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Inheritance of single copy nuclear genes (SCNGs) in artificial hybrids of *Hesperocyparis arizonica* x *H. macrocarpa*: Potential for utilization in the detection of hybridization in natural populations

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ABSTRACT

Analyses were performed on 18 artificial hybrids from a cross of *Hesperocyparis arizonica* (male parent) *x H. macrocarpa* (female parent) using 9 single copy nuclear genes (SCNGs). Three SCNG were found to be informative: myb, 4CL and CnAIB2. Gene myb contained 5 variable sites, of which site 89 was homozygous (CC, TT) as was site 261 (GG, AA) and useful for the detection of hybridization. All 18 hybrids were heterozygous (CT and GA) at these 2 sites as predicted in hybrids. 4CL contained 8 variable sites, of which 1 site (591) was homozygous (TT, CC) and all 18 hybrids were heterozygous (TC) at this site as expected. CnAIP2 had two variable sites: 301 (AA, AC) and 554 (AG, AA). For site 301, 8 hybrids were AA, and 10 were AC as expected. For site 554, 10 hybrids were AA and 8 were AG, so neither would be useful for unequivocally identifying hybrids. The inheritance of variable sites for the three SCNGs followed simple co-occurrence. Examination of myb in the 18 hybrids revealed 2 cases of cross-over in the pollen gametes. Published on-line www.phytologia.org *Phytologia 101(1):58-66 (March 21, 2019).* ISSN 030319430.

KEY WORDS: *Hesperocyparis arizonica, H. macrocarpa,* Cupressaceae, hybrids, single copy nuclear genes, SCNG, inheritance.

Recently, Adams, Miller and Low (2016) analyzed the inheritance of nrDNA in artificial hybrids between *Hesperocyparis arizonica* and *H. macrocarpa* from New Zealand. Sequencing nrDNA of parents (*Hesperocyparis arizonica, H. macrocarpa)* found their nrDNA differed at 8 sites. Analysis of 18 artificial hybrids, revealed each of the hybrids had nrDNA that was heterozygous at each of the 8 sites. However, the peak ratios in the chromatograms were not 1:1 as expected, but varied from 1:1 to 3:1, usually being more like *H. arizonica*. Principle Coordinates Ordination (PCO) of the variation in the peak heights revealed four groups of hybrids that seemed to be associated with chromosome inheritance. However, PCO clearly distinguished the parents and the hybrids. But, the ordination of hybrids closer to *H. arizonica*, could lead one to interpret that some introgression was occurring, when, in fact, there were only hybrids in the PCO.

However, nrDNA spacer regions have been reported to exhibit some oddities in inheritance. The conserved nature of the multi-copy nrDNA (thousands of copies per cell) seemed to be due to concerted evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Because these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA. Thus, nrDNA (ITS) has often been used for the analysis of hybridization.

There have been several reports where nrDNA in hybrids (and backcrosses?) has been more like one of the parents than a hybrid (i. e., heterozygous at every informative site). Chaing et al. (2001) reported that in artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (maternal), nrDNA was predominantly like that of the maternal parent, *B. formosana*. This is disturbing because having equal parts of nrDNA from both parents is a principle that is critical for the classification of plants as hybrids, or backcrosses. Thus, the predominate similarity to the maternal parent, *B. formosana*, would lead one to erroneously conclude that the hybrid was a backcross.

Volkov et al. (1999) reported that one of the parental nrDNAs was eliminated in the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time. These reports clearly cause concern about the use of nrDNA for the detection of hybrids and introgression. However, it is noteworthy, that they do supply examples of the asymmetrical inheritance between parents, favoring one of the parents in hybridization.

Artificial hybrids were made between *Armeria villosa* ssp. *longiaristata* and *A. colorata*, then examined the inheritance of nrDNA in F_1 and F_2 generations (Aguilar et al. 1999). They found the expected additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2 generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization toward the recurrent parent for five of the six polymorphic sites to the recurrent parent. This asymmetrical inheritance of nrDNA clearly skewed the pattern such that backcrosses might be erroneously interpreted.

Introgression in *Mitella* was studied using nrDNA ITS and ETS, and cpDNA and found that cpDNA revealed the most introgression, ITS regions showed a moderate amount of introgression and the ETS region gave no evidence of introgression (Okuyama et al. 2005). They concluded that non-uniform concerted evolution between the ETS region and ITS regions explained these different patterns of introgression.

These reports clearly cause concern about the use of nrDNA for the detection of hybrids and introgression due to concerted evolution (Liao, 1999; Okuyama et al. 2005), maternally influenced inheritance (Chaing et al. 2001), and the exclusion of one parent's nrDNA in allopolyploid tobacco (Volkov et al. 1999). Each of the mechanisms for the homogenizing heterozygous nrDNA may explain the abnormalities of inheritance of nrDNA in hybrids and they provide mechanisms helpful in explaining cases of chloroplast capture in taxa derived by ancient hybridization (Adams, Schwarzbach and Tashev, 2016).

Due to the occasional asymmetrical inheritance of nrDNA (see above), there has recently been an expansion in the utilization of Single Copy Nuclear Genes (SCNGs), although in the Cupressaceae, there are few studies using SCNGs. A great example is that of Moreno-Letelier, Mastretta-Yanes and Barraclough (2014) who used six SCNGs proved useful in a study lineage divergence in *Juniperus blancoi*.

Adams (2015a, b) found, in field studies of *J. maritima* R. P. Adams x *J. scopulorum* Sarg. hybridization, that nrDNA identified 15 hybrids, whereas, maldehy, a single copy nuclear gene (SCNG), detected 25 hybrids. The nrDNA data frequently appeared to be the same as one of the parents, whereas the SCNG (maldehy) was heterozygous at both (2) informative sites, indicating the plant(s) were of hybrid origin. These studies Adams (2015a, b) were in natural populations, so it is often difficult to be completely confident that hybrids are being analyzed (as opposed to backcrosses, or F_2 plants). That factor has led us to examine inheritance of single copy nuclear genes (SCNGs) from the cypress cross analyzed by Adams, Miller and Low (2016).

In the Cupressaceae, breeding programs are rare, so the existence of parents and artificial (verified) hybrids is an important resource for studies on inheritance. Scion Research Institute, Rotorua, New Zealand has a breeding program that involves crossing *Cupressus* and *Hesperocyparis* species. The breeding program afforded an unusual opportunity to examine the inheritance of SCNGs in hybrids in the Cupressaceae. The purpose of this paper is to report on the inheritance of SCNGs in artificial hybrids of *H. arizonica* x *H. macrocarpa* and determine if their inheritance (as heterozygous in hybrids) validates their use in determining hybrids in natural populations.

MATERIALS AND METHODS

Plant material: Crosses were made at the Scion Research Institute, Rotorua, New Zealand using pollen of *H. arizonica* (2003.017) onto receptive seed cones of *H. macrocarpa* (896.752). Seedlings were obtained and greenhouse grown to 50-80 cm, then field planted. Leaf samples were taken after approximately one year in the field (plants about 1 m tall). Parents: *Adams 14854 H. arizonica* (2003.017), *Adams 14856 H. macrocarpa* (896.752), (leaves in silica gel), eighteen (18) Hybrids (leaves in silica gel) (lab accession #): *Adams 14914 - Adams 14931*.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20º C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. Nine single copy nuclear genes (SCNGs): *LHCA*4 (type IV chlorophyll binding protein), *maldehy* (malate dehydrogenase), *myb* (Myb transcription factor), *ABI3* (ABI3-interacting protein gene, *4CL* (4-coumarate CoA ligase), *CnAIP2* (*Callitropsis nootkatensis* abscisic acid-insensitive 2), *cc13333* (GTP binding protein gene), *chs* (chalcone synthase) and *hsp* (heat shock protein) (Adams et al. 2009, Moreno-Letelier et al. 2014, Zheng, et al. 2013) were sequenced for each of the parents (*H. arizonica* (2003.017; *H. macrocarpa* (896.752) to determine if they were informative in distinguishing the parents. Three SCNGs (*4CL, CnAIP3* and *myb*) were found to be potentially informative and these were sequenced for each of the18 hybrids.

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

RESULTS AND DISCUSSION

Sequencing myb of the parents (*H. arizonica* (2003.017; *H. macrocarpa* (896.752) revealed 5 sites with some heterozygosity: 89, 261, 338, 748, and 849. However, only two sites (89, 261) were homozygous in both parents (89 CC TT; 261 GG, AA, Table 1) and thus, potentially useful for the detection of hybrids in natural populations. Each of the 18 artificial hybrids was heterozygous at loci 89 and 261 (Table 1) and these myb loci are useful in the detection of natural hybrids between *H. arizonica* and *H. macrocarpa* **if** analyses of *H. arizonica* and *H. macrocarpa* in the natural population being studied proved these taxa are always homozygous at sites 89 and 261. Our study of this cross indicates, but does not prove that myb, sites 89 and 261 would be useful in the study of a natural population (it might be noted that these two species are not sympatric in nature, so the discussion is somewhat academic).

The inheritance of the alleles at the other 3 sites (338, 748, 849) is interesting to examine. Only 4 of the 8 possible pollen haplotypes were present among the 18 hybrids (Table 1). The hybrids were found to be of 4 genotypes (Table 1, group 1: CT, GA, GG, CC, TC, with 9 plants; group 2: CT, GA, AG, AC, CC, with 6 plants; group 3: CT, GA, GG, CC, CC, with 2 plants; and group 4: CT, GA, AG, CC, TC, with 1 plant).

Group 1 pollen haplotype CGGCT was found in 9 hybrids, and group 2 pollen haplotype CGAAC was in 6 hybrids. It seems likely that these haplotypes are on 2 different chromosomes of *H. arizonica* (the male parent). Pollen haplotype, CGGCC, (Gp. 3, Table 1) was present in only 2 hybrids, and appears to be the result of cross-over between CGGC**T** and CGAA**C** between sites 748 and 849 (100 bp distance) to produce the CGGC**C** haplotype.

The haplotype, CGACT, (Gp. 4, Table 1) was found in only 1 hybrid. It appears be the product of cross-over between CGA**AC** and CGG**CT** at sites 338 and 749 (410 bp) to produce the CGACT haplotype.

It should be noted that there was only one egg haplotype (TAGCC), because *H. macrocarpa* was homozygous at all 5 sites (TT, AA, GG, CC, CC, Table 1) and this facilitated the analyses of the pollen haplotypes.

Sequencing 4CL of the parents discovered 8 sites with some heterozygosity: 507, 529, 531, 533, 591, 612, 638 and 644. Only one site (591) was homozygous in both parents (591: TT, CC, Table 2) and likely useful for the detection of hybrids. Each of the 18 artificial hybrids was heterozygous at locus 591 (Table 2). Thus, the 4CL locus may be useful in the detection of natural hybrids between *H. arizonica* and *H. macrocarpa* **if** analyses of *H. arizonica* and *H. macrocarpa* in the natural population being studied proved these taxa are always homozygous at site 591.

The inheritance of the alleles at the other 7 sites (507, 529, 531, 533, 612,638, 644) is interesting to examine. Notice how physically close these sites are (529, 531, 533; and 638, 644). Only 2 of the 8 possible pollen haplotypes were present among the 18 hybrids (Table 2). The hybrids were found to be of 4 genotypes (Table 2, group 1: AA, CC, CT, AA, TC, CG, AG, AA, with 9 plants; group 2: TA, CT, CC, GA, TC, CC, AA, GA, with 5 plants; group 3: AA, CT, CC, AA, TC, CC, AA, AA, with 3 plants; and group 4: TA, CC, CT, GA, TC, CG, AG, GA, with 1 plant).

Group 1 pollen haplotype (ACCATCAA) was found in 12 hybrids, and pollen haplotype TCCGTCAG (Group 2) was in 6 hybrids. It seems likely that these haplotypes are on 2 different chromosomes of *H. macrocarpa* (the female parent).

Two egg haplotypes were found: Group 1 egg haplotype (ACTACGGA in 10 hybrids, and egg haplotype ATCACCAA (Group 2) was in 8 hybrids, implying these haplotypes are on 2 chromosomes of *H. macrocarpa* (the female parent, Table 2).

Sequencing CnAIP2 of the parents (*H. arizonica* (2003.017; *H. macrocarpa* (896.752) revealed two sites with some heterozygosity: 301 and 554, but neither of these were useful for hybrid detection (Table 3).

The inheritance of the alleles at the two sites (301, 554) is interesting to examine. Only 2 of the 4 possible pollen haplotypes and 2 of the 4 egg possible haplotypes were present among the 18 hybrids (Table 3). The hybrids were found to be of 4 genotypes (Table 3, group 1: AA, AA, in 6 plants; group 2: AC, AG, in 6 plants; group 3:AC, AA, in 4 plants; and group 4: AA, AG in 2 plants).

Group 1 pollen haplotype (AA) was found in 10 hybrids, and pollen haplotype AG (Group 2) was in 8 hybrids, implying these haplotypes are on 2 chromosomes of *H. arizonica* (the male parent).

Two egg haplotypes were found: Group 1 egg haplotype (AA) in 8 hybrids, and egg haplotype CA (Group 2) was in 10 hybrids, implying these haplotypes are on 2 chromosomes of *H. macrocarpa* (the female parent).

In summary, the survey of 9 SCNGs using only two parents (*H. arizonica, H. macrocarpa*), yielded only 3 candidate genes, of which only 2 proved useful. These 2 genes had only three informative sites (myb, 2; 4CL, 1). However, these three sites showed perfectly clean chromatograms that were always heterozygous in all 18 hybrids. It is possible that the other 6 'SCNGs' were, at least in this instance, multi-copy genes.

Two novel pollen haplotypes were discovered in myb in *H. arizonica* pollen. Haplotype CGGCC, present in 2 hybrids, appears to from a cross-over between sites 748 and 849 (100 bp gap). The second haplotype, CGACT, in 1 plant, seems to have arisen by a cross-over between sites 338 and 748 (409 bp gap).

In *H. macrocarpa*, gene 4CL had a deletion (5 bp) beginning at position 90 and a deletion at 151 (70 bp) and *H. arizonica* had a deletion (49 bp) at 323 that led slippage in the hybrid's sequences and un-callable bases. This can be addressed by NextGen sequencing.

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Table1. Variable myb sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross. Parent differ at 8 sites. Site numbering is from the 5' end. Hybrids all full-sibs. Pollen haplotypes not found: CGGAT,CGACC, CGAAT, CGGAC.

5 sites analyzed	89[1]	261[2]	338[3]	748[4]	849[5]	
pollen haplotypes found:	*arizonica* genotype (male)					Frequency by group
	CC	GG	GA	CA	TC	
CGGCT	C	G	G	C	T	Gp. 1(9)
CGAAC	C	G	A	A	C	Gp. 2(6)
CGGCC	C	G	G	C	C	Gp. 3(2)
CGACT	C	G	A	C	T	Gp. 4(1)
	macrocarpa genotype (female)					
egg haplotype	TT	AA	GG	CC	CC	
TAGCC	T	A	G	C	C	All Gps.18/18
Groups found						
Gp. 1, pollen	C	G	G	C	T	CGGCT
egg	T	A	G	C	C	TAGCC
14914 Gp. 1(9)	CT	GA	GG	CC	TC	2/5 # homozygous
14915	CT	GA	GG	CC	TC	2/5
14918	CT	GA	GG	CC	TC	2/5
14919	CT	GA	GG	CC	TC	2/5
14920	CT	GA	GG	CC	TC	2/5
14925	CT	GA	GG	CC	TC	2/5
14926	CT	GA	GG	CC	TC	2/5
14928	CT	GA	GG	CC	TC	2/5
14931	CT	GA	GG	CC	TC	2/5
Gp. 2, pollen	C	G	A	A	C	CGAAC
egg	T	A	G	C	C	TAGCC
14917 Gp. 2(6)	CT	GA	AG	AC	CC	1/5
14921	CT	GA	AG	AC	CC	1/5
14923	CT	GA	AG	AC	CC	1/5
14927	CT	GA	AG	AC	CC	1/5
14929	CT	GA	AG	AC	CC	1/5
14930	CT	GA	AG	AC	CC	1/5
Gp. 3. pollen	C	G	G	C	C	CGGCC
egg	T	A	G	C	C	TAGCC
14922 Gp. 3(2)	CT	GA	GG	CC	CC	3/5
14924	CT	GA	GG	CC	CC	3/5
Gp. 4.pollen	C	G	A	C	T	CGACT
egg	T	A	G	C	C	TAGCC
14916 Gp. 4(1)	CT	GA	AG	CC	TC	1/5
summary of genotypes	all CT homozy	all GA homozy	11 GG, 7 AG	12 CC 6 AC	8 CC 10 CT	
heterozygous/ total	18/ 18	18/ 18	7/ 18	6/ 18	10/ 18	

[1] left of GCTATTAAG, [2] left of GCGATTTTA, [3] left of CCGGGGTCA
[4] left of CGGAGCGTT, [5] left of CCCCTTTTC

Table 2. Variable 4CL sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross that differ at 8 sites. Site numbering is from the 5' end.

8 sites analyzed	507[1]	529[2]	531[3]	533[4]	591[5]	612[6]	638[7]	644[8]	
pollen haplotypes found:	*arizonica* genotype (male)								Frequency by groups
	A/T	C/C	C/C	A/G	T/T	C/C	A/A	A/G	
ACCATCAA	A	C	C	A	T	C	A	A	Gp 1(9), Gp 3(3)
TCCGTCAG	T	C	C	G	T	C	A	G	Gp 2(5), Gp 4(1)
egg haplotypes found:	*macrocarpa* genotype (female)								
	A/A	C/C	C/T	A/A	C/C	C/G	A/G	A/A	
ACTACGGA	A	C	T	A	C	G	G	A	Gp 1(9), Gp4(1)
ATCACCAA	A	T	C	A	C	C	A	A	Gp 2(5), Gp 3(3)
Groups found									
Gp 1. pollen	A	C	C	A	T	C	A	A	ACCATCAA
egg	A	C	T	A	C	G	G	A	ACTACGGA
14914 Gp. 1(9)	AA	CC	CT	AA	TC	CG	AG	AA	4/8 # homozygous
14919	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14920	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14921	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14923	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14928	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14929	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14930	AA	CC	CT	AA	TC	CG	AG	AA	4/8
14931	AA	CC	CT	AA	TC	CG	AG	AA	4/8
Gp 2. pollen	T	C	C	G	T	C	A	G	TCCGTCAG
egg	A	T	C	A	C	C	A	A	ATCACCAA
14915 Gp. 2(5)	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14922	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14917	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14926	TA	CT	CC	GA	TC	CC	AA	GA	3/8
14927	TA	CT	CC	GA	TC	CC	AA	GA	3/8
Gp 3. pollen	A	C	C	A	T	C	A	A	ACCATCAA
egg	A	T	C	A	C	C	A	A	ATCACCAA
14916 Gp. 3(3)	AA	CT	CC	AA	TC	CC	AA	AA	6/8
14918	AA	CT	CC	AA	TC	CC	AA	AA	6/8
14924	AA	CT	CC	AA	TC	CC	AA	AA	6/8
Gp 4. pollen	T	C	C	G	T	C	A	G	TCCGTCAG
egg	A	C	T	A	C	G	G	A	ACTACGGA
14925 Gp. 4(1)	TA	CC	CT	GA	TC	CG	AG	GA	1/8
summary of genotypes	11 AA 7 AT	10 CC 8 CT	10 CT 8 CC	12 AA 6 AG	all TC homo zy	10 CG 8 CC	10 AG 8 AA	12 AA 6 AG	
# homozygous/ heterozygous	11/ 7	10/ 8	8/ 10	12/ 6	0/ 18	8/ 10	8/ 10	12/ 6	

[1] left of CATTCATTA, [2] right of GAGTAGTT, [3] left of TACACAATT, [4] left of CACAATTCG, [5] left of TCTAAAAA, [6] left of TAGAACAAT, [7] right of CTTTCAAC, [8] left of GTACCCTTT,

Table 3. Variable CnAIP2 sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross which differ at 2 sites. Site numbering is from the 5' end.

2 sites analyzed	301[1]	554[2]	
	arizonica genotype (male)		
pollen haplotypes	AA	AG	freq. by group
AA	A	A	Gp. 1 (6), Gp. 3 (4)
AG	A	G	Gp. 2 (6), Gp. 4 (2)
	macrocarpa genotype (female)		
egg haplotypes	AC	AA	
AA	A	A	Gp. 1 (6), Gp. 4(2)
CA	C	A	Gp. 2 (6), Gp. 3 (4)
Groups found			
Gp. 1. pollen	A	A	AA
egg	A	A	AA
14914 Gp 1. (6)	AA	AA	4/4
14920	AA	AA	4/4
14924	AA	AA	4/4
14925	AA	AA	4/4
14930	AA	AA	4/4
14931	AA	AA	4/4
Gp. 2. pollen	A	G	AG
egg	C	A	CA
14915 Gp 2 (6)	AC	AG	2/4
14919	AC	AG	2/4
14921	AC	AG	2/4
14923	AC	AG	2/4
14922	AC	AG	2/4
14928	AC	AG	2/4
Gp. 3. pollen	A	A	AA
egg	C	A	CA
14917 Gp 3. (4)	AC	AA	1/4
14918	AC	AA	1/4
14927	AC	AA	1/4
14929	AC	AA	1/4
Gp. 4. pollen	A	G	AG
egg	A	A	AA
14916 Gp 4. (2)	AA	AG	1/4
14926	AA	AG	1/4
summary of genotypes	8 AA 10 AC	10 AA 8 AG	
homozygous/ heterozygous	8/ 10	10/ 8	

[1] left of ATGTGCTT, [2] left of CAGCATCT