Brief Communications

AFLP Analysis of the Critically Endangered *Limonium cavanillesii* (Plumbaginaceae)

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Genetic variation in the only remaining population of *Limonium cavanillesii* has been assayed by means of AFLP analysis. The use of three different primers provided 231 marker bands, 13 of which were polymorphic among the 29 individuals assayed, thus allowing their classification into 11 distinct, but very closely related phenotypes. The low genetic variability found in *L. cavanillesii* could be explained both by the apomictic reproductive system of this species and by the passage through a severe bottleneck in recent times, after which there has been no chance for mutation to restore higher genetic variation. These results are compared to previous data obtained with RAPDs using the same *L. cavanillesii* individuals. In this case, AFLPs seem to represent a significantly more variable class of markers than RAPDs, as no variation was found with the later. Consequently a genetic marker is now available for the implementation and monitoring of conservation measures for this critically endangered species.

The AFLP methodology shares with other PCR-based genome fingerprinting methods (RAPDs, AP-PCR, DAF, etc.) several advantages over other techniques (like RFLPs, microsatellites, PCR-RFLPs, DNA fingerprinting, etc.), especially for assessing genetic variation in conservation studies (Gibbs et al. 1994; Nusser et al. 1996; Palacios and González-Candelas 1997a; Rossetto et al. 1995; Travis et al. 1996). The technique requires no prior sequence knowledge and/or investments in primer design or characterization of probes. The requirements in the quantity and quality of DNA are minimal: no more than 500 ng of DNA, purified to ensure complete digestion. The whole genome is sampled and the number of markers obtained can be very high. Moreover, AFLPs overcome the main technical drawbacks of other methods, as they have repeatedly been reported as insensitive to changes in DNA concentrations, type of DNA polymerase, and other factors (Janssen et al. 1996; Vos et al. 1995). The flexibility of the AFLP technique is also remarkable. The complexity of AFLP fingerprints can be adjusted for different genome sizes and their possible bias in G+C content through the use of different enzymes and/or the change in the selective bases present in the 3′ end of the primers (Janssen et al. 1996). The technique has started to evolve toward almost full automation by the use of fluorescent-dye-labeled primers and automated sequencers. By the combination of these features, the potential number of markers that can be detected simultaneously with this technology is superior to other techniques, at least in complex genomes (Vos et al. 1995).

Due to the dominant nature of these markers, limits to the AFLP technique lay in data analysis. The assumptions usually applied to RAPD data analysis are also pertinent here (Lynch and Milligan 1994). In spite of their drawbacks, the use of appropriate statistical methods (Huff et al. 1993; Rossetto et al. 1995; Travis et al. 1996) has allowed improved evaluation of intraspecific diversity using these markers (Clark and Lanigan, 1993; Noli et al. 1997; Pakniyat et al. 1997; Palacios and González-Candelas 1997a; Stewart and Excoffier, 1996).

Limonium cavanillesii Erben is a perennial species with leaves arranged in a basal rosette and racemose inflorescences, containing both sterile and fertile branches, with pink flowers. The triploid chromosome number ($2n = 27$), the very high presence (>95%) of sterile and malformed pollen grains, and the presence of a self-incompatible pollen-stigma combination (Erben 1979) have led researchers to consider *L. cavanillesii* an essentially apomictic species (Rosselló JA et al., unpublished data). It is endemic to a restricted part of the Spanish Mediterranean coast in the Valencia region and was declared under protection by the local government in 1986 (DOGV 336, Generalitat Valenciana, Spain). Despite this protection, *L. cavanillesii* was thought to be extinct in nature. The old populations had been destroyed by human activities or sea erosion and it had not been detected for two decades (Laguna et al. 1994). In 1994 the species was rediscovered in Serra d'Irta, near Peñíscola (150 km north of Valencia), as a population of 29 individuals distributed in a small area around Torreón de Badún.

Because of its current demographic status and the continuous reduction of its habitat, *L. cavanillesii* is one of the most endangered species of this genus (Laguna et al. 1994). Some of the measures taken to preserve the species consist of a program to better understand the biology of the species, the causes leading to its decline, and the genetic variability of the only remaining population. The area where *L. cavanillesii* lives is currently under protection within the Programa de Creación de Microrreservas de Flora de la Comunidad Valenciana, funded by the European Union through the LIFE program (Laguna E, personal communication). Further management measures should consider ex situ conservation and the establishment of new self-sustaining populations. Both measures would largely benefit from finding polymorphic loci that would allow the sampling of seeds and/or individuals and the monitoring of their evolution.

To assess genetic variation, a study was carried out using RAPDs as molecular

markers (Palacios and González-Candelas 1997b). A complete lack of genetic variation in the whole population was found, even though a considerable number of markers (131 bands) were screened. Therefore AFLP markers were used as an alternative method to analyze the genetic diversity of *L. cavanillesii*.

Materials and Methods

DNA Samples and AFLP Procedure

DNA from the 29 individuals of *L. cavanillesii* living in the wild was extracted following a modified CTAB method (Doyle 1991). Further details are described in Palacios and González-Candelas (1997b). These DNAs are the same employed in the previous RAPD study.

AFLP assays were performed with the Perkin-Elmer/Applied Biosystems (PE/ABI) AFLP™ plant mapping kit for small genomes according to the manufacturer's instructions. It is based on the method of Vos et al. (1995), but it uses nonradioactive, fluorescent dyes to label the primers, thus enabling the multiplexing of up to three different reactions from one individual in a single gel lane. The kit uses *Mse*I-C in the preselective amplification (PSA) and Dye-*Eco*RI-AX/TX and *Mse*I-CXX primers in the selective amplification (SA), where X represents any of the four nucleotides. Following Janssen et al. (1996), a preliminary study was done to determine which primer combination would be more appropriate. Primer selection was based only on the number of fragments amplified, and there was no bias in favor of those primers that gave more polymorphism, which is important in order to make comparisons among different techniques or with other species (Clark and Lanigan, 1993). Three primer combinations were selected: *Mse*I-CTG/*Eco*RI-AC, *Mse*I-CAA/*Eco*RI-AG, and *Mse*I-CTG/*Eco*RI-AT, labeled with blue, green, and yellow dyes, respectively.

Modifications to the original protocol were minimal. The number of cycles in the final step of the SA, usually 23, was increased for the green and yellow fluorescent dyes to 25 and 27, respectively. The SA reactions were multiplexed in order to load the three primer combinations from one individual in a single lane in the gel. Multiplexing was carried out by adding an uneven quantity of each SA (5 μl of the blue dye, 10 μl of the green dye, and 20 μl of the yellow dye) and 0.3 μl of the Genescan 500 ROX size marker. Electrophoresis was performed on 4% polyacrylamide gels at constant voltage (3000 V) for 3 hours at 51°C on an automated DNA sequencer (Model 377, PE/ABI) equipped with GeneScan analysis software (version 2.1, PE/ABI).

A multifactorial experiment was carried out to determine the influence on the final AFLP profiles of DNA concentration and possible random differences in the three steps of each AFLP reaction. DNA quantities of 50, 100, and 350 ng were tested, and no differences were observed in the profiles obtained among the 14 reactions performed for each DNA concentration. Consequently the amount of DNA used per AFLP reaction was 5.5 μL of the original extraction, which implies variation in DNA concentrations in the range mentioned above. Reproducibility was further tested for five individuals by repeating the complete AFLP procedure, including running the samples on different gels. Samples from another 12 individuals were run twice on different gels.

Data Analysis

The programs GeneScan and Genotyper (version 1.1, PE/ABI) were used to assemble a matrix of AFLP phenotypes and to further inspect the electrophoretograms to manually correct for any misinterpretations from both programs. The resulting presence/absence data matrix was analyzed using the package RAPDistance (Armstrong et al. 1996). Estimates of pairwise distances were made using the Euclidean distance proposed by Excoffier et al. (1992). Given the nature of the variation revealed by AFLPs, we used another distance measure originally developed for estimating nucleotide divergence from restriction fragment data (Nei and Li 1979). We assume that differences in banding patterns among AFLP phenotypes result from at least one single nucleotide substitution occurring in either the target sequence recognized by the restriction enzymes or in the selective (3′) ends of the adapters. This assumption is equivalent to that considered in Nei and Li's model for RFLP data analysis. Given the number of bases recognized by the corresponding endonucleases in each digestion and those incorporated as selective bases in the adapters, we have used a value for the length of the recognition sequence of $r = 7.5$. The resulting distance matrices were used to construct a dendrogram using the neighbor-joining procedure (Saitou and Nei 1987) with program NEIGHBOR in the PHYLIP package (Felsenstein 1993). We have used PAUP* (Swofford 1998) to perform a parsimony analysis of the AFLP phenotypes.

Results

Plant materials used in this study were the same as in the previous survey with RAPDs (Palacios and González-Candelas 1997b). A clear difference between both techniques was already detected in the preliminary study. No variation among the AFLP profiles was found when significantly different amounts of DNA were used, as opposed to the different profiles obtained with RAPDs with smaller differences in the amount of DNA. Furthermore, the remaining reproducibility tests carried out in the preliminary study also gave identical results for each individual tested. Hence the reliability of the AFLP technique at its different steps was confirmed in this study.

The combined analysis with three AFLP primer combinations generated a total of 231 fragments, ranging from 75 to 500 bp, with an average of 222.6 fragments per individual and 77 bands per primer combination. Only 13 (5.6%) AFLP markers were polymorphic, and they were able to distinguish 11 different AFLP phenotypes in *L. cavanillesii* (Table 1), but the variability observed was relatively low, which is in agreement with the previous RAPD study where no polymorphic markers were obtained.

The absolute frequency of each pattern and the estimates of pairwise distances among AFLP phenotypes obtained using the Excoffier and the Nei and Li methods are also shown in Table 1. The most abundant patterns are 5 and 6, separated by only one mutational step. The remaining patterns appear with a lower frequency, and only one or two mutational steps separate them from each other or from the most common patterns. Hence all individuals are very closely related. The average number of nucleotide substitutions per site among AFLP phenotypes is 0.00041, with values ranging from 0.00010 to 0.00111. The corresponding dendrogram obtained with the neighbor-joining procedure is shown in Figure 1. The topology is coincident with that obtained using the Euclidean measure. The parsimony analysis provided one single tree, 14 steps long, whose topology was coincident with the one derived by neighbor-joining (Figure 1). Only one character (band F in Table 1 and Figure 1) was homoplasious in the tree. The consistency index was 0.929, the homoplasy index was 0.071, and the reten-

Table 1. Presence and absence data matrix for the 13 polymorphic AFLP bands (A–M) of the 11 phenotypes, absolute frequency of each phenotype (N) in *L. cavanillesii*.

Phenotype	N	Polymorphic bands A	B	C	D	E	F	G	H	I	J	K	L	M	Distance										
1	3	0	0	0	0	0	1	0	1	0	0	1	1	0	—	3	1	1	6	5	6	8	4	1	3
2	1	0	0	0	0	1	1	1	1	0	0	1	0	0	3.0	—	4	4	3	2	3	5	1	2	6
3	1	0	0	0	0	0	1	0	1	0	0	1	1	1	1.0	4.0	—	2	7	6	7	9	5	2	2
4	3	0	0	0	0	0	1	0	1	1	0	1	1	0	1.0	4.0	2.0	—	7	6	7	9	5	2	4
5	11	0	0	0	1	1	0	1	1	0	0	0	0	0	6.1	3.0	7.1	7.1	—	1	2	2	4	5	9
6	5	0	0	0	1	1	0	1	1	0	0	1	0	0	5.0	2.0	6.0	6.0	1.0	—	3	3	3	4	8
7	1	0	0	1	1	1	1	1	1	0	0	0	0	0	6.0	3.0	7.0	7.0	2.0	3.0	—	2	4	5	9
8	1	0	0	1	1	1	0	1	0	0	0	0	0	0	8.1	5.0	9.1	9.1	2.0	3.0	2.0	—	6	7	11
9	1	1	0	0	0	1	1	1	1	0	0	1	0	0	4.0	1.0	5.0	5.0	4.0	3.0	4.0	6.0	—	3	7
10	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1.0	2.0	2.0	2.0	5.0	4.0	5.0	7.1	3.0	—	4
11	1	0	1	0	0	0	1	0	1	0	1	1	1	1	3.0	6.0	2.0	4.0	9.1	8.0	9.0	11.1	7.0	4.0	—

Matrices obtained using Excoffier (upper hemimatrix) and Nei and Li nucleotide divergence ($\times 10^{-4}$, lower hemimatrix) distances among *L. cavanillesii* AFLP phenotypes.

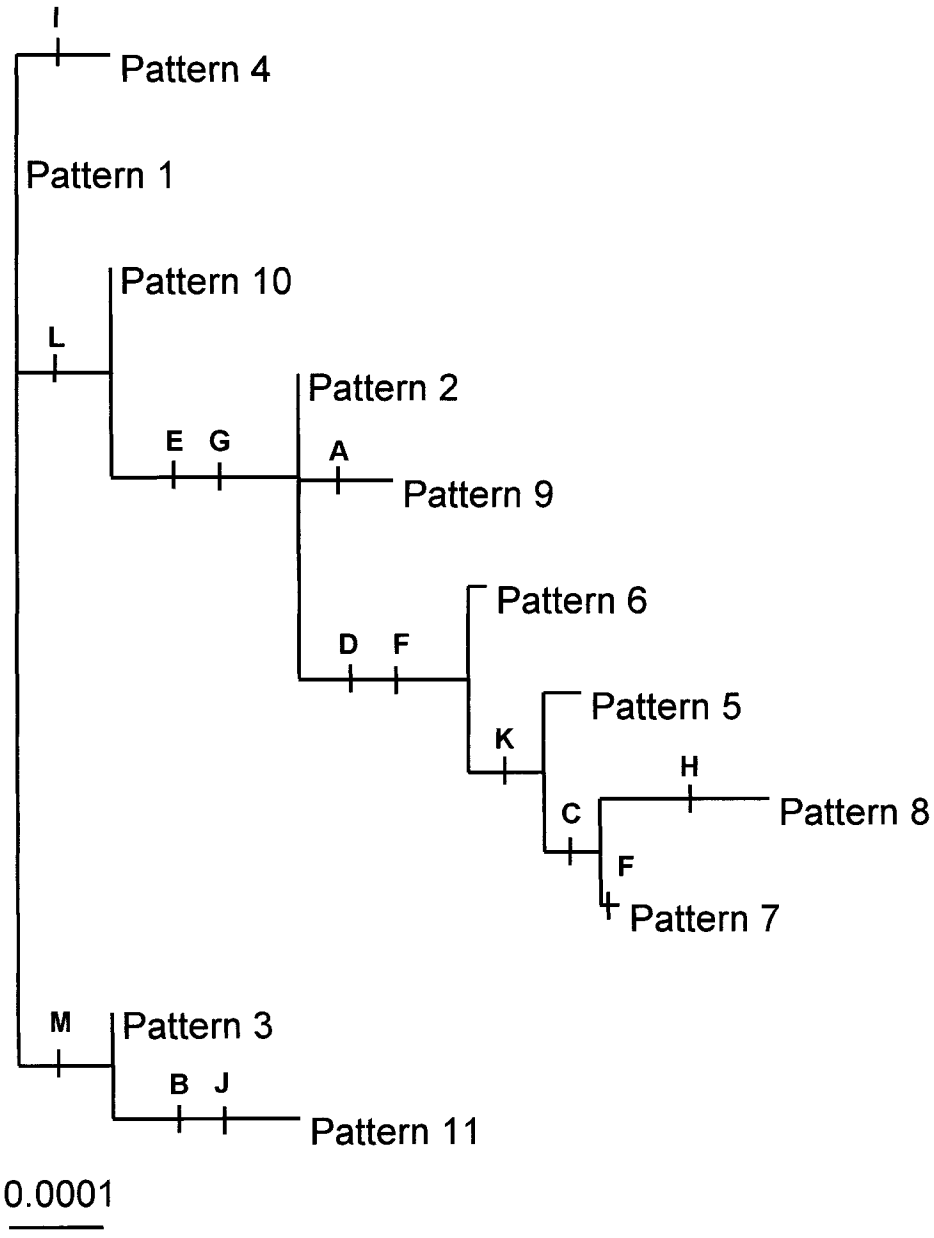

Figure 1. Unrooted neighbor-joining dendrogram based on pairwise differences among the AFLP patterns from *L. cavanillesii* obtained using Nei and Li distance. The same topology was obtained in the only tree found by parsimony analysis using PAUP*. Marks along branches indicate the minimum changes in the corresponding bands (see Table 1) accounting for the most parsimonious tree.

tion index was 0.950. All these measures indicate the high reliability of the relationships reflected in the tree.

Discussion

The need for polymorphic markers for planning recovery strategies in *L. cavanillesii* is important for its ex situ conservation in germplasm collections and the adequate establishment of new natural populations. In both cases, samples taken from nature should represent the variability found in the species. The ability to recognize individual plants is also desirable to monitor the success of each phenotype within the new populations (Rossetto et al. 1995). AFLPs provide a suitable class of markers for this goal, with the advantages previously noted over other kinds of markers. In contrast, RAPDs seem to present lower variability per primer than AFLPs in this particular species (cf. Mackill et al. 1996; Palacios and González-Candelas 1997b).

The lack of knowledge about the genomic organization and mapping location of these markers in this species prevents one from drawing further conclusions regarding the explanation for this observation. However, as more studies employing both techniques appear, it will be possible to determine whether this is a particular or general phenomenon. Nevertheless, it seems likely that variability levels similar to those found in this assay with AFLPs could only be achieved by the use of a significantly larger number of RAPD primers, with the concomitant increase in cost and time.

The advantages attributed to AFLPs over other PCR-based DNA fingerprinting methods have been confirmed in the present study. Our preliminary study and others have demonstrated the reliability of these markers, which makes AFLPs a very

efficient method, as it is not necessary to establish the reproducibility of every fragment being considered and because of the larger amount of data available with this technique. Reproducibility and reliability are at the very heart of the most common criticisms of RAPDs and other DNA fingerprinting techniques. In summary, when PCR-based DNA fingerprinting methods are chosen as the most appropriate technique for a particular problem, such as the analysis of variability in an endangered species or of species for which there is an indication of very low genetic variation, AFLP fingerprinting has several features that make it advantageous over others of these methods.

The low levels of genetic variability found in *L. cavanillesii* could be explained either by the effects of the apomictic reproductive system of this species, or the passage through a severe bottleneck in recent times, after which there has been no chance for mutation to restore significant genetic variation. This recent bottleneck is confirmed by the absence of *L. cavanillesii* from past periodic surveys of this location (Laguna E, personal communication). The analyses performed show that *L. cavanillesii* individuals are very closely related, as nucleotide divergence values among AFLP phenotypes (0.0001–0.00091) are rather low as compared to other species. Estimates of nucleotide divergence among individuals of the same species have been provided mainly from RFLP studies. Soltis et al. (1992) and Böhle et al. (1994) reported intraspecific nucleotide divergence values from 0.000 to 0.003 in surveys of cpDNA variation, whereas Milligan (1991) reported values commonly ranging from 0.0003 to 0.0015. Our results obtained in another *Limonium* species (Palacios et al. 1999), *L. dufourii,* give nucleotide divergence estimates between pairs of AFLP patterns ranging from 0.0001 to 0.0024, with an average of 0.0009 substitutions per nucleotide.

Once some knowledge about the levels of genetic variation in *L. cavanillesii* and a genetic marker that allows some discrimination among population variants is available, measures for in situ and ex situ conservation and their progress can be better monitored (Rossetto et al. 1995). The adoption of conservation measures for apomictic species can be controversial (Kraft and Nybom, 1995; Kraft et al. 1996; van Heusden et al. 1991, as their taxonomic status is questionable, and there is no consensus on whether to preserve all and each variant (population, clone, or individual) arising in the species, or only a representative sample of them. A conservative policy should preserve as much of the current diversity as possible, especially when dealing with species in such low numbers as *L. cavanillesii.* Given the near genetic homogeneity of the only extant population of this species, it seems obvious that the whole population should be preserved. Some authors have stressed the potential value of small reserves to provide a wider choice of sites for protection and emphasized that they can play an important role in plant conservation (Lesica and Allendorf, 1991; Reznicek, 1987). Reestablishment of the species in suitable and ecologically secure areas, creating new self-sustaining populations, is desirable. In this respect, AFLPs provide a suitable marker for monitoring the possible role of genetic differences in the establishment and recovery of *L. cavanillesii* populations.

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References

Armstrong J, Gibbs A, Peakall R, and Weiller G, 1996. RAPDistance programs: version 1.04 for the analysis of patterns of RAPD fragments. Camberra: Australian National University.

Böhle UR, Hilger H, Cerff R, and Martin WF, 1994. Noncoding chloroplast DNA for plant molecular systematics at the infrageneric level. In: Molecular ecology and evolution: approaches and applications (Schierwater B, Streit B, Wagner GP, and DeSalle R, eds). Basel: Birkhäuser-Verlag; 391–404.

Clark AG and Lanigan CMS, 1993. Prospects for estimating nucleotide divergence with RAPDs. Mol Biol Evol 10:1096–1111.

Doyle JJ, 1991. DNA protocols for plants. In: Molecular techniques in taxonomy (Hewitt GM, Johnston AWB, and Young JPW, eds). Berlin: Springer Verlag; 101–115.

Erben M, 1979. Karyotype differentiation and its consequences in Mediterranean *Limonium.* Webbin 34:409–417.

Excoffier L, Smouse PE, and Quattro JM, 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Felsenstein J, 1993. PHYLIP: phylogenetic inference package, version 3.5. Seattle: University of Washington.

Gibbs HL, Prior KA, and Weatherhead PJ, 1994. Genetic analysis of populations of threatened snake species using RAPD markers. Mol Ecol 3:329–337.

Huff DR, Peakall R, and Smouse PE, 1993. RAPD variation within and among natural populations of outcrossing buffalograss [*Buchlöe dactyloides* (Nutt.) Engelm.] Theor Appl Genet 86:927–934.

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M, and Kersters K, 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142:1881–1893.

Kraft T and Nybom H, 1995. DNA fingerprinting and biometry can solve some taxonomic problems in apomictic blackberries (*Rubus* subgen. *Rubus*). Watsonia 20:329–343.

Kraft T, Nybom H, and Werlemark G, 1996. DNA fingerprint variation in some blackberry species (*Rubus* subg. *Rubus, Rosaceae*). Plant Syst Evol 199:93–108.

Laguna E, Aguilella A, Carretero JL, Crespo MB, Figuerola R, and Gonzalo M, 1994. *Libro de la flora vascular rara, endémica o amenazada de la comunidad Valenciana.* Valencia: Generalitat Valenciana.

Lesica P and Allendorf FW, 1991. Are small populations of plants worth preserving? Conserv Biol 5:182–185.

Lynch M and Milligan BG, 1994. Analysis of population genetic structure with RAPD markers. Mol Ecol 3:91–99.

Mackill DJ, Zhang Z, Redoña ED, and Colowit PM, 1996. Levels of polymorphism and genetic mapping of AFLP markers in rice. Genome 39:969–977.

Milligan BG, 1991. Chloroplast DNA diversity within and among populations of *Trifolium pratense.* Curr Genet 19:411–416.

Nei M and Li WH, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273.

Noli E, Salvi S, and Tuberosa R, 1997. Comparative analysis of genetic relationships in barley based on RFLP and RAPD markers. Genome 40:607–616.

Nusser JA, Goto RM, Ledig DB, Flesicher RC, and Miller MM, 1996. RAPD analysis reveals low genetic variability in the endangered light-footed clapper rail. Mol Ecol 5: 463–472.

Pakniyat H, Powell W, Baird E, Handley LL, Robinson D, Scrimgeour CM, Nevo E, Hackett CA, Caligari PDS, and Forster BP, 1997. AFLP variation in wild barley (*Hordeum spontaneum* C. Koch) with reference to salt tolerance and associated ecogeography. Genome 40: 332–341.

Palacios C and González-Candelas F, 1997a. Analysis of population genetic structure and variability using RAPD markers in the endemic and endangered *Limonium dufourii* (*Plumbaginaceae*). Mol Ecol 6:1107–1121.

Palacios C and González-Candelas F, 1997b. Lack of genetic variability in the rare and endangered *Limonium cavanillesii* (*Plumbaginaceae*) using RAPD markers. Mol Ecol 6:671–675.

Palacios C, Kresovich S, and González-Candelas F, 1999. A population genetic study of the endangered plant species *Limonium dufourii* (*Plumbaginaceae*) based on AFLPs. Mol Ecol 8:645–657.

Reznicek AA, 1987. Are small reserves worthwhile for plants? Endanger Species Update 5:1–3.

Rossetto M, Weaver PK, and Dixon KW, 1995. Use of RAPD analysis in devising conservation strategies for the rare and endangered *Grevillea scapigera* (*Proteaceae*). Mol Ecol 4:321–329.

Saitou N and Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Soltis DE, Soltis PS, and Milligan BG, 1992. Intraspecific chloroplast DNA variation: systematic and phylogenetic implications. In: Molecular systematics of plants (Soltis PS, Soltis DE, and Doyle JJ, eds). New York: Chapman & Hall; 117–150.

Stewart JCN and Excoffier L, 1996. Assessing population genetic structure and variability with RAPD data: application to *Vaccinium macrocarpon* (American cranberry). J Evol Biol 9:153–171.

Swofford DL, 1998. PAUP*, version 4.0b1. Sunderland, MA: Sinauer.

Travis SE, Maschinski J, and Keim P, 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax,* a critically endangered plant, using AFLP markers. Mol Ecol 5:735–745.

van Heusden AW, Rouppe van der Voort J, and Bachmann K, 1991. Oligo-(GATA) fingerprints identify clones in asexual dandelions *(Taraxacum, Asteraceae*). Fingerprint News 3:13–15.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, and Zabeau M, 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414.

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High-Resolution Mapping and Genetic Characterization of the *Lazy-2* Gravitropic Mutant of Tomato

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Mutation of the *Lazy-2* (*Lz-2*) gene in tomato (*Lycopersicon esculentum* Mill.) produces a phytochrome-dependent reversal of shoot gravitropism, providing a unique genetic resource for investigating how signals from light modulate gravitropism. We mapped the *Lz-2* gene using RFLPs and a PCR-based technique to assess the feasibility of positional cloning. Analysis of a 1338 plant backcross population between *L. esculentum* and *L. pennellii* placed *Lz-2* within a 1.2 cM interval on chromosome 5, 0.4 cM from TG504, and 0.8 cM from CT201A. We did not observe crossovers between several other markers in the TG504-CT201A interval. The inability to resolve these markers indicates that *Lz-2* resides in a centromeric region in which recombination is highly suppressed. *Lazy-2* is tightly linked to but does not encode the gene for ACC4, an enzyme involved in ethylene biosynthesis. We also observed that *lz-2* is partially dominant under certain conditions and stages of development.

The *lazy-2* (*lz-2*) gravitropic mutant of tomato displays downward shoot growth in light but not in darkness. The *lz-2* allele was isolated from a tomato (*Lycopersicon esculentum* Mill. cv. San Marzano) population mutagenized with ethyl methane sulfonate (EMS) and was classified as recessive by Soressi and Cravedi (1967). The reversal of shoot gravitropism has been shown to be regulated by phytochrome based on physiological (Gaiser and Lomax 1993) and genetic studies (Behringer and Lomax 1999). Phytochrome photoreceptors transduce signals from red, far-red, and blue light over a wide range of photon fluences, allowing plants to optimize development in response to numerous and diverse environmental signals (Smith 1994). Because the reversed gravitropic response of *lz-2* plants depends on phytochrome, the *lz-2* mutant provides a unique opportunity to gain insight into the mechanisms by which phytochrome activation influences gravitropism.

Gravitropism is a complex physiological process that likely involves a number of factors that combine to control cell expansion for optimizing shoot angle. A number of studies have shown that light, acting through phytochrome, can modulate gravitropism (Hangarter 1997). Compared to many phytochrome-mediated responses, however, the role of phytochrome in influencing gravitropism is poorly understood. Perhaps the best-studied example of this interaction is that of maize cv. Merit, in which roots are triggered to change from horizontal to downward growth by the activation of phytochrome (Lu et al. 1996; Mandoli et al. 1984). The roots of certain other maize cultivars (Johnson et al. 1991) and *Convolvulus arvensis* (Tepfer and Bonnett 1972) respond similarly. Light also plays a role in stem orientation in *Oplismenus* and *Tradescantia* (Digby and Firn 1995). In *Arabidopsis,* light acting through phytochrome blocks negative gravitropism (upward growth) of hypocotyls (Poppe et al. 1996; Robson and Smith 1996). In tomato, several gravitropic mutants have been identified. Probably the best known is *diageotropica* (*dgt*), in which the horizontal gravitropic response of the mutant is restored to normal by minute concentrations of ethylene (Jackson 1979; Madlung et al., in press; Zobel 1973, 1974). In contrast to the *dgt* mutant, which is altered in auxin signaling in general (Coenen and Lomax 1998; Kelly and Bradford 1986; Mito and Bennett 1995), *lz-2* does not appear to be altered in auxin responsiveness (Gaiser and Lomax 1993). In addition, the pleiotropic *dgt* phenotype is expressed in the dark, whereas the reversed gravitropic response in the *lz-2* mutant is specifically triggered by light via phytochrome. The *Lz-2* gene appears likely to encode a component of the transduction chain that integrates signals from gravity and light to orient shoot growth (Gaiser and Lomax 1993).

To uncover the biochemical function of the *Lz-2* gene product, we determined the physical map location of the mutation and assessed the suitability of positional cloning as a means to isolate the gene. Tomato is suitable for map-based cloning because of its extensive RFLP map, relatively small genome size, and the availability of YAC and BAC libraries (Tanksley et al. 1995). Positional cloning has been successfully used to clone the *Pto* gene which confers bacterial resistance in tomato (Martin et al. 1993a). Here we placed *Lz-2* on the tomato RFLP map (Tanksley et al. 1992) and then identified the two closest flanking markers from existing RFLPs. In the process of mapping we observed that heterozygous *lz-2*/+ plants displayed an intermediate gravitropic response under certain lighting conditions and at specific stages of development. Mapping results also showed that the *Lz-2* gene is adjacent to but does not encode *ACC4,* the gene for an enzyme involved in ethylene biosynthesis.

Materials and Methods

The *lz-2* allele introgressed into cv. Ailsa Craig (AC) through repeated backcrossing (Rick C, personal communication) was used in these experiments. For mapping experiments, an F_2 population was derived by introducing pollen from *L. pennellii* LA716 (Tanksley et al. 1992) onto emasculated *lz-2* flowers, followed by selfing of the F_1 plants. A backcross (BC) population was obtained by crossing F_1 and *lz-2* plants, with *lz-2* plants as the pollen recipient. For experiments to test partial dominance, *lz-2* and AC plants were crossed and the F_1 and F_2 plants were tested for gravitropic characteristics. Plants were grown in the greenhouse at 22°C–27°C with supplemental lighting during the winter months, except for the seedlings used to test partial dominance, which were germinated and grown at 22°C–24°C under red light with a fluence rate of 0.8 $\mu mol/m^2/s$ (640–680 nm), as described by Gaiser and Lomax (1993).

RFLP analysis was performed using CTAB-extracted DNA (Rogers and Bendich 1994) prepared from young expanding leaves and flower buds. The restriction enzymes for generating RFLPs were selected based on information provided by the Solgenes website (http://probe.nalusda.gov:8300/cgi_bin/

browse/solgenes) or by screening enzymes with survey filters. Southern blots were prepared by digesting approximately 10 μg of DNA with the appropriate restriction enzyme and fractionating the samples through 0.7% agarose. The DNA was depurinated with 0.25 N HCl (2 × 10 min) and transferred to Hybond N^+ membranes (Amersham) by alkaline transfer (Sambrook et al. 1989). Following transfer, membranes were washed for 20 min with 5× SSPE and stored at 4°C.

Probes used in RFLP analysis were prepared by PCR amplification of RFLP clones (supplied by S. Tanksley, Cornell University) using M13 forward and reverse primers in a standard PCR reaction. The PCR products were purified by spin column and run on an agarose gel to confirm the size and to quantitate the amount of DNA. Probes were labeled with ^{32}P-dCTP using random primers (Ambion, Decaprime kit). DNA blots were prehybridized in a 1 M NaCl, 10% dextran sulfate solution at 65°C for 30 to 60 min. Labeled probe (10^6 cpm/ml prehybridization solution) was boiled with salmon sperm DNA (100 mg/ml prehybridization solution) and added to the prehybridized blots. After hybridization at 65°C for 12–24 h, blots were washed with 0.1× SSPE at 65°C for 1–2 h and then exposed to Kodak X-OMAT XAR or BIOMAX MR film for 4–24 h.

DNA samples from several plants were pooled to facilitate initial RFLP marker analysis. Reliable detection of putative recombinants was confirmed by combining leaf material from an *L. esculentum*/*L. pennellii* heterozygote with equal amounts of leaf material from two *L. esculentum* plants. In these control experiments the heterozygous plant was reliably scored. Subsequently equal amounts of leaf material from three BC_1 plants exhibiting the *lz-2* phenotype were pooled prior to DNA extraction.

RFLPs closely linked to *lz-2* were converted to a PCR-based scoring procedure termed CAPS (cleavable amplified polymorphic sequences; Konieczny and Ausubel 1993). In brief, the ends of the RFLP probes were sequenced by automated dideoxy sequencing and primers were designed to amplify the RFLP loci. Primer pairs were used only if they amplified the target sequence equally from both *L. esculentum* and *L. pennellii* genomic DNA. The products derived from *L. esculentum* and *L. pennellii* DNA were screened with restriction enzymes to identify a site unique to one of the two products. Plants were genotyped by comparison with parental controls after electrophoresis of digested PCR products.

Table 1. Primers, PCR profiles, and expected PCR product sizes in the CAPS analysis of the *Lz-2* region of chromosome 5[a]

Locus	Primer sequence (5′ → 3′)	PCR profile	Enzyme	Product size (kb) *esc*[b]	Product size (kb) *penn*[b]
TG504	TAATGTTCTTGACCGAGCTTC ATGGACTAAAATATGGCCTTAG	2	*Msp*I	550 450	1000
TG619	TACCCAAGTACTTCGTGACTG ATTCACCCTCAGCTGCATATG	2	*Xho*I	800	550 250
CT201A	CCTTTCACACACCAATAGTTG CAGTTCTTCAATACCTCAAGG	1	*Dra*I	1200	800 400
ACC4	TGAATTGTAGCGGTGAAAGTC AAATACATGCCAATGTAAGCCA	2	*Xba*I	1000 150	1150
CT202	GGACGAACAATCACTGGATGT CATTAGCCACACAACCTCATG	2	*Dra*I	1100	700 400
CT318	TTGTGTGCTTCATTTCCATGG CTATTTGCATGCATACTTACAG	3	*Xho*I	1350	900 450

[a] Details of the PCR reaction conditions (profiles 1–3) and restriction enzyme analysis of the PCR products are given in Materials and Methods.

[b] *esc* = *L. esculentum*, *penn* = *L. pennellii*.

For CAPS analysis, a rapid DNA prep was used to extract DNA from approximately 20 mg of cotyledons or leaves (Cheung et al. 1993; Rogers et al. 1996). The DNA was resuspended in 40 μl sterile water and 1 μl of each sample was amplified in a 25 μl reaction containing buffer, 50 μM of each dNTP, 2.5 mM $MgCl_2$, 2.5 pmole of each primer and 1 unit *Taq* polymerase (Gibco-BRL). The PCR parameters were 1 cycle at 94°C for 1 min; 4 cycles at 94°C for 30 s, "*n*°C" for 1 min and 72°C for 1 min; 36 cycles at 94°C for 10 s, "*m*°C" for 1 min and 72°C for 1 min, followed by 4 min at 72°C for final extension; where $n = m = 55$ (profile 1), $n = m = 50$ (profile 2), and $n = 45$ and $m = 50$ (profile 3). Restriction endonucleases were added either directly to the completed PCR reaction (*Dra*I and *Msp*I) or to 20 μl reactions containing 8 μl of the PCR product. Digests were performed with 2.5 units of enzyme for a minimum of 4 h prior to separation of the DNA and visualization of bands with ethidium bromide. The sizes of the various amplification products are summarized in Table 1 along with primers, PCR conditions, and the enzymes used to score each locus.

Map distances between markers and *Lz-2* were computed using the Kosambi mapping function (Kosambi 1944) and ordered with a LOD > 3.0 using the computer program G-Mendel v 3.0 (Holloway and Knapp 1994).

Results and Discussion

Initial Mapping of *Lz-2*

Initial experiments, which used an F_2 mapping population from a cross between *Lz-2* (in *L. esculentum*) and *L. pennellii,* placed *lz-2* near the centromere of chromosome 5, with strong linkage to markers CT201A and CT202 (Figure 1A). However, of the 372 F_2 plants scored, only 44 grew downward and could be classified as *lz-2*/*lz-2*. This large departure from the expected number of downward-growing plants (93) was statistically significant ($\chi^2 = 34.4$, $P < .001$). The majority of scored plants grew at an intermediate angle (i.e., between upright and horizontal, data not shown), suggesting that *L. pennellii* contains modifiers capable of affecting *Lz-2* expression. The distorted segregation and variable phenotypes caused plant misclassification and indicated that an F_2 population between *L. esculentum* and *L. pennellii* was not suitable for fine-mapping.

Fine-Mapping *Lz-2* in a BC_1 Population

To clarify phenotype scoring, a population backcrossed to *lz-2* was produced. The BC_1 seedlings segregated with 678 seedlings growing upward and 660 growing downward, correlating very well with an expected 1:1 ratio. Analysis of the BC_1 population with CAPS markers (Table 1) placed *lz-2* within a 1.2 cM interval defined by TG504 and CT201A (Figure 1B). The location of the *Lz-2* gene on chromosome 5 clearly differentiates it from the *dgt* mutation, which maps to chromosome 1 (Tanksley et al. 1992; Ellard-Ivey et al., personal communication). This map location also defined *Lz-2* as a novel locus controlling photomorphogenesis since *Lz-2* did not map to the loci of any of the five phytochrome genes in tomato (van Tuinen et al. 1997).

Mapping *Lz-2* to the TG504-CT201A interval also revealed that *Lz-2* resides in a

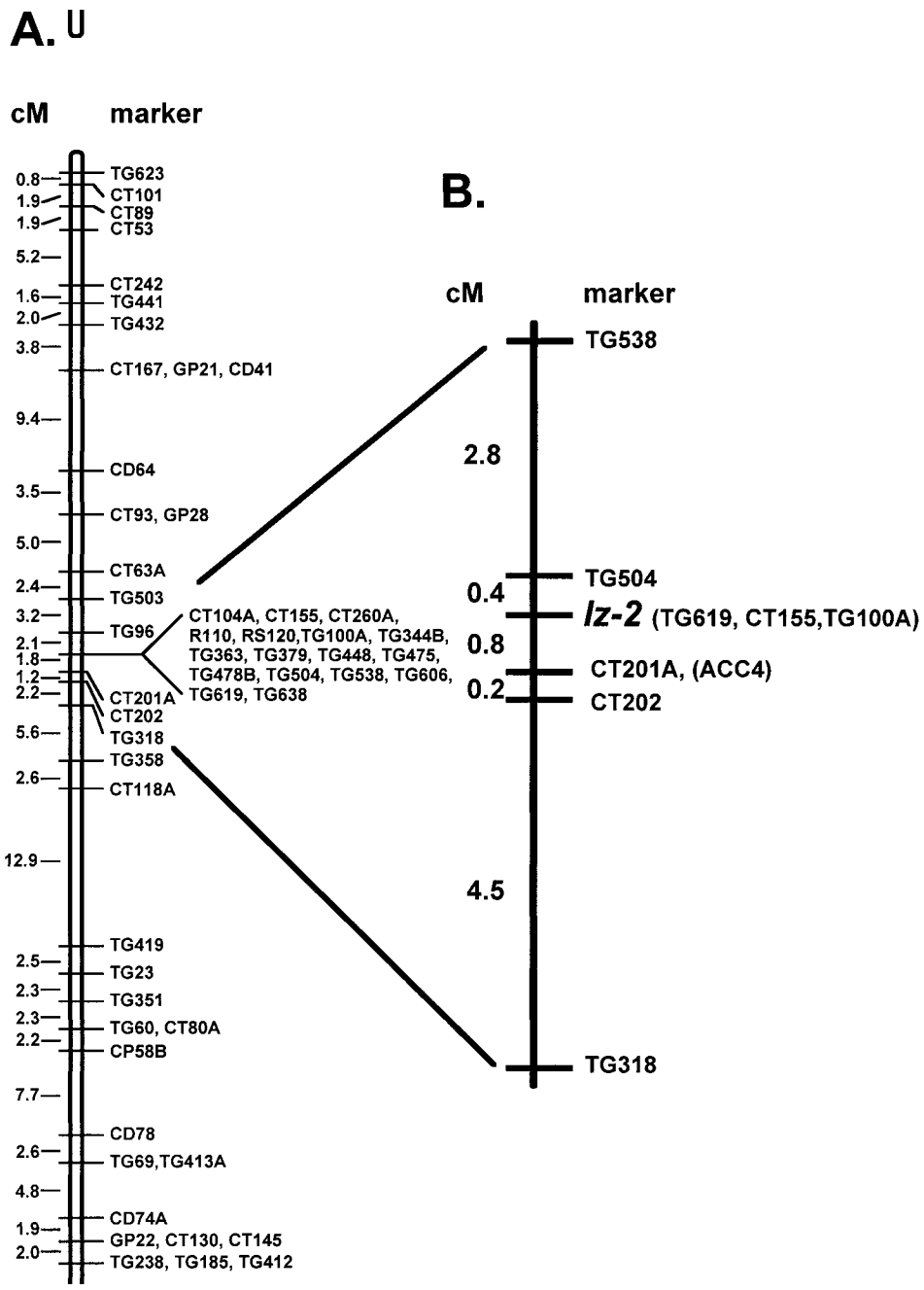

Figure 1. Localization of *Lz-2* in the centromeric region of chromosome 5. **(A)** Map of markers placed on chromosome 5 ordered with a LOD > 3 (adapted with permission from Martin et al. 1993b). **(B)** Placement of *Lz-2* between TG504 and CT201A using a BC population (n = 1338). Map distances and order were computed with the program *G-Mendel* with a LOD > 3.0. Markers enclosed in parentheses were assigned their position based on analysis of subsets of plants and were not incorporated in the computer mapping. The entire BC population was scored for TG504, CT201A, and CT202 with less than 2% of the plants not classified for a particular locus. For TG538 and TG318, 251 and 189 plants were scored, respectively. The *ACC4* gene was mapped in 81 *L. esculentum* × *L. pennellii* F_2 plants. The RFLP markers CT155, TG100, and TG619 were mapped in the subset of BC plants that displayed recombination between *lz-2* and the flanking markers TG504 and CT201A.

centromeric region. The lack of recombination between markers in this region of chromosome 5 indicates potential difficulty with using positional cloning to recover this gene. High suppression of recombination rates (Frary et al. 1996; Khush and Rick 1968; Sherman and Stack 1995) and large physical distances between even very tightly linked genetic markers (Pillen et al. 1996) in centromeric regions are contributing factors. However, rates of recombination can vary abruptly along a chromosome (Gorman et al. 1996). If the gene of interest resides in a recombination hotspot, then the possibility exists that markers will be found that are tightly linked both genetically and physically. The very low frequency of recombination among the markers CT201A, CT202, TG318, and ACC4 agrees well with the map presented by Tanksley et al. (1992; Figure 1A). However, we observed enhanced recombination between TG504 and TG538, something not seen by Tanksley et al. (1992; Figure 1A).

The increased recombination between TG504 and TG538 in our mapping population indicated that at least a subset of the markers clustered in this region can be resolved and that these markers might be helpful in more precisely defining the location of *Lz-2.* Of interest, Martin et al. (1993b) observed an even greater crossover frequency between TG504 and TG538 and several other markers in this region. We therefore attempted to further resolve *Lz-2* by testing for recombination between *Lz-2* and several of these markers—TG619, CT155, and TG100A. These markers map 0.2 cM apart from each other within the TG504-CT201A interval in an *L. esculentum* population carrying a small *L. pimpinellifolium* introgression (Martin et al. 1993b). However, no crossovers between the *Lz-2* locus and any of these markers were detected in the 13 BC plants previously confirmed to be recombinant between TG504 and CT201A (Figure 1B). Thus without the use of the introgression line to generate a mapping population (Martin et al. 1993b), these markers do not appear to be separable.

We also initiated analysis of an F_2 population generated from a cross between *lz-2* plants (*L. esculentum*) and *L. pimpinellifolium* to enhance recombination rates over those observed in the BC_1 population. Rick (1969) has suggested that interspecific crosses between more closely related species increase recombination events. In addition, recombination rates have been reported to be slightly higher in megagametogenesis as compared to microgametogenesis (de Vicente and Tanksley 1991). Since F_1 plants were used as pollen donors, our BC_1 population did not take advantage of recombination events possible within an F_1 ovum. We did observe a 3:1 ratio of wild-type-appearing to *lz-2* plants in the *L. esculentum* × *L. pimpinellifolium* F_2 population, indicating the lack of background effects on *lz-2* expression in *L. pimpinellifolium.* In such closely related species, however, restriction enzyme site differences necessary for CAPS analysis were difficult to find. Sequence

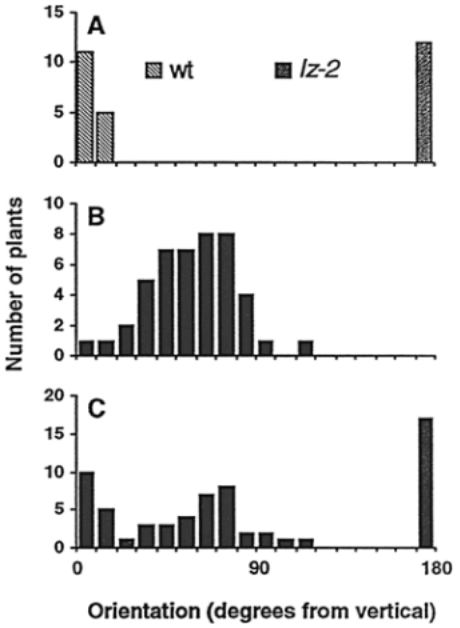

Figure 2. Distribution of growth angles of +/+, *lz-2*/+, and *lz-2*/*lz-2* seedlings under red light. **(A)** Wild-type and *lz-2,* **(B)** wild-type × *lz-2* F_1, and **(C)** F_2 seedlings were germinated and grown under red light for 10 days. The hypocotyl orientation was measured, with 0 degrees defined as upright (vertical) and 180 degrees as downward.

analysis of the TG504 and TG619 loci (1 kb each) from both *L. esculentum* and *L. pimpinelifolium* confirmed sequence similarities. Only a 0.1% difference was observed between sequences from the two species. Obviously an alternative method that can differentiate single-base differences will be required to permit rapid scoring of this cross.

The *Lz-2* Allele Is Partially Dominant

During the generation of the backcross mapping population, a number of mature BC_1 plants were observed to grow at angles that deviated from vertical, some growing horizontally. This observation raised the question of whether *lz-2* is actually recessive. To test this hypothesis, segregation of the *lz-2* phenotype was measured in an F_1 population resulting from a cross between *lz-2* and wild type (Ailsa Craig). Seedlings were grown in red light instead of white light to remove the blue-light component of the spectrum and thus eliminate the effects of phototropism. Under these conditions, 100% of the wild-type seedlings grew upright (0–20 degrees from vertical), while 100% of the homozygous *lz-2* plants reoriented downward (180 degrees; Figure 2A). The majority of the F_1 seedlings grew at some intermediate angle (Figure 2B). When F_2 seedlings resulting from selfing the same cross were grown in red light, seedlings could be grouped into three classes with one-fourth of the plants growing upward, one-fourth growing downward, and one-half growing at an angle of 20–100 degrees from vertical (Figure 2C). These phenotype segregation patterns indicate that *lz-2* is semidominant under these growth conditions.

Mature vegetative and flowering plants were also examined for partial dominance (data not shown). Eighteen F_2 plants were grown during the summer under natural light. Three plants were recognizable as *lz-2*/*lz-2* at an early stage. The rest were indistinguishable until the flowering stage, at which time the shoots of 10 plants began to grow at an angle (approximately 45–90 degrees from upright). The remaining five vertically growing plants were reoriented to a horizontal position. All returned to upward growth within 8 h, indicating that these plants were fully responsive to gravity at this stage. Progeny testing showed that all but 1 of the 10 plants observed to grow at an angle segregated 3:1 for normal versus downward-growing plants. This result indicated a strong correlation between intermediate growth angles and heterozygosity.

In a third experiment, upright 4-week-old F_2 plants were tested for gravitropic responsiveness by placing them horizontally under red light. The plants reoriented their shoots uniformly within 4–5 h and it was not possible to differentiate heterozygotes from the wild-type plants using this assay (data not shown). This experiment confirmed that the partial dominance of the *lz-2* allele is subtle and apparent only under specific environmental conditions and stages of development. In particular, germination and growth under low fluence red light is required for reliable scoring of heterozygotes. As plants mature, partial dominance may easily be missed. In situations where segregating plants are grown in natural lighting, phototropism is also active and masks the effect of an *lz-2*/+ heterozygote, particularly in seedlings. Of interest, the effects of *lz-2* overcome those of phototropism during flowering. The necessity of staking plants in confined growing conditions also makes it difficult to see the effect of *lz-2*/+ on the gravitropic response of mature plants. At this time the basis for partial dominance of *lz-2* is unknown. It would be interesting to determine whether only one copy of the wild-type allele results in insufficient levels of the gene product, as is common with regulatory components including phytochrome (van Tuinen et al. 1995), or if the *lz-2* mutation results in the gain of a function.

Testing the Possible Role of Ethylene in *lz-2* Plants

The mechanism underlying the reversal of gravitropic curvature by mutation of the *Lz-2* gene is unknown. The mapping studies described above placed *Lz-2* on chromosome 5 near *ACC4,* the locus of a member of the gene family encoding ACC synthase, a key enzyme in ethylene regulation (Rottmann et al. 1991). This proximity raised the possibility that the *lz-2* mutation is in or near the *ACC4* gene and modifies its expression such that shoot gravitropism is reversed. This hypothesis seemed plausible since ethylene has been shown to affect gravitropism (e.g., Golan et al. 1996; Wheeler and Salisbury 1981; Zobel 1973, 1974), ethylene levels can be regulated by phytochrome (Finlayson et al. 1998; Golan et al. 1996), and ethylene can alter transport of auxin, which is involved in establishing plant tropisms (Lomax et al. 1995). A relevant example of the interaction of ethylene, auxin, and phytochrome in affecting gravitropism is the maintenance of apical hooks of dark-grown plants (Schwark and Bopp 1993). Our mapping results demonstrated recombination between *Lz-2* and *ACC4,* indicating these two genes are on separate loci (Figure 1B). However, while the *Lz-2* gene does not encode *ACC4,* this does not preclude the possibility that *Lz-2* affects ethylene physiology by some other means. Indeed, the reversed gravitropic response of *lz-2* plants has recently been demonstrated to have enhanced sensitivity to ethylene (Madlung et al., in press).

In summary, using previously established RFLP markers, we mapped *Lz-2* to the centromeric region of chromosome 5. Because recombination rates are highly suppressed in heterochromatin, which is found near centromeres (Frary et al. 1996; Khush and Rick 1968; Pillen et al. 1996; Sherman and Stack 1995), the identification of markers sufficient for positional cloning would require scoring a prohibitively large mapping population. Since *Lz-2* and other transcriptionally active genes are in centromeric regions (Khush and Rick 1967), alternate approaches to localizing such loci and gene cloning are desirable. Knowing the location of *Lz-2* will greatly aid implementation of these approaches. Some of these options include targeting a transposable element to this locus (Burbridge et al. 1995; Carroll et al. 1995) and screening for cDNAs from this

chromosomal region using differential display and introgression lines (Eshed and Zamir 1994; Hannapple et al. 1995). We are also generating new alleles of *lz-2* to aid in cloning and understanding the basis for the partial dominance of this allele.

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References

Behringer FJ and Lomax TL, 1999. Genetic analysis of the roles of phytochromes A and B1 in the reversed gravitropic response of the *lz-2* tomato mutant. Plant Cell Environ 22:551–558.

Burbridge A, Grieve TM, Woodman KJ, and Taylor IB, 1995. Strategies for targeted transposon tagging of ABA biosynthetic mutants in tomato. Theor Appl Genet 91: 1022–1031.

Carroll BJ, Klimyuk VI, Thomas CM, Bishop GJ, Harrison K, Scofield SR, and Jones JDG, 1995. Germinal transpositions of the maize element *Dissociation* from T-DNA loci in tomato. Genetics 139:407–420.

Cheung WY, Hubert N, and Landry BS, 1993. A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and other PCR analysis. PCR Methods Appl 3:69–70.

Coenen C and Lomax TL, 1998. The *Diageotropica* gene differentially affects auxin and cytokinin responses throughout development in tomato. Plant Physiol 117: 63–72.

de Vicente MC and Tanksley SD, 1991. Genome-wide reduction in recombination of back-cross progeny derived from male versus female gametes in an interspecific cross of tomato. Theor Appl Genet 83:173–178.

Digby J and Firn RD, 1995. The gravitropic set-point angle (GSA): the identification of an important developmentally controlled variable governing plant architecture. Plant Cell Environ 18:1434–1440.

Eshed Y and Zamir D, 1994. A genomic library of *Lycopersicon pennellii* in *L. esculentum*: a tool for fine mapping of genes. Euphytica 79:175–179.

Finlayson SA, Lee I-J, and Morgan PW, 1998. Phytochrome B and the regulation of circadian ethylene production in sorghum. Plant Physiol 116:17–25.

Frary A, Presting GG, and Tanksley SD, 1996. Molecular mapping of the centromeres of tomato chromosomes 7 and 9. Mol Gen Genet 250:295–304.

Gaiser JC and Lomax TL, 1993. The altered gravitropic response of the *lazy-2* mutant of tomato is phytochrome regulated. Plant Physiol 102:339–344.

Golan A, Tepper M, Soudry E, Horowitz BA, and Gepstein S, 1996. Cytokinin, acting through ethylene, restores gravitropism to *Arabidopsis* seedlings grown under red light. Plant Physiol 112:901–904.

Gorman SW, Banasiak D, Fairley C, and McCormick S, 1996. A 610 kb YAC clone harbors 7 cM of tomato (*Lycopersicon esculentum*) DNA that includes the male sterile 14 gene and a hotspot for recombination. Mol Gen Genet 251:52–59.

Hangarter RP, 1997. Gravity, light and plant form. Plant Cell Environ 20:796–800.

Hannappel U, Balzer HJ, and Ganal MW, 1995. Direct isolation of cDNA sequences from specific chromosomal regions of the tomato genome by the differential display technique. Mol Gen Genet 249:25–36.

Holloway JL and Knapp SJ, 1994. G-Mendel 3.0: software for the analysis of genetic markers and maps. Corvallis, Oregon: Oregon State University; 1–130.

Jackson MB, 1979. Is the diageotropic tomato ethylene deficient? Physiol Plant 46:347–351.

Johnson EM, Pao LI, and Feldman LJ, 1991. Regulation of phytochrome message abundance in root caps of maize: spatial, environmental, and genetic specificity. Plant Physiol 95:544–550.

Kelly MO and Bradford KJ, 1986. Insensitivity of the *diageotropica* tomato mutant to auxin. Plant Physiol 82: 713–717.

Khush GS and Rick CM, 1967. Studies on the linkage map of chromosome 4 of the tomato and on the transmission of induced deficiencies. Genetica 38:74–94.

Khush GS and Rick CM, 1968. Cytogenetic analysis of the tomato genome by means of induced deficiencies. Chromosoma 23:452–484.

Konieczny A and Ausubel FM, 1993. A procedure for mapping *Arabidopsis* mutations using codominant ecotype-specific PCR-based markers. Plant J 4:403–410.

Kosambi DD, 1944. The estimation of map distances from recombination values. Ann Eugen 12:172–175.

Lomax TL, Muday GK, and Rubery PH, 1995. Auxin transport. In: Plant hormones, physiology, biochemistry and molecular biology, 2nd ed (Davies PJ, ed). Dordrecht: Kluwer Academic; 509–530.

Lu Y, Hidaka H, and Feldman LJ, 1996. Characterization of a calcium/calmodulin-dependent protein kinase homolog from maize roots showing light-regulated gravitropism. Planta 199:18–24.

Madlung A, Behringer FJ, and Lomax TL, in press. Ethylene plays multiple non-primary roles in modulating the gravitropic response in tomato. Plant Physiol.

Mandoli DF, Tepperman J, Huala E, and Briggs WR, 1984. Photobiology of diagravitropic maize roots. Plant Physiol 75:359–363.

Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, and Tanksley SD, 1993a. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432–1436.

Martin GB, de Vicente MC, and Tanksley SD, 1993b. High-resolution linkage analysis and physical characterization of the *Pto* bacterial resistance locus in tomato. Mol Plant Microbiol Interact 6:26–34.

Mito N and Bennett A, 1995. The *diageotropica* mutation and synthetic auxin differentially affect the expression of auxin-regulated genes in tomato. Plant Physiol 109:293–297.

Pillen K, Ganal MW, and Tanksley SD, 1996. Construction of a high-resolution genetic map and YAC-contigs in the tomato Tm-2a region. Theor Appl Genet 93:228–233.

Poppe C, Hangarter RP, Sharrock R, Nagy F, and Schafer E, 1996. The light-induced reduction of the gravitropic growth-orientation of seedlings of *Arabidopsis thaliana* (L.) Heynh. is a photomorphic response mediated synergistically by the far-red-absorbing forms of phytochromes A and B. Planta 199:511–514.

Rick CM, 1969. Controlled introgression of chromosomes of *Solanum pennellii* into *Lycopersicon esculentum*: segregation and recombination. Genetics 62:753–786.

Robson PRH and Smith H, 1996. Genetic and transgenic evidence that phytochromes A and B act to modulate the gravitropic orientation of *Arabidopsis thaliana* hypocotyls. Plant Physiol 110:211–216.

Rogers SO and Bendich AJ, 1994. Extraction of total cellular DNA from plants, algae, and fungi. In: Plant molecular biology manual, 2nd ed (Gelvin SB and Schilperoort RA, eds). Dordrecht: Kluwer Academic; D1–D8.

Rogers HJ, Burns NA, and Parkes HC, 1996. Comparison of small-scale methods for the rapid extraction of plant DNA suitable for PCR analysis. Plant Mol Biol Rep 14: 170–183.

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, and Theologis A, 1991. 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol 222:937–961.

Sambrook J, Fritsch EF, and Maniatis T, 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schwark A and Bopp M, 1993 Interaction of ethylene and auxin in the regulation of hook growth. II. The role of ethylene in different growing regions of the hook of *Phaseolus vulgaris.* J Plant Physiol 140:585–592.

Sherman JD and Stack SM, 1995. Physical map of crossover frequency on synaptonemal complexes from tomato primary microsporocytes. Tomato Genet Coop Rep 45:42–43.

Smith H, 1994. Sensing the light environment: the functions of the phytochrome family. In: Photomorphogenesis in plants, 2nd ed (Kendrick RE and Kronenberg GHM, eds). Dordrecht: Kluwer Academic; 377–416.

Soressi GP and Cravedi P, 1967. Tomato mutants obtained by means of X-ray and ethylmethanesulfonate (EMS) treatments. Tomato Genet Coop Rep 17:51.

Tanksley SD, Ganal MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roeder MS, Wing RA, Wu W, and Young ND, 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141–1160.

Tanksley SD, Ganal MW, and Martin GB, 1995. Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11:63–68.

Tepfer DA and Bonnett HT, 1972. The role of phytochrome in the geotropic behavior of roots of *Convolvulus arvensis.* Planta 106:311–324.

van Tuinen A, Cordonnier-Pratt MM, Pratt LH, Verkerk R, Zabel P, and Koornneef M, 1997. The mapping of phytochrome genes and photomorphogenic mutants of tomato. Theor Appl Genet 94:115–122.

van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE, and Koornneef M, 1995. A temporarily red light-insensitive mutant of tomato lacks a light-stable, B-like phytochrome. Plant Physiol 108:939–947.

Wheeler RM and Salisbury FB, 1981. Gravitropism in higher plant shoots. I. A role for ethylene. Plant Physiol 67:686–690.

Zobel RW, 1973. Some physiological characteristics of the ethylene-requiring tomato mutant diageotropica. Plant Physiol 52:385–389.

Zobel RW, 1974. Control of morphogenesis in the ethylene-requiring tomato mutant, diageotropica. Can J Bot 52:735–741.

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Comparative Mapping of the DiGeorge Region in the Dog and Exclusion of Linkage to Inherited Canine Conotruncal Heart Defects

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Conotruncal defects (CTDs) of the heart are a frequent component of DiGeorge, velocardiofacial, or other syndromes caused by deletions of the human chromosome 22q11 region (HSA22q11). In addition, some human patients with isolated nonsyndromic CTDs have been reported to have deletions of this region. Taken together, these findings lead to the conclusion that deletions of an HSA22q11 locus or loci produce abnormalities in cardiac development leading to CTDs. A spontaneous model of isolated inherited conotruncal malformations occurs in the keeshond dog. We have previously shown in experimental matings that nonsyndromic CTDs in the keeshond are inherited in a manner consistent with a major underlying locus. In the studies described in this article we tested two hypotheses: (1) the region of HSA22q11 commonly deleted in DiGeorge and related syndromes is evolutionarily conserved in the dog, and (2) a locus in this region is linked to hereditary CTD in the keeshond. Two loci within the minimal DiGeorge critical region (MDGCR) and two loci that lie telomeric to the MDGCR, one of which is commonly deleted in DiGeorge patients, were mapped in the dog using a combination of linkage analysis and fluorescence in situ hybridization (FISH). The results confirm conserved synteny of the loci DGS-I, CTP, D22S788 (N41), and IGLC on the telomeric end of canine chromosome 26 (CFA26). The group of four syntenic gene loci, which spans a genetic distance of 2.5 cM is the first to be mapped to this small acrocentric canine chromosome and adds gene-associated polymorphic markers to the developing dog linkage map. Linkage of loci in this region to hereditary CTD in the keeshond was excluded.

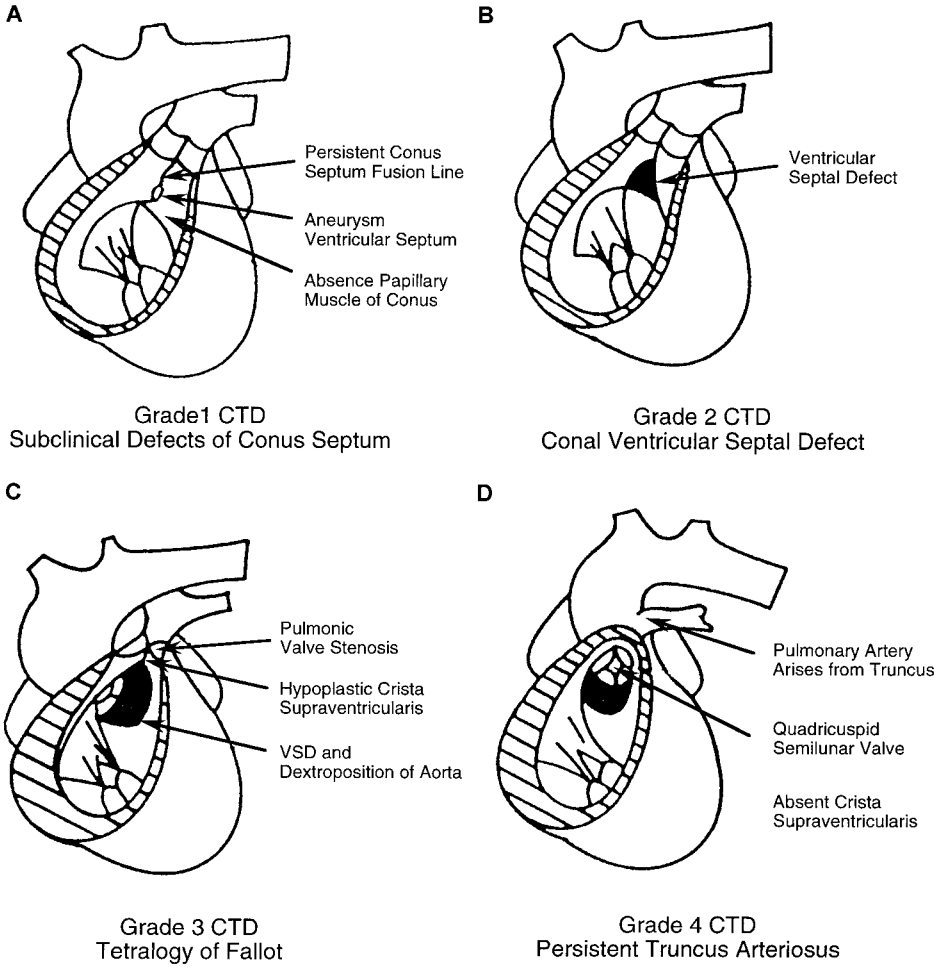

Figure 1. Anatomy of conotruncal anomalies in the CTD line of keeshond dogs. The heart is viewed in the frontal plane, with the right ventricular wall removed.

Deletions and other rearrangements in the human chromosome 22q11 region (HSA22q11) are responsible for DiGeorge syndrome (DGS), velocardiofacial, and conotruncal anomaly face syndromes (reviewed in Burn and Goodship 1997; Emanuel et al. 1998). Conotruncal heart defects (CTDs) are a frequent component of these syndromes, suggesting that the HSA22q11 syndromes are due to haploinsufficiency of one or a number of genes in this region, including a gene required for normal conotruncal development. Reports that isolated conotruncal anomalies in some human patients are associated with microdeletions in HSA22q11 also support this hypothesis (e.g., Goldmuntz et al. 1993; Wilson et al. 1991).

In earlier studies of congenital heart disease in dogs, we showed that isolated (nonsyndromic) cardiovascular malformations involving the conotruncal region are inherited in the keeshond breed (Patterson 1968; Patterson et al. 1974). The cardiac defects include ventricular septal defects of the conal type, tetralogy of Fallot, and persistent truncus arteriosus (Figure 1). Subsequent embryologic studies supported the concept that the anatomic defects in this group of cardiac malformations are genetically related and are all explainable by varying degrees of hypoplasia and failure of fusion of the conotruncal septum (Patterson et al. 1974; Van Mierop and Patterson 1978; Van Mierop et al. 1977). Fraser and Hunter (1975) reported that the same defects found in related keeshonds cluster in human families and this observation has been verified in a number of subsequent studies of congenital heart disease in humans (Corone et al. 1983; Debrus et al. 1996; Digilio et al. 1997; Ferencz et al. 1985; 1989; le Marec et al. 1989; Miller and Smith 1979; Pierpont et al. 1988). More recently we reported Mendelian breeding experiments supporting the conclusion that a major locus underlies CTD in the keeshond CTD line (Patterson et al. 1993). In our attempts to map the canine CTD locus in the keeshond, one of the candidate gene regions we examined was the dog homolog of the HSA22q11 region involved in DiGeorge and related syndromes. The finding that the minimal DiGeorge critical region (MDGCR) is conserved in the mouse (Botta et al. 1997; Galili et al. 1997; Puech et al. 1997) sug-

gested that this region might also be conserved in the dog.

The canine genome map is in an active but early stage of development. Microsatellite markers suitable for linkage analysis have been reported (Lingaas et al. 1997; Ostrander et al. 1993, 1995) and a first-generation microsatellite map with linked groups has appeared (Mellersh et al. 1997). A panel of canine-rodent hybrid cell lines has been reported (Langston et al. 1997). However, to date, the chromosomal locations of only a few gene loci are known in the dog, and published reports integrating gene and microsatellite markers have only now begun to appear (Dutra et al. 1996; Guevara-Fujita et al. 1996; Keller et al. 1998; Werner et al. 1997, 1998). Using physical and linkage mapping in the dog, we tested two hypotheses: (1) the DiGeorge region (DGR) is conserved in the dog, and (2) the CTD locus lies within or near the dog homolog of the DGR. Using methods described in a recent article in which we mapped HSA17 loci to the homologous chromosomal regions in the dog (Werner et al. 1997), we constructed physical and linkage maps of the canine homolog of the DGR and examined the question of linkage of CTD to canine genes in this region.

Methods

The CTD Line

Dogs with CTD from a partially inbred line of purebred keeshonds (CTD line) were mated to unaffected beagles and the F_1 offspring were backcrossed to the CTD line to produce an F_1 backcross generation for linkage analysis (Patterson et al. 1993). All of the dogs were eventually euthanized and their hearts examined postmortem to establish their cardiac phenotypes as previously described (Patterson et al. 1993). The conotruncal anomalies found in the CTD line are shown diagrammatically in Figure 1. Of the 108 F_1 backcross offspring included in the analysis, 60 had normal hearts and the remaining 48 had conotruncal malformations classified according to degree of severity as grade 1 (30), grade 2 (15), and grade 3 (3).

Probes

Human cDNA probes for CTP (Goldmuntz et al. 1996), IGLC (clone plambda8) (Budarf et al. 1996), D22S788 (clone LN41) (Budarf et al. 1992), and DGS-I (Gong et al. 1997) were used for Southern blot analysis and library screening (Table 1).

Table 1. Probes from the HSA22q11 region used for mapping in the dog

Human cDNA probes	Source	RFLP in the CTD line	Allele size (kb)
CTP	Goldmuntz et al. (1996)	(FISH only)	
DGS-I	Gong et al. (1997)	*Hind*III	25/23/22
D22S788 (N41)	Budarf et al. (1992)	*Msp*I	7.5/7
IGLC	Budarf et al. (1996)	*Bam*HI	2.9/2.3

Genotyping

Genomic DNA was isolated from peripheral blood or tissue samples by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. RFLPs segregating in the CTD-line were initially found with the probes for DGS-I using *Hin*dIII, IGLC using *Bam*HI, and D22S788 (N41) using *Msp*I. Subsequently 5 μg of genomic DNA were digested with these enzymes, run on a 0.8% agarose gel, and transferred onto a Hybond-N+ nylon membrane (Amersham). The human cDNA probes were radiolabeled with α-^{32}P-dCTP by random labeling and hybridization was carried out at 60°C overnight. The membranes were washed to a final stringency of 0.5× SSC/0.1%SDS and autoradiographed at −70°C.

A simple sequence repeat was later found in one of the bacteriophage clones specific for the canine homolog of D22S788 (N41) through screening the clone with simple repeat oligonucleotides. The hybridizing fragment was subcloned, sequenced, and flanking primers were constructed. N41.2F (5′ AAAGATGCGGGACAGTTCAGC 3′) and N41.1R (5′ CGCGTGCGTGCAGATAGAC 3′) amplify a 223 bp product containing a $(CA)_{11}$ repeat. The PCR reaction was carried out at an annealing temperature of 63°C in a volume of 25 μl using 50 ng of genomic DNA and 0.2 μM of each primer under the following conditions: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 5 mM $MgCl_2$, 0.8 mM dNTP, and 1 U *Taq* polymerase (Promega, Madison, Wisconsin). The products were run on a 6% nondenaturing polyacrylamide gel which was then stained with ethidium bromide.

Library Screening/FISH

A canine spleen genomic library in the lambda DASH vector or a canine genomic cosmid library in pWE15 (both from Stratagene, La Jolla, California) was screened for gene-specific clones which were used for FISH. A more detailed description of the methods used for screening, chromosome preparation, and FISH was reported earlier (Werner et al. 1997). A human total chromosome painting probe for HSA22 (Oncor, Gaithersburg, Maryland) was used for hybridization to canine chromosomes. Identification of chromosomes is based on the Giemsa-banded standard canine karyotype reported by Selden et al. (1975).

Linkage Analysis

Genotyping information from 83 to 88 F_1 backcross dogs was used to calculate maximum LOD scores and recombination fractions by performing two-point linkage analysis with the computer program Map Manager (Manly 1993). Based on the findings in the study by Patterson et al. (1993), an autosomal recessive inheritance of the major locus causing CTD was assumed for the linkage studies (all grades of CTD having equal weight).

Results

The MDGCR on HSA22q11 maps to the centromeric end of a larger region of about 2 Mb that is deleted in the majority of patients with DiGeorge and related syndromes (Gong et al. 1996). We mapped two canine homologs of loci that lie within the human MDGCR, DGS-I (transcription unit from the MDGCR), and CTP (citrate transport protein). Two loci that flank the MDGCR on its telomeric side in humans were also mapped in the dog: D22S788 (N41) and a marker from the immunoglobulin lambda gene cluster (IGLC). D22S788 lies within the region commonly deleted in DGS patients, while IGLC lies immediately telomeric to the commonly deleted region. Human cDNA probes representing these loci were used for Southern blot analysis of genomic dog DNA as well as to screen dog genomic bacteriophage or cosmid libraries and locus-specific clones were isolated as previously described (Werner et al. 1997). The clones were then hybridized with the corresponding human cDNA to verify their identity and the verified dog cosmids/bacteriophages were used as probes in FISH studies to physically map the loci to canine chromosomes as described previously (Werner et al. 1997; 1998). All four of the clones hybridized to the telomeric half of dog chromosome 26 (CFA26) within the same region (Figures 2

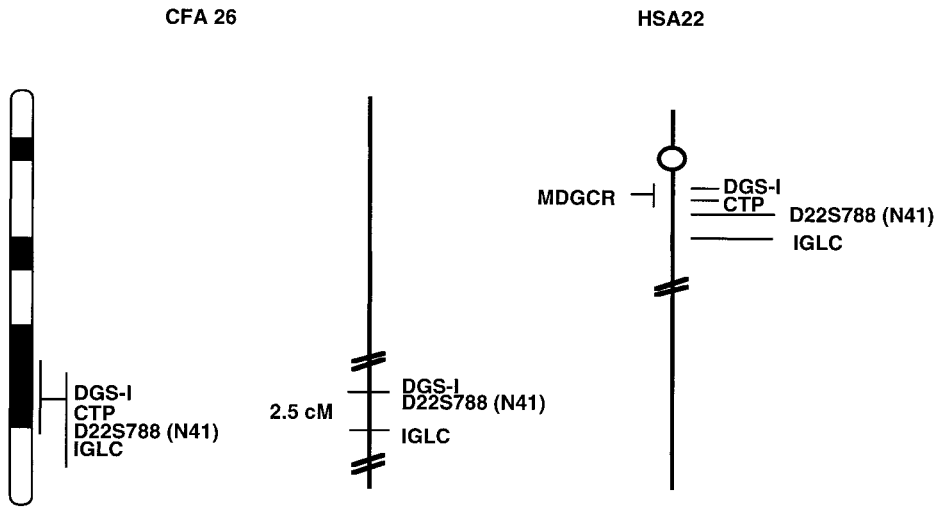

Figure 2. Linkage and physical map of the dog homolog of the commonly deleted region on HSA22q11 in DiGeorge- and velocardiofacial syndromes on CFA26 (MDGCR: minimal DiGeorge critical region). A definite order of DGS-I and D22S788 with respect to each other, to IGLC, and to the centromere was not determined.

and 3). The same region on CFA26 hybridized to a human total chromosome 22 painting probe (not shown).

Linkage Analysis

Using human cDNAs of the HSA22q11 loci as probes, we initially screened Southern blots of genomic DNA from the founder dogs of the CTD backcross pedigree using 20 to 27 different restriction enzymes. These studies revealed segregating RFLPs that were informative for linkage analysis using three of the four locus-specific probes. The probe for DGS-I displayed a *Hin*dIII polymorphism, the D22S788 (N41) probe revealed two segregating alleles following digestion with *Msp*I, and the IGLC probe detected a *Bam*HI polymorphism (Table 1). To evaluate linkage between the loci, 83 to 88 offspring from the F_1 backcross CTD line matings were genotyped at these loci. Analysis of linkage is summarized in Table 2. Later an additional gene-associated simple sequence repeat polymorphism (SSRP) was detected in the D22S788 (N41) clone and the genotyping results for this locus derived from RFLP data were confirmed. The three loci comprise a closely linked group spanning 2.5 cM with two-point LOD scores of 15–20 (Figure 2, Table 2). No recombinants were found between D22S788 (N41) and DGS-I in 64 meioses analyzed. That is consistent with evidence that DGS-I and D22S788 are separated by a physical distance of only about 1 Mb in humans. IGLC mapped 2.5 cM distal to D22S788, a finding consistent with the estimated physical distance of 2–3 Mb between these two loci in humans. Analysis of cosegregation of CTD line alleles at the D22S788, DGS-I, and IGLC loci with CTD showed 44–50% recombination, excluding close linkage of these three loci to CTD (Table 2).

Table 2. Recombination fractions with standard error and two point LOD scores (bold)

	IGLC	D22S788 (N41)	DGS-I
D22S788 (N41)	2/81		
	2.5 ± 1.7		
	20.3		
DGS-I	2/63	0/64	
	3.2 ± 2.2	0.0	
	15.1	**19.3**	
CTD	40/81	40/81	38/86
	49.4 ± 5.6	49.4 ± 5.6	44.2 ± 5.4
	0.0	**0.0**	**0.3**

Each listing describes data on linkage between the locus on the *x* and *y* axis. From the top of each listing, the fraction gives the number of recombinants/total meioses scored, followed by the distance in centi-Morgans ± its standard error, calculated according to Green (1981), and the LOD scores (in bold).

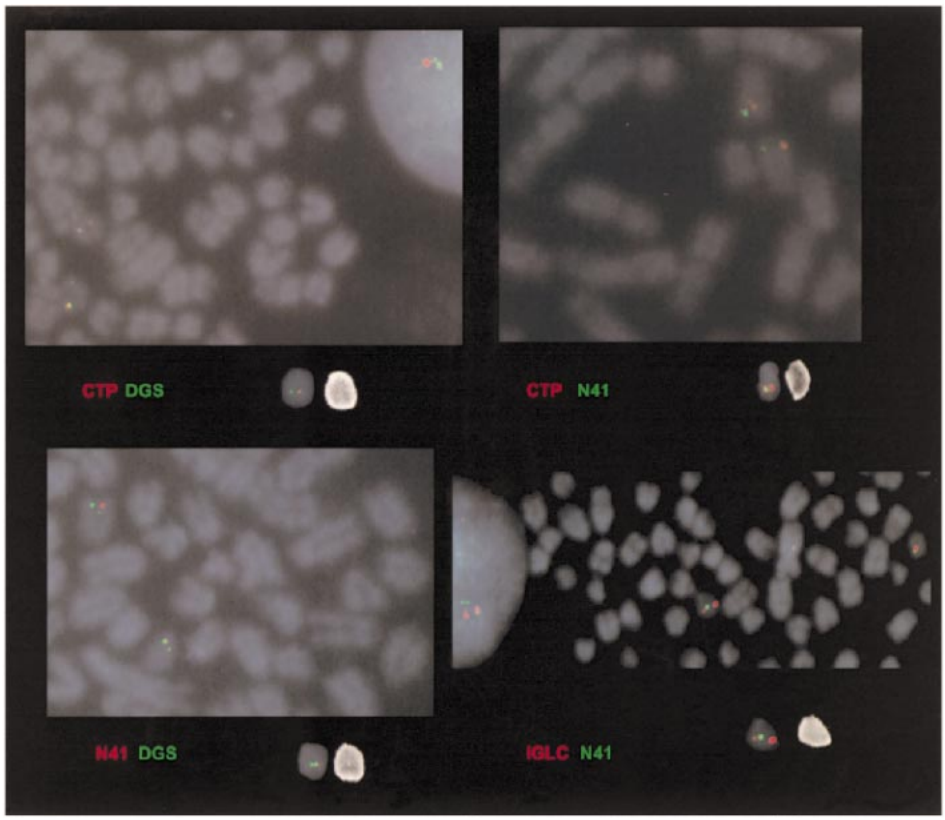

Figure 3. FISH of dog-specific cosmid or bacteriophage clones as single-locus probes to canine metaphase spreads. Single chromosomes, G-banded, and the corresponding FISH-positive chromosome, partially from different spreads are shown below. The probes used are listed below each spread and marked in the color corresponding to the hybridization signal. Yellow indicates a region where both signals overlap.

Discussion

Our findings from FISH and linkage studies show that two loci from the MDGCR on HSA22q11 (DGS-I and CTP), as well as two loci that flank this region on its telomeric side and are deleted in some DGS-patients (D22S788 and IGLC), comprise a syntenically conserved group of loci on the telomeric end of CFA26. These are the first loci to be assigned to this small acrocentric canine chromosome. The identification of gene-associated polymorphisms for these loci is an additional contribution to the developing dog linkage map. While the DGR

appears to be conserved in the dog, we failed to find evidence of linkage between keeshond CTD and the canine gene loci mapped within this region. This provides strong evidence that the CTD locus lies elsewhere in the canine genome. Using a panel of 83 to 88 F_1 backcross meioses informative for linkage between CTD and loci within the canine DGR, we should have detected significant linkage (LOD score ≥ 3) if the CTD locus lies within 24 cM of the canine DGR loci.

Conotruncal defects are among the most common cardiac defects in humans as well as dogs and nonsyndromic forms have also been reported to aggregate in human families (Corone et al. 1983; Ferencz et al. 1985, 1989; Gelb et al. 1991; Miller and Smith 1979; Pierpont et al. 1988; Rein et al. 1990; reviewed in OMIM #217095, 1998). Familial CTD in humans has been shown, as in the dog, to have an autosomal recessive mode of transmission in some families with consanguineous matings (le Marec et al. 1989; Rein et al. 1990; Rein and Sheffer 1994). In at least some affected families, deletions of HSA22q11 are not present (e.g., Debrus et al. 1996; Digilio et al. 1997; Pierpont et al. 1988), supporting the view that in humans, as well as dogs, at least one locus underlying nonsyndromic conotruncal defects lies in an as yet unmapped location outside the DGR. Further studies utilizing recently developed anonymous and gene-associated markers (Mellersh et al. 1997; Werner et al. 1999), as well as additional candidate gene approaches are currently being used to map the canine CTD locus. These studies may also shed light on the identity of a locus or loci underlying conotruncal defects in humans.

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References

Botta A, Lindsay EA, Jurecic V, and Baldini A, 1997. Comparative mapping of the DiGeorge syndrome region in mouse shows inconsistent gene order and differential degree of gene conservation. Mamm Genome 8:890–895.

Budarf ML, Baldwin S, Biegel J, McDermid H, Emanuel BS, and Driscoll D, 1992. Isolation and characterization of a cDNA from 22q11.2 that maps to the DiGeorge syndrome critical region (DGCR). Am J Hum Genet 51: A119.

Budarf ML, Eckman B, Michaud D, McDonald T, Gavigan S, Buetow KH, Tatsumura Y, Liu Z, Hilliard C, Driscoll D, Goldmuntz E, Meese E, Zwarthoff EC, Williams S, McDermid H, Dumanski JP, Biegel J, Bell CJ, and Emanuel BS, 1996. Regional localization of over 300 loci on human chromosome 22 using a somatic cell hybrid mapping panel. Genomics 35:275–288.

Burn J and Goodship J, 1997. Congenital heart disease. In: Principles and practice of medical genetics (Rimoin DL, Connor JM, and Pyeritz RE, eds). New York: Churchill Livingstone; 767–801.

Corone P, Bonaiti C, Feingold J, Fromont S, and Berthet-Bondet D, 1983. Familial congenital heart disease: how are the various types related. Am J Cardiol 51:942–945.

Debrus S, Berger G, de Meeus A, Sauer U, Guillaumont S, Voisin M, Bozio A, Demczuk S, Aurias A, and Bouvagnet P, 1996. Familial non-syndromic conotruncal defects are not associated with a 22q11 microdeletion. Hum Genet 97:138–44.

Digilio MC, Marino B, Giannotti A, Toscano A, and Dallapiccola B, 1997. Recurrence risk figures for isolated tetralogy of Fallot after screening for 22q11 microdeletion. J Med Genet 34:188–190.

Dutra AS, Mignot E, and Puck JM, 1996. Gene localization and syntenic mapping by FISH in the dog. Cytogenet Cell Genet 74:113–117.

Emanuel BD, Budarf ML, and Scambler PJ, 1998. The chromosome 22q11.2 deletion. In: Heart development (Harvey R and Rosenthal N, eds). New York: Academic Press.

Ferencz C, Boughman JA, Neill CA, Brenner JI, and Perry LW, 1989. Congenital cardiovascular malformations: questions on inheritance. Baltimore-Washington Infant Study Group. J Am Coll Cardiol 14:756–763.

Ferencz C, Rubin JD, McCarter RJ, Brenner JI, Neill CA, Perry LW, Hepner SI, and Downing JW, 1985. Congenital heart disease: prevalence at livebirth. The Baltimore-Washington Infant Study. Am J Epidemiol 121:31–36.

Fraser FC and Hunter ADW, 1975. Etiologic relations among categories of congenital heart malformations. Am J Cardiol 36:793–796.

Galili N, Baldwin HS, Lund J, Reeves R, Gong W, Wang Z, Roe BA, Emanuel BS, Nayak S, Mickanin C, Budarf ML, and Buck CA, 1997. A region of mouse chromosome 16 is syntenic to the DiGeorge, velocardiofacial syndrome minimal critical region. Genome Res 7:17–26.

Gelb BD, Towbin JA, McCabe ER, and Sujansky E, 1991. San Luis Valley recombinant chromosome 8 and tetralogy of Fallot: a review of chromosome 8 anomalies and congenital heart disease. Am J Med Genet 40:471–476.

Goldmuntz E, Driscoll DA, Budarf ML, Zackai EH, McDonald-McGinn DM, Biegel JA, and Emanuel BS, 1993. Microdeletions of chromosomal region 22q11 in patients with congenital conotruncal cardiac defects. J Med Genet 30:807–812.

Goldmuntz E, Wang Z, Roe BA, and Budarf ML, 1996. Cloning, genomic organization, and chromosomal localization of human citrate transport protein to the DiGeorge/velocardiofacial syndrome minimal critical region. Genomics 33:271–276.

Gong W, Emanuel BS, Collins J, Kim DH, Wang Z, Chen F, Zhang G, Roe B, and Budarf ML, 1996. A transcription map of the DiGeorge and velo-cardio-facial syndrome minimal critical region on 22q11. Hum Mol Genet 5:789–800.

Gong W, Emanuel BS, Galili N, Kim DH, Roe B, Driscoll DA, and Budarf ML, 1997. Structural and mutational analysis of a conserved gene (DGSI) from the minimal DiGeorge syndrome critical region. Hum Mol Genet 6: 267–276.

Green EL, 1981. Genetics and probability. New York: Oxford University Press.

Guevara-Fujita ML, Loechel R, Venta PJ, Yuzbasiyan-Gurkan V, and Brewer GJ, 1996. Chromosomal assignment of seven genes on canine chromosomes by fluorescence in situ hybridization. Mamm Genome 7:268–270.

Keller RC, Switonski M, Jörg H, Ladon D, Arnold S, and Schelling C, 1998. Chromosomal assignment of two putative canine keratin gene clusters. Anim Genet 29:141–143.

Langston AA, Mellersh CS, Neal CL, Ray K, Acland GM, Gibbs M, Aguirre GD, Fournier REK, and Ostrander EA, 1997. Construction of a panel of canine-rodent hybrid cell lines for use in partitioning of the canine genome. Genomics 46:317–325.

le Marec B, Odent S, Almange C, Journel H, Roussey M, and Defawe G, 1989. Truncus arteriosus: an autosomal recessive disease? J Genet Hum 37:225–230.

Lingaas F, Sorensen A, Juneja RK, Johansson S, Fredholm M, Wintero AK, Sampson J, Mellersh C, Curzon A, Holmes NG, Binns MM, Dickens HF, Ryder EJ, Gerlach J, Baumle E, and Dolf G, 1997. Towards construction of a canine linkage map: establishment of 16 linkage groups. Mamm Genome 8:218–221.

Manly KF, 1993. A Macintosh program for storage and analysis of experimental genetic mapping data form recombinant inbred strains. Mamm Genome 4:303–313.

Mellersh ES, Langston AA, Acland AF, Fleming MA, Ray K, Wiegand NA, Francisco LV, Gibbs M, Aguirre GD, and Ostrander EA, 1997. A linkage map of the canine genome. Genomics 46:326–336.

Miller ME and Smith DW, 1979. Conotruncal malformation complex:examples of possible monogenic inheritance. Pediatrics 63:890–893.

Online Mendelian Inheritance in Man, OMIM (TM). Johns Hopkins University, Baltimore, MD. MIM Number: 217095: 8.24.1998. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim.

Ostrander EA, Mapa FA, Yee M, and Rine J, 1995. One hundred and one new simple sequence repeat-based markers for the canine genome. Mamm. Genome 6:192–195.

Ostrander EA, Sprague GF, and Rine J, 1993. Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in dog. Genomics 16:207–213.

Patterson DF, 1968. Epidemiologic and genetic studies of congenital heart disease in the dog. Circ Res 23:171–202.

Patterson DF, Pexieder T, Schnarr WR, Navratil T, and Alaili R, 1993. A single major-gene defect underlying cardiac conotruncal malformations interferes with myocardial growth during embryonic development: studies in the CTD line of keeshond dogs. Am J Hum Genet 52:388–397.

Patterson DF, Pyle RL, Van Mierop L, Melbin J, and Olson M, 1974. Hereditary defects of the conotruncal septum in keeshond dogs: pathologic and genetic studies. Am J Cardiol 34:187–205.

Pierpont ME, Gobel JW, Moller JH, and Edwards JE, 1988. Cardiac malformations in relatives of children with truncus arteriosus or interruption of the aortic arch. Am J Cardiol 61:423–427.

Puech A, Saint-Jore B, Funke B, Gilbert DJ, Sirotkin H, Copeland NG, Jenkins NA, Kucherlapati R, Morrow B,

and Skoultchi AI, 1997. Comparative mapping of the human 22q11 chromosomal region and the orthologous region in mice reveals complex changes in gene organization. Proc Natl Acad Sci USA 94:14608–14613.

Rein AJ, Dollberg S, and Gale R, 1990. Genetics of conotruncal malformations: review of the literature and report of a consanguineous kindred with various conotruncal malformations. Am J Med Genet 36:353–355.

Rein AJ and Sheffer R, 1994. Genetics of conotruncal malformations: further evidence of autosomal recessive inheritance. Am J Med Genet 50:302–303.

Selden JR, Moorehead PS, Oehlert ML, and Patterson DF, 1975. The Giemsa banding pattern of the canine karyotype. Cytogenet Cell Genet 15:380–387.

Van Mierop LHS and Patterson DF, 1978. The pathogenesis of spontaneously occurring anomalies of the ventricular outflow tract in keeshond dogs: embryologic studies. Birth Defects 14:361–375.

Van Mierop LHS, Patterson DF, and Schnarr WR, 1977. Hereditary conotruncal septal defects in keeshond dogs: embryologic studies. Am J Cardiol 40:936–950.

Werner P, Raducha MG, Prociuk U, Henthorn PS, and Patterson DF, 1997. Physical and linkage mapping of human chromosome 17 loci to dog chromosome 9 and 5. Genomics 42:74–82.

Werner P, Raducha MG, Prociuk U, Lyons LA, Kehler JS, Henthorn PS, and Patterson DF, 1998. RXRA and HSPA5 map to the telomeric end of dog chromosome 9. Anim Genet 29:220–223.

Werner P, Mellersh CS, Raducha MG, DeRose S, Acland GM, Prociuk U, Wiegand N, Aguirre GD, Henthorn PS, Patterson DF, and Ostrander EA, in press. Anchoring of canine linkage groups with specific markers. Mamm Genome.

Wilson DI, Cross IE, Goodship JA, Coulthard S, Carey AH, Scambler PJ, Bain HH, Hunter AS, Carter PE, and Burn J, 1991. DiGeorge syndrome with isolated aortic coarctation and isolated ventricular septal defect in three sibs with a 22q11 deletion of maternal origin. Br Heart J 66:308–312.

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