Relationships among 3 Kochia species based on PCR-generated molecular sequences and molecular cytogenetics

B.S. Lee, M.Y. Kim, R.R.-C. Wang, and B.L. Waldron

Abstract: Forage kochia (Kochia prostrata ssp. virescens 'Immigrant') is native to the arid and semiarid regions of central Eurasia. It was introduced into the United States in 1966 as PI 314929 and released as a perennial forage shrub in 1984. Kochia americana is a perennial native to the United States, whereas Kochia scoparia is an introduced annual species that became a weed. To assess both the breeding potential and the possibility of genetic contamination, relationships among the 3 Kochia species were analyzed using random amplified polymorphic DNA (RAPD) markers, sequence tagged site (STS) marker sequences of the chloroplast NADH dehydrogenase gene (ndhF), genomic in situ hybridization (GISH), and multicolor fluorescence in situ hybridization (MC-FISH). Seventy decamer random primers yielded 458 polymorphic bands from 9 plants of K. americana, 20 plants of K. prostrata, and 7 plants of K. scoparia. Fifty-four and 55 species-specific RAPD markers were identified for K. americana and K. prostrata, whereas 80 RAPD markers were specific to K. scoparia. Based on the presence or absence of informative RAPD markers, the 3 species always grouped into 3 distinct clusters in a NTSYSpc2.01b-generated dendrogram. The same relationships were found among the 3 Kochia species based on ndhF DNA sequence divergence. Using a set of 7 STS markers that can identify each Kochia species, we did not find a single interspecific hybrid from artificial hybridizations among the 3 Kochia species. In GISH studies, chromosomes of 1 species fluoresced in green only when they were probed by genomic DNA of the same species. Cross-hybridization by genomic DNA of another species was not observed. In FISH studies using pTa71 (for 18S–5.8S–26S rDNAs) and pScT7 (for 5S rDNA) as probes, there were 1, 1 and 3 pTa71 sites and 2, 1, and 1 pScT7 sites in each haplome of K. prostrata, K. americana, and K. scoparia, respectively. It is concluded that these 3 Kochia species are so genomically distinct that gene introgression among them would be extremely rare.

Key words: RAPD, STS, ndhF, GISH, FISH, mixoploidy, forage kochia.

Introduction

Forage kochia (Kochia prostrata (L.) Scrad.), sometimes called prostrate kochia, prostrate summer cypress, or Bassia prostrata, is a long-lived, perennial, semievergreen half-shrub native to the heavily grazed arid and semiarid rangeland regions of central Eurasia (Harrison et al. 2000). It was first introduced into the United States in 1966 and became well adapted to semiarid rangelands in western states (McArthur et al. 1974, 1996; Blauer et al. 1993; Harrison et al. 2000). Many consider forage kochia an important plant to restore function and desired use to degraded western rangelands because of its high salt and alkali tolerance (Francois 1976; Romo and Haferkamp 1987) and its competitiveness against the annual noxious weeds cheatgrass (Bromus tectorum L.) and halogeton (Halogeton glomeratus [Stephen ex Biebr.] C.A. Mey.) (McArthur et al. 1990; Stevens and McArthur 1990; Clements et al. 1997; Harrison et al. 2000; Monaco et al. 2003). For centuries, forage kochia has been an important fall and winter forage for sheep, cattle, horses, camels, and wildlife in Kazakhstan, Uzbekistan, and surrounding republics (Balyan 1972; Waldron et al. 2001a, 2001b; Waldron et al. 2005). Its nutritive value includes high crude protein during the critical fall–winter grazing period (Davis and Welch 1985; McKell et al. 1990), low nontoxic levels of oxalates (Davis 1979), and relatively high digestibility and preference (Nemati 1977; Welch and Davis 1984; Stevens et al. 1985; McKell et al. 1990). Recent research in the United States has evaluated livestock performance when grazing forage kochia in the fall–winter and has shown that forage kochia can reduce winter feeding costs, potentially increasing the sustainability of livestock production in rural areas (Zobell et al. 2003).

Forage kochia has also been proven to be very effective in greenstrips used to stop wildfires in western states (Monsen 1994; Pellant 1994; Harrison et al. 2002). It is effective in suppressing wildfires because of its active summer growth and ability to compete against cheatgrass and reduce dry fuel loads. Forage kochia is one of a few species that can be established on frequently burned and cheatgrass-infested rangelands (Monaco et al. 2003), so it may be a useful bridge to reestablish of native species.

Forage kochia should not be confused with annual kochia (Kochia scoparia (L.) Scrad.). Annual kochia, while sometimes used as forage, is considered an agricultural weed throughout the western and midwestern United States (Whitson 2000). Forage kochia differs from annual kochia in that forage kochia is a perennial and does not have potential nitrate and oxalate toxicity. Harrison et al. (2000) conducted a review of forage kochia adaptation, potential uses in the United States, and potential weediness. They concluded that forage kochia, unlike annual kochia, is not an invasive weed and does not invade undisturbed perennial plant communities. Forage kochia is known to recruit into and stabilize disturbed sites and alkali playas where it competes against annual species.

Greenmolly or perennial summer cypress (Kochia americana S. Wats.) is probably the US native equivalent of forage kochia (Clements et al. 1997; Stubbendieck et al. 1997). Greenmolly, like forage kochia, is a drought- and saline-tolerant perennial semishrub in the Chenopodiaceae family that has value as a fall–winter forage. However, greenmolly has a limited range of adaptation, is difficult to establish, has low productivity, and is not widely dispersed throughout western rangelands (Clements et al. 1997).

Kochia prostrata ‘Immigrant’ released in 1984 (Stevens et al. 1985) is currently the only cultivar of forage kochia in North America. The US Department of Agriculture has initiated a breeding program to develop types of forage kochia with increased stature and forage production using ecotypes from Kazakhstan (Waldron et al. 2001b) and Uzbekistan (Waldron et al. 2005). Forage kochia is extremely polymorphic with several subspecies and forms a polyploid series with $2n = 18$, $4n = 36$, and $6n = 54$ types (Pope and McArthur 1977; Zakharyeva and Soskov 1981; Shakhnov and Sagimbaev 1982; McArthur et al. 1996).

Molecular cytogenetic techniques such as genomic in situ hybridization (GISH) have been successfully applied for chromosomal identifications and genome recognition in many plants (Schwarzacher et al. 1989, 1992; Anamthawat-Jonsson et al. 1990; Mukai et al. 1991; Leitch and Heslop-Harrison 1992; Schwarzacher 2003). A large number of repetitive DNA sequences from plant species were physically mapped by in situ hybridization. Physical maps of genes by fluorescent in situ hybridization (FISH) represent a potentially new source of chromosomal characters that may be phylogenetically informative (Jiang and Gill 1994; Hanson et al. 1996; Zhang and Sang 1999). The most frequently mapped genes are the 45S (18S–5.8S–26S) and 5S ribosomal RNA genes (rDNA) in plant chromosomes. Evolutionary implications of variation in number and location of rDNA loci have been explored in many polyploid plants (Fukui et al. 1994; Xu and Earle 1996; Zhang and Sang 1999; Lee and Kim 2000; Mishima et al. 2002; Goo and Kim 2003). Determination of the number and location of rDNA loci in Lycoris provided insights into the evolutionary mechanism of allopolyploid species (Lee and Kim 2000).

Genomic relationships among K. americana, K. prostrata, and K. scoparia have not been studied previously. To assess whether the annual weed K. scoparia may contaminate forage kochia, K. prostrata, and if the perennial native species K. americana can serve as a gene pool for breeding forage...
kochia, species relationships among *K. americana*, *K. prostrata*, and *K. scoparia* were assessed using the random amplified polymorphic DNA (RAPD) technique as well as sequence divergence analysis of the ndhF gene in their chloroplast genomes. Furthermore, GISH and multicolor FISH (MC-FISH) were carried out using genomic DNA and cloned pTa71 (for 45S rDNA) plus pScT7 (for 5S rDNA) DNAs as probes, respectively. Determining these relationships will also clarify the potential for introgression of the nonnative *K. prostrata* into native *K. americana*.

**Materials and methods**

**Studies of molecular sequences**

Nine plants of *K. americana*, 20 plants of *K. prostrata*, and 7 plants of *K. scoparia* (Table 1) were used in this RAPD study. These studied plants sample the chromosome races of *K. prostrata* from diploid (2x) to hexaploid (6x) (Table 1). Only diploid was known in *K. scoparia* (Goldblatt 1981; Goldblatt and Johnson 1991, 1996, 2000). For *K. americana*, both diploid and tetraploid (4x) populations are known (S.C. Sanderson and E.D. McArthur, unpublished).

Genomic DNA was extracted from leaves using the CTAB method of Doyle and Doyle (1987). Only DNA of *K. americana* required the removal of polysaccharides using an OmniPrep kit (GenoTech, St. Louis, Missouri). However, the polysaccharide associated with *K. americana* was not completely removed by the OmniPrep kit. Template DNA was quantified by a Hoefer DyNA Quant200 fluorometer and diluted to a final concentration of 20 ng/µL.

Eighty decamer primers from random primer kits of Operon Technologies (Alameda, California) were used for amplification of RAPD fragments. The optimized amplification reaction mixture (25 µL) contained 13.8 µL of sterile double-distilled water, 2.5 µL of 10× buffer, 2 µL of 8 mmol dNTP/µL, 2 µL of 10 µmol/L primer, 3 µL of 25 mmol MgCl2/µL, 0.2 µL (2 units) of AmpliTaq Stoffel fragment.
Fig. 1. Agarose profiles for RAPD products amplified from primers (a) OPAP15 and (b) OPAP16 using 9, 20, and 7 plants of Kochia americana (lanes 1–9), Kochia prostrata (lanes 10–29), and Kochia scoparia (lanes 30–36), respectively, as templates. Species-specific markers are indicated by arrows. M indicates the bands for (top to bottom) 1500-, 1200-, 1000-, 900-, 800-, 700-, 600-, 500-, 400-, 300-, 200-, and 100-bp size markers.

(Applied Biosystem, Foster City, Calif.), 0.5 µL of DMSO, and 1 µL of template DNA (20 ng/µL).

Only the reproducible bands were scored for their presence (1) or absence (0) in the 36 plants of 3 Kochia species. A dendrogram was generated based on the presence and absence of 458 informative RAPD markers using the SAHN (Sequential, Agglomerative, Hierarchical, and Nested Clustering) program of the NTSYSpc2 software (Rohlf 1994). Similarity coefficients were generated and analyzed using SIMQUAL (Similarity for Qualitative Data) of NTSYSpc2.

Four species-specific RAPD markers for each species of Kochia were selected for cloning and sequencing. Procedures for cloning and sequencing followed those used by Li et al. (2002a). Based on sequences of these species-specific RAPD markers, primers for sequence tagged site (STS) markers were designed using the web-based software Primer3 (available at http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi) and then tested for species specificity. Because these STS markers were converted from most unique and consistent RAPD markers specific to 1 of 3 Kochia species, only 3 randomly selected plants from each species were tested for their species specificity.

The STS marker sequence for the chloroplast NADH dehydrogenase gene (ndhF) was amplified from the total genomic DNA of each Kochia species using the primer pair GGATTAACGATTTTATGTTTGG and CCCCTAYAGTTTGT of Olmstead and Sweere (1994). PCR conditions were the same as those reported in Jones et al. (2000). Amplified fragments were cloned into plasmid pBlueScript and sequenced. The DNA sequences of the ndhF gene in the 3 Kochia species were aligned along with those of 2 Chenopodiaceae species, Amaranthus quitensis (GenBank accession AF194822) and Amaranthus palmeri (GenBank accession No. AF194821), using EMBL-EBI ClustalW (http://www.ebi.ac.uk/clustalw/index.html) to generate a phylogram.

Molecular cytogenetic studies

Root tips from plants of diploid K. prostrata (2x = 18) and K. scoparia (2x = 18) and tetraploid K. americana (2n = 4x = 36) were used for GISH and MC-FISH studies. Mitotic chromosome preparation followed the same procedures as those for wheat except that an enzyme treatment was essential for Kochia. Cell walls were digested in enzyme mixtures using the method of Fukui et al. (1994) before root tip cells were squashed on slides. The 10-mL enzyme mixture contained 0.2 g of cellulase Onozuka RS (Yukult Pharmaceutical, Tokyo, Japan), 0.15 g of macerozyme R-10 (Yakult Pharmaceutical), 0.03 g of pectolase Y-23 (Kyowa Chemical Products, Osaka, Japan), 200 µL of EDTA (0.5 mol/L), and 9.8 mL of double-distilled water. The root tips were immersed in 80 µL of enzyme mixture in a 0.7-mL microcentrifuge tube for approximately 3 min at 37 °C.

Total genomic DNA was extracted from 0.5–1.0 g of fresh leaves using the CTAB method (Doyle and Doyle 1987) and mechanically sheared to 10- to 12-kb fragments by vortexing for 5 min. For GISH, DNA was labeled with biotin-16-dUTP with a BioNick translation kit (Life Technologies Inc., Rockville, Maryland) as previously described (Zhang et al. 1996; Wang et al. 2003). Chromosomes of each species were probed by biotin-labeled genomic DNA of 1 species and blocked by unlabeled genomic DNA of the other 1 or 2 species. To prepare the genomic blocking DNA, DNA fragments of 100–250 bp length were obtained by autoclaving the total genomic DNA from each of the Kochia species. The hybridization mixture consisted of 5 µg biotinylated genomic DNA/µL, 50% deionized formamide, 10% dextran sulphate, 500 µg/µL sonicated salmon sperm, 50 µg blocking DNA/µL and 2× SSC. Detection of hybridization sites of the biotinylated DNA probe was described by Leitch et al. (1994) using 10 µg fluorescein isothiocyanate (FITC) – avidin DN/mL (Vector Laboratories Inc., Burlingame, Calif.) and amplified once with 20 µg biotinylated antiavidin D/mL.
(Vector Laboratories, Inc.) followed by 10 µg FITC – avidin DN/mL. Chromosomes were counterstained using 2 µg propidium iodide (PI)/mL and 1 µg the 4’6-diamidino-2-phenylindole (DAPI) in antifade solution Vectashield (Vector Laboratories Inc.). The fluorescent signals were detected using a Zeiss Axioplan 2 microscope equipped with Chroma filter set 31001 (excitation 480 nm) for simultaneous detection of FITC–avidin and PI and filter set 31000 (excitation 360 nm) for DAPI.

For MC-FISH, digoxigenin-labeled pTa71 (for 18S–5.8S–26S rDNAs of *Triticum aestivum* L.; Gerlach and Bedbrook 1979) and biotin-labeled pScT7 (for 5S rDNAs of *Secale cereale* L.; Scoles et al. 1987) were simultaneously used as probes. The FISH procedures of Leitch et al. (1994) were used with some modifications (Lee and Kim 2000). Hybridizations with the 2 probes were detected by the red and green color from rhodamine and FITC, respectively, against the blue color of chromosomes counterstained by DAPI under the triple-pass filter set 61000V2 for DAPI–FITC–TRITC.

**Crosses among 3 Kochia species**

To determine if interspecific hybrids could be obtained, inflorescences of *K. americana* and *K. prostrata* were bagged without emasculation of their anthers because in these 2 species, anthers were dehisced before pistils became receptive. Crosses were made in combinations of *K. americana × K. prostrata*, *K. americana × K. scoparia*, *K. prostrata × K. americana*, and *K. prostrata × K. scoparia* (female × male). Annual *K. scoparia* is highly autogamous; thus, crosses using it as maternal parent were not attempted.

**Results and discussion**

**RAPD and STS markers**

Of the 80 primers tested, 70 Operon random primers produced 458 polymorphic fragments among plants of the 3 *Kochia* species (see supplementary data²). Number of polymorphic bands per primer ranged from 1 for AQ19 to 13 for AQ04. Some primers resulted in species-specific fragments

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²Supplementary data for this article are available on the Web site or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 4042. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.
Table 2. STS markers specific for 3 Kochia species.

<table>
<thead>
<tr>
<th>Species</th>
<th>RAPD marker</th>
<th>STS marker (GenBank accession No.)</th>
<th>Primer sequences</th>
<th>$T_A$ (°C)</th>
<th>Cycles</th>
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<tr>
<td><em>K. americana</em></td>
<td>OPBB19&lt;sub&gt;628&lt;/sub&gt;</td>
<td>KA-BB19&lt;sub&gt;616&lt;/sub&gt; (BV210892)</td>
<td>TTGCAGCACAGAAATAAGGTC</td>
<td>56</td>
<td>30</td>
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<td></td>
<td></td>
<td></td>
<td>AGAACCAAGACGGCAACAGT</td>
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<td></td>
<td>GTGCCGAATGCATTAAAAATT</td>
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<td></td>
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<td></td>
<td>ACAATTGATGATTTCTATTTAGGCAATGG</td>
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<tr>
<td><em>K. prostrata</em></td>
<td>OPBB19&lt;sub&gt;388&lt;/sub&gt;</td>
<td>KP-BB19&lt;sub&gt;385&lt;/sub&gt; (BV210893)</td>
<td>CCGACAGACGATGGTAAAGGA</td>
<td>56</td>
<td>30</td>
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<td></td>
<td></td>
<td></td>
<td>TTGCCGACAGCATGAATAAC</td>
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<td>CCCYTCAAGACCATGACG</td>
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<td></td>
<td>AACTGGCCCACATGAGAC</td>
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<td></td>
<td></td>
<td></td>
<td>ATGCCGACATTYARAGCGACR</td>
<td></td>
<td></td>
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<tr>
<td><em>K. scoparia</em></td>
<td>OPAP18&lt;sub&gt;600&lt;/sub&gt;</td>
<td>KS-AP18&lt;sub&gt;599&lt;/sub&gt; (BV210888)</td>
<td>GTGCTGACAGAGACAGAGAG</td>
<td>56</td>
<td>30</td>
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<td></td>
<td></td>
<td></td>
<td>TCGTGACAGTTTTCTTTATG</td>
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<td></td>
<td></td>
<td>CTCGCACTTTAGTGGATGG</td>
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*RAPD markers are named using the codes for decamers of Operon Technologies' primer kits followed by the length of the sequence (bp) as a subscript.

*STS markers are named for the species primer code with the length of target sequences (bp) as a subscript.

Fig. 3. STS markers (a) KA-BB19<sub>616</sub>, (b) KP-BB19<sub>385</sub>, (c) KP-AQ15<sub>379</sub>, and (d) KS-AP18<sub>599</sub>. Template DNAs are *Kochia americana* (lanes 1–3), *Kochia prostrata* (lanes 4–6), and *Kochia scoparia* (lanes 7–9). M indicates the bands for (top to bottom) 1500-, 1200-, 1000-, 900-, 800-, 700-, 600-, 500-, 400-, 300-, 200-, and 100-bp size markers.
in 1 or more species. Primers AP15 and AP16 produced 0, 2, and 2 and 2, 1, and 1 species-specific fragments for *K. americana*, *K. prostrata*, and *K. scoparia*, respectively (Fig. 1). A total of 54, 55, and 80 species-specific fragments were identified for *K. americana*, *K. prostrata*, and *K. scoparia*, respectively.

Based on RAPD markers, *K. americana*, *K. prostrata*, and *K. scoparia* are easily separated into 3 clusters using the NTSYS-UPGMA program. The relative positions of the 3 clusters varied depending on the data subset. Cophenetic similarity coefficient values were 0.44 between clusters of *K. americana* and *K. prostrata* and 0.38 between the cluster of these 2 species and that of *K. scoparia* when all 458 markers were analyzed as a single data set (Fig. 2; supplemental data). Annual *K. scoparia* is more distantly related to perennial *K. americana* and *K. prostrata* than the 2
perennials are to each other. These cophenetic similarity coefficient values are lower than those (0.64 and 0.55) found among 3 species of *Delphinium* (Li et al. 2002b). It is also worthy to note that *K. scoparia* exhibited the least genetic variability among plants, reflecting its self-pollinating mode of reproduction (R.R.-C. Wang, unpublished). Only S1 and S6 plants of *K. scoparia*, collected from 2 different locations, were genetically identical (Fig. 2). If apomixis had occurred in this species, S1 to S3 or S4 to S7 would have been identical. Our RAPD data excluded apomixis as a possible mode of reproduction in *Kochia* species. Because of their low similarity coefficients (Fig. 2), there is no evidence for genetic introgression among the 3 *Kochia* species in recent history.

STS markers were converted from most unique and consistent RAPD markers specific to each 1 of 3 *Kochia* species. A set of 8 STS markers was developed to identify the 3 *Kochia* species (Table 2). Four STS markers are shown in Fig. 3. Although some primer pairs amplified PCR fragments from nontarget species, only the target species yielded 1 fragment of the correct length at high copy numbers, whereas nontarget species produced different profiles with multiple bands. Therefore, the 8 STS markers listed in Table 2 are species specific in that they can produce the target fragment more efficiently and precisely only from the target species. These STS markers are useful in the identification of any interspecific hybrids, between any 2 species of the 3 studied *Kochia* species, that may occur in nature or that may be synthesized artificially.

From a controlled-selfing study of the 3 *Kochia* species, we noted variation in the degree of autogamy among plants of *K. prostrata*, the male sterility or dioecy (lacking anthers in all flowers of a plant) in some plants of *K. americana*, and high autogamy of *K. scoparia* (data not shown). Therefore, *K. scoparia* was used only as the paternal parent in artificial hybridization attempted to obtain interspecific hybrids. In an effort of controlled pollination without emasculation, 78 seeds from *K. americana × K. prostrata*, 40 seeds from *K. americana × K. scoparia*, 359 seeds from *K. prostrata × K. americana*, and 59 seeds from *K. prostrata × K. scoparia* were obtained. Only 1, 0, 85, and 19 seedlings were established for these crosses. These plants were tested with STS markers for respective parental genomes. All of them exhibited the presence of STS markers for their maternal species and the absence of those for their putative paternal species. It is concluded that all established plants were raised from seeds resulting from intraplant pollination. Therefore, the risk of gene exchange due to interspecific hybridization among the 3 studied *Kochia* species in nature is considered to be nil. This situation is different from that of 2 larkspur species, *Delphinium barbeyi* (L) Huth and *Delphinium occidentale* S. Watts, which had a higher cophenetic similarity coefficient value (0.64) and natural interspecific hybrids (Li et al. 2002b).

In the PCRs using the primer pair for the chloroplast ndhF gene, both *K. americana* and *K. prostrata* produced 1 fragment of approximately 950 bp, while *K. scoparia* produced 2 fragments of approximately 950 and >1500 bp. These fragments were cloned and sequenced. Sequences in the same direction from the 3 *Kochia* species were aligned with the known ndhF gene from *A. quitensis* (AmqndhF; GenBank accession AF194822) and *A. palmeri* (AmpndhF; GenBank accession AF194821) in Fig. 4. A phylogram was generated from the alignment using the ClustalW function, depicting relationships among the 3 *Kochia* species (Fig. 5) similar to those from RAPD analysis (Fig. 2), except that *K. scoparia* was grouped closer to *K. prostrata* based on the chloroplast gene sequence. However, the fact that both *K. prostrata* and *K. americana* produced only 1 PCR product whereas *K. scoparia* produced 2 PCR products indicates that the former 2 species are more closely related. There was 1 duplication–transposition event in *K. scoparia* that resulted in the amplification of 2 products from the primer pair used for the chloroplast ndhF gene. The other end of the long fragment of the PCR product from *K. scoparia* shows no homology to the ndhF gene; thus, the short fragment was
duplicated and transposed to another site of the chloroplast genome.

Cytogenetics, GISH, and MC-FISH

With proper enzyme treatments to digest cell walls in the root tip, chromosomes of Kochia species could be squashed to give satisfactory slide preparations with well-spread cells having clear chromosome definitions (Fig. 6). Chromosomes in tetraploid K. americana are slightly shorter than those in diploid K. prostrata and K. scoparia. All chromosomes appear to be metacentric and similar in length, making chromosome karyotyping difficult. Mixoploidy (cells with different ploidy levels in the same plant) was observed in forage kochia root tips (Table 1). Five out of 20 plants of K. prostrata had cells with different chromosome numbers: 2 had 2x, 4x, and 6x cells in the same root tips of the plants, whereas the other 3 had only 2x and 4x cells in the same root tips. The occurrence of mixoploidy could be attributed to endoreplication; thus, the polyploid plants of forage kochia could only be autopolyploids.

In the GISH studies, chromosomes of K. prostrata were strongly hybridized by its own genomic DNA in the presence of blocking DNA of K. americana and K. scoparia (Table 3; Fig. 7b). No hybridization could be detected when K. americana or K. scoparia DNA was used as probe and K. scoparia or K. americana was used as the block, respectively. Similarly, chromosomes of K. americana and K. scoparia were more strongly hybridized by probe DNA of their own species than the other species (Table 3; Figs. 7d and 7f). The polysaccharide associated with K. americana was not completely removed by the OmniPrep kit. Although this impurity was not a problem for PCR used to amplify genomic DNA fragments, it resulted in a low efficiency in both DNA hybridization and blocking in GISH. Thus, a low level of in situ hybridization of K. americana chromosomes by K. americana DNA probe (Fig. 7d) and an incomplete blocking of K. scoparia chromosomes by K. americana DNA block while probed with K. prostrata DNA (Table 3) were observed.

The GISH results revealed that the 3 Kochia species have such distinct genomes that the genome of each species was preferentially hybridized by only its own genomic DNA. This conclusion agrees with data obtained from studies of molecular markers in both nuclear and chloroplast genomes.

Studies on the 45S and 5S rDNA also revealed differences in genomic organization of the 3 Kochia species. The diploid K. prostrata had 2 chromosomes with strong signals for pTa71 sites (45S rDNA, red color) at telomeric ends, 2 chromosomes with weak pScT7 sites (5S rDNA, green color) at metacentric regions, and other 2 chromosomes with weak pScT7 sites at telomeric ends (Fig. 8a). Four telomeric 45S and 4 metacentric 5S sites, all displaying relatively strong signals and occurring on different chromosomes, were observed in the tetraploid K. americana (Fig. 8b). Different from both K. prostrata and K. americana, diploid K. scoparia exhibited 6 (4 strong and 2 weak signals) 45S and 2 strong 5S sites (Fig. 8c). However, the 5S site was located at the metacentric region of 1 chromosome that also had a strong telomeric 45S site. These FISH results indicate that the basic genome (haplome) in K. prostrata, K. americana, and K. scoparia has 1, 1, and 3 45S rDNA sites and 2, 1, and 1 5S rDNA sites, respectively. Changes in number and location of rDNA loci are significant features of genome differentiation (Fukui et al. 1994; Xu and Earle 1996; Zhang and Sang 1999; Lee and Kim 2000; Mishima et al. 2002; Goo and Kim 2003). Therefore, both GISH and FISH studies support the conclusion that the 3 species have different basic genomes. These results definitely establish species status for K. americana and K. prostrata, an issue challenged by Blackwell et al. (1978), who stated “It remains to be finally demonstrated just how distinct K. americana and K. prostrata are”.

Conclusions

Three Kochia species were investigated using various approaches. Results from molecular cytogenetic studies were congruent with those from molecular assessment of species
relationships among the 3 Kochia species. Low cophenetic similarity coefficient values (0.44 and 0.38) were obtained from analyses of 458 RAPD markers among 3 Kochia species. Using 8 species-specific RAPD-derived STS markers to identify parental genomes, not a single interspecific hybrid was found in progenies of controlled crosses. STS–PCR products from the 3 Kochia species for the chloroplast ndhF gene were also different. In the GISH experiments, genomic DNAs of the other 2 species could not hybridize with chromosomes of the target species. In addition, genomes of the 3 Kochia species differed in both number and location of 45S and 5S rDNAs. It is concluded that the 3 Kochia species have different basic genomes. It is quite certain that genetic contamination of forage kochia by the weedy annual kochia would be rare, if possible at all. It is also quite certain that forage kochia will not contaminate the native greenmolly.

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