RESEARCH NOTE

Molecular markers unravel intraspecific and interspecific genetic variability in *Plantago ovata* and some of its wild allies

SHIVANJALI KOTWAL, MANOJ K. DHAR, BALBIR KOUR, KULDEEP RAJ and SANJANA KAUL*

Genome Research Laboratory, School of Biotechnology, University of Jammu, Jammu 180 006, India

[Kotwal S., Dhar M. K., Kour B., Raj K. and Kaul S. 2013 Molecular markers unravel intraspecific and interspecific genetic variability in *Plantago ovata* and some of its wild allies. *J. Genet.* **92**, 293–298]

Introduction

Genus *Plantago* of family Plantaginaceae consists of ~200 species (Rahn 1996). *Plantago ovata* Forsk. is a medicinal herb cultivated extensively in western India for seed husk known as blonde psyllium or isabgol (in Hindi). Besides being used as laxative, psyllium is also used in ice creams, chocolates, cosmetics and in printing and finishing. It lowers blood cholesterol levels considerably and has been used as an indigenous Ayurvedic and Unani medicine for a wide range of bowel problems including chronic constipation, amoebic dysentery and diarrhea (Dhar *et al.* 2011).

Plantago ovata has a small genome of 621 Mb based on four ($2n = 2x = 8$) heterochromatin rich chromosomes. It has a narrow genetic base and therefore lacks in-built variation (Dhar *et al.* 2009). Thus, it exhibits low chiasmata frequency and low recombination index. Wild species of *Plantago* are potential sources of several important genes, which if transferred to *P. ovata*, can revolutionize the production of psyllium (Dhar *et al.* 2011). However, to develop efficient interspecific crosses it is essential to determine their phylogenetic relationships. Although some workers have tried nuclear ribosomal DNA internal transcribed spacer (ITS) regions for this purpose (Ronsted *et al.* 2002; Dhar *et al.* 2006), it would be worthwhile to use molecular markers, especially amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers, which can later be followed in hybrids. The present investigation was aimed at detection of genetic variability in this species and several of its wild allies using molecular markers which was not otherwise possible through conventional methods. If successful, such a study would provide a platform for further genetic studies including the development of a mapping population.

*For correspondence. E-mail: sanrozie@rediffmail.com.

Keywords. AFLP; SSR; genetic diversity; wild allies; *Plantago ovata*.

Material and methods

Plant material

Genomic DNA was isolated from young leaves of accessions of *P. ovata* and some of its wild allies (table 1) by following the protocol of Doyle and Doyle (1987) with slight modifications. DNA was purified and quantified using standard procedures.

AFLP analysis

For AFLP analysis, AFLP kit (Invitrogen Life Technologies, New York, USA) was used following manufacturer's instructions. Among 20 primer pair combinations, eight combinations (*Eco*RI-AGC/*Mse*I-CAT, *Eco*RI-AGG/*Mse*I-CAA, *Eco*RI-ACT/*Mse*I-CTC, *Eco*RI-ACC/*Mse*I-CTA, *Eco*RI-ACT/*Mse*I-CTG, *Eco*RI-AGG/*Mse*I-CAT, *Eco*RI-AAC/*Mse*I-CAC and *Eco*RI-ACG/*Mse*I-CAG) were finally used. Genomic DNA (0.5 μg) was first digested with 5 units each of *Eco*RI and *Mse*I for 1 h and then 500 pmol *Mse*I adapter and 50 pmol of *Eco*RI adapter were ligated to the restriction fragments with 1 unit T4 DNA ligase for 3 h. Restriction and ligation were performed in 1× RL buffer at 37°C. The PCR conditions for preamplification and selective amplification were as described by Vos *et al.* (1995). The amplified product was resolved on 10% polyacrylamide gel (PAGE) and visualized autoradiographically.

SSR analysis

For SSR analysis, various accessions of *P. ovata* and its wild allies were used (table 1). Initially, 18 SSR primers; three primers reported earlier for *Plantago* sp. (Squirrell and Wolff 2001) and 15 primers based on *Malus* sp. and *Phaseolus* sp. (unpublished data) were used for PCR amplification on five samples. Finally, 12 primers A3, A4, A6, BB1, BB2,

Table 1. *Plantago ovata* and its wild relatives used for genetic diversity analysis.

Accession no.	Species	Original source	Marker analysis	
			AFLP	SSR
Pov78	*Plantago ovata*	USDA	+	−
Pov79	*P. ovata*	USDA	+	+
Pov80	*P. ovata*	USDA	+	−
Pov83	*P. ovata*	USDA	+	+
Pov85	*P. ovata*	USDA	+	−
Pov86	*P. ovata*	USDA	+	+
Pov90	*P. ovata*	USDA	+	+
Pov93	*P. ovata*	USDA	+	+
Pov25	*P. ovata*	Jammu, India	+	+
A2	*P. ovata*	Jammu, India	−	+
B3	*P. ovata*	Jammu, India	−	+
B5	*P. ovata*	Jammu, India	−	+
Pov26	*P. ovata*	Gujarat, India	+	−
Par	*P. arenaria*	RBG, Kew	+	+
Pse01	*P. serraria*	RBG, Kew	+	+
Pco01	*P. coronopus*	RBG, Kew	+	−
Pin01	*P. indica*	RBG, Kew	+	+
Pla	*P. lagopus*	RBG, Kew	+	+
Pma01	*P. maritima*	RBG, Kew	+	+
Prh01	*P. rhodosperma*	Kansas, USA	+	−
Pru01	*P. rugelii*	Kansas, USA	+	−
Pex01	*P. exigua*	Jammu, India	+	+
Pmj01	*P. major*	Jammu, India	+	−
Pln01	*P. lanceolata*	Jammu, India	+	−

+, indicates the presence of taxa in the particular study; −, indicates absence of taxa.

BB3, BB4, BB5, BB6, BB7, BB8 and BB9 showing best amplification were selected. DNA ladder, 100 bp was used as the marker. Polyacrylamide gel (PAGE), 10% was used to resolve the amplified products and silver staining was done for fragment visualization.

Data analysis

For AFLP and SSR analyses, separate binary data matrices were generated indicating the presence or absence of the specific band as 1 or 0, respectively. The genetic association among taxa was evaluated by calculating the Jaccard's similarity coefficient (Jaccard 1908) (see tables 1, 2 and 3 in electronic supplementary material at http://www.ias.ac.in/jgenet/) for pairwise comparisons based on the proportion of shared bands produced by the primers. A distance matrix was generated using Nei and Li similarity index (Nei and Li 1985), which provides the genetic distance between two samples. The distance matrix was further analysed using PHYLIP 3.5 program (Felsenstein 1981). The tree was prepared using TreeView software (taxonomy.zoology.gla.ac.uk/rod/treeview.html). The polymorphic information content (PIC) was calculated as proposed by Botstein *et al.* (1980) as $\text{PIC} = 1 - \Sigma p_i^2$, where p_i^2 is the allele frequency of the ith allele. The marker index (MI) was calculated by using the formula $\text{MI} = EH_{\text{av}}$, where E is effective multiplex ratio and H_{av} average heterozygosity. The marker index is the product of diversity index and effective multiplex ratio (EMR), where EMR (E) is the number of loci polymorphic in the germplasm of interest analysed per experiment. Resolving power (Prevost and Wilkinson 1999) was used to identify the primers that could distinguish samples most efficiently. For bootstrapping analysis FreeTree software was used and TreeView (www.natur.cuni.cz/flegs/programs/freetree.htm) was used for representation of the trees.

Results and discussion

Several primer combinations with different numbers of selective bases at the 3′ end were initially tested for generating AFLP fingerprints. The combination having three selective bases revealed excellent polymorphism. *Eco*RI + 3 combined with *Mse*I + 3 resulted in amplification of fairly high number of bands which could be easily separated on a sequencing gel. Out of eight primer combinations tested, the band profile of six primer combinations was unambiguous, therefore, final scoring was based on six primer combinations (table 2). In different accessions of *P. ovata*, primer combination *Eco*RI-ACT/*Mse*I-CTG resulted in a minimum of 104 bands (figure 1a) and primer combination *Eco*RI-AGC/*Mse*I-CAT resulted in the maximum number of bands

Table 2. Analysis of polymorphism generated by AFLP markers among different accessions of *Plantago ovata* and its wild allies.

Primer	Monomorphic bands		Polymorphic bands		Total bands		% Polymorphism		PIC		MI	
	Plantago ovata	Wild allies	*Plantago ovata*	Wild allies	*Plantago ovata*	Wild allies	*Plantago ovata*	Wild allies	*Plantago ovata*	Wild allies	*Plantago ovata*	Wild allies
A	61	0	101	194	162	194	62.3	100.0	0.58	0.86	58.6	166.8
B	84	13	51	161	135	174	37.8	92.5	0.41	0.60	20.9	96.6
C	108	11	10	114	118	125	8.5	91.2	0.18	0.67	1.8	76.3
D	55	07	66	131	121	138	54.5	94.9	0.34	0.66	22.4	86.4
E	26	0	78	158	104	158	75.0	100.0	0.54	0.89	42.1	140.6
F	24	01	95	139	119	140	79.8	99.2	0.72	0.92	68.4	127.8

Primer combinations: A, *Eco*RI-AGC/*Mse*I-CAT; B, *Eco*RI-AGG/*Mse*I-CAA; C, *Eco*RI-ACT/*Mse*I-CTC; D, *Eco*RI-ACC/*Mse*I-CTA; E, *Eco*RI-ACT/*Mse*I-CTG; F, *Eco*RI-AGG/*Mse*I-CAT.

(figure 1b). The percentage polymorphism of all the primers varied between 8.5 to 79.8%. The PIC value for AFLP primers ranged from 0.60 to 0.89 in different species of *Plantago*, whereas, as it ranged from 0.18 to 0.72 in different accessions of *P. ovata.*

The results of SSR analysis were interesting. Variable number of alleles (4–11) was observed in the samples used for the present study. The highest number of alleles was detected by primer pair A6 while the primer pair BB7 resulted in the detection of minimum number of alleles (table 3). The allelic variation was also observed in various accessions of *P. ovata* and it ranged from 3 to 7. In the present study primer pair BB8 helped in discriminating various *P. ovata* accessions more efficiently than the others (figure 1, c–e). PIC values ranged from 0.34 to 0.77. In addition to the PIC, expected heterozygosity (H_e) and observed heterozygosity (H_o) were also calculated for each primer pair (table 3).

TreeView program was used to generate dendrogram of the AFLP scoring data of different accessions (figure 2a). Out of the 10 accessions of *P. ovata* used in the study, Pov83 forms an outgroup. Four major clusters were formed of the remaining nine accessions; the first cluster includes Pov80 and Pov90. Second cluster includes accessions Pov85 and Pov86. Third cluster consists of two accessions Pov26 and Pov79. Fourth cluster is made of three accessions, Pov78, Pov25 and Pov93. Out of these, the last two are genetically closer than the first. Overall, the genetic variability in the set of accessions of *P. ovata* is limited. However, AFLP seems to bring out whatever little variability exists but is hidden.

Analysis of the dendrogram constructed on the basis of AFLP scoring data of different species of *Plantago* revealed interesting results: only four clusters were formed. The first cluster consists of *P. coronopus* and *P. serraria* which is in agreement with the ITS data of Ronsted *et al.* (2002). Both these species belong to subgenus *Coronopus*. The second cluster consists of two species, namely *P. lagopus* and *P. lanceolata*. Both these species belong to subgenus *Bougueria* section *Lanceifolia* (Rahn 1996) and thus were expected to group together. The third cluster consists of four species, namely *P. indica*, *P. major*, *P. rhodosperma* and *P. rugelii*. This result is in complete agreement with the data on ITS regions generated by Dhar *et al.* (2006) and Ronsted *et al.* (2002). The fourth cluster is of *P. arenaria* and *P. exigua*, which is also in line with the phylogenetic work of Rahn (1996), wherein he has placed both these species in subgenus *Psyllium*. Placement of *P. maritima* in the present tree is surprising where it forms an outgroup. The species belongs to subgenus *Coronopus* and should have therefore clustered with species in the first cluster, which would also be in agreement with the results obtained on the basis of ITS regions and morphological data. Since ITS represents a specific region of the genome, while AFLP markers are randomly distributed in the genome, therefore, *P. maritima* may be an example where the two markers do not agree with the data on morphology and other characteristics. Another possibility is that it may be a case of mistaken identity (figure 2b). The AFLP molecular marker technique is considered to be the most suitable, reliable and appropriate method for the analysis of genomes for which limited information is available, because it is more reproducible than other molecular markers and moreover, AFLP profile does not alter with minor variations in experimental conditions (Tomkins *et al.* 2001).

SSRs, like AFLP are PCR-based markers are codominant in nature, multiallelic and uniformly dispersed throughout plant genomes. SSR primers show cross genus and cross species amplification (Liebhard *et al.* 2002; Yamamoto *et al.* 2004). Yamamoto *et al.* (2004) examined SSRs derived from pear and apple for cross genus amplification in quince (*Cydonia oblonga* Mill). In their study, 50% pear markers and 73% apple markers showed amplification in quince. In the present study, 83.3% of the primers showed cross amplification in majority of the *Plantago* species while 50% of them showed polymorphism.

During the present investigation, the dendrogram constructed on the basis of SSR scoring data (figure 2c) clearly revealed *P. indica* as an outgroup. The remaining taxa could be divided into two groups; one based on single species,

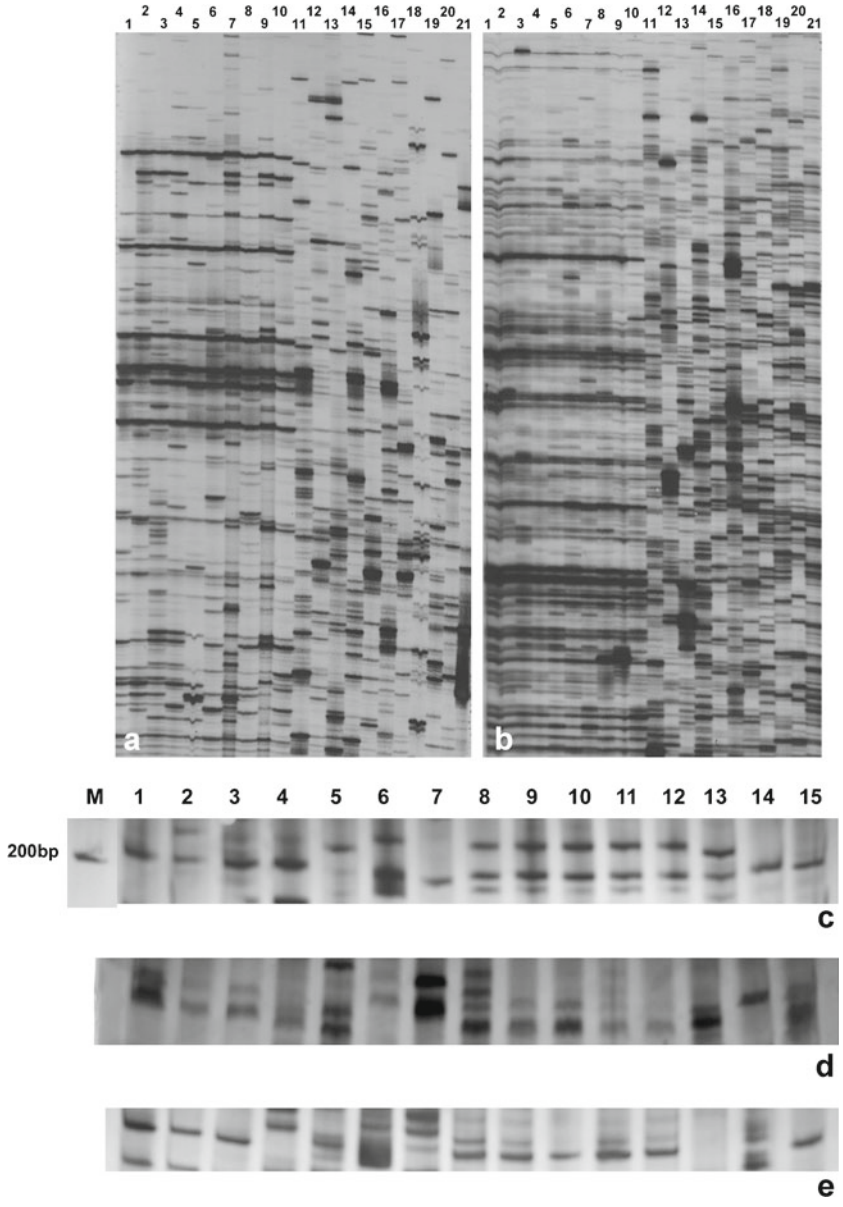

Figure 1. (a, b) AFLP profiles generated with two different primer combinations. Lanes 1 to 10 represent different accessions of *P. ovata* namely, Pov80, Pov90, Pov86, Pov85, Pov79, Pov26, Pov78, Pov25, Pov83 and Pov93, respectively. Lanes 11–19 represent different species of *Plantago* namely, *P. coronopus*, *P. lanceolata*, *P. lagopus*, *P. serraria*, *P. exigua*, *P. maritima*, *P. arenaria*, *P. indica*, *P. major*, *P. rugelii* and *P. rhodosperma* respectively. (c, d, e) Amplification of SSR loci in different species of *Plantago* using using A6 (1c), A3 (1d) and A4 (1e) primers. Lane 1, *Plantago ovata* (Po-A2); 2, *P. ovata* (Po-B3); 3, *P. ovata* (Po-B5); 4, *P. lagopus* (P-L); 5, *P. exigua* (P-E); 6, *P. indica* (P-I); 7, *P. serraria* (P-S); 8, *P. ovata* (Po-86); 9, *P. ovata* (Po-93); 10, *P. ovata* (Po-90); 11, *P. ovata* (Po-79); 12, *P. ovata* (Po-83); 13, *P. arenaria* (P-A); 14, *P. maritima* (P-M); 15, *P. ovata* (Po-25); M, 100-bp ladder.

namely *P. maritima* while the other group comprised of *P. ovata*, *P. arenaria* and *P. lagopus*. This finding is in accordance with the work conducted by Ronsted *et al.* (2002) and Dhar *et al.* (2006). *P. maritima* and *P. serraria* belong to the subgenus *Coronopus* and the remaining taxa belong to subgenera *Psyllium* and *Albicans* (Rahn 1996). Therefore, *P. maritima* and *P. serraria* were expected to group together. However, surprisingly the two were found distant from each other. This observation can be explained on the basis of their different chromosome numbers; while, *P. maritima* has $2n = 2 \times = 12$; *P. serraria* has $2n = 2 \times = 10$. Rahn (1996) has drawn similar conclusions on the basis of morphological, embryological and chemical data while comparing these two species. In his study, *P. maritima* and *P. serraria* did not fall under one clade and he has placed them into different sections i.e. *Maritima* and *Coronopus* respectively.

The larger group of species could be further divided into three smaller subgroups. The first subgroup includes all the

Table 3. Polymorphic information content (PIC), expected (H_e) and observed (H_o) heterozygosity values of different SSR primers.

	Primer	Total number of alleles	PIC	H_e	H_o
1	A3	7	0.65	0.65	0.89
2	A4	9	0.73	0.73	0.81
3	A6	11	0.34	0.34	0.26
4	BB1	6	0.62	0.73	0.97
5	BB2	9	0.60	0.60	0.61
6	BB3	5	0.49	0.59	0.87
7	BB4	5	0.34	0.70	0.57
8	BB5	7	0.57	0.75	0.87
9	BB6	5	0.49	0.57	0.87
10	BB7	4	0.36	0.72	0.76
11	BB8	10	0.77	0.77	0.80
12	BB9	6	0.39	0.39	0.55

accessions of *P. ovata* as was expected and also confirms our earlier observations of low variability within the species (Dhar *et al.* 2011). The second group was based on *P. lagopus* and *P. serraria.* These two species are grouped together in this study, which is a sign of their genetic affinity but in Rahn's (1996) study the two species did not fall under one clade and were placed into different subgenera *Albicans* and *Coronopus*, respectively. The last subgroup consists of *P. arenaria* and *P. exigua*, and it can be inferred that these two species are closely related to each other. Their placement is in agreement with the work done by Rahn (1996).

A significant feature of the present study is that except primers based on *P. lagopus*, which is a relative of *P. ovata*, primers based on unrelated genera like *Malus* and *Phaseolus* turned out to be highly successful. The primers designed by Squirrell and Wolff (2001) on the basis of *P. major* showed limited cross amplification in other species of *Plantago*. We also observed limited transferability of these primers in *P. ovata*. Same is true of few earlier workers who used these primers for other *Plantago* species (Koorevaar *et al*. 2002; Wolff *et al*. 2009).

The presence of unique SSR and AFLP markers among various *Plantago* species indicates the usefulness of this approach for fingerprinting purposes. AFLP primers showed values from 0.18 to 0.92 and the PIC values obtained in case of SSR primers ranged from 0.34 to 0.77. AFLP proved to be more efficient in unraveling the intraspecific and interspecific genetic diversity in the genus *Plantago*. Similar types

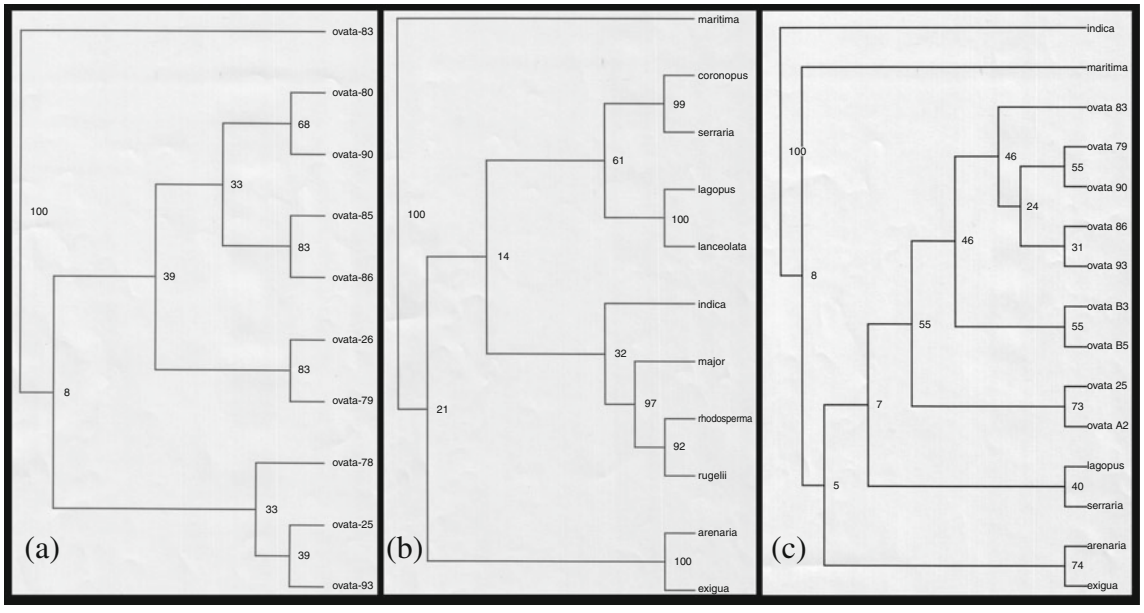

Figure 2. Dendrograms based on AFLP (a) different accessions of *P. ovate*, (b) different *Plantago* species, and (c) SSR data.

of results have also been reported in other studies as well (Skrede *et al.* 2009; Biswas *et al.* 2011). AFLP markers are considered to be markers of choice because no prior information of the genome is necessary and a very high level of polymorphism can be detected. It has been further documented that for the large scale intraspecific and interspecific studies AFLP is the most appropriate molecular marker (Woodhead *et al.* 2005).

Thus, it can be concluded that AFLP and SSR markers are highly useful for not only unraveling and assessing genetic diversity in *P. ovata* and some of its wild allies at molecular level but also for determining their phylogenetic relationships. For developing mapping population it would be worthwhile to select parents from accessions, Pov80 and Pov79 based upon the AFLP molecular characterization. Once the mapping population is obtained, the next step can be the development of a genetic map, which has not yet been generated in *P. ovata* or any of its wild allies. This can prove to be a major breakthrough for the genetic improvement of this plant.

Acknowledgements

We are grateful to USDA and Royal Botanic Gardens, Kew for initially providing seed samples of some accessions of *P. ovata* and its wild species. Authors are grateful to Department of Biotechnology (DBT), Govt. of India, for financial assistance in the form of research projects to MKD. The authors also thank the Coordinator, Bioinformatics Centre (DBT-BIF), School of Biotechnology for providing necessary facilities to carry out some part of this work.

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Received 13 September 2012, in revised form 29 October 2012; accepted 8 February 2013
Published on the Web: 25 June 2013