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Identification of plastid genotypes in *Oenothera* subsect. *Munzia* by restriction fragment length polymorphisms (RFLPs)

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Abstract Five discrete plastid genotypes (plastomes), designated I–V and typified by *Oenothera Hookeri*, *biennis*, *Lamarckiana*, *parviflora* and *argillicola* respectively, have been previously characterized within the European subsect. *Euoenothera*. The evolutionarily more-derived plastome types (I, II and V) are generally less tolerant of new hybridization events than the ancestral types (III and IV), and were first identified based on their incompatibility reactions with standard hybrid nuclei. Restriction maps for all five plastomes are available for the enzymes *Pvu*II, *Sal*I, *Kpn*I and *Pst*I (Gordon et al. 1982). The present study employs *Pvu*II and *Kpn*I restriction digests to compare 28 of the 45 species of subsect. *Munzia* with *Euoenothera* plastomes I–V. The results of plastome RFLP fingerprinting show uniform divergence of the South American taxa from their European congeners; all share the previously documented 45-kb inversion in the large single-copy region reported by Hachtel et al. (1991). However, at least six new plastome types have evolved within subsect. *Munzia*, giving rise to small-fragment size differences of 0.1–0.7 kb. In two of these cases (*Oe. featherstonei* and *Oe. longiflora*) unique fragments occurred. For *Oe. featherstonei* the unique *Kpn*I fragment resulted from a novel 2.2 kb insertion, whereas in *Oe. longiflora* an additional *Pvu*II restriction site has been created.

Key words Chloroplast DNAs · Hybridization · Coevolution · Indels

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Introduction

The widespread use of chloroplast DNA restriction-site mapping in angiosperm systematics has been discussed in several reviews (Palmer 1987; Olmstead et al. 1994; Sytsma and Hahn 1994; Hillis and Moritz 1997). Advantages of restriction-site mapping for phylogenetic studies include its technological simplicity, the "random sampling" nature of enzymatic cleavage (e.g., independence of characters), and the low level of homoplasy associated with RFLP data sets of congeneric species (Palmer 1987).

In addition to its well-recognized systematic utility, however, genetic fingerprinting of the chloroplast DNA has particular relevance in *Oenothera* hybridization studies. The existence of different cytoplasmic genotypes within the European oenotheras was first noted based on their different reactions to hybridization (Stubbe 1959). Fitness consequences of cytoplasmic intolerance to hybridization can include chlorosis, pollen abortion and embryonic lethality. Since hybridization is the most common mode of speciation in *Oenothera*, the importance of the cytoplasm as an evolutionary selective factor cannot be overemphasized. While these intolerance syndromes may be due either to mitochondrial (chondriome) or to chloroplast (plastome) incompatibility with the hybrid nuclear genome, most studies to-date have focused on the plastome. Angiosperm mitochondrial genomes vary in size by a factor of ten and contain many sequences of chloroplast origin (Gillham 1994). Plastomes of green flowering plants, by contrast, are all between 120 and 220 kb in size and are remarkably consistent in organization (circular, typically with 20–25 kb inverted repeats and no genes of mitochondrial origin; Gillham 1994). Hence, the majority of comparative studies have focused on the plastome.

Both chondriome and plastome differ strikingly from the nuclear genome in their mechanisms of gene

expression, replication and transmission. These organelles exert intracellular selection through interaction with the nucleus, and thus can determine the relative success of an incipient species even under the best environmental conditions (Chapman and Mulcahy 1997).

Five individual European plastome types (those of *Oenothera Hookeri*, *biennis*, *Lamarckiana*, *parviflora* and *argillicola*) were first characterized based on their variable reactions to combinations of three standard Renner complexes (Stubbe 1959). A restriction map for plastome IV using *Sal*I, *Pst*I and *Kpn*I enzymes was obtained (Gordon et al. 1981), following which mapping was accomplished for the other four *Euoenothera* plastomes by a comparison of cleavage products with those of plastome IV. While the overall fragment order was found to be the same for all five plastomes, 11 size differences attributable to insertion/deletion events were noted in the other plastomes when compared with IV (Gordon et al. 1982).

South American *Oenothera villaricae* and *Oenothera picensis* ssp. *picensis* of subsect. *Munzia* are known to differ in their plastid hybridization tolerance (Schwemmle 1938). Analysis of the chloroplast-DNA restriction maps generated for these two species (vom Stein and Hachtel 1986) showed them to differ slightly in the sizes of five regions but to contain identical restriction sites. A more detailed interspecific comparison by the cloning and sequencing of polymorphic restriction fragments revealed these regions of the plastome to be notable for insertion/deletion differences, short inverted repeats and frame shift-mutated open reading frames (vom Stein and Hachtel 1988 a, b).

An RFLP comparison of plastomes I–V from sect. *Euoenothera* with a representative of the *Munzia* subsection shows evidence of comparable divergence in fragment sizes, as well as the previously reported 45-kb inversion in the large single-copy region which marks the divergence of European from New World taxa (Hachtel et al. 1991). To further characterize the extent of plastome divergence within the exclusively South American subsect. *Munzia*, RFLPs of 28 *Munzia Oenothera* species were obtained using the enzymes *Pvu*II and *Kpn*I.

Materials and methods

Seeds of the *Oenothera* species shown in Table 1 were germinated in growth chambers and raised to vegetative maturity (rosette stage) in the greenhouse at the University of Massachusetts, Amherst. After 2 days light-starvation to reduce carbohydrates, 5 g of leaf tissue were harvested in triplicate from young rosette leaves, wrapped in aluminum foil and immediately frozen in liquid nitrogen, then stored at -80°C.

DNA extractions

Total DNA was extracted from frozen tissue in CTAB buffer after the method of Chang et al. (1996). Chloroplast DNA was 70% enriched from crude extracts using the cesium chloride/bisbenzimid gradient technique of Chiu et al. (1990). Chloroplast DNA used as a probe for Southerns (isolated from *Oenothera argillicola*) was similarly prepared, except that three serial gradients were employed to yield a calculated cpDNA enrichment of 97%. Final concentrations of cpDNA samples were calculated from optical densities at 260 nm obtained by UV spectroscopy. Samples were stored at $+4^{\circ}$C to avoid shearing of the chloroplast genomes in the freeze/thaw cycles.

Table 1 *Oenothera* species of subsections *Munzia* and *Euoenothera* and acronyms used in this study

Species	Acronym	Subsection	Series
Oe. hookeri	HOO (I)	*Euoenothera*	NA
Oe. biennis	BIS (II)	”	”
Oe. Lamarckiana	LAM (III)	”	”
Oe. parviflora	PRV (IV)	”	”
Oe. argillicola	ARG (V)	”	”
Oe. peruana	PER	*Munzia*	*Renneria*
Oe. tarijensis	TAR	”	”
Oe. lasiocarpa	LAS	”	”
Oe. longituba	LGT	”	”
Oe. tafiensis	TAF	”	”
Oe. pedunculifolia	PED	”	”
Oe. rubida	RUB	”	”
Oe. recurva	REC	”	”
Oe. sandiana var. *campylocalyx*	CYX	”	”
Oe. nana	NAN	”	”
Oe. magellanica	MAG	”	*Clelandia*
Oe. villaricae	VIL	”	”
Oe. elongata	ELO	”	”
Oe. pseudoelongata	PSE	”	”
Oe. cordobensis	COR	”	”
Oe. punae	PUN	”	”
Oe. mendociensis	MEN	”	*Allochroa*
Oe. longiflora ssp. *grandiflora*	LGR	”	”
Oe. catharensis	CAT	”	”
Oe. indecora	IND	”	”
Oe. affinis	AFF	”	”
Oe. mollissima	MOL	”	”
Oe. stricta	STR	”	”
Oe. picensis ssp. *picensis*	PIC	”	”
Oe. picensis ssp. *bonarensis*	PBO	”	”
Oe. picensis ssp. *brasiliensis*	PBR	”	”
Oe. montevidensis	MON	”	”
Oe. nocturna	NOC	”	”
Oe. parodiana	PAR	”	”
Oe. featherstonei	FEA	”	”

Restriction digests

Appropriate volumes of DNA samples to yield 1 μg of DNA were digested using *Pvu*II or *Kpn*I enzymes according to the manufacturer's instructions (Promega). Digested samples were precipitated using one-tenth vol 3 M sodium acetate and 2 vol of cold ethanol, followed by 1-hour incubation at -20°C. The precipitates were collected by microcentrifugation at 15 000 rpm. Pellets were re-suspended in 20 μl of TE buffer. The restriction fragments were separated by electrophoresis in 0.7% agarose with a total

tracking dye migration of 19 cm. Lambda size-markers digested with *Hin*dIII, or a combination of lambda fragments produced by separate digestions with *Sal*I, *Sma*I, *Hin*dIII, *Eco*RI + *Kpn*I and *Eco*RI + *Hin*dIII, were included on all gels.

Southern blots were performed after the method of Carpenter et al. (1994). Briefly, the gels were stained with ethidium bromide and photographed, then treated with 0.4 N hydrochloric acid for 10 min followed by 0.6 N NaOH/NaCl for 30 min. DNAs were blotted onto Zetabind membranes and allowed to transfer overnight. Then the membranes were removed, washed twice with 2 × SSC and allowed to dry. Following blotting, gels were re-stained and re-photographed to document DNA transfer (data not shown).

Southern hybridizations

Membranes were pre-hybridized for 3 h at 65°C in the hybridization buffer of Carpenter et al (1994). ^{32}P-labeled cpDNA and lambda probes were prepared by random-primer labeling of cpDNA from *Oe. argillicola* according to the manufacturer's instructions (Promega). To estimate the incorporation of label, 1/300 dilutions of probe were made before and after Sephadex filtration, and activity was measured with a Geiger counter; the average incorporation level was approximately 30%. Probe DNAs were then denatured for 5 min at 100°C and added to the hybridization buffer. Hybridization was allowed to proceed at 65°C overnight. Following hybridization, membranes were washed 2–5 × (depending on signal strength) with 2 × SSC, 0.1% SDS at 65°C and wrapped in Saran Wrap. Wrapped membranes were autoradiographed at −80°C for 2–14 days depending on the final level of the radioactive signal.

Results

The chloroplast DNA restriction fragment patterns associated with all *Oenothera* species investigated are presented in Table 2. Five sect. *Euoenothera* species, representing plastomes I–V, were included as controls. Though some *Euoenothera* bands are missing from Figs. 1 and 2, their presence was ascertained from a control comparison study where plastomes I–V cut with *Pvu*II and *Kpn*I were run on the same gel (data not shown). *Pvu*II and *Kpn*I single-enzyme digests resolved 14 and 9–10 fragments, respectively, for all species except *Oe. picensis* ssp. *bonarensis* (PBO, which has lost a *Kpn*I fragment) and *Oe. longiflora* ssp. *grandiflora* (LGR, which has an additional *Pvu*II site). Fragments smaller than about 1.9 kb migrated off the gel. None of the *Pvu*II digests and only 0.3% of the *Kpn*I digests as judged by the maps of VIL (*Oe. villaricae* = *Oe. berteriana*, vom Stein and Hachtel 1986) should have been lost in this way. Additional RFLP fingerprints (data not shown) bring the total number of *Munzia* species analyzed to 28. Lambda size-markers (Figs. 1 and 2, lane 1) were used to generate cpDNA fragment sizes.

The most striking RFLP differences occurred between the European control species (*Oe. Hookeri*, *biennis*, *Lamarckiana*, *parviflora and argillicola*; Figs. 1 and 2, lanes 28–34) and the South American *Munzia* species (Figs. 1 and 2, lanes 2–27). These differences are primarily attributable to the known 45-kb inversion (Hachtel et al. 1991). For example, the *Pvu*II 11.8-kb fragment represented in almost all *Munzia* species (Table 2 b) is the location of one end of the inversion found in the *Euoenothera* plastome. Hence, *Euoenothera* species (Table 2 a) lack this band. Individual fragment sizes, as well as the total plastome size of approximately 156 kb, are consistent with previously reported estimates (Gordon et al. 1982; Hachtel et al. 1991).

Euoenothera plastomes I, IV and V are distinguished from each other and from II and III by PvuII RFLPs, involving size differences of up to 0.4-kb in the 19-kb (2nd largest) fragment. Among the *Munzia* species, divergent plastomes appeared in all three series. Departures from the most common pattern A, typified by species of the series *Renneria* such as *Oe. peruana* and *lasiocarpa* (PER, LAS), are designated B through E. *Pvu*II class B (LGT) shows evidence of a 0.2-kb deletion to yield 9.8- and 9.6-kb bands in place of the doublet of comparable size found in A. Class C (LGR) has a 9.8-kb doublet, a 9.5-kb singlet and a unique 5.3-kb band, but lacks the 14.6-kb fragment of classes A and B, suggesting that a novel restriction site evolved within that fragment. The largest insertion occurs in class D (FEA), where a 2.2-kb fragment has been inserted into the 14.6-kb fragment resulting in a 16.8-kb band. Class E (PBR) shows evidence of a 0.6-kb deletion in the 14.6-kb fragment of class A.

Control plastomes II, III and IV are distinguished from each other and from I and V by *Kpn*I RFLPs. Fragment size differences (0.1–0.8 kb) were noted among the *Euoenothera* species involving the 9.7-kb doublet found in I and V. The majority of species in subsection *Munzia* conform to the pattern typified by VIL and NAN (F). Divergent RFLPs (G,H,J,K) again appeared in all three series, involving slight (<0.4 kb) size differences in the bands that range from 3.8 to 4.2 kb in various species. Class J (FEA) shows evidence of a unique 6.4-kb band and loss of the 4.2-kb band of class G. This results from a 2.2-kb insertion. Class K (PBR) lacks the 3.8-kb band found in class F.

Discussion

Combined analysis of *Pvu*II (Table 2 b, A–E) and *Kpn*I RFLPs (Table 2 d, F–K) within the subsection *Munzia* suggests that at least six new plastome types (A/G, A/H, B/F, C/G, D/J and E/K; Table 2 e) have diverged from the ancestral pattern (A/F) since the Miocene colonization of South America by *Oenothera* subsection *Munzia* (Raven and Axelrod 1974). The majority of new plastome types appear in representatives of *Allochroa*, the most geographically widespread of the three series. The primary mode of plastome evolution appears to be small insertions and deletions (0.1–0.4 kb). Notable exceptions to this trend are *Oe. featherstonei* (FEA, plastome D/J) and *Oe. longiflora* ssp. *grandiflora* (LGR,

Table 2 Restriction fragment patterns associated with species of *Oenothera* section *Euoenothera* and subs. *Munzia*. Roman numerals after acronyms indicate plastome types for species as follows: HOO(I) = *Oe. hookeri*; BIS(II) = *Oe. biennis*; LAM(III) = *Oe. lamarckiana*; PRV(IV) = *Oe. parviflora*; ARG(V) = *Oe. argillicola*. Fragment sizes in kilobases. Parentheses indicate doublets

a *Pvu*II digests (*Euoenothera* species)

HOO(I)	BIS(II)	LAM(III)	PRV(IV)	ARG(V)
26.0	26.0	26.0	26.0	26.0
19.0	19.4	19.4	19.0	19.0
18.5	18.5	18.5	18.5	18.5
18.1	18.1	18.1	18.1	18.1
15.5	15.7	15.7	15.7	15.7
(9.8)	(10.1)	(10.1)	(10.1)	9.8
–	–	–	–	9.3
7.9	8.0	8.0	8.0	7.8
(7.4)	(7.4)	(7.4)	(7.4)	(7.4)
(4.0)	(4.0)	(4.0)	(4.0)	(4.0)
(3.0)	(3.0)	(3.0)	(2.9)	(2.9)
Sum: 153.4	154.7	154.7	154.1	152.8

b *Pvu*II digests (*Munzia* species). Letters indicate plastome types (A = {AFF[a], CAT, COR, CYX, ELO[a], IND, LAS, MAG, MEN, MOL[a], MON, NAN, NOC, PAR, PED, PER, PIC, PSE[a], PUN, REC, RUB, STR, TAF, TAR, VIL}; B = LGT; C = LGR; D = FEA; E = PBR)

A	B	C	D	E
28.0	28.0	28.0	28.0	28.0
18.5	18.5	18.5	18.5	18.5
18.0	18.0	18.0	18.0	18.0
–	–	–	16.8	–
15.9	15.9	15.9	15.9	15.9
14.6	14.6	–	–	14.0
11.8	11.8	11.6	11.3	11.8
(9.8)	9.8	(9.8)	(9.8)	(9.8)
–	9.3	9.3	–	–
7.9	7.9	7.9	7.9	7.9
7.2	7.2	7.2	7.2	7.2
–	–	5.3	–	–
(4.0)	(4.0)	(4.0)	(4.0)	(4.0)
(3.0)	(3.0)	(3.0)	(3.0)	(3.0)
Sum: 155.5	155.0	155.2	157.2	154.9

[a] Data not shown in Fig. 1

c *Kpn*I digests (*Euoenothera* species)

HOO(I)	BIS(II)	LAM(III)	PRV(IV)	ARG(V)
47.0	47.0	47.0	47.0	47.0
28.0	28.0	28.0	28.0	28.0
27.5	27.5	27.5	27.5	27.5
24.9	24.9	24.9	24.9	24.9
(9.7)	9.8	(9.8)	9.8	(9.7)
–	9.5	–	–	–
–	–	–	8.6	–
4.6	4.6	4.6	4.6	4.6
3.45	3.45	3.45	3.45	3.45
2.25	2.25	2.35	2.35	2.35
Sum: 157.1	157.0	157.4	156.2	157.2

d *Kpn*I digests (*Munzia* species). Letters indicate plastome types [F = (AFF[a], CYX, COR, ELO[a], IND, LAS, LGT, MAG, MOL[a], NAN, NOC, PED, PER, PSE[a], PUN, REC, RUB, STR, TAR, VIL); G = (CAT, LGR, MEN, PIC); H = TAF; J = FEA; K = PBR]

F	G	H	J	K
45.0	45.0	45.0	45.0	45.0
29.8	29.8	29.8	29.8	29.8
24.2	24.2	24.2	24.2	24.2
18.6	18.6	18.6	18.6	18.6
13.0	13.0	13.0	13.0	13.0
9.75	9.75	9.75	9.75	9.75
–	–	–	6.4	–
4.6	4.6	4.6	4.6	4.6
3.8	4.2	4.0	–	–
3.45	3.45	3.45	3.45	3.45
2.35	2.35	2.35	2.35	2.35
Sum: 154.6	154.9	154.8	157.2	150.8

[a] Data not shown in Fig. 2

e Plastome types of subs. *Munzia*. Combined RFLP data from **b** and **d** above

Series	Class						
	A/F	A/G	A/H	B/F	C/G	D/J	E/K
Renneria	CYX LAS NAN PED PER REC RUB TAR		TAF	LGT			
Clelandia	COR ELO MAG PSE PUN VIL						
Allochroa	AFF IND MOL NOC STR PBO	CAT MEN PIC	MON PAR		LGR	FEA	PBR

plastome C/G). In *Oe. longiflora* the two novel *Pvu*II bands (9.3 and 5.3 kb) suggest that a new restriction site has formed, mapping to the 14.6-kb *Pvu*II fragment of the A and B types. In *Oe. featherstonei*, an increase of about 2.2 kb to produce the unique 6.4-kb *Kpn*I fragment (represented by one 3.8–4.2-kb band in the other species) maps to the same area where a unique 16.8-kb *Pvu*II fragment is found. This fragment is also approximately 2 kb larger than the 14.6-kb fragment found in other *Munzia* species, thus verifying that an insertion of that size has occurred. This insertion maps to the same part of the genome for both enzymes according to the data of vom Stein and Hachtel (1986). In *Oe. picensis*

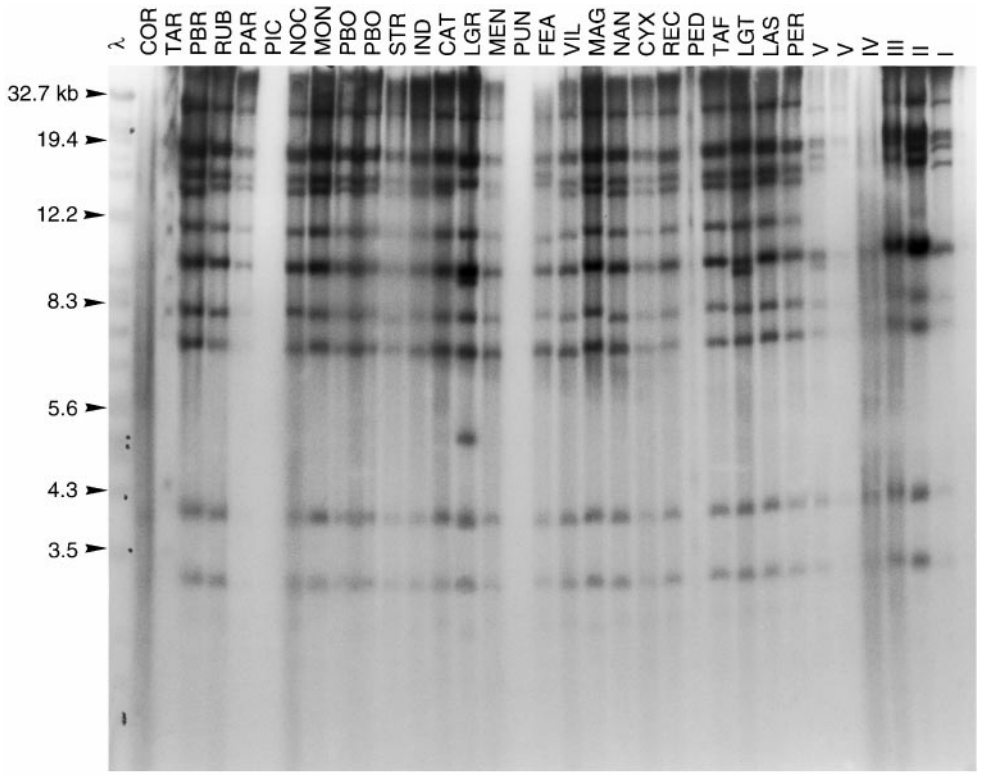

Fig. 1 RFLP comparison of *Pvu*II-digested chloroplast DNAs from *Oenothera* species. *Lane 1*: size markers; a mixture of the following Lambda restriction digests: *Sal*I, *Sma*I, *Hin*dIII, *Eco*RI + *Kpn*I and *Eco*RI + *Hin*dIII. *Lanes 2–27*: *Munzia* species. *Lanes 28–33*: *Euoenothera* controls (some fragments not visible)

ssp. *bonarensis* (PBR), the complete absence of the 3.8-kb *Kpn*I band found in class A/F is puzzling. On comparison of the *Pvu*II and *Kpn*I data, the 4.1-kb discrepancy in total plastome size for PBR approximates the size of the missing fragment. Its loss may be due to the occurrence of a new *Kpn*I restriction site within the region, yielding fragments smaller than 2 kb which ran off the gel.

In contrast to PBR, FEA and LGR, all other cpDNA polymorphisms detected among the *Munzia* species are attributable to small insertions or deletions. Surprisingly, the 3.3- and 3.8-kb *Pvu*II fragments reported by vom Stein and Hachtel (1986) were not detected in the present study; these were reported as faint bands in the original paper, and were not visible in their gel profile. From the perspective of stoichiometry, these bands should be about 50% of their very bright 2.65-MDa (our 4-kb) doublet band (Fig. 1 in vom Stein and Hachtel 1986). Our method of hybridizing purified cpDNA of *Oe. argillicola* (V) to that plastome readily detects bands in this size range, as seen in Fig. 1, so the lack of any signal representing these two *Pvu*II fragments is puzzling.

Oenothera chloroplast DNA mutations in response to the nucleus are well documented. In the plastome mutator (*plm*) system, plants homozygous for a nuclear mutation display a wide range of green and chlorotic phenotypes associated with deletion events in the plastome (Chiu et al. 1990). Since plastome RFLPs of chlorotic deletion mutants were identical to those of green mutants, it is unlikely that deletions were directly responsible for the chlorosis; rather, a developmental mechanism is implicated (Chiu et al. 1990). Conventional breeding studies are needed to document any incompatibility reactions which may occur on hybridization of *Munzia* species with dissimilar plastomes.

Inasmuch as the most common *Munzia* plastome type is that typified by *Renneria* species PED and TAR, and this type has persisted in the evolutionarily derived series *Allochroa* and *Clelandia* (e.g., COR, ELO, IND, AFF, MOL), it is probably quite tolerant to hybridization. A likely ideal maternal candidate for hybridization is PED (*Oe. pedunculifolia*, Dietrich 1977). It is structurally homozygous (7.II meiotic configuration) with large, conspicuous flowers (petals 2 cm), and carries the plastome type described above. Species with this morphology are attractive to pollinators; their genetic load is insignificant compared with translocation ring-formers (Cleland 1972), and their plastome is geographically dominant. Our prediction is that no nucleocytoplasmic barriers should exist to hybridization with the PED maternal lineage.

In *Munzia Oenotheras* of hybrid origin whose Renner complexes have been identified to progenitor species (Dietrich 1977), cpDNA RFLP analysis can sometimes be used to identify the direction of hybridization. For

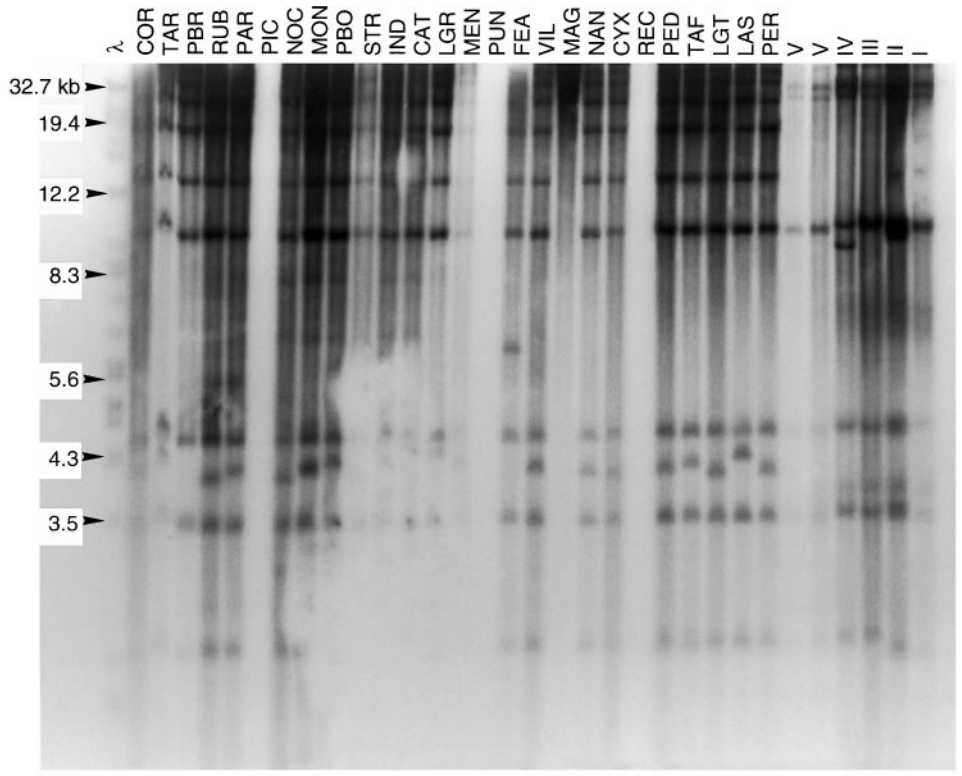

Fig. 2 RFLP comparison of *Kpn*I-digested chloroplast DNAs from *Oenothera* species. *Lane 1*: size markers; a mixture of the following Lambda restriction digests: *Sal*I, *Sma*I, *Hin*dIII, *Eco*RI + *Kpn*I and *Eco*RI + *Hin*dIII. *Lanes 2–27*: *Munzia* species. *Lanes 28–33*: *Euoenothera* controls (some fragments not visible)

example, the Renner complexes of ELO are known to be derived from LGR and AFF (Dietrich 1977). The *Pvu*II cpDNA fingerprint for ELO conforms to that of AFF (the probable ancestral type for subsect. *Munzia*; Table 2 a), suggesting that AFF or a closely related species was the maternal parent in the cross which gave rise to ELO. In other cases, the plastome appears to have diverged from the common parental type subsequent to the hybridization event. An example is MON, whose Renner complexes are derived from IND and AFF (Dietrich 1977). The plastome type of MON is dissimilar to either IND or AFF (Table 2 e). The interesting case of *Oe. picensis* shows plastome divergence within a species: of the three subspecies tested, only *Oe. picensis* ssp. *bonarensis* (PBO) conforms to the dominant restriction profile (A/F). Further crosses and cpDNA studies are desirable to identify the plastome types of the remaining species in subsect. *Munzia* and their maternal lineages.

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