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Physical Mapping of Ribosomal Genes in New World Members of the Genus Chenopodium Using Fluorescence in Situ Hybridization

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PHYSICAL MAPPING OF RIBOSOMAL RNA GENES IN NEW WORLD MEMBERS OF THE GENUS *CHENOPODIUM* USING FLUORESCENCE IN SITU HYBRIDIZATION

by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Maria C Sederberg in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative figures including tables, charts, and graphs are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

PHYSICAL MAPPING OF RIBOSOMAL RNA GENES IN NEW WORLD MEMBERS OF THE GENUS *CHENOPODIUM* USING FLUORESCENCE IN SITU HYBRIDIZATION

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The genus *Chenopodium* contains many economically important species in the New World, but is relatively understudied and poorly understood, especially in terms of evolutionary relationships. A better understanding of the structure of this genus could significantly help in breeding efforts on its cultivated members, notably the tetraploid *C. quinoa* and also certain varieties of *C. berlandieri*, also tetraploid. Of special concern is determining which diploid weed species are the most likely ancestors for *C. quinoa*, *C. berlandieri*, and the other tetraploid members of subsection *Cellulata*. The phylogeny can be understood in part by examining the ribosomal RNA loci and observing how many

copies of the 5S and 45S loci each New World species contains. In this work, the 5S and 45S ribosomal RNA loci are characterized by means of fluorescence *in situ* hybridizat in 23 *Chenopodium* species collected in the New World, with the 5S locus labeled red and the 45S locus labeled green. Based on these results, the pool of most likely candidate ancestor species for *C. quinoa* and *C. berlandieri* includes *C. fremontii, C. incanum, C. neomexicanum,* and *C. watsonii*.

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CHAPTER 1: PHYSICAL MAPPING OF RIBOSOMAL RNA GENES IN NEW WORLD MEMBERS OF THE GENUS *CHENOPODIUM* USING FLUORESCENCE IN SITU HYBRIDIZATION

Introduction

The genus *Chenopodium* L. (x=8,9) consists of a relatively under-studied group of dicotyledonous plants with a worldwide distribution. The genus consists mostly of uncultivated and weed species, although members have been cultivated in many areas: *C. album* L. in Europe; *C. giganteum* D.Don, or tree spinach, in Central Asia; *C. berlandieri* Moq. var. *nuttaliae* in Mesoamerica; and *C. pallidicaule* Heller and *C. quinoa* Willd., or quinoa, in South America (Maughan *et al.*, 2006). Quinoa is the most important economically, possessing many characteristics that make it a potentially ideal crop. It is adapted to grow in regions of low temperature and high salinity (Stevens, 2006). Like the other cultivated chenopods, quinoa's seed protein content, about 12-18%, is higher than the true cereals and has a superior balance of essential amino acids, making it a valuable addition to vegetarian and subsistence farming diets (Galwey *et al*., 1993; Konishi *et al*., 2004). Because of quinoa's lack of importance as a commodity of the Developed World, breeding efforts have mostly been restricted to small programs in the Andean region.

Some headway has been made towards a better understanding of quinoa's genetic structure. Molecular genetic resources have been made for quinoa, including AFLP and SSR-based markers (Maughan *et al.*, 2004; Mason *et al.*, 2005; Jarvis *et al*, 2008); ESTs (Coles *et al.*, 2005); BACs (Stevens *et al.*, 2006); and rRNA gene markers (Maughan *et*

al., 2006). These tools are being utilized to measure genetic variation in genetic resource collections of quinoa (Christensen *et al.,* 2007; Fuentes *et al.,* 2008).

One untapped and poorly characterized resource is the germplasm available within the other New World members of the genus *Chenopodium*. Since quinoa, cañahua (*C. pallidicaule*), Mexican huazontle, chia roja, and quelite (*C. berlandieri* var. *nuttaliae*) are the only modern cultivated New World members of the genus, very little information is available about the evolutionary history of this group of crops. This is somewhat surprising in light of the economic damage attributed to several *Chenopodium* weeds. However, these weeds and some 20+ wild native species (Table 1) carry a trove of valuable genes that might be bred into quinoa and the Mexican domesticated *Chenopodium* as a source of desirable phenotypes (Maughan *et al.,* 2006).

In general, studying large-scale chromosomal variations and alterations is a quick and inexpensive way to begin determining how different species have diverged within a genus. However, *Chenopodium* chromosomes are very small and regular, differing in overall size by less than a micron (Palomino *et al.,* 2008), and do not produce distinctive banding patterns after C-banding (Parkinson, 2001), making it difficult to use conventional methods to search for rearrangements at the chromosomal level. The small, uniform nature of these chromosomes makes fluorescence in situ hybridization, or FISH, an ideal technique for studying them. A previous study identified quinoa and the weed *C. berlendieri* var. *zschackei* as having one 45S locus and two 5S rDNA loci (Maughan *et al.,* 2006). This study, along with Palomino *et al.* (2008), indicate that Mexican huazontle had two 45S loci and three 5S loci. Maughan *et al.* (2006) also identified Mexican quelite as having one 45S locus and three 5S loci.

In this study, I used fluorescence probes designed against ribosomal DNA loci to determine number of rDNA loci in 23 *Chenopodium* species, mostly native to the New World. This information will clarify the secondary gene pool for improving quinoa and huazontle through sexual hybridization. It will also shed useful light on the phylogenetic origins of a historically important group of crops in the highland Andean, Southern Chilean, Mesoamerica, and Easter North American civilization zones of the New World.

Materials and Methods

Chromosome preparation

All tissue used to obtain chromosome spreads for this project was grown from seed gathered on collecting trips between in 2004-2008 (Table 1). Pericarps of vitreous-seeded materials were manually scored or chipped using a scalpel and soaked in 80-100 ppm GA to germinate, and even post-scarification often exhibited poor germination. After the seedlings had developed at least two sets of true leaves, tissue samples were taken from apical and lateral meristems, placed immediately in 100 µl of phosphate buffer and arrested in metaphase using 180 psi N_2O (Kato, 1999) for 6 h, or placed directly into a mixture of 0.002 M 8-hydroxyquinoline/10 µM amiprophos methyl (Austratec Pty Ltd, Mooroolbark, Australia) for 6 h. Tissue preparations were then fixed in 90% acetic acid and further treated as per Kato (1999), Kato *et al*. (2004 and 2006), and Lamb et al. (2006) with the following modifications.

Prior to tissue digestion, samples were rinsed in cold 1X citric buffer 2-3 times to remove any acetic acid or ethanol. Samples were then placed under a dissecting scope and accessory tissue to the meristem was removed. The remaining tissue was placed in 40 µl of cellulose-pectolyase solution (0.3 g pectolyase Y-23, from MP Biomedicals (Solon,

OH), and 0.4 g cellulose onozuka R-10 (Yakult Honsha, Minato-ku, Tokyo, Japan) in 10 ml citric buffer; 40 µl aliquoted into thin-walled PCR tubes) and incubated at 37˚C for 2 hours to overnight. Reaction was terminated by flooding the tube with cold TE.

After digestion, the tissue was prepared for cytogenetic analysis as described by Kato *et al*. (2004), with the exception that 10 µl were dropped on each microscope slide. 10 µl Vectashield (Vector, Burlingame, CA) with DAPI was added, covered with a coverslip and observed under a Zeiss Axioskop microscope. If two good slides were observed from the sample, the remaining slides were made up by dropping 10 µl solution onto the slide, allowing to dry, adding 5 µl 45% acetic acid, squashing, and placing on dry ice for at least 5 minutes. Coverslips were removed, and slides were then UV crosslinked and stored at -20˚C until use.

Probe preparation

45S probe

The 45S probe template DNA was obtained by PCR-amplifying genomic quinoa DNA, from the Chilean lowland accession NL-6 and the Peruvian highland accession 0654. Because of the highly repetitive nature of the 45S locus, only the 18S portion of the gene was amplified for use as a probe. Since the only portion of the 45S locus that has been sequenced in quinoa is the intergenic spacer region, primers were designed around the 18S region of the gene using the sequenced data in Genbank for *Cicer arietenum* L. (Genbank accession number AJ577394). Two sets of primers, 18S1 and 18S2, were used (Table 2); both worked equally well on NL-6 and slightly less well on 0654 (Figure 1). The PCR product was cleaned with a Qiagen QIAquick PCR purification kit (Valencia, CA) and labeled using standard nick translation.

5S probe

A plasmid containing the complete 5S gene, including the spacer, was created prior to this research with a Promega PGem-T Easy cloning kit (San Luis Obispo, CA). Bacterial cultures containing the KU-2 2 gene (GenBank accession no. DQ187981) and the 0654 1 gene (GenBank accession no. DQ187979) were grown in LB broth containing ampicillin overnight and the plasmid was harvested using a Promega Wizard DNA Cleanup Miniprep Kit (San Luis Obispo, CA). The insert was amplified using M13 primers in a Qiagen MasterMix PCR reaction. The PCR product was purified using a Qiagen cleanup kit and labeled with the same nick translation protocol as the 45S probe, except using PerkinElmer Texas Red-dUTP-5 (Waltham, MA).

Hybridization

Hybridization was carried out in the manner prescribed in Kato *et al.* (2004), with two general modifications. Instead of using 5 µl of probe and block, 10 µl of a .5X concentration were used to ensure there was enough liquid to cover the area of interest on the slide, and once the hybridization and wash were completed, ten µl of Vectashield with DAPI were added to each slide before the coverslip was added.

Visualization

Slides were visualized under a light microscope. Pictures of slide preparations were taken with a color camera attached to the microscope and analyzed for probe detection. Individual pictures from DAPI, Texas Red, and fluorescein filters were compiled in Adobe Photoshop (Adobe, San Jose, CA) using layering and brightness/contrast enhancement functions.

Results and Discussion

The taxonomy of *Chenopodium* is in considerable disarray. Many, if not most, of the species have been reclassified since Linnaeus categorized the first *Chenopodium* (Wilson and Heiser, 1979), and many species are difficult to classify. This taxonomic uncertainty, while not a major impediment, was a cause of some concern and lack of conclusiveness. The taxonomic treatment which we chose to follow is the Online Flora of North America (www.efloras.org)

The results, summarized in Table 3 and Figure 1, were as follows.

C. album and *C. hircinum*

Two accessions of *C. album* were examined and contained different ploidy levels and rDNA locus counts (Table 3). This variation in chromosome numbers confirms what was observed by B. Kolano (personal communication) while studying Old World *Chenopodium*: namely, that material classified as *C. album* exists at the diploid, tetraploid, and hexaploid levels. It is possible that the *C. hircinum* included in this analysis was actually *C. album*; inspection of photographs of seed from the plants collected at Canchones Research Station in northern Chile revealed larger, smooth fruits (*album*-type) were mixed with smaller, alveolate fruits (*hircinum*-type). This is a common situation in Chilean fields, as noted previously by (1980).

C. atrovirens Rydb.

Our study of accession BYU 536 from pinyon-juniper shrub-steppe near Duchesne, Utah, indicates that *C. atrovirens* is a diploid, as previously thought, and

contains one 5S locus and one 45S locus. However, cytological inspection of a second accession of *C. atrovirens* from the Book Cliffs, BYU 837, (results not shown) revealed two 45S and four 5S loci in a tetraploid genetic background.

C. berlandieri vars. *boscianum* and *macrocalycium* (Aellen)

We observed a single 45S and three 5S loci for these two geographically diverse (Louisiana Gulf Coast and Maine Atlantic Coast) accessions. This pattern is consistent with what was observed by Maughan *et al.* (2006) for domesticated quelite, and suggests a possible third phylogenetic origin for the *C. berlandieri* group having this rRNA locus distribution.

C. berlandieri var. *sinuatum* (Murr) Wahl

We observed two 45S and four 5S loci in accession BYU 411 from the San Bernardino Mountains of California. It is therefore distinct from the other accessions and botanical varieties of *C. berlandieri* examined thus far for numbers of these rRNA gene loci (Maughan *et al.*, 2006).

C. californicum S. Watson and *C. foliosum* Moench.

These two morphologically similar diploid species showed a base chromosome count of $n = 8$, with only 16 chromosomes visible per nucleus. Both species contained one 45S locus and one 5S locus. *C. foliosum* was studied by B. Kolano (personal communication) and found to have 18 chromosomes and two copies of the 45S locus. *C. capitatum* is very similar in appearance to *C. foliosum*, and it is possible that there was a

misclassification. However, since Kolano's specimens were not collected in the New World, it is also possible that there has been some divergence in *C. foliosum* since it arrived in the New World. These two plants are also significantly different from the other species in this study morphologically, with leaves growing as a whorled rosette from a basal stem.

C. carnosolum Moq.

There is some ambiguity between *C. carnosolum* and *C. murale* since the two plants are morphologically similar. Since the locus count number for *C. carnosolum* is the same as that found by Kolano for *C. murale* (one 45S locus, one 5S locus), it is possible that these two accessions represent different ecotypes of the same species.

C. fremontii

In the present study we observed that *C. fremontii* is a diploid containing one 45S locus and two 5S loci. This species is the most widespread of all the native *Chenopodium* diploids of western North America. The fact that it contains two 5S loci, and its sympatric association with subsect. *Cellulata* diploids along the southern Colorado Plateau, elevates it as a candidate-ancestor of the domesticated Mesoamerican chenopods (Maughan *et al.*, 2006).

C. glaucum L. var. *salinum* (Standley) B. Boivin

The accession examined in this study, taken from the shore of Utah Lake, had one 45S and one 5S loci. This species is one that was studied by B. Kolano, but from an Old

World accession (*C. glaucum* L. var. *glaucum*). Our results confirmed hers; both diploids possessed single 45S and 5S loci (B. Kolano, personal communication).

C. hians Standl.

The range of this putative diploid is limited to patches throughout the mountainous southwest of the United States; plants matching the description of *C. hians* have recently been collected along the southern margins of the Colorado Plateau, in an area considered to be a potential center of origin for *C. berlandieri.*

However, the accession of putative *C. hians* examined in the present study, BYU 417, were collected in 2004 in the San Gabriel Mountains of California. This accession contained three 45S loci and three 5S loci. There is a possibility that the accession number for this sample of *C. hians* was misclassified *C. album.*

C. incanum S. Watson

Before this work began, it was thought that *C. incanum* was a likely candidate for one of the progenitor diploids that gave rise to the ancestral *C. berlandieri* of the Mexican tetraploid *C. berlandieri* var. *nuttaliae* complex (huazontle, chia roja, and quelite).
C. incanum belongs to the subsection *Leiosperma,* and is closely allied with *C. fremontii,* although its range overlaps the subsect. *Cellulata* diploids *C. watsonii* and neomexicanum. However, the Mexican berlandieri complex contains at a minimum two 45S and three 5S loci, indicating that one of its ancestors must contain a second 5S locus. Since a single 5S locus and a single 45S locus were identified in C. *incanum*, this makes the possibility of *C. incanum* as an ancestor to the Mexican domesticates highly unlikely.

C. macrospermum Hooker

In taxonomic studies, *C. macrospermum* was reported to be a tetraploid. No definitive chromosome counts were possible from our samples taken, but the tentative measurements along with the fact that the plant contains only a single copy of each of the rDNA loci indicates that it is probably diploid in this study (Flora of North America, 1982). We are confident that our taxonomic identification of *C. macrospermum* is accurate, although it is possible that the species exists in both diploid and tetraploid forms.

C. murale

C. murale is an Old World species studied by Kolano, who found it to contain one 45S locus and one 5S locus. This is in contrast to our results, which showed one 45S locus and two 5S loci. While the species originated in Europe and North Africa, it now claims one of the most global distributions of any member of the genus. It is possible that the species has evolved different rRNA locus numbers since arriving in the Americas, although this species is one that deserves closer inspection to verify its taxonomy.

C. neomexicanum

Kolano studied this species (personal communication), and, as in our results, found the presence of 1 45S locus and 1 5S locus in this diploid. This species is the closest morphologically to *C. berlandieri* at this ploidy level.

C. oahuense Aellen

Among the *Chenopodium* studied here, *C. oahuense* is unique in its niche, having evolved in isolation from what was likely a very limited, trans-oceanic, incidental introduction to the Hawaiian Archipelago. We ascertained that it is a diploid with three 45S loci and three 5S loci, confirming its evolutionary isolation.

C. petiolare (C. frigidum Phil)

C. petiolare has been previously classified by B. Kolano as a hexaploid with two 45S loci and four 5S loci (Kolano, personal communication). However, the identity of the plant used by Kolano is most likely not accurate; it seems likely that the plant labeled *C. petiolare* was in actuality *C. album*. Since then, the *C. petiolare* in the present study was collected by Drs. Fuentes, Jellen and Maughan in northern Chile (personal communication). The accession studied herein was a diploid containing one 45S and one 5S loci. There is, nonetheless, potentially confounded taxonomic information, with the online Flora of Chile (www.efloras.org) listing *C. frigidum* similarly to the material listed here as *C. petiolare.*

C. rubrum L. var. *humile* (Hooker) S. Watson

The botanical variety of *C. rubrum* var. *humile* we examined was collected along Utah Lake and was tentatively indicated as a diploid entity native to the New World in the Online Flora of North America (1982). Our study revealed that this accession had single 45S and 5S loci. However, Kolano found that Old World *C. rubrum* var. *rubrum* was a tetraploid with two 5S loci (B. Kolano, personal communication). These results

support the hypothesis that these two varieties are truly native to their respective hemispheres and merit subspecies designations at the least, if not separate species as per Welsh *et al* (2003).

C. simplex Raf.

The Online Flora of North America (www.efloras.org) lists *C. simplex* as a closely related New World relative of *C. hybridum* L. Kolano (personal communication) examined putative Old World *C. hybridum* cytologically using the same probe sequences and found the diploid number with single 45S and 5S loci. However, we found that there were 36 chromosomes in the Utah accession we examined, along with two 45S and 5S loci.

C. strictum Roth

Originally, it was thought that the variants of *C. strictum* found in North America were native, but that is almost universally disputed now (www.efloras.org). In this instance, our findings supported those of Kolano: *C. strictum* contained one 45S locus and two 5S loci. If the species were a New World native, this would place it in the category of potential ancestors for the New World tetraploids, but since it is a Eurasian native, it is not.

C. watsonii A. Nelson

Because of its placement in the subsection *Cellulata*, *C. watsonii* is a contender for the position of one of the diploid ancestors of the domesticated New World tetraploid

chenopods. However, we found that *C. watsonii* contains only one 45S locus and one 5S locus, indicating that chances are slim that *C. watsonii* and *C. neomexicanum* anciently hybridized to form the progenitor of the *C. berlandieri* complex.

Summary

From these data, it is possible to continue narrowing the field on which species are the most likely candidates for the ancestors of quinoa, cañahua, quelite, huazontle, and chia roja. Maughan *et al.* (2006) observed one 45S locus and two 5S loci in *C. berlandieri* var. *zschackei,* two 45S locus and three 5S loci in huazontle, and one 45S locus and three 5S loci in quelite. This indicates that if the formation of the *Chenopodium* section *Chenopodium* subsect. *Cellulata* tetraploids came from a monophyletic event; one of the diploid ancestors must have contained at least two copies of the 5S locus.

Among the species in this study, only four fit that classification. *Chenopodium strictum* and *C. murale* can be discounted because of their Old World origins. *Chenopodium fremontii,* however, is a strong candidate. This is especially interesting in light of Walter's observation (1988) that *C. neomexicanum,* a strong candidate for the single 5S locus donor ancestor, readily forms viable offspring when crossed with *C. fremontii*, even though *C. fremontii* does not belong to subsect. *Cellulata*. The third species of interest, *C. atrovirens,* consists definitely in diploid (single 45S and 5S loci) and possibly also autotetraploid forms (data not shown). Others have noted that polyploidization in the genus *Chenopodium* may be fairly easily achieved (Kolano, personal communication; Sanderson *et al.*, 1990).

Studying the distribution of these candidates strengthens the possibility of their position as potential ancestors. *Chenopodium fremontii* and *C. neomexicanum* overlap in

their ranges along the southern Colorado Plateau. During collecting trips in this area, R. Jellen has observed it to be rich in diversity for *Chenopodium* diploids and tetraploids, containing many hybrid swarms. Regardless, these data call for closer examination of *C. fremontii* and its allied species complex, along with *C. neomexicanum*, as candidate diploid ancestors for *C. quinoa* and the other 4*x* subsect. *Cellulata* taxa. The other cross that seemed likely was that of *C. neomexicanum* and *C. watsonii*, which are both diploids in *Cellulata*. However, since both of these diploids contain 1 45S locus and 1 5S locus, this combination can be readily discounted as the ancestral cross that created *C. berlandieri* var. *nuttaliae* and/or its direct ancestor – though not necessarily *C. berlandieri* var. *zschackei* and *C. quinoa* (Maughan *et al.*, 2006).

Further Research

Unfortunately, from this work it is not possible to speak conclusively in favor of the validity of either the monophyletic or the polyphyletic tetraploidization event hypothesis. There is still a significant amount of work to be done in establishing a reliable phylogeny for the genus *Chenopodium*, both Old and New World species. These efforts should include sequencing data as well as additional FISH information.

For the purposes of constructing a phylogeny, the most useful FISH information will be genome-painting probes that can help continue the quest as to which diploids are candidate ancestors. These probes will come from the utilization of profusely repetitive elements, such as 18-24J.

The repetitive elements themselves will be extracted from quinoa as part of the development of some small repeat-rich libraries and mined for sequences that are

substantially dominant in one of quinoa's subgenomes. Successful genome-painting probes will be applied to the diploids that are the most likely ancestor candidates- notably *C. neomexicanum* and *C. fremontii,* also keeping in consideration *C. watsonii*, *C. incanum*, and possibly also *C. atrovirens.*

Further information that can be gathered through FISH includes obtaining rDNA locus information for other New World species that were unavailable at the time of this study. This includes several native subsect. *Leiosperma* diploids from east of the Rocky Mountains: *C. cycloides*, *C. foggii*, *C. pallescens*, *C. standleyanum*, and *C. subglabrum*.

Since the sequence data gathered from chloroplast gene introns has proved to be less than useful for delineating ancestry in allopolyploid *Chenopodium*, a new tack of sequencing introns from single-copy nuclear genes, similar to the technique used by Kim *et al.* (2008) may produce more beneficial data. Current efforts are focusing on intron 2 of the *leafy* locus and various introns of *SOS1* orthologs using expressed sequence tags and genomic sequence data from quinoa.

When this cytogenetic and sequencing data is successfully brought together and added to the growing stockpile of *Chenopodium* data, a clearer picture will emerge of how the New World *Chenopodium* species evolved, leading to the formation of goosefoot and quinoa. This information will be very useful for determining which species can be successfully cross-bred with quinoa and will provide us with further information on the path towards developing quinoa into an optimized, homogenous crop.

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CHAPTER 2: LITERATURE REVIEW

History of New World Chenopodium Species

Quinoa has been cultivated as a crop for millennia. Before the Spanish conquest of Central and South America, it served as a staple in the Incan diet, along with the potato and *Phaseolus* beans. However, unlike the other Incan staples, it failed to make an impact in Renaissance Europe. This can most likely be attributed to two major factors. First, it is possible that the explorers tried to bring quinoa back to Europe along with the other new crops. However, quinoa is very susceptible to mildew and other diseases and is otherwise highly susceptible to heat stress that would have limited its productivity in lowland Western Europe. Second, quinoa was a very significant part of Incan religion. Every year, the emperor would plant the first quinoa seed with a golden spade as an offering to the gods. When the Catholic Conquistadores arrived in America, professing a desire to convert the Incans to Christianity, they probably saw quinoa as a barbaric part of a heathen religion that needed to be stamped out to make room for Catholicism. Thus, quinoa was relegated to obscurity until the mid 20th Century, thriving only in the poorest, most desolate high mountain regions where the Europeans did not care to settle (Galwey *et al.,* 1993).

With the rediscovery of quinoa as a nutritious, versatile, and hardy crop in the mid-1970s came a series of biochemical and morphology-based systematic studies on quinoa (Varriano-Marston and DeFrancisco 1984; Koziol 1992; Brinegar and Goundan 1993; Jacobsen and Stolen 1993; Prego *et al.,* 1998; Caperuto *et al.,* 2000; Bertero *et al.,* 2004; Konishi *et al.*, 2004; Ng *et al.*, 2007) Currently, quinoa can be found in health food stores in the United States, and it is quickly gaining popularity. It has also been selected

as a potential crop to introduce into poverty- and drought-stricken countries such as Morocco and India, and is finding a niche in Europe now that modern plant breeding is improving the crop's disease and heat-stress resistance (Dijkstra *et al.*, 2003; Benlhabib *et al*., 2004, unpublished; Bhargava 2006;). In order to ensure the future of this crop, a continued emphasis on breeding, aided by molecular techniques, is needed. When compared to mainstream crops, there is still a preponderance of genetic work to be done with quinoa.

One area of significant interest is the elucidation of a phylogenetic tree of the genus. Many of quinoa's wild and weedy relatives contain traits of potential interest for cross-breeding purposes. As quinoa is allotetraploid (Ward, 2000), discovering which two diploid *Chenopodium* species crossed to produce quinoa would be especially valuable.

When quinoa was originally classified in 1797, it was thought to be the sole domesticated species in the New World section of the genus. It was not until 1917 that the other major cultivated *Chenopodium* tetraploid was discovered in Mesoamerica (Wilson and Heiser, 1979). This plant was originally classified by Safford as *C. nuttaliae*. The plant consists of three different culitvars- huzontle, which is eaten for the immature inflorescences, chia roja, which is eaten for the seed, and quelite, which is consumed for the leaves.

Since its original classification, this plant has been reclassified several times, including a period of time when it was considered to be conspecific with quinoa. It is currently considered to be part of the *C. berlandieri* complex, and is known as *C. berlandieri* var. *nuttaliae* (Wilson and Heiser, 1979). Because of the unique position

these two species carry of being the only domesticated New World *Chenopodium*, it is widely accepted that they must share a heritage, although various studies have indicated that they were most likely cultivated independent of each other (Heiser and Nelson, 1974).

When genetic research on quinoa was initiated in the late 1970s, quinoa was widely believed to have originated in South America, descended of highland diploid *Chenopodium* species such as *C. pallidicaule* (cultivated cañahua), *C. petiolare* Kunth, and *C. carnosolum* Moq., through the South American weedy tetraploid *C. hircinum* Schrad., or *C. quinoa* var. *melanosperma* (Mujica and Jacobsen 2000). An alternative hypothesis, forwarded originally by Wilson and Heiser (1979), is that quinoa descended from the North American tetraploid *C. berlandieri*. Shortly after the Mexican berlandieri complex was first described, it was considered to be conspecific with quinoa. Further research led taxonomists to place them in their own species (Wilson and Heiser, 1979), although they are morphologically and genetically very similar. The current prevailing paradigm is that *C. quinoa* evolved from an early tetraploid *C. berlandieri*, most likely *C. berlandieri* var. *zschackei*, but that the Mexican domesticated tetraploids are descended from *C. berlandieri* var. *sinuatum*. This idea is supported by morphology, cross-breeding experiments, isozyme studies, and limited genetic analysis (Heiser and Nelson 1974; Wilson and Heiser 1979; Wilson 1980; Walters 1988; Maughan *et al*., 2006). If this hypothesis is correct, it would indicate that *C. quinoa's* tetraploid origins are in North America, from an ancestor similar to *C. berlandieri* var. *zschackei*. It most likely moved down the continent to South America with the aid of human migration as a weed, probably *C. hircinum*, where it was then domesticated as *C. quinoa.*

Based largely on morphological characteristics, quinoa is classified in section *Chenopodium,* subsection *Cellulata*, along with the *C. berlandieri* complex, the South American weedy tetraploid *C. hircinum*, and the North American diploids *C. neomexicanum* Standl. and *C. watsonii* A. Nels. (Aellen and Just 1929; Wilson 1980). This categorization, based primarily on the alveolate seed coat morphology, indicates that *C. neomexicanum* and *C. watsonii* are strong potential ancestors for their domesticated tetraploid counterparts. Wilson (1988) showed that crosses made across the boundaries of this subsection produce few viable offspring, although it should be noted that he found *C. neomexicanum* is more compatible with the smooth-fruited diploid *C. fremontii* S. Wats., subsect. *Leiosperma,* than it is with *C. watsonii.*

Although there has lately been an increase of *Chenopodium* studies, more information is needed to finish piecing together a plausible phylogeny for the genus, specifically as it relates to quinoa and its close relatives in the species *C. berlandieri*. One excellent way to do this is through chromosomal studies of quinoa, Mexican berlandieri, and their possible diploid ancestors.

Cytogenetics

Chenopodium cytology is a largely untouched area, due to the fact that *Chenopodium* metaphase chromosomes are all roughly the same size. Within *C. quinoa* and *C. berlandieri,* the chromosomal size range is a mere 1-3.3 µM, and almost all of the chromosomes are metacentric, which makes them hard to distinguish by measuring arm length and centromere location (Kolano *et al.,* 2001; Palomino *et al.*, 2008). They also do not display distinctive banding patterns for useful analysis by C-banding (Parkinson

2001). Other food crops such as potato and feed crops such as *Medicago* also face this problem (Dong et al. 2000; Kukilova et al. 2001; McKnight Foundation CCRP annual report 2002, unpublished). An additional obstacle is that *Chenopodium* often express mixoploidy, having sectors with diploid, tetraploid, octoploid, and even decaploid tissues (Sanderson et al. 1990; Kolano, personal communication). Mixoploidy has been observed at frequencies at or above 60% in the terminal root meristems of cultivated *Chenopodium* as well as wild ones, with much lower rates in shoot apices and laminar meristems. This has necessitated the use of the latter tissue types for accurate cytogenetic analysis (Maughan *et al*., 2006; Kolano, personal communication).

Because of the challenges of producing a useful karyotype, *Chenopodium* chromosomes have yet to be numbered, and minimal work has been done to locate physical markers on the chromosomes. However, because of the small, uniform chromosomes size, in-situ hybridization is an ideal technique for studying *Chenopodium* cytogenetics.

In situ Hybridization

In the 1970's, the birth of *in situ* hybridization, or ISH, techniques provided a new way of studying chromosomes (Gall and Pardue, 1969). The methodology behind all *in situ* hybridization techniques is very similar to that of Southern blotting: if genomic DNA is made single-stranded, and a single-stranded probe DNA is added, the two will hybridize, highlighting the region of interest (Schwarzacher, 2003). ISH utilizes specific DNA sequences, taken from such sources as BAC libraries, genomic clones, EST libraries, and PCR products like SSRs to probe against chromosome squashes that have

been prepared on microscope slides (Kim *et al.*,2002; Zhang *et al.*, 2004; Kato *et al.*, 2005; Lamb and Birchler, 2006).

The earliest ISH assays used radioactively labeled probes. These assays provided for the detection of repetitive elements, but not single-copy sequences, in plants (Harper and Sunders**,** 1981; Hutchinson and Londsdale, 1982**).** However, the technique was cumbersome and time-consuming, requiring a liquid silver emulsion or film sheet to cover the slides, which then required days or even weeks to get sufficient exposure for developing ^{3}H-labeled probes (Schwarzacher, 2003), resulting in pictures that may or may not be bright enough to analyze.

Fluorescence *in situ* Hybridization

Fluorescence *in situ* hybridization has several advantages over enzyme- and radioisotope-based probes. The detection molecules are fluorochromes such as coumarin (blue), fluorescin isothiocyanate or FITC (green), tetramethyl rhodamine (red), and cyanine 5 (far red). The fluorochromes provide a much tidier visual image than can be obtained by both enzymatic or radioisotopic detection; and different probes can be labeled with different fluorochromes in the same assay, permitting the detection of multiple targets within the same assay (Wang *et al.*, 2006).

Probes are usually created in a nick translation procedure that utilizes the enzyme activity of DNAse I to randomly nick the probe, and DNA polymerase I to excise the nicked portions of the molecule and replace them with added dNTPs, some of which are fluorescent analogs. Although other procedures, such as PCR and random oligonucleotide primer labeling, can be used, the advantage of nick translation is that the size of the probe can be more carefully controlled. Typically, probe sizes of between 50

and 300 nucleotides are optimal for FISH, as longer probes have been reported to result in higher amounts of background fluorescence (Levsky and Singer, 2003; Schwarzacher, 2003; Kato *et al.*, 2006; Birchler, personal communication).

FISH has been used with very high resolution results, particularly in chromosome preparations from mammalian cells. In humans, single-copy targets as small as 1 kb have been successfully identified (Jiang *et al.*, 1995). The resolution level to date in plants is considerably lower; it is very difficult to detect single-copy targets that are smaller than 3 kb (Lamb *et al.*, 2006; Wang *et al.*, 2006), which is widely attributed to the additional debris that comes from cell walls (Jiang *et al.*, 1995). The ability to detect probes as small as 3 kb is a great improvement over past abilities, however, that includes several alterations in the "standard" FISH protocol, including increasing to 10-50X the amount of DNA polymerase I; direct labeling using relatively "short-armed" dNTP-hapten conjugates that incorporate better into the probe than haptens that protrude farther out from the deoxyribonucleotide; elimination of signal-amplifying antibodies that increase background fluorescence; and the use of pressurized nitrous oxide gas (150-180 psi) for increasing the mitotic index (Kato *et al.* 2005).

FISH can be used to assign physical locations to genes that have been genetically mapped, to locate chromosomal aberrations (Kim *et al.*, 2002), to create karyotypes for interspecific comparisons (Islam-Faridi *et al.*, 2002), to confirm the order of loci as determined by genetic mapping, and to identify different genomes in a polyploidy, a process known as genome painting (Mukai *et al.*, 1993, Zhang *et al.*, 2004). Regardless of the resolution of the probe, FISH provides a means for studying genotypes that are unavailable from any other assay. Although it is impossible to tell how many sequences

are represented in a band on a gel, if the band is used as a cytological probe on a chromosomes spread, it becomes plausible to understand the sequence diversity in the band (Schwarzacher, 2003). It is also much easier to understand the physical genomic layout of genes and gene families. New techniques for high-resolution FISH are useful in identifying the location of t-DNA inserts, which supplies useful data about how the quantity and chromosomal location of transgenic segments affects their expression (Khrustaleva and Kik, 2001). These techniques can also be used to compare gene loci across species to see where chromosomal rearrangements occur. For plant breeders, high-resolution FISH is becoming a valuable tool for identifying the introgressed DNA in nearly isogenic lines.

Because of the size of and limited knowledge about quinoa chromosomes, this level of work is not feasible at the present time. However, it is feasible and useful to study high-copy sequences to elucidate how *Chenopodium*, specifically subsect. *Cellulata* evolved.

Two physical markers, from probes 18-24J and 12-13P, have been successfully hybridized to the quinoa genome using fluorescence *in situ* hybridization, or FISH, although at present, all other attempts to determine novel sequences for use as physical probes in quinoa have been unsuccessful (Kolano, personal communication; Sederberg, unpublished).

Although the search for novel sequence probes has proved mostly unfruitful at present, there are a handful of well-established DNA sequences that, as FISH probes, should successfully produce evolutionary data about New World *Chenopodium*. The most prominent of these are the ribosomal RNA genes.

Ribosomal DNA

In most eukaryotes, there are four different segments of ribosomal DNA: the 5S, 5.8S and 25S, which are part of the large ribosomal subunit, and the 18S rDNA, which contributes to the small ribosomal subunit. The 5.8S, 18S, and 25S are all transcribed as one large RNA molecule, called the 45S, prior to posttranscriptional processing. 45S loci are the sites of nucleolar organizing regions (NORs). The 5S genes are spatially separate from the 45S locus and exist in tandem repeats, each repeat separated by a non-transcribed spacer region (NTS). The 45S gene also exists in high copy numbers, with an intergenic spacer region (IGS) between the 18S and 25S subunits (Rogers and Bendich 1987; Flavell *et al.,* 1986; Kolano *et al*., 2001; Kato *et al*., 2005). A typical animal cell will have from 100 to 1000 copies of rDNA in its genome, but plant cells generally carry 500 to 40,000 rDNA repeats, although most are inactive (Rogers and Bendich 1987). A major concern when performing FISH on plant cells is the size of the probe, because sequences smaller than ~3.1 kb are generally undetectable (Jiang *et al*., 1995; Wang *et al*., 2006; Lamb *et al*., 2007;). The high copy number of the rDNA genes makes them ideal FISH probes for the small, uniform chromosomes of *Chenopodium*.

Ribosomal DNA probes (both 5 and 45S) have been applied to twenty Old World *Chenopodium* (Kolano, unpublished), to *C. quinoa*, and to three subspecies of *C. berlandieri* (Maughan *et al.,* 2006; Palomino *et al.,* 2008). Quinoa contained two interstitial 5S loci but only a single 45S locus. The three *C. berlandieri* genotypes, however, showed surprising divergence. *C. berlandieri* var. *zschackei* contained one 45S and two 5S loci; huazontle contained two 45S loci and three 5S loci; and quelite contained one 45S locus and three 5S loci (Maughan *et al.,* 2006).

There are two plausible hypotheses at present that successfully explain the difference between the 5S locus number in *C. berlandieri* var. *zschackei* and the locus number in quelite and huazontle. First, it is possible that the ancestor to the plants currently classified as *C. berlandieri* and *C. quinoa* was formed in a monophyletic event, from one diploid with a single copy of both the 45s and the 5S loci and a second diploid with a single 45S locus and two 5S loci. In this scenario, the primitive tetraploid contained at least two 45S loci and three 5S loci. Consequently, one 5S and one 45S locus were deleted before the divergence of *C. berlandieri* var. *zschackei* and the following development of quinoa. If this hypothesis is correct, one would expect that of the diploid ancestors of these tetraploid *Chenopodium,* one would contain a single copy of either locus while the second would contain two 5S loci.

However, according to the data that Wilson produced in his studies of subsect. *Cellulata* (1980), it is equally possible that the creation of the *Cellulata* tetraploid group was a polyphyletic event, in which case quinoa and *C. berlandieri* var. *zschackei* would not share the same two diploid ancestors with the Mexican cultivated tetraploids. Application of the genome-painting repetitive FISH probe 18-24J to both quinoa and huazontle resulted in strong hybridization to half the chromosomes of either species, indicating that the two tetraploids share at least one common ancestor. This probe also hybridized strongly to the entire complement of chromosomes in *C. neomexicanum*, marking it as a putative diploid ancestor for the cultivated tetraploid *Chenopodium* (Jellen and Kolano, unpublished). The probe will need to be further utilized against other diploids to strengthen the possibility of *C. neomexicanum* as an ancestor. Under the first hypothesis this would indicate that it is most likely that quinoa's other diploid ancestor

contains two 5S loci. Under either hypothesis, diploids containing two 5S loci are strong candidates for ancestors of these tetraploid *Chenopodium* species.

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Tables and Figures

Table 1. Wild and weedy Chenopodium species found in the Americas. Explanations: * designates N. American native species; # South American native species.

Species	BYU Accession No.	Collection Site(s)
C. album L.	412, 415, 416, 430, 431, 558, 559, 560, 573, 576, 598, 601, 612, 652, 654, 655, 662, 857, 871	UT, Chile, AZ, CA,
C. atrovirens Rydberg*	457, 460, 617, 618, 644, 658, 659, 722, 824, 837	UT, CA
C. berlandieri Moq-Tand*	402, 422, 423, 457, 466, 586, 652, 653, 661, 702, 717, 718, 720, 801, 841, 880	CA, UT, AZ, NM
C. berlandieri var. *boscianum* (Moq-Tand) Wahl*	802	LA
C. berlandieri var. *macrocalycium* (Aellen) Cronquist*	803	ME
C. berlandieri var. *nuttaliae**	567, 570, 571, 668,	Mexico
C. berlandieri var. *sinuatum* (Murr) Wahl*	411, 575, 579, 587, 588, 596, 840,845	CA, AZ
C. berlandieri var. *zschackei* (Murr) Murr ex Graebner*	467, 518, 519,540,578, 606, 622, 629, 637,641,642, 656, 657, 664, 862, 870, 874, 876, 878	AZ, NV, CO, UT
C. californicum (S. Watson) S. Watson*	501	CA
C. carnosolum Moquin.#	556, 561, 711, 712	Chile Reg. I
C. desiccatum A. Nelson*	639, 834,835	CO, NV
C. foliosum (Moench) Asch	603,812,825	UT
C. fremontii S. Watson*	404,410,420,424,437,438,439,445.5,446,448,449,450,461,462,465,472, 525, 532,535,551,590, 615,626,646, 660,666, 817,836,851,855, 866	UT, AZ, NM, NV, ID, CA
C. glaucum L. var. *salinum* (Stand) B. Boivin	534, 811	UT
C. hians Standley*	417,456,595, 631, 634, 636, 842,850, 854,	UT, CA
C. hircinum Schrad.#	565,566, 713,	Chile Reg. I
C. incanum (S. Watson) A. Heller*	429, 442,542, 572, 593, 820, 822, 823, 830, 833, 844, 846, 853, 861, 864,	CA, UT, AZ, CO, NM, WY
C. leptophyllum (Moquin-Tandon) Nuttall ex S. Watson*	469,507,533,589, 647, 716, 719, 829, 848, 856,	AZ, UT, KS, NM, NV
C. macrospermum Hooker*#	426,427,428,428.5, 433, 574, 701, 838, 858, 859, 860,	CA, UT
C. murale L.	432, 557	CA, Chile Reg. I
C. nevadense Stand*	816,818,819	NV
C. oahuense	651	HI
C. petiolare #	562,563,564,706,707,708, 709,710, 813	Chile Reg. I
C. pratericola Rydberg*	458, 568,569,577, 604,623,624, 625, 638, 814,815,832	AZ, CO, UT, ID, KS, NV, WY,
C. quinoa Willd#	602	Chile Reg. IX
C. rubrum L. var. *rubrum*	725	UT
C. rubrum L. var. *humile* (Hooker) S. Watson*	665	UT
C. simplex (Torrey) Rafinesque*	619,640,723	UT
C. strictum Roth	434,435,436	CA
C. watsonii A. Nelson*	470,508,516,517,539,541, 543,552,591,592,839,847, 863,867,873	AZ-NM

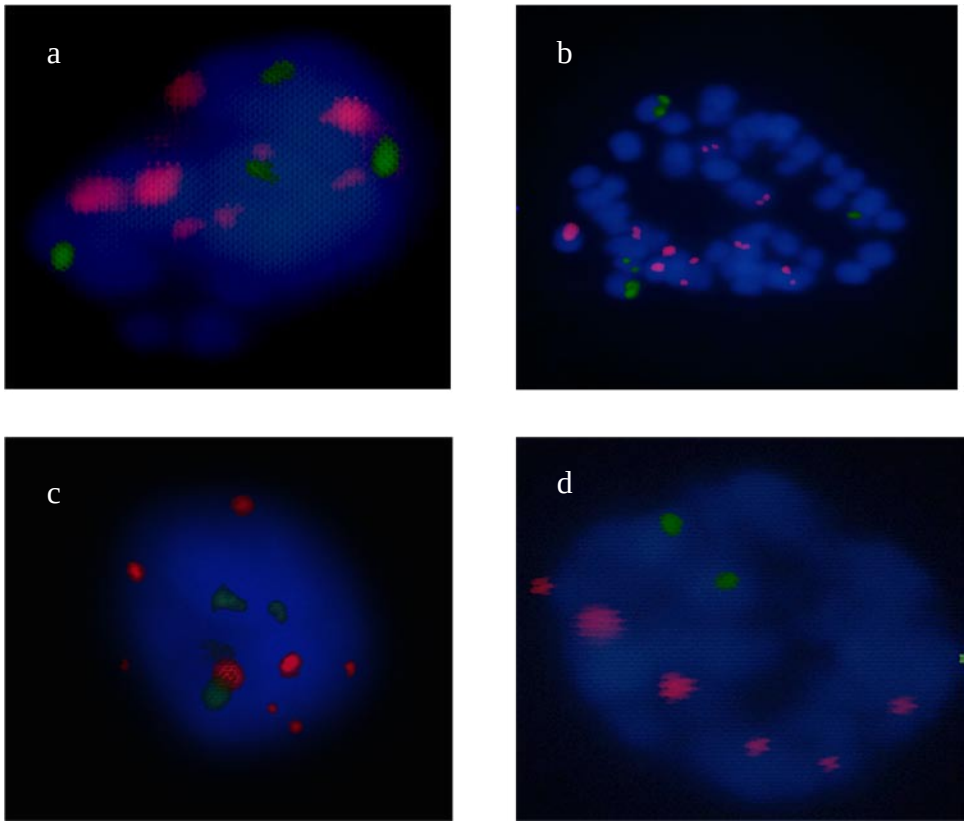
Table 2. Primers used to amplify the 18S portion of the 45S gene in quinoa

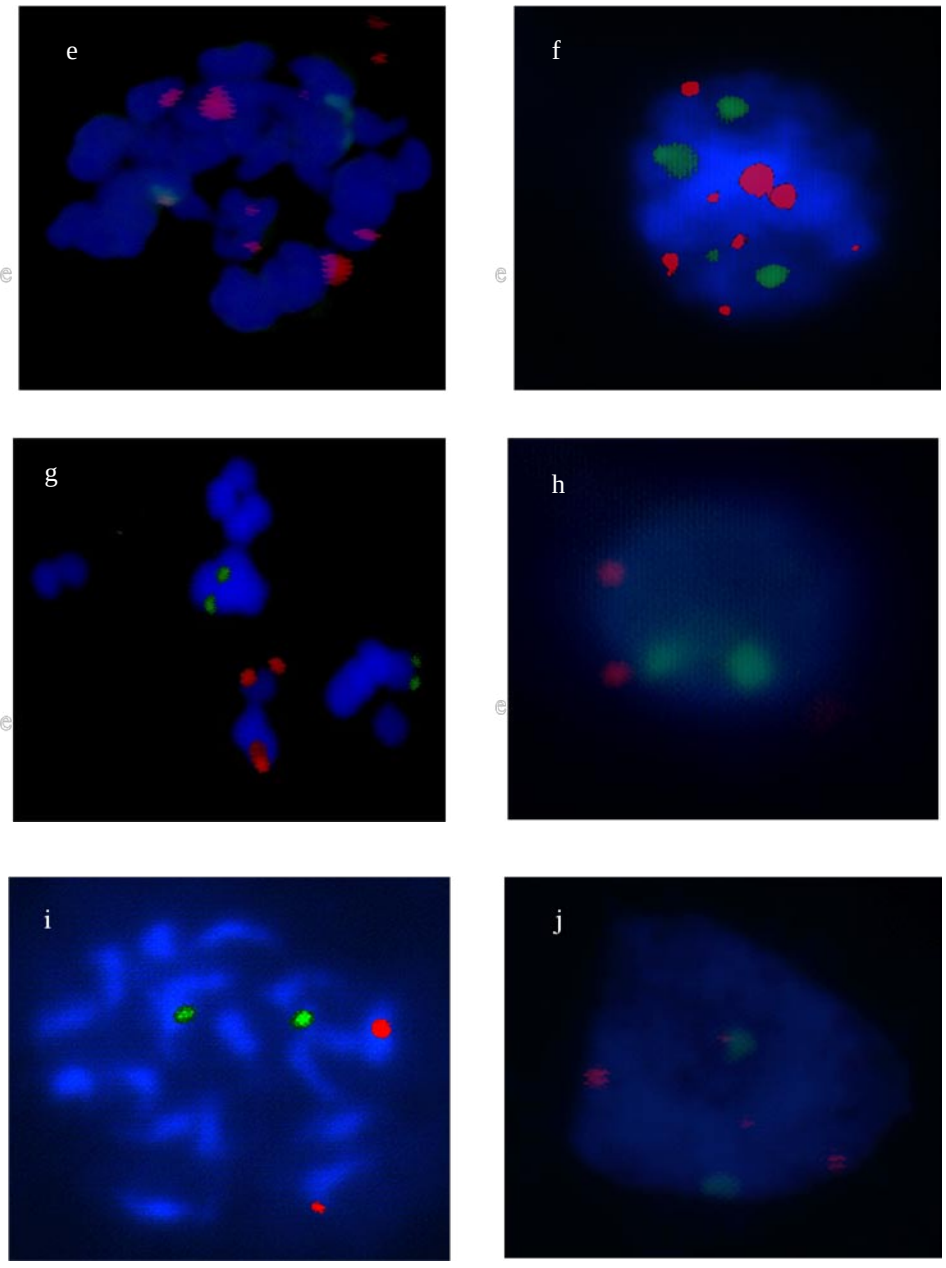
18S1-R	aacacttcaccggaccattc	18S2-R	ggaccattcaatcggtagga
18S1-L	gactgtgaaactgcgaatgg	18S2-L	aactgcgaatggctcattaaa

Table 3. rDNA locus counts, chromosome counts (where possible), and accession numbers of species studied

Species	**Accession #**	**Chromosome count (2n)**	**45S loci**	**5S loci**
C. californicum	BYU 501	16	1	1
C. foliosum	BYU 603	16	1	1
C. atrovirens	BYU 536	18	1	1
C. carnosolum	BYU 561	18	1	1
C. glaucum var. *salinum*	BYU 534	18	1	1
C. incanum	BYU 822	18	1	1
C. macrospermum	BYU 427	18	1	1
C. neomexicanum	Field	18	1	1
C. petiolare	BYU 709	18	1	1
C. rubrum var. *humile*	BYU 665	18	1	1
C. watsonii	BYU 539	18?	1	1
C. fremontii	BYU 817	18	1	2
C. murale	BYU 432	18	1	2
C. strictum	BYU 434	18?	1	2
C. hians	BYU 417	18?	3	3
C. oahuense	BYU 651	18	3	3
C. berlandieri var. boscianum	BYU 802	36	1	3
C. berlandieri var. macrocalycium	BYU 803	36	1	3
C. simplex	BYU 640	36	2	2
C. album	BYU 652	36	2	4
C. berlandieri var. *sinuatum*	BYU 411	36	2	4
C. hircinum	BYU 558	36	2	4
C. album	BYU 430	52	2	4

Figure 1. Results of FISH probes for 45S and 5S loci in Chenopodium species. Green signals, obtained with fluorescein, show 45S hits; red signals, obtained with Texas Red, show 5S hits. (a) *C. album* (tetraploid); (b) *C. album* (hexaploid); (c) *C. atrovirens;* (d) *C. berlandieri* var. *boscianum;* (e) *C. berlandieri var. macrocalycium;* (f) *C. berlandieri var.sinuatum;* (g) *C. californicum;* (h) *C. carnosolum;* (i) *C. foliosum;* (j) *C. fremontii;* (k) *C. glaucum salinum;* (l) *C. hians;* (m) *C. hircinum;* (n) *C. incanum;* (o) *C. macrospermum;* (p) *C. murale;* (q) *C. neomexicanum;* (r) *C. oahuense;* (s) *C. petiolare;* (t) *C. rubrum humile;* (u) *C. simplex;* (v) *C. strictum;* (w) *C. watsonii*

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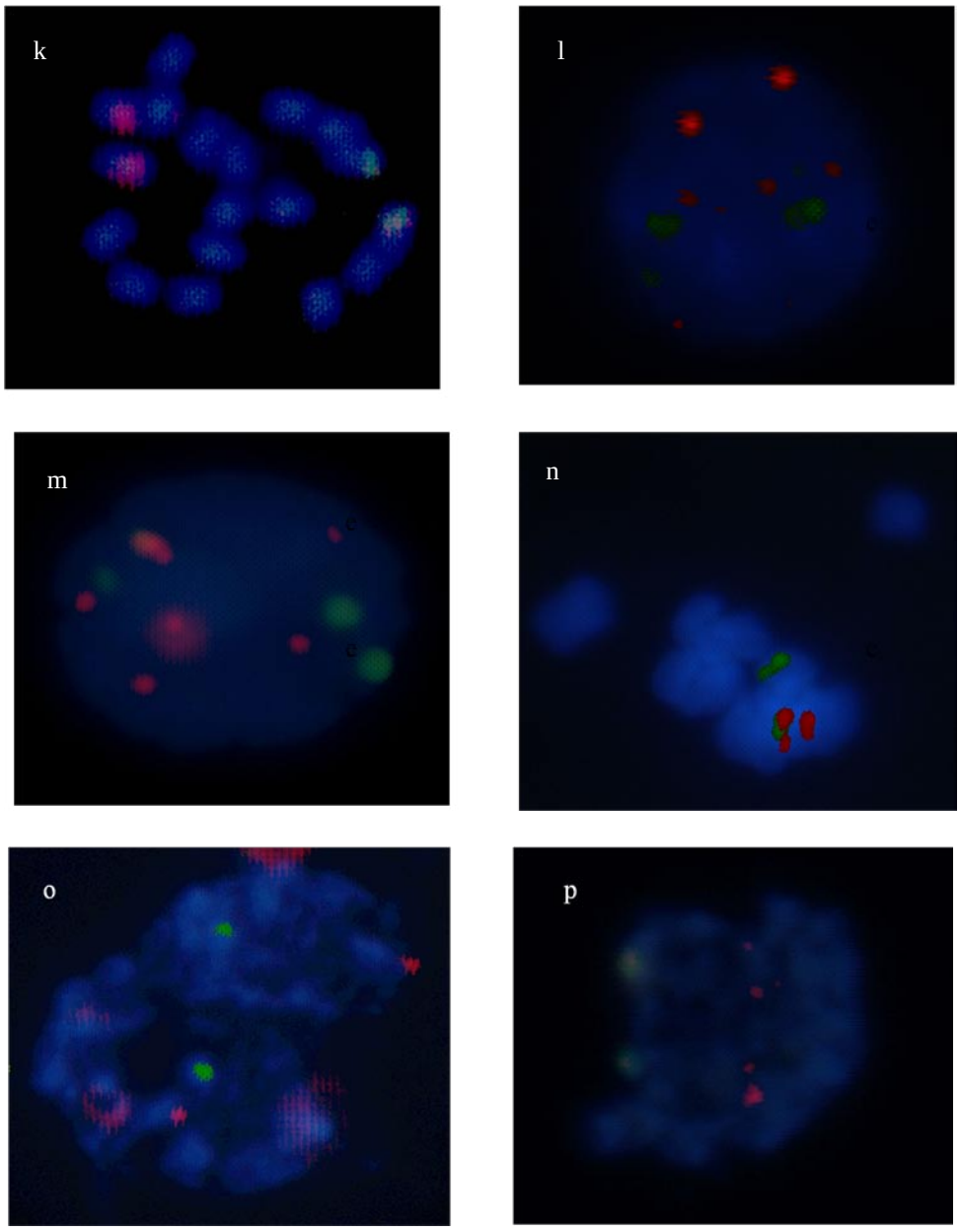
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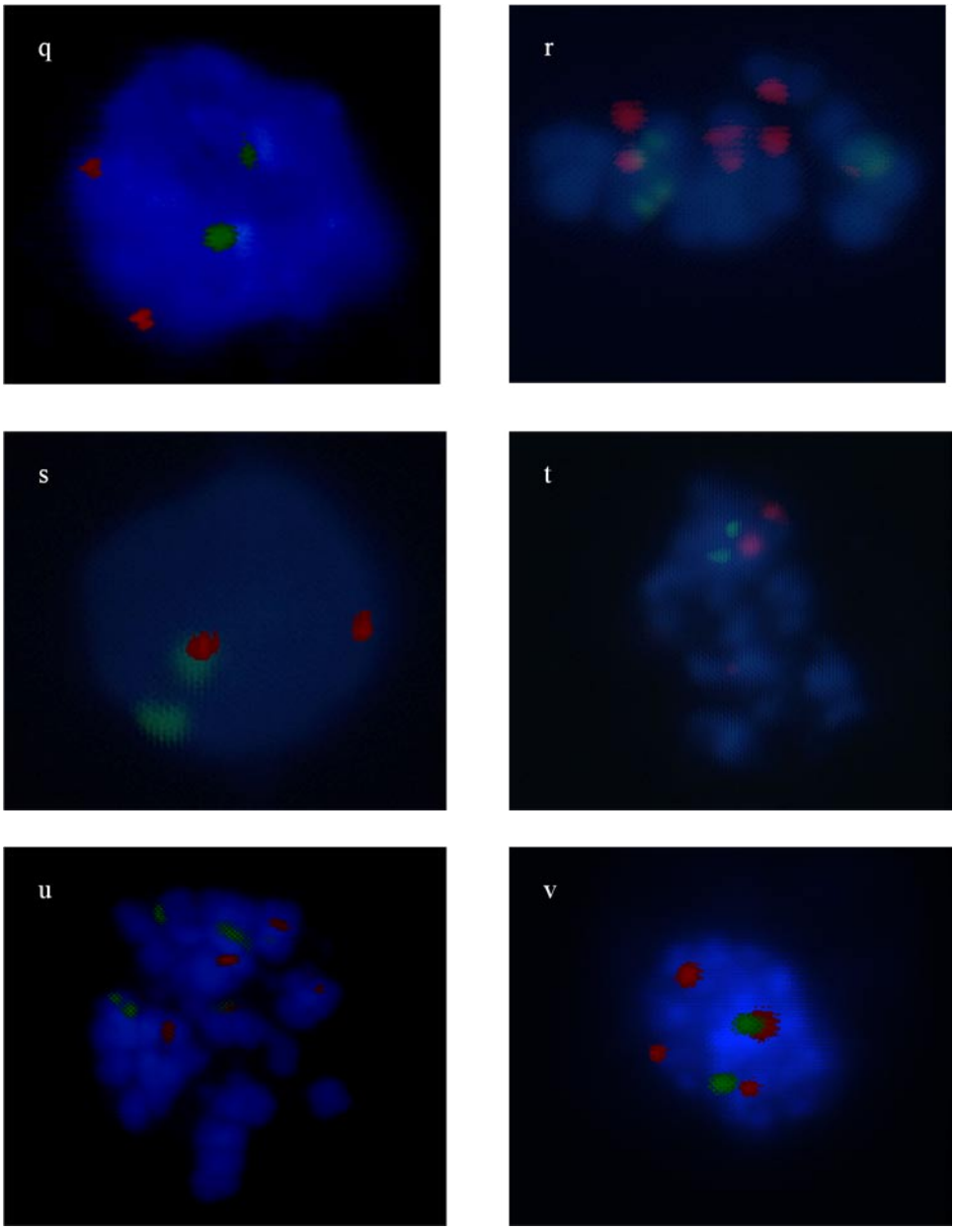
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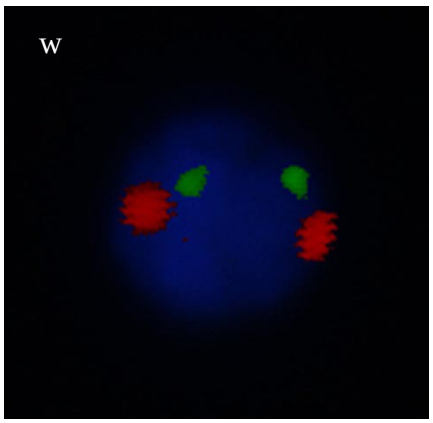
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