e. *Alu*I, *Apa*I, *Bst*EII, *Hae*III, *Hinf*I, *Pvu*I and *Rsa*I. Where differences in RFLP profiles were detected, a larger set of native accessions was examined with the relevant enzyme:probe combination(s) to determine whether differences were consistent between material sampled from the Iberian and Turkish regions, and also between material sampled from different localities within the Iberian region. Following this, a comparison was made between this material of *R. ponticum* and accessions of the other 15 *Rhododendron* species listed above to detect interspecific differences in cpDNA, using 28 enzyme:probe combinations as follows: *Bam*HI, *Bcl*II, *Bgl*III, *Cla*I, *Dra*I, *Eco*RI, *Hae*III, *Hind*III, *Hpa*II, *Rsa*I, *Sal*I, *Sma*I, and *Stu*I digests probed with pLsC7–10; *Bcl*II, *Bgl*III, *Cla*I, *Eco*RI, *Hpa*II, *Sma*I and *Stu*I digests probed with pLsC6; *Bcl*II, *Bgl*III, *Hpa*II, and *Stu*I digests probed with pLsC4; *Bam*HI and *Hpa*II digests probed with pLsC2; and *Bcl*II and *Bgl*III digests probed with the combined probes pLsC5, 11, 12 and 13 (5–13). Ultimately a small set of enzyme:probe combinations was employed that distinguished individual cpDNA haplotypes for material of *R. ponticum* from Spain, Portugal and Turkey, and also identified unique haplotypes for each of the other *Rhododendron* species examined. This allowed cpDNA haplotypes found in British naturalized material to be assigned to either *R. ponticum* from one of the stated native areas, or to one of the other *Rhododendron* species examined, or to an unidentified species not included in the analysis.

For rDNA RFLP analysis, the same material was screened with 13 restriction enzymes (*Bam*HI, *Bcl*II, *Bgl*III, *Cla*I, *Dra*I, *Eco*RI, *Hae*III, *Hind*III, *Hpa*II, *Rsa*I, *Sal*I, *Sma*I, *Stu*I) in combination with the rDNA probe pTa71. This was to identify rDNA polymorphisms within *R. ponticum* and rDNA fragments that distinguished *R. ponticum* from the other *Rhododendron* species surveyed, particularly those

**Table 2** Chloroplast DNA restriction fragment length polymorphisms (RFLPs) detected among Turkish and Iberian accessions of *Rhododendron ponticum*, and the character states of haplotypes defined. Haplotypes TA, TB, TD and TE were found only in Turkish accessions, while haplotypes IL and IE were present only in Iberian accessions

Enzyme	Probe (pLsC)	State	Fragments present	Number of accessions containing fragment(s)			Haplotypes in which state occurs*						
				Turkey	Portugal	Spain	TA	TB	TD	TE	IL	IE	
				(C.)	(S.)	(SW.)							
<i>Bgl</i> III	4	0	1.55	21	0	0	0	+	+	+	+		
		1	1.45	0	4	8	4					+	+
<i>Bgl</i> III	4	0	9.79	21	0	0	0	+	+	+	+		
		1	4.44 + 5.27	0	4	8	4					+	+
<i>Bcl</i> I	6	0	6.27 & 8.50	29	0	0	0	+				+	
		1	7.34 & 9.94	1	0	0	0		+				
		2	5.23 & 7.65†	1	0	0	0				+		
		3	5.23 & 7.65	0	10	30	18					+	+
<i>Bgl</i> III	6	0	5.81	20	0	0	0	+	+	+			
		1	6.75	1	0	0	0				+		
		2	4.53	0	4	8	4					+	+
<i>Sma</i> I	6	0	4.15	9	0	0	0	+	?	?	+		
		1	3.53	0	1	6	1		?	?		+	+
<i>Hind</i> III	6	0	12.25 & 14.91	4	0	0	0	+	?	?	?		
		1	10.41 & 12.98	0	1	2	1		?	?	?	+	+
<i>Hpa</i> II	7–10	0	2.13	60	10	32	0	+	+	+	+	+	
		1	2.09	0	0	1	26						+
<i>Hae</i> III‡	7–10	0	1.42	5	3	13	0	+	?	?	?	+	
		1	1.38	0	0	1	6		?	?	?		+

\*? indicates no accessions with this haplotype were examined with this combination.

†These fragment sizes are not certainly identical to those in Iberian material (see text).

‡*Hpa*II/pLsC7–10 and *Hae*III/pLsC7–10 detect the same length polymorphism.

species which morphological or historical evidence indicates were likely to have contributed germplasm to *R. ponticum* naturalized in the British Isles.

#### *cpDNA and rDNA types in R. ponticum naturalized in the British Isles*

With regard to cpDNA, all accessions sampled in the British Isles were first screened with a single enzyme:probe combination, *Hpa*II/pLsC7–10, which had been found to detect an apomorphy whose presence defined the most common haplotype among naturalized accessions. All accessions not possessing this haplotype were examined with a further six enzyme:probe combinations, i.e. *Bcl*I/pLsC6, *Bgl*III/pLsC4, *Bgl*III/pLsC6, *Dra*I/pLsC7–10, *Eco*RI/pLsC7–10, *Sma*I/pLsC6.

To determine rDNA type, all British accessions were examined with two enzymes, *Dra*I and *Sst*I, in combination with the rDNA probe pTa71, while a subset of 40 accessions was also examined with this probe and a third enzyme, *Eco*RV.

## Results

### *cpDNA variation in Iberian and Turkish material of Rhododendron ponticum*

Differences in cpDNA type between Iberian and Turkish accessions of *R. ponticum* (Table 2), were detected with the enzyme:probe combinations: *Bcl*I/pLsC6 (confirmed in 58 Iberian and 31 Turkish accessions); *Bgl*III/pLsC4 (confirmed in 16 Iberian and 21 Turkish accessions, based on two independent mutations); *Bgl*III/pLsC6 (confirmed in 16 Iberian and 21 Turkish accessions); *Hind*III/pLsC6 (confirmed in four Iberian and four Turkish accessions); and *Sma*I/pLsC6 (confirmed in eight Iberian and nine Turkish accessions). The following enzyme:probe combinations also appeared to detect fragment polymorphisms between the initial two accessions of *R. ponticum* surveyed, but were not investigated further using additional accessions: *Bcl*I/pLsC4; *Cl*aI/pLsC2 + 6; *Dra*I/pLsC2 + 6; *Hpa*II/pLsC6; *Sal*I/pLsC2 + 6; *Sst*I/pLsC4, *Sst*I/pLsC2 + 6, *Xba*I/pLsC2 + 6, and *Xba*I/pLsC1 + 16.

**Table 3** Sizes (kb) of two variable restriction fragments detected with *BclII*/pLsC6 and the difference between them for each *Rhododendron* taxon examined

Taxon	Length of smaller fragment	Length of larger fragment	Difference
<i>R. degrobianum heptamerum heptamerum</i>	5.14	7.30	2.16
<i>R. degrobianum yakushimanum</i>	5.14	7.30	2.16
<i>R. ponticum</i> (Iberian)	5.23	7.65	2.42
<i>R. smirnovii</i>	5.28	7.56	2.28
<i>R. hyperythrum</i>	5.42	7.65	2.23
<i>R. aureum</i>	5.47	7.85	2.38
<i>R. degrobianum heptamerum hondoense</i>	5.96	7.85	1.89
<i>R. makinonii</i>	5.96	7.85	1.89
<i>R. maximum</i>	5.78	8.16	2.38
<i>R. brachycarpum</i>	5.90	8.16	2.26
<i>R. caucasicum</i>	5.96	8.27	2.31
<i>R. ponticum</i> (Turkish)	6.27	8.50	2.23
<i>R. macrophyllum</i>	6.27	8.50	2.23
<i>R. catawbiense</i>	6.54	8.74	2.20
<i>R. ungerii</i>	6.97	8.74	1.77
<i>R. degrobianum degrobianum</i>	7.13	9.00	1.87
<i>R. arboreum</i>	5.14	7.30	2.16
<i>R. adenopodium</i>	5.28	7.30	2.02
<i>R. argyrophyllum</i>	5.28	7.30	2.02

*R. fortunei* was not examined with the *BclII*/pLsC6 enzyme:probe combination.

Of particular interest were restriction fragment profiles generated by *BclII*/pLsC6, in which two different fragments varied in size between Iberian and Turkish material. In Iberian material the respective size of these two fragments was 5.23 and 7.65 kb, whereas in Turkish material it was normally 6.27 and 8.50 kb, respectively (Table 3). These two fragments also varied in size from 5.14 and 7.30 kb to 7.13 and 9.00 kb, respectively, among the other *Rhododendron* taxa analysed (Table 3), and it was evident that their size variation was strongly correlated ( $r = 0.964$ ,  $P < 0.0001$ ), with the larger fragment estimated to be between 1.77 and 2.42 kb greater in size than the smaller fragment over all taxa examined (Table 3). In addition, the size of a single variable fragment within *BgIII*/pLsC6 profiles of all taxa was strongly correlated with that of both the larger ( $r = 0.911$ ;  $P < 0.0001$ ) and the smaller ( $r = 0.958$ ;  $P < 0.0001$ ) *BclII*/pLsC6 fragments. The correlated size variation between the fragments detected by *BclII*/pLsC6 and *BgIII*/pLsC6 is most probably due to a region of hypervariable length in the pLsC6 region of the cpDNA molecule, while the correlated size variation between the two *BclII*/pLsC6 fragments is probably caused by the probe pLsC6 falling within the IR region of the cpDNA molecule (Jansen & Palmer 1987). If a fragment was produced containing part of the IR probed by pLsC6 and also part of the adjacent LSC region, then a complementary fragment would also be produced containing the corresponding part of the other IR copy, and extending into the other end of the LSC region. These fragments would be terminated by sites in different positions in the LSC

region, causing them to differ in length. Associated variation in the sizes of the two fragments could then be generated by length or site changes in the part of the IR which the fragments contain.

However, among the accessions of *R. ponticum* examined from Turkey, one was detected with a unique size of the *BgIII*/pLsC6 variable fragment only (haplotype TE), one was detected with a unique size of the *BclII*/pLsC6 variable fragments only (TB), and one was detected which possessed a *BclII*/pLsC6 profile very similar to that of Iberian material (TD, Table 2). These additional findings indicate that among these rare haplotypes variation of pLsC6 fragments is not always associated, most probably due to site mutations occurring in the pLsC6 region and contributing to the observed polymorphisms.

Therefore it is likely, although not certain, that a single length polymorphism is responsible for all the differences in the pLsC6 profiles between Turkish and Iberian material detected by the restriction enzymes *BgIII*, *BclII*, *HindIII* and *SmaI*. In addition, two polymorphisms detected by *BgIII*/pLsC4 also distinguish material from the two regions; of these polymorphisms one is almost certainly a site gain in Iberian material; the other is uncertain in type (Table 2).

#### *cpDNA variation within Iberian material of R. ponticum*

In addition to the cpDNA polymorphisms that distinguished Iberian from Turkish material of *R. ponticum*, a single cpDNA polymorphism was detected among material

**Table 4** Chloroplast DNA (cpDNA) haplotypes present in accessions of *Rhododendron ponticum* from the British Isles and their source of origin

cpDNA haplotype	Number of accessions of <i>R. ponticum</i> from the British Isles	Percentage of total	Source of origin
IE	229	88.08	<i>R. ponticum</i> (mainly Spain)
IL	26	10.00	<i>R. ponticum</i> (Portugal)
TA	0	00.00	<i>R. ponticum</i> (Turkey)
CA	1	00.38	<i>R. catawbiense</i> (North America)
CB	1	00.38	<i>R. catawbiense</i> (North America)
XX	1	00.38	<i>R. maximum</i> (North America)
QA	1	00.38	Unknown, not <i>R. ponticum</i>
QB	1	00.38	Unknown, but possibly Iberian <i>R. ponticum</i>

from different regions within the Iberian Peninsula. This related to the presence or absence of a deletion of 0.04 kb in the pLsC7–10 region detected by *HaeIII* and *HpaII* (Table 2). This deletion was present in all 26 accessions examined from Spain and a single accession from southern Portugal. It was absent, however, from the cpDNA of the remaining 32 accessions surveyed from southern Portugal, from all 10 accessions examined from central Portugal, from all 60 accessions examined from Turkey and also from all other *Rhododendron* species examined. Iberian material which possessed the deletion was designated as haplotype IE, whereas Iberian material which lacked the deletion was designated as IL, while the common Turkish haplotype was designated as TA (Table 2).

#### cpDNA variation between *R. ponticum* and related taxa

Two enzyme:probe combinations, *DraI*/pLsC7–10 and *EcoRI*/pLsC7–10, resolved considerable cpDNA RFLP variation among the 16 *Rhododendron* species examined. *DraI*/pLsC7–10 treatment yielded 16 different fragment profiles, while *EcoRI*/pLsC7–10 resolved 13 different profiles. Using these enzyme:probe combinations together, it was possible to distinguish the cpDNA of all species from one another, in nearly all cases by at least two independent polymorphisms. For example, with *DraI*/pLsC7–10, *R. ponticum* was distinguished from other *Rhododendron* species by the presence of unique 3.03 and 2.79 kb fragments in its restriction profile. Similarly, using *EcoRI*/pLsC7–10, the American species *R. catawbiense* and *R. maximum* could be distinguished from other species by the replacement of a 2.58 kb fragment by a 1.93 kb fragment, and from each other by the presence of a unique 5.68 kb fragment in *R. maximum*.

#### cpDNA variation in naturalized British material of *R. ponticum*

Treatment of DNA extracts with *HpaII*/pLsC7–10 showed

that 229 of the 260 accessions examined of naturalized British *R. ponticum* possessed cpDNA haplotype IE. Treatment of extracts of the remaining 31 accessions with *BclI*/pLsC6, *BglIII*/pLsC4, *BglIII*/pLsC6, *DraI*/pLsC7–10, *EcoRI*/pLsC7–10 and *SmaII*/pLsC6, showed whether these accessions contained a different haplotype of *R. ponticum* or a haplotype of one of the other *Rhododendron* species examined. Of these accessions, 26 were shown to possess haplotype IL, one possessed a haplotype (QB) not detected in native material of *R. ponticum*, but which differed by only one mutation from haplotype IL (identified by *BclI*/pLsC6), and the remaining four accessions had haplotypes from species other than *R. ponticum*: two of these four accessions had cpDNA haplotypes (CA and CB) which matched those in material examined of *R. catawbiense*; one had a haplotype (XX) which matched that of *R. maximum*; and one had a haplotype (QA) acquired from an unidentified species (Table 4).

The common haplotype IE predominated in all areas and populations of the British Isles from which accessions of *R. ponticum* were sampled (Tables 5 and 6). The rarer haplotype IL was detected in all but two of the areas in Britain and in both areas of Ireland surveyed (Table 5). In Scotland, this haplotype was present in five of the eight populations examined (Table 6). The IE and IL haplotypes were shown to be randomly distributed among areas (Table 5;  $\chi^2 = 5.10$ ;  $P > 0.05$ ) and populations (Table 6;  $\chi^2 = 10.70$ ;  $P > 0.05$ ) in the British Isles.

#### rDNA variation between *R. ponticum* and related taxa

Iberian material of *R. ponticum* produced a *DraI* rDNA profile which distinguished it from all other taxa examined. Its rDNA also differed from Turkish material of *R. ponticum* in that, in common with other species of subsection Pontica, Iberian material possessed a 5.49 kb *DraI* fragment (confirmed in 59 accessions) which was replaced in Turkish material by a unique 6.30 kb fragment (confirmed in 40 accessions) (Milne 1997; Milne *et al.* 1999).

Area of British Isles	Number of accessions	cpDNA haplotype			rDNA type		
		IE	IL	Other	PP	CC	Other
Western Scotland	28	25	3	0	25	3	0
Scottish Islands	6	5	1	0	4	2	0
Kintyre	8	8	0	0	8	0	0
Eastern Scotland	24*	20	2	2 (CB, QB)	17	5	1 (QQ)
Fife & Lowlands	13	12	1	0	13	0	0
Northern England	11	10	1	0	10	1	0
Lake District	11	8	2	1 (QA)†	9	1	1 (QQ)†
Wales	14	13	1	0	13	1	0
East Anglia	7	5	2	0	7	0	0
Southern England	8	8	0	0	8	0	0
Southeast England	18	16	2	0	17	1	0
Killarney, Ireland	13	12	1	0	12	0	1 (PS)
Connemara, Ireland	16	14	2	0	15	0	1 (PS)
Total	177	156	18	3	158	14	4

\* Only 23 accessions were successfully examined with respect to rDNA type from this area.

†The individual with cpDNA type QA also possessed rDNA type QQ.

**Table 5** Distribution of chloroplast DNA (cpDNA) haplotypes and ribosomal DNA (rDNA) types in *Rhododendron ponticum* from different areas of the British Isles

Location	Number of accessions	cpDNA haplotype			rDNA type		
		IE	IL	Other	PP	CC	Other
Eastern Scotland							
Killin	5	3	2	0	3	2	0
Craigtoun	6	4	1	1 (CA)*	5	1	0
Loch Katrine	8	8	0	0	7	0	1 (MX)
Killiekrankie	9	6	2	1 (XX)	7	2	0
Western Scotland							
Gaerloch	12	10	2	0	6	6	0
Lochranza	14	13	1	0	14	0	0
Knapdale	14	14	0	0	14	0	0
Torridon	15	15	0	0	13	1	1 (PS)

\*This accession also possessed rDNA type CC.

**Table 6** Distribution of chloroplast DNA (cpDNA) haplotypes and ribosomal DNA (rDNA) types in Scottish populations of *Rhododendron ponticum*

*R. catawbiense* differed from Iberian material of *R. ponticum* by the presence in *R. catawbiense* of a unique 4.43 kb *DraI* fragment. In addition, all five accessions of *R. catawbiense* surveyed possessed a 3.95 kb *SstI* fragment that was present in only two of 59 Iberian accessions of *R. ponticum* examined. Comparison of the rDNA fragment profiles of native *R. ponticum* and *R. maximum* showed that they differed in the presence of two *EcoRV* bands (3.42 and 5.07 kb) in *R. ponticum* that were absent from *R. maximum* profiles. The rDNA of *R. arboreum* could also be distinguished from that of *R. ponticum*, and indeed all other *Rhododendron* species examined, by the presence of a unique *DraI* 4.51 kb fragment. All of *R. caucasicum*, *R. smirnovii*, *R. ungerii*, *R. macrophyllum*, *R. aureum*, and *R. hyperythrum* each produced unique rDNA profiles that

contained fragments not present in *R. ponticum*. The other taxa examined had rDNA profiles containing fragments which distinguished them from *R. ponticum* but not in all cases from one another (Milne *et al.* 1999; R. Milne, unpublished).

#### *rDNA variation in naturalized material of R. ponticum*

In no naturalized British accession of *R. ponticum* was the 6.30 kb *DraI* fragment characteristic of Turkish material of *R. ponticum* detected. Therefore all naturalized material either contained rDNA profiles which matched Iberian *R. ponticum* or appeared to be those of another species or an additive profile of another species and Iberian *R. ponticum*.

rDNA type	Fragments (kb) present or absent for three restriction enzymes:							Source of origin
	<i>Sst</i> I	<i>Sst</i> I	<i>Sst</i> I	<i>Dra</i> I	<i>Dra</i> I	<i>Eco</i> RV	<i>Eco</i> RV	
	3.95	4.93	2.81	4.43	6.52	3.42*	5.07*	
PP	-	-	-	-	-	+/?	+/?	<i>R. ponticum</i>
PS	+	-	-	-	-	+/?	+/?	<i>R. ponticum</i>
CC	+	-	-	+	-	+/?	+/?	<i>R. catawbiense</i>
QQ	-	+	+	-	+	+/?	+/?	Unknown
MX	-	-	-	-	-	-	-	<i>R. maximum</i>

\*? indicates that some accessions were not examined with *Eco*RV and in these the presence/absence of this fragment was not recorded.

Accessions of *R. ponticum* from the British Isles with rDNA restriction profiles containing both the 4.43 kb *Dra*I and 3.95 kb *Sst*I fragments that characterized the rDNA profiles of native *R. catawbiense* were considered to be introgressed with *R. catawbiense* rDNA and were labelled CC (Table 7). Accessions containing only the 3.95 kb *Sst*I fragment were labelled PS, while those containing neither of these two fragments were designated PP (Table 7). Of the 259 accessions of *R. ponticum* from the British Isles that were screened successfully for rDNA type, 26 were classified as CC type, three were of PS type, and 227 (i.e. all but three of the remaining accessions) were designated as nonintrogressed PP type (Tables 5 and 6).

As the rDNA profile of *R. maximum* only differed from that of *R. ponticum* in that the former lacked two *Eco*RV fragments, an additive profile of the two species could not be distinguished from that of *R. ponticum*. However, one accession of naturalized *R. ponticum* had an *Eco*RV rDNA profile which lacked both fragments that distinguish *R. ponticum* from *R. maximum* and hence matched that of *R. maximum* (i.e. was of MX rDNA type, Table 7). This accession was therefore considered to be introgressed with rDNA from *R. maximum*. The two remaining accessions both contained an rDNA type designated as QQ. The *Dra*I and *Sst*I profiles of this type included all fragments that characterized PP rDNA, but in addition contained a 6.52 kb *Dra*I fragment, and also two *Sst*I fragments (4.93 and 2.81 kb in length, Table 7). These profiles did not match those generated for any other *Rhododendron* species examined, and therefore a possible donor for this type of rDNA could not be determined. One of the two accessions with this rDNA type also possessed the cpDNA haplotype QA.

For no accession was a *Dra*I profile produced which contained the distinctive 4.51 kb fragment of *R. arboreum*, or the diagnostic fragments for any of the other *Rhododendron* species which were examined. Consequently no evidence was obtained of naturalized *R. ponticum* in the British Isles being introgressed with rDNA from *R. arboreum* or

**Table 7** Ribosomal DNA (rDNA) types present in naturalized material of *Rhododendron ponticum* from the British Isles based on restriction fragment profiles

any species of subsection Pontica other than *R. maximum* and *R. catawbiense*.

#### *Frequency and distribution of introgressed individuals of R. ponticum in the British Isles*

Based on the results of both cpDNA and rDNA analyses, it was evident that 27 accessions of *R. ponticum* from the British Isles were introgressed with genetic material from *R. catawbiense* (i.e. possessed either cpDNA type CA or CB and/or rDNA type CC; Table 8). Individuals of PS rDNA type might also contain introgressed material from *R. catawbiense* but are excluded from the introgressed category as type PS occurs in native *R. ponticum*. In addition, two accessions were introgressed with genetic material from *R. maximum*, and a further two were introgressed with material from an unknown species. The geographical distribution of accessions introgressed with *R. catawbiense* genetic material did not deviate from a random one between areas in Britain ( $\chi^2 = 11.29$ ,  $P > 0.05$ ). However, among the eight Scottish populations surveyed the distribution of introgressed individuals was clearly nonrandom ( $\chi^2 = 18.6$ ,  $P < 0.02$ ). Tests were conducted using the binomial distribution to determine whether the observed frequency of introgressed plants within a given area of Britain or within a particular population was significantly greater than the proportion of accessions throughout Britain showing introgression from *R. catawbiense* (Table 8). This proportion was taken to be the proportion of British individual accessions (excluding those introgressed by other species) introgressed by *R. catawbiense*, i.e.  $15$  of  $145 = 0.103$  (Table 8). As a sample size of 30 or more was required to show that introgressed types were significantly less frequent than average in any population or area, this part of the analysis could not be attempted. The binomial analysis demonstrated a significantly high (6 of 22 = 27%;  $P < 0.025$ ) proportion of introgressed accessions in the eastern Scotland area (Table 8). Apart from eastern Scotland, all the other areas in which

Area	Total no. of accessions*	No. of accessions possessing CC rDNA type and/or CA or C cpDNA haplotype	<i>P</i>
Britain			
Western Scotland	28	3	0.565
Scottish Islands	6	2	0.121
Kintyre	8	0	1
Eastern Scotland	22	6	0.021
Fife & Lowlands	13	0	1
Northern England	11	1	0.699
Lake district	10	1	0.664
Wales	14	1	0.783
East Anglia	7	0	1
Southern England	8	0	1
Southeast England	18	1	0.860
Total	145	15	
Ireland			
Killarney	13	0	1
Connemara	16	0	1
Scottish populations			
Killin	5	2	0.087
Craigtoun	6	1	0.481
Loch Katrine	7	0	1
Killiekrankie	8	2	0.197
Gaerloch	12	6	0.0007
Lochranza	14	0	1
Knapdale	14	0	1
Torridon	15	1	0.806
Overall total	255	27	

\*Excluding accessions for which there is evidence of introgression from species other than *R. catawbiense*.

introgressed types comprised at least 10% of accessions surveyed were in the northern half of Britain (i.e. the Lake District, northern England, western Scotland, Scottish Islands; Table 8). Indeed, introgressed types appeared to be three times more common in the northern than the southern half of Britain (12 of 86 accessions compared with two of 45). However, the difference was not significant ( $\chi^2 = 1.90$ ,  $P > 0.05$ ). Among the eight Scottish populations surveyed, the sample from Gaerloch had a significantly higher proportion (50%) of introgressed types than expected ( $P < 0.001$ ; Table 8). Because only four accessions of *R. ponticum* showed introgression from species other than *R. catawbiense* (i.e. possessed cpDNA types QA or XX and/or rDNA types QQ or MX) no detailed analysis was made of the distribution of these accessions throughout the British Isles.

No individuals possessing the CC rDNA type were found among the 29 accessions of *R. ponticum* examined from Ireland (Table 5); however, there were insufficient accessions to prove statistically that individuals of this type were rarer there than in other parts of the British

**Table 8** Distribution of accessions possessing CC ribosomal DNA (rDNA) type and/or chloroplast DNA (cpDNA) haplotype CA or CB in naturalized material of *Rhododendron ponticum* in the British Isles. The probability (*P*) that the observed frequency of such individuals in each area is not greater than that throughout the British Isles is also given

Isles. In contrast, rDNA type PS was more frequent among Irish accessions than among those from Britain (Tables 5 and 6), i.e. two of 29 in Ireland compared with one of 201 accessions examined in Britain excluding CC, QQ and MX rDNA types ( $P = 0.0408$ ).

## Discussion

### *Source of Rhododendron ponticum naturalized in the British Isles*

The survey of cpDNA and rDNA variation reported here provides strong evidence that material of *R. ponticum* that is naturalized in the British Isles is entirely Iberian in origin. Any *R. ponticum* of Turkish origin introduced and cultivated in the British Isles has contributed no cpDNA or rDNA, and very probably no other nuclear genetic material, to the naturalized populations. The finding that all naturalized material appears to be of Iberian origin might be because this material was introduced to Britain first and was plentiful in cultivation by the time Turkish

material arrived (Curtis 1803). Alternatively, it may be because Iberian material seems hardier and easier to grow in the British Isles (Royal Botanic Gardens Edinburgh staff, personal communication). Two cpDNA haplotypes, IE and IL, were detected within Iberian material and both were present in naturalized *R. ponticum* in the British Isles. The IE haplotype, which was far more common in naturalized material, was present in all 26 individuals surveyed from southern Spain, but in only one of 33 accessions sampled from southern Portugal. The remaining 32 accessions from southern Portugal possessed the IL cpDNA haplotype, as did all 10 accessions examined from central Portugal. Given the rarity of haplotype IE among Portuguese accessions, it is likely that British material of *R. ponticum* with haplotype IE is of Spanish descent. Thus, molecular evidence supports historical records that introductions (including the original one) were made from Spain (Coats 1963). In Spain, *R. ponticum* occurs only in the extreme southwest, i.e. within a narrow stretch of land up to 10 km wide and a little under 40 km in length that runs from the coast at Tarifa to the Sierra del Aljibe inland (Sales 1997). The 26 accessions surveyed from this area were sampled from four sites in the southern and two sites in the northern half of the species distribution (Table 1). As less sites were sampled than in Portugal, it remains possible that haplotype IE occurs in low frequency in Spain as does haplotype IL in Portugal. It thus cannot wholly be discounted that British material with haplotype IL was derived from one plant of this haplotype introduced from Spain among others with type IE. However, the more likely hypothesis is that whereas all naturalized material which possessed the IE haplotype (i.e. 89% of accessions surveyed) is of Spanish origin, naturalized material containing the IL haplotype (i.e. 10% of accessions examined) is Portuguese in origin.

#### *Distribution of cpDNA haplotypes in R. ponticum naturalized in the British Isles*

If the relative frequencies of cpDNA haplotypes IL or IE in *R. ponticum* had been found to vary significantly between areas or populations in the British Isles it might indicate that introductions from different sources or cultivated stock had occurred, as was reported for the invasive alien *Bromus tectorum* in North America based on randomly amplified polymorphic DNA (RAPD) markers (Novak & Mack 1993; Novak *et al.* 1993). However, no evidence of such was found in the present study, and the data are consistent with all material having arisen from the same pool of cultivated stock. The rare haplotype IL probably occurs in all areas of the British distribution of *R. ponticum*, having been detected at low frequency in all regions except two (southern England and the Kintyre Peninsula, western Scotland) where its apparent absence

is probably due to small sample numbers (eight accessions in each case). The presence of both haplotypes in five of the eight Scottish populations from which multiple accessions were examined, indicates that plantings which gave rise to such populations often comprised both haplotypes. Thus it is feasible that multiple introductions were made to the British Isles, and at an early point in the species' cultivated history all such material was allowed to mix and interbreed in what was effectively a single population. From this original stock, plantings were made giving rise in time to naturalized material. Most populations appear to have arisen independently from a single planting, so it is unsurprising that no regional trend is observed. The possibility of a single cultivated cpDNA pool gains further support from the survey of cpDNA variation in Irish material. Although only 29 accessions were examined from Ireland, it is notable that the proportion of IL haplotypes (9.67%) is very similar to that on the British mainland (10%), which indicates that British and Irish material were probably derived from the same original cultivated or wild source, rather than deriving from separate introductions into cultivation. That said, the higher proportion of rDNA type PS in Irish material indicates a possible difference in nuclear composition between Irish and British material, which could result, for example, from a bottleneck caused by the introduction of a relatively small number of cultivated individuals from Britain to Ireland.

#### *Evidence for introgression from R. catawbiense*

Twenty-six accessions of naturalized *R. ponticum* possessed the CC rDNA type, that was diagnostic of native *R. catawbiense* germplasm, and were therefore considered as introgressed with *R. catawbiense* rDNA. Allozyme markers have been shown to exhibit Mendelian inheritance in complex *Rhododendron* hybrids (Krebs 1996) and it is possible that rDNA markers are inherited in the same way. If so, then *R. catawbiense* would be homozygous for the allele, C, which causes possession of the two fragments which characterize its rDNA type, while *R. ponticum* would be homozygous for the recessive allele causing absence of the two fragments. If genotype frequencies in naturalized material of *R. ponticum* are at Hardy–Weinberg equilibrium with respect to these two alleles, then nearly all observed CC rDNA types would be heterozygous, and as these form 10% of naturalized material, the C allele must be present at just over 5% frequency. However, if the C allele is unevenly distributed, the proportion of homozygotes and thus of the C allele would be slightly higher. This proportion of the C allele provides an estimate of the average proportion of *R. catawbiense* germplasm in naturalized British material of *R. ponticum*, i.e. just over 5%. If Hardy–Weinberg equilibrium is assumed within

areas where CC types are significantly more frequent than others, then estimates of 15% *R. catawbiense* rDNA in the eastern Scotland region and 29% in the Gaerloch population are obtained.

In contrast, the two cpDNA haplotypes of *R. catawbiense* were each detected in single naturalized *R. ponticum* accessions only (from Perthshire, eastern Scotland, and the Craigtoun population, Fife, eastern Scotland). Therefore, less than 1% of British accessions had the cpDNA of *R. catawbiense* compared with a 5% proportion of nuclear rDNA. This discrepancy can best be explained by *R. ponticum* being the favoured maternal parent in crosses with *R. catawbiense*. Possible explanations for this are: *R. ponticum* might have been chosen by breeders as the seed parent; it might set seed more readily; nuclear material of *R. ponticum* might be less compatible with *R. catawbiense* cytoplasm than the reverse; or introgression might occur following pollen transfer from cultivated *R. catawbiense* individuals to naturalized *R. ponticum* plants.

Further evidence that naturalized *R. ponticum* in the British Isles is introgressed with *R. catawbiense* germplasm is provided by the morphological characteristic of ovary hair. Ovary hair is absent in native material of *R. ponticum*, but is present in most other *Rhododendron* species, including *R. catawbiense* (Chamberlain 1982). Its presence in naturalized *R. ponticum* in the British Isles has been suggested to indicate introgression from *R. catawbiense* (Clapham *et al.* 1987). Of the accessions collected from Scottish populations, almost half (41 of 83) had some degree of ovary hair. In the Gaerloch population, where six accessions had the CC rDNA type and six the PP rDNA type, ovary hair was present in two accessions with CC rDNA but four accessions with PP rDNA type. The presence of ovary hair and CC rDNA were also not correlated in their occurrence in the other Scottish populations. If these characters in British *R. ponticum* have the same origin through introgression, then these characters have to an extent become dissociated from one another in their occurrence. Their combined analysis, however, indicates that introgression from *R. catawbiense* in this population is possibly universal, rather than concentrated in a few individuals.

It is of interest that the data from individual accessions of naturalized *R. ponticum* suggest that the level of introgression from *R. catawbiense* is greater in eastern Scotland than elsewhere in the British Isles. The mean January temperature in eastern Scotland is the lowest of the areas surveyed, 2–2.5 °C (adjusted to sea level; Brown *et al.* 1992), whereas the range for Iberian localities of native *R. ponticum* is around 10–12 °C (de Blij & Muller 1993). *R. catawbiense* has better cold tolerance than *R. ponticum* (Davidian 1982; Nilsen 1991), and consequently introgression from *R. catawbiense* may confer increased cold tolerance on *R. ponticum*, which could in turn provide a selective advantage for introgressed individuals in colder

areas such as eastern Scotland. It is possible that the observed increased frequency of introgressed individuals in eastern Scotland results from horticultural selection, i.e. growers were aware that 'hardy hybrids' (i.e. cultivars of *R. ponticum* crossed with *R. catawbiense*, *R. maximum* and sometimes *R. arboreum*; Bean 1976) or other introgressed cultivars of *R. ponticum* were more hardy in colder areas and stocked their gardens accordingly. Alternatively, natural selection may have acted on the *R. ponticum* populations during and after naturalization, favouring individuals with a higher level of introgression from *R. catawbiense* in colder regions only. A third possibility, however, is that the trend results simply from differing aesthetic tastes or breeding programmes in different parts of the British Isles. Further detailed investigation is required to prove that natural selection has acted to favour introgressed material in colder parts of the British Isles since naturalization. The findings of such research will be of considerable value given the current paucity of information on the adaptive significance of introgressive hybridization. Increased temperature tolerance due to introgression has been demonstrated in the fruitfly *Dacus tryoni* (Lewontin & Birch 1966), although as both species involved had similar original temperature tolerances this is probably an example of transgressive segregation (Rieseberg *et al.* 2000) rather than adaptive trait transference. Introgression accompanied by broadened habitat tolerance has also been observed in the native plant species *Cypripedium pubescens* (Klier *et al.* 1991) and *Helianthus divaricatus* (Heiser 1979), and the introduced or invasive species *H. annuus* (Heiser 1951; Rieseberg *et al.* 1990a), *Lythrum salicaria* (Strefeler *et al.* 1996) and *Bothriochloa intermedia* (Harlan & deWet 1963), although in no case has a direct link been proved.

#### *Introgression from other Rhododendron species*

Evidence that naturalized *R. ponticum* is introgressed with genetic material from two other *Rhododendron* species (i.e. *R. maximum* and an unidentified species) was also detected in the present study. The cpDNA of *R. maximum* was clearly identified in one accession of naturalized *R. ponticum*, and its rDNA type was detected in another accession. Both accessions were from eastern Scotland. Because time did not allow the majority of accessions to be examined with *EcoRV* in combination with the rDNA probe, and an additive *EcoRV* rDNA profile of *R. ponticum* and *R. maximum* could not be distinguished from that of pure *R. ponticum*, it is probable that further accessions which contained introgressed germplasm of *R. maximum* were overlooked in this survey. This is borne out by the results of morphological examination (Milne 1997). Neither *R. ponticum* nor *R. catawbiense* normally have calyx lobes 1 mm long or more, whereas those of *R. maximum* are typically 3–5 mm (Chamberlain 1982). Among the eight

Scottish populations examined, 20 of 83 accessions had calyx lobes between 2 and 8 mm long, and a further 19 had lobes of 1–1.5 mm. This indicates that *R. maximum*, or another species with long corolla lobes, contributed to their ancestry.

Germplasm from an unidentified *Rhododendron* species was detected in two accessions from well-separated localities (the Lake District, northwest England; and Glenshee, eastern Scotland). As these widely separated accessions most probably result from separate naturalization events, it is probable that the unidentified species in question is one which has been deliberately crossed with *R. ponticum*. It is less likely, with so many *Rhododendron* species in cultivation, that chance introgression would have occurred from the same cultivated species in two separate events.

No molecular evidence of introgression from *R. arboreum* was detected. However, dark red colouring on the corolla and red to crimson corolla markings were noted in, respectively, 23 and 20 of 83 accessions from Scottish populations examined (Milne 1997), and as these characteristics do not occur within subsection Pontica (Chamberlain 1982), their presence in naturalized *R. ponticum* must result from introgression from a species outside this subsection, e.g. *R. arboreum*. Further molecular analysis is required to confirm whether *R. arboreum* has been involved in the ancestry of naturalized *R. ponticum* in the British Isles.

## Conclusions

This study has demonstrated the suitability of cpDNA markers for tracing the origins of an introduced species. Based on our finding that all naturalized *Rhododendron ponticum* in the British Isles is derived from material introduced from the Iberian Peninsula, it is recommended that future studies on the ecology, and possible control measures for, *R. ponticum*, involve comparisons between the British and Iberian material and do not consider Turkish material, except as a possible source for control organisms. The presence of two Iberian cpDNA haplotypes among naturalized material indicates that the latter probably contains more genetic variability than any single native Iberian population. Moreover, naturalized material has gained additional genetic variation through crossing with at least three other species, of which the most important is likely to have been *R. catawbiense*. It is suggested that increased cold tolerance has been conferred through this introgression, and that this may have facilitated the colonization of colder parts of the British Isles.

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This work formed part of the research conducted for a PhD by Richard Milne on the molecular systematics of *Rhododendron ponticum* and its close allies. Also investigated were phylogenetic relationships within this group of species, and the occurrence of hybridization between *Rhododendron* species in Turkey. Richard Abbott investigates evolutionary questions concerning introgression, hybrid speciation and phylogeography.

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