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Association between chloroplast DNA and mitochondrial DNA haplotypes in *Prunus spinosa* L. (Rosaceae) populations across Europe

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Running title: Association of cpDNA and mtDNA haplotypes in *Prunus spinosa*

Abstract

Chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) were studied in 24 populations of *Prunus spinosa* sampled across Europe. The cpDNA and mtDNA fragments were amplified using universal primers and subsequently digested with restriction enzymes to obtain the polymorphisms. Combination of all the polymorphisms resulted in 33 cpDNA haplotypes and two mtDNA haplotypes. Strict association between the cpDNA haplotypes and mtDNA haplotypes was detected in most cases, indicating conjoint inheritance of the two genomes. The most frequent and abundant cpDNA haplotype (C20, frequency = 51%) is always associated with the more frequent and abundant mtDNA haplotype (M1, frequency = 84%). The cpDNA haplotypes (except two) associated with the less frequent mtDNA haplotype (M2) are private haplotypes. These private haplotypes are phylogenetically related but geographically unrelated. They form a separate cluster on the minimum-length spanning tree.

Key words: cpDNA haplotypes, mtDNA haplotypes, PCR-RFLP, phylogenetic relationship, *Prunus spinosa* L.

INTRODUCTION

In most angiosperms, chloroplast and mitochondrial genomes are both inherited maternally (Reboud and Zeyl, 1994), and therefore are expected to remain completely linked (Schnabel and Asmussen, 1989). There are studies where chloroplast and mitochondrial genomes have been investigated simultaneously (Berthou *et al.*, 1983; Laurent *et al.*, 1993; Shu *et al.*, 1993; Tsunewaki, 1993; Lee *et al.*, 1994; Caha *et al.*, 1998). However, there are only few studies describing the association between the two organelle genomes in angiosperms (e.g., Dumolin-Lapègue *et al.*, 1998; Desplanque *et al.*, 2000; Olson and McCauley, 2000; Belahbib *et al.*, 2001).

The chloroplast genome is well characterized and structurally very stable (Clegg *et al.*, 1994). The variations detected in chloroplast DNA (cpDNA) using the PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) technique are useful for population genetic studies both at interspecific and intraspecific level (McCauley, 1995; Newton *et al.*, 1999; Demesure *et al.*, 1996; El Mousadik and Petit, 1996; Dumolin-Lapègue *et al.*, 1997a; King and Ferris, 1998; Fineschi *et al.*, 2000; Dutech *et al.*, 2000). In some studies, mitochondrial DNA (mtDNA) variations have been very informative too; mtDNA polymorphisms are geographically structured at local scale in *Thymus vulgaris* (Manicacci *et al.*, 1996), and at regional scale in *Hevea brasiliensis* (Luo *et al.*, 1995), *Fagus crenata* (Tomaru *et al.*, 1998) and *Beta vulgaris* ssp. *maritima* (Desplanque *et al.*, 2000). In *Theobroma cacao* and *Glycine soja*, mitochondrial haplotypes have widespread geographical distribution, and do not present any geographic structuring (Laurent *et al.*, 1993; Tozuka *et al.*, 1998).

The present investigation is carried out in *Prunus spinosa* L., a wild shrub of European deciduous forests, that is grown as a hedge plant. A preliminary study of seven populations of *P. spinosa* has revealed high cpDNA diversity (Mohanty *et al.*, 2000). In another study, three regions of cpDNA (approx. 8250 bp) were analysed for a population genetic analysis of 25 populations from European deciduous forests. The study revealed low genetic differentiation among populations and an absence of phylogeographic structure (Mohanty *et al.*, 2002). In the

present study, 24 populations were analyzed with an additional region of cpDNA of approx. 3800 bp and three regions of mtDNA. The main objectives were to study the extent of mtDNA variations and phylogenetic and geographic relationship between cpDNA and mtDNA haplotypes in populations of *P. spinosa*.

MATERIALS AND METHODS

Plant material

Twenty-four wild populations, which included 157 individuals of *P. spinosa*, were sampled from deciduous forests across Europe (Table 1). Only 157 of the 203 samples from a previous study (Mohanty *et al.*, 2002) have been analysed by studying an additional region of cpDNA and three regions of mtDNA. All 203 individuals could not be included in the present study as CD fragment could be amplified only in 157 samples.

DNA extraction, amplification and digestion

DNA was extracted from frozen leaves following the protocol of Torres *et al.* (1993), and then standardized (4 ng μl^{-1}). The final results have been interpreted by combining the data obtained by using four cpDNA primer pairs, HK, K1K2, VL (previous study; Mohanty *et al.*, 2002) and CD (present study), and three mtDNA primer pairs, *nad*1/B-C, *nad*4/2-3, and *rps*14-*cob* (present study). The four cpDNA primer pairs and three mtDNA primer pairs are described in Demesure *et al.* (1995) and Dumolin-Lapègue *et al.* (1997b). The details of amplification with the cpDNA primers are described in Mohanty *et al.* (2000). The amplifications using mtDNA primers were performed in 30 µl of reaction mixture, consisting of 0.2 µM of each primer, 200 µM of each of the four dNTP, 2 mM $MgCl_2$, 0·5-1·0 U Eco*Taq* DNA polymerase in the buffer provided by the manufacturers of the enzyme (ECOGEN, S.R.L., Barcelona, Spain), and 12 ng of genomic DNA. The PCR amplifications were carried out in a PTC-100 thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA) with heated lid, using an initial

cycle of 4 min at 94 ºC, followed by 30 cycles of 45 s at 94 ºC, 45 s at 55 ºC and 3 min (for *nad*1/B-C and *rps*14-*cob*) or 4 min 30 s (for *nad*4/2-3) at 72 ºC, and finally a 10 min extension at 72 ºC.

Amplified products, obtained using cpDNA and mtDNA primers, were digested with the restriction enzymes *Hin*fI and *Taq*I (Amersham Corporation, Buckinghanmshire, UK). In addition, *Alu*I was used with the primer pairs VL, *nad*1/B-C, and *rps*14-*cob*. The digestion conditions are detailed in Mohanty *et al.* (2000). Restriction fragments were separated on 2·6 % agarose gels in Tris-borate-EDTA buffer (1X), run at 3 V/cm for 4 h, stained with ethidium bromide and visualized under UV light. Size of the polymorphic bands were analysed using Kodak Digital Science 1D Image Analysis software, and a 50 bp ladder (Pharmacia Biotech., Brussels, Belgium) was used as a molecular size marker.

DNA sequencing

The two detected mtDNA haplotypes were sequenced in order to determine the exact nature of mutation. The amplified fragments obtained using the mtDNA primer pair *rps*14-*cob*, for two individuals of each mtDNA haplotype, were cloned into plasmid of pGEM-T (Promega Corporation, Madison, Wisconsin, USA). Automated sequencing of the recombinant plasmids was performed using fluorescence-base labeling with ABI PRISM system (Perkin-Elmer Corporation, Norwalk, Connecticut, USA). The sequencing strategy involved the use of the plasmid-specific SP6 and T7 primers (synthesized by Pharmacia Biotech., Brussels, Belgium), which are located on the vector from both ends of the inserts. The sequencing was performed in both directions until sequences from the two ends overlapped. Analysis of DNA sequences was carried out with the SeqMan and Mapdraw Lasergene programs (DNASTAR Inc., Madison Wisconsin, USA).

Nucleotide sequence data reported in this paper has been submitted to the GenBank, EMBL and DDBJ databases under the accession number AF464899 for mtDNA haplotype 1 (M1), and AF464900 for mtDNA haplotype 2 (M2).

Analysis of data

The program HAPLONST (Pons and Petit, 1996) was used to calculate the parameters of cpDNA diversity (H_T = total diversity, H_S = average intrapopulation diversity, and G_{ST} = level of population subdivision using unordered alleles, and N_{ST} = level of population subdivision using ordered alleles).

The number of mutational differences between haplotypes of wild populations was calculated to produce a minimum-length spanning tree of haplotypes, using the program NTSYS-pc (Rohlf, 1992). The procedure is used to connect points (haplotypes) by direct links having the smallest possible total length (Prim, 1957). Minimum spanning networks are alternatives to Wagner parsimony trees, and better convey the connections between haplotypes (Excoffier and Smouse, 1994).

RESULTS

cpDNA haplotypes

PCR-RFLP of cpDNA fragment obtained using the primer pair CD, resulted in eight polymorphic fragments (Table 2). The mutations detected using the primer pairs HK, K1K2 and VL (Mohanty *et al.*, 2002) and CD (present study) were combined to define the cpDNA haplotypes C1 to C33 (Table 3). Of the 33 cpDNA haplotypes distinguished, nine were shared by two or more populations and the rest (24) were private (as denominated by Slatkin, 1985) or unique haplotypes (Table 3). C20 was the most frequent haplotype (frequency = 51 %). It was represented in 80 of 157 individuals studied (Table 3).

Analysis of diversity using the HAPLONST program revealed high total diversity (H_T = 0·76), of which a major portion was located within populations (H_S = 0·50). The level of population subdivision using unordered and ordered alleles were G_{ST} = 0·34 and N_{ST} = 0·46, respectively. The difference between N_{ST} and G_{ST} was non-significant (U test = 0·74, P = 0·05; Pons and Petit, 1996).

mtDNA haplotypes

Of the three mtDNA primer pairs used, *nad*1/B-C and *rps*14/*cob* showed good amplification, and the sizes of the amplified fragments were approximately 1300 bp and 1280 bp, respectively. There was no amplification with *nad*4/2-3. The restriction digestions of the amplified fragments with *Alu*I, *Hin*fI, and *Taq*I revealed no polymorphisms in the amplified fragment of *nad*1/B-C. The combination *rps*14/*cob*-*Taq*I resulting in two mtDNA haplotypes (M1 and M2). The haplotype-M1 showed a restricted fragment of approx. 210 bp, and the other haplotype (M2) showed a 170 bp fragment. The mutation appeared to be an indel of approximately 40 bp, on the agarose gel; however, it was actually a restriction site mutation (revealed on sequencing), which appeared as an indel mutation because of the 40 bp fragment migrating out of the gel. Sequencing of mtDNA amplified fragment of the two haplotypes showed a length of 1286 bp. Sequencing also revealed only a 1 bp substitution at position 72, with a thymine (T) in haplotype-M1 and guanine (G) in haplotype-M2, resulting in gain/loss of a restriction site for the restriction enzyme *Taq*I. M1 was the more abundant haplotype, represented in 132 individuals (frequency = 84 %), and M2 in 25 individuals (frequency = 16 %). Of the twenty-four populations surveyed, eight (8, 9, 13, 14, 16, 20, 21, 24) were polymorphic, with both mtDNA haplotypes M1 and M2; fourteen populations (1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 15, 17, 18, 22) are monomorphic for M1, and two populations (19, 23) are monomorphic for M2 (Table 3).

Association between cpDNA and mtDNA haplotypes

The cpDNA haplotypes C1 to C33 with their corresponding mtDNA haplotypes (M1 and/or M2) are given in Table 3, and shown in Fig. 1. Twenty cpDNA haplotypes are associated with mtDNA haplotype-M1, and 11 cpDNA haplotypes with M2. Two cpDNA haplotypes (C9 and C10) are associated with both mtDNA haplotypes, M1 as well as M2. Of the two individuals representing C9, one has mtDNA haplotype-M1 and the other has M2 (Table 3). Similarly, of the nine individuals with C10, five have M1 and four have M2 (Table 3). All individuals with

haplotypes C9-M2 and C10-M2 are from population 23 (Greece). All cpDNA haplotypes (except C9 and C10) which are associated with mtDNA haplotype-M2 are private haplotypes (Table 3).

The minimum-length spanning tree showing the phylogenetic relationships of cpDNA haplotypes is shown in Fig. 1. The distribution of mtDNA haplotypes (M1 or/and M2) associated with each cpDNA haplotype are also plotted on the tree. C10 and C20 form two main nodes of the tree. The node represented by C20 harbours a group of cpDNA haplotypes, all of which are associated with mtDNA haplotype-M1 (Cluster I). The other node represented by C10 has two groups of cpDNA haplotypes: Cluster II, associated with mtDNA haplotype-M1, and Cluster III, associated with mtDNA haplotype-M2, except C9, which is associated with M1 also (Fig. 1). In each cluster, the phylogenetically related cpDNA haplotypes are mostly geographically unrelated.

DISCUSSION

P. spinosa is a wild allotetraploid shrub (Reynders-Aloisi and Grellet, 1994) which represents one of the possible ancestors of *P. domestica* (Watkins, 1976, 1981). The shrub has wide range of environmental adaptability including resistance to calcareous soils and drought. Its fruits are important for preparation of an alcoholic drink (Pacharan) in Spain. All these attributes of *P. spinosa* are important for improvement of rootstocks or varieties through interspecific hybridization. For such improvement programs, assessment of genetic variability in populations is very useful. A population genetic analysis of this shrub using cpDNA markers has already provided some information about genetically diverse populations (Mohanty *et al*., 2002). In the present investigation, cpDNA and mtDNA diversity and phylogenetic and geographic relationship between them has been discussed which can be useful for identifying populations for conservation and to formulate their management strategies.

In our previous study (Mohanty *et al.*, 2002), three regions of cpDNA (approx. 8250 bp in total) were analysed for a population genetic study. In the present study, another region of the

chloroplast genome (approx. 3800 bp) was analysed and found to be very polymorphic, with eight polymorphic fragments when restricted with two restriction enzymes. The combination of all the mutations detected in the regions HK, K1K2, VL (previous study), and CD (present study) resulted in 33 haplotypes in 157 individuals from 24 populations, implying a mean of 1·38 haplotypes per population. Of the 33 cpDNA haplotypes, only nine are shared between two or more populations and 24 are private haplotypes. There is only one haplotype (C20; frequency = 51 %) which is abundant and geographically widespread. This cpDNA haplotype may correspond to the ancestral type. Analysis of diversity showed that differentiation among the populations was low (G_{ST} = 0·34), which is very close to that obtained when only three regions of cpDNA was analysed (G_{ST} = 0·33; Mohanty *et al.*, 2002). The N_{ST} value was not significantly higher than G_{ST}, which indicated an absence of phylogeographic structure. Thus, the additional analysis of another fragment CD of cpDNA showed several more polymorphisms but it did not change the overall result (i.e., low genetic differentiation among populations and absence of phylogeographic structure) from that obtained in the previous study (Mohanty *et al.*, 2002). Absence of phylogeographic structure could be due to efficient seed dispersal by mammals and birds who ingest the fruits of *P. spinosa*. The intensive seed movements can decrease genetic heterogeneity among populations and erase the phylogeographic structure (Mohanty *et al.* 2002).

In contrast to high cpDNA diversity (33 haplotypes), the mtDNA showed lower levels of variation (only 2 haplotypes). The low variation of mtDNA maybe explained by the fact that rate of nucleotide substitution is at least three times slower in mtDNA than cpDNA (Wolfe *et al.*, 1987; Palmer, 1992). However, there are studies where lower cpDNA variations and higher mtDNA variations have been observed (Laurent *et al.*, 1993; Caha *et al.*, 1998).

Of the 33 cpDNA haplotypes, 15 haplotypes are shared between two or more individuals and 18 are represented in one individual each. Of these 15 shared cpDNA haplotypes, 13 show strict association with their mtDNA haplotype. Most prominent is the example of C20 (represented in more than 50 % of the individuals studied, and also geographically the most widespread), which

is always coupled with mtDNA haplotype M1. The strong association between the two genomes suggests the same inheritance pattern for both organelles, which is assumed to be maternal. In *P. spinosa* there is no previous study demonstrating maternal inheritance of the two cytoplasmic genomes, but maternal inheritance of cpDNA has been demonstrated in *Prunus cerasus* (Brettin *et al.*, 2000). So, if C20 represents an ancient haplotype, then M1 (which is strictly associated with C20) may also be considered to be of older origin compared to M2.

There are only two cases of uncoupling of cpDNA with its corresponding mtDNA. The dissociations are in the cpDNA haplotypes C9 and C10. The cpDNA haplotype C10 is associated with M1 in five individuals and with M2 in four individuals. The second case of dissociation is seen in cpDNA haplotype C9; it is associated with M1 in one individual (population 3) and with M2 in one individual of population 23 (Greece). There maybe three possible explanations. The first hypothesis is homoplasy in mtDNA, however, this seems unlikely because of very low polymorphism observed in mtDNA (implying a low mutation rate). In the second hypothesis, it maybe considered that mtDNA mutation (a substitution) must have occurred only once, thereafter C9 was derived twice independently from C10. This is more likely because only two indel mutations distinguish C9 from C10. It appears that a recurrent mutation event has occurred in cpDNA (since the two mtDNA haplotypes are both found in two cpDNA types). These cpDNA haplotypes are probably similar by state but not by descent A third hypothesis of paternal leakage of either cpDNA or mtDNA as a causal factor for dissociation of cpDNA and mtDNA also cannot be ignored.

The minimum-length spanning tree of cpDNA haplotypes, with the mtDNA haplotypes plotted on it shows three clusters: cpDNA haplotypes coupled to M1 are distributed in Cluster I and Cluster II, whereas Cluster III consists of all cpDNA haplotypes coupled to M2, except C9, which is associated with both M1 and M2. C20 represents a node for Cluster I, while Cluster II and III arise from the node represented by C10. Besides, C9 and C10, all the cpDNA haplotypes associated with M2 are private haplotypes. Although, these private cpDNA haplotypes are geographically unrelated (phylogeographic structure is absent), phylogenetically they are related

and have the same mtDNA haplotype M2. Cluster II does not separate from Cluster III in the absence of mtDNA marker (a non homoplasious marker in the present study). All the three clusters have phylogenetically related but mostly geographically unrelated cpDNA haplotypes.

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Figure legend

FIG. 1. Minimum-length spanning tree of 33 cpDNA haplotypes of *Prunus spinosa* with the mtDNA haplotypes plotted on it. The length of segments joining the circles is proportional to the number of mutations between haplotypes. The empty circles, black circles, and half filled circles represent the cpDNA haplotypes associated with the mtDNA haplotype M1, M2, and both M1 and M2, respectively.

1 TABLE 1. Populations, origin and number of individuals studied in *Prunus spinosa*

Population code	Collection site	Origin	Longitude, Latitude	No. of individuals
01	Glen Afric	Great Britain	04º83′W, 57º32′N	6
02	Lake District	Great Britain	03º00′W, 54º27′N	6
03	Forest of Dean	Great Britain	02º65′W, 51º83′N	7
04	Stenshuvud	Sweden	14º25′E, 55º65′N	6
05	Halltorps Hage	Sweden	16º53′E, 56º75′N	8
06	Shönberg	Germany	07º83′E, 47º96′N	6
07	Bovenden	Germany	10º05′E, 51º57′N	7
08	Kelheim	Germany	11º83′E, 48º93′N	8
09	Fointainebleau	France	02º67′E, 48º42′N	6
10	Chizé	France	00º40′W, 46º14′N	8
11	Seillon	France	05º00′E, 46º00′N	7
12	Valbonne	France	04º55′E, 44º24′N	8
13	State Forest of Aitone	France	08º88′E, 42º28′N	7
14	Devesa da Rogueira	Spain	07º08′W, 42º25′N	7
15	Valle de Salazar	Spain	00º92′W, 42º83′N	8
16	Montejo de la Sierra	Spain	03º50′W, 41º13′N	8
17	Parco Naz. Foreste Casentinesi	Italy	11º80′E, 43º78′N	6
18	Alto Garda Bresciano	Italy	10º88′E, 45º80′N	5
19	Park of Calabria	Italy	16º58′E, 39º00′N	8
20	Mt. Medvenica	Croatia	15º95′E, 45º87′N	6
21	Savarsin	Romania	22º23′E, 46º02′N	5
22	Boki	Slovakia	19º12′E, 48º57′N	4
23	Paleochori	Greece	23º69′E, 40º51′N	5
24	Voronez Reserve	Russia	39º50′E, 51º83′N	5

TABLE 2. Major patterns and variants (in bp) in the polymorphic fragments obtained with the primer pair CD and two restriction enzymes (*Hin*fI and *Taq*I) in *Prunus spinosa*. A, B, C, D, E, and F are the polymorphisms in each polymorphic fragment

Polymorphic fragments	Major pattern →Variant
CD-(*Hin*fI)1	760(C) → 850(A), 830(B), 730(D), 510(E), 440 + 320(F)
CD-(*Hin*fI)2	380(B) → 420(A), 370(C), 350(D)
CD-(*Hin*fI)3	325(B) → 335(A), 240(C), 220(D), 0(E)
CD-(*Hin*fI)4	290(A) → 0(B)
CD-(*Taq*I)1	1400(A) → 1100(B)
CD-(*Taq*I)2	850(C) → 940(A), 880(B)
CD-(*Taq*I)3	520(C) → 580(A), 550(B)
CD-(*Taq*I)4	330(B) → 345(A), 320(C)

TABLE 3. Distribution of cpDNA and associated mtDNA haplotypes in the 24 populations of *Prunus spinosa* studied (see population code in Table 1)

cpDNA (mtDNA) haplotype		Population code (No. of individuals with a particular haplotype)																								Total no. of individuals with haplotype
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
C1	(M1)	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C2	(M1)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C3	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	3
C4	(M2)	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C5	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
C6	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
C7	(M2)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C8	(M2)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C9	(M1)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C9	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
C10	(M1)	0	1	0	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0	1	0	0	5
C10	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4
C11	(M1)	0	1	1	0	0	0	0	0	1	1	0	0	0	2	3	0	0	0	0	0	0	0	0	0	9
C12	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
C13	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
C14	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
C15	(M1)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C16	(M1)	0	0	0	0	1	0	0	1	0	0	0	0	0	4	1	1	0	0	0	0	0	0	0	0	8
C17	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
C18	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3
C19	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
C20	(M1)	6	4	4	6	7	0	7	2	4	5	7	8	2	0	1	5	4	5	0	2	1	0	0	0	80
C21	(M1)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
C22	(M1)	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C23	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
C24	(M1)	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	3
C25	(M1)	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
C26	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
C27	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
C28	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2
C29	(M1)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3
C30	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
C31	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
C32	(M1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
C33	(M2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	8
Totals		6	6	7	6	8	6	7	8	6	8	7	8	7	7	8	8	6	5	8	6	5	4	5	5	157

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