CHARACTERIZATION AND ADAPTIVE EVOLUTION OF α-TUBULIN GENES IN THE *MISCANHTUS SINENSIS* COMPLEX (POACEAE)<sup>1</sup>

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To investigate the organization of and mode of selection in the α-tubulin genes, full-length α-tubulin genes were cloned from four intraspecific taxa of *Miscanthus sinensis* and its close relatives *M. floridulus* and *M. condensatus* using standard polymerase chain reaction (PCR) and rapid amplification of genomic ends (RAGE)-PCR strategies. Genealogical analysis of angiosperms recovered a monophyletic group of *Miscanthus* α-tubulin genes, which is homologous to the *tau* locus of maize. Two clusters of nearly equal frequency revealed paralogy within each *Miscanthus* taxon. Between-cluster recombination was frequent. Additional evidence for co-occurrence of two haplotypes within individuals and a large-scale crossover all suggested a likely allelic relationship between the *Miscanthus* clusters. Given a long between-species divergence time in *Miscanthus*, wide occurrence of the trans-species polymorphisms in α-tubulin genes and the approximately equal frequency of each allelic type make it extremely unlikely that α-tubulin diversity has been maintained under neutrality. Balancing selection may have contributed to such an apportioning of genetic variability as well as to high levels of genetic variation in α-tubulin and higher substitution rates at synonymous sites of exons than at intron bases of *M. sinensis*. In addition, certain effects of demographic oscillation may have distorted the scenario of a functional locus operating under balancing selection.

**Key words:** allelic recombination; α-tubulin; balancing selection; demographic history; *Miscanthus sinensis* complex; Poaceae; trans-species polymorphisms.

Microtubules are one of the major cytoskeletal filaments of eukaryotic cells and participate in the control of cell shape, cell division, and intracellular transport (Stotz and Long, 1999). They are also principal components of mitotic and meiotic spindles, cilia, and flagella (Dustin, 1984). All microtubules are primarily composed of two polypeptides of ca. 50 kD, α- and β-tubulins, each of which has a sequence that has been highly conserved throughout the evolution of plants. Functionally, these highly conserved regions are involved in basic tubulin functions, such as polymerization and dimer interaction.

In the past 15 yr, various dinitroaniline (antimicrotubule) herbicides for eliminating weeds have been designed to bind the tubulins and inhibit polymerization (Morejohn et al., 1987). However, striking differences in the sensitivity of weeds to antimicrotubule drugs, such as the herbicide oryzalin (dinitroaniline) and the fungicide benomyl, have been well documented (Mysore and Baird, 1995; Zeng and Baird, 1997, 1999; Baird et al., 2000).

The α-tubulins are known to be coded by multiple genes; the number varies between two and 20 for α-tubulin gene families of various organisms. In higher plants, relatively large multigene families comprising 3–9 dispersed tubulin genes have been described, with 11, six, three, and four α-tubulin genes for tobacco (Smertenko et al., 1997), *Arabidopsis* (Kopczak et al., 1992), maize (Montoliu et al., 1990, 1992), and soybean (Brierly et al., 1995), respectively. The phylogenetic analysis of Villemur et al. (1992) revealed polyphyly of α-tubulin genes within most plant genera and species. Why a great number of α-tubulin genes are maintained in organisms, with only slight sequence differences, remains an open question.

Recently, α-tubulin genes have received increasing attention and provide an ideal model for understanding structure, genomic organization, and adaptive evolution at the molecular level of genes coding for proteins with basic metabolic or structural functions. Some members of these genes exhibit strong developmental regulation and/or tissue-specific expression (Jeon et al., 2000), partly explaining the within-individual polymorphisms. In addition, missense mutations in the α-tubulin genes have been found to be correlated with the antimicrotubule drug resistance in goosegrass (*Eleusine indica*) (Anthony et al., 1998; Yamamoto et al., 1998; Yamamoto and Baird, 1999), indicating that advantageous mutations may be favored.

*Miscanthus* is typical of many dominant grasses of eastern Asia. Vegetative propagation via rhizomes is the most common means by which patches can expand and thereby make them difficult to exterminate. In the past decade, antimicrotubule herbicides have been widely used to control the troublesome weeds of *Miscanthus* (Chou et al., 1999). *Miscanthus sinensis* Anders. is composed of morphologically distinct intraspecific taxa that are distributed along a latitudinal gradient (Hodkinson et al., 2002b). Besides the widespread variety *sinensis* of the Chinese mainland and Japan, taxa that are distributed in high mountains (var. *transmorrisonensis* (Hayata) Lee), middle-elevation grassland (var. *formosanu* Hack.), and low-elevation wasteland (var. *glaber* (Nakai) Lee) in Taiwan have been recorded. Our previous study, based on evidence from the *Adh1* gene and the *atPB-rbcL* intergenic spacer, recovered a history of postglacial bottlenecks and re-
cent population expansions of \textit{M. sinensis} (Chiang et al., 2003). Other molecular studies have examined the evolution of allopolyplaid \textit{Miscanthus \times giganteus} using DNA sequences of nuclear ribosomal DNA and fluorescent in situ hybridization (Hodkinson et al., 2002c); the phylogeny of \textit{Miscanthus, Saccharum}, and allied genera using the internal transcribed spacer of nuclear ribosomal DNA and the \textit{trnL-F} regions of plastid DNA (Hodkinson et al., 2002a); and intraspecific variation within \textit{Miscanthus} using amplified fragment length polymorphism and inter-SSR (single sequence repeat) PCR (Hodkinson et al., 2002b).

The objectives of the current study were to answer the following questions. Because antimicrotubule hericides may act as a selective force, are \(\alpha\)-tubulin genes of the \textit{M. sinensis} complex shaped by positive selection, as shown in other plants? Does \textit{M. sinensis} possess \(\alpha\)-tubulin genes of multiple origins like most angiosperms? Because the number of \(\alpha\)-tubulin genes varies across different species, what is the evolutionary implication of the frequent gain-loss of gene events? Because the \textit{M. sinensis} complex experienced historical population fluctuations, what is the relative effect of selection vs. population demography on molecular evolution of the \(\alpha\)-tubulin genes?

\textbf{MATERIALS AND METHODS}

\textbf{Plant materials}—Plants of four intraspecific taxa of the \textit{M. sinensis} Anders. complex, i.e., var. \textit{sinensis}, var. \textit{glaber} (Nakai) Lee, var. \textit{formosanus} Hack., and var. \textit{transmorrisonensis} (Hayata) Lee, were collected from the field in Taiwan and mainland China. Outcrossing \textit{M. floridulus} (Labill.) Warb. and inbreeding \textit{M. condensatus} Hack. were chosen as outgroups for examining the effects of the mating system. Sources of all plant materials and GenBank accession numbers for DNA sequences are available as supplementary material accompanying the online version of this article. Healthy, young leaves were collected in the field and were stored at \(-80^\circ\text{C}\) until they were processed. Leaf tissue was ground to a powder in liquid nitrogen. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980).

\textbf{Amplification, cloning, and sequencing of \(\alpha\)-tubulin genes}—To clone the \(\alpha\)-tubulin genes present in \textit{Miscanthus}, standard PCR and rapid amplification of genomic ends (RAGE)-PCR strategies were employed (Cormack and Somssich, 1997). A conserved portion of the genes was first PCR-amplified with 100 ng genomic DNA using degenerate primers \textit{Po}T1 (5'-ATGAGGGA-GWTCATYASSAT-3') and \textit{Po}T2 (5'-CCRACYTCTCCTAGTCTTCTTC-3'), which were designed based on conserved nucleotide sequence among 18 \(\alpha\)-tubulin genes of plants, including different types occurring in \textit{Arabidopsis}, maize, and rice (Fig. 1). The PCR reactions were performed under the following conditions: 0.2 mmol/L primers, 2.0 mmol/L MgCl2, 0.2 mmol/L dNTP, and 5 units/50 \(\mu\)L \textit{Taq} DNA polymerase (Roche Diagnostics, Mannheim, Germany). After 5 min at 95°C, the following cycle was repeated 25 times: 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 3 min. After the cycles were complete, samples were incubated at 72°C for 10 min and cooled to 4°C. The PCR products were purified from agarose gel electrophoresis using the Pre-A Gene DNA purification kit (Biorad, Hercules, California, USA) and cloned directly into the pGEM-T vector system (Promega, Madison, Wisconsin, USA). From each species, 10–12 clones were sequenced on both strands by standard methods with the \textit{Taq} Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer, Palo Alto, California, USA) on an ABI 377A automated sequencer (ABI Applied Biosystems, Foster, California, USA). Universal primers of the vector (M13 forward and reverse primers) and internal primers were used for the sequence determination of clones. Each nucleotide sequence was compared with available sequences in databases to verify the gene identity. As expected, we found that these \textit{Miscanthus} \(\alpha\)-tubulins were approximately 46 amino acids short in the 3' portion.

To obtain further sequences 3' to those obtained with primers \textit{Po}T1 and \textit{Po}T2, a method of rapid amplification of genomic ends (RAGE) was used (Cormack and Somssich, 1997). Genomic DNA (3 \(\mu\)g) of \textit{M. sinensis} var. \textit{transmorrisonensis} and var. \textit{glaber} was digested to completion with HindIII and \textit{PstI}, respectively, followed by denaturation and polyadenylation of their free 3' ends with terminal transferase (50 units) (Roche Diagnostics). A subsequent PCR was performed using 100 ng of the polyadenylated genomic DNA as template, 100 pmol of gene-specific primer \textit{GSP1} (5'-CA-CCTCCTGTGTTCCCTTGAG-3') (Fig. 1), 100 pmol of Universal-\textit{T}17 primer (5'-GTAAAACGACGGCAGTCGAC-3'), 200 \(\mu\)mol/L dNTPs and 5 units of \textit{Taq} DNA polymerase (Roche Diagnostics) in 100 \(\mu\)L volume. The PCR was carried out at 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min cycled 35 times using an MJ Thermal Cycler (PTC 100, MJ Research, Watertown, Massachusetts, USA). Following the reaction, 1 \(\mu\)L of amplified product was then used as template for a second round of PCR under the same conditions, except that 100 pmol of gene-specific primer \textit{GSP2} (5'-GTCGGTGAGGGTATGGAAAGAGG-3'), downstream of the first primer \textit{GSP1}, and 100 pmol of universal primer (5'-GTAAAACGACGGCGT-3') were used. A third
PCR reaction was performed using 1 µL of product from the second reaction, the same primers, and 2.5 units of Pfu DNA polymerase (Stratagene, La Jolla, California, USA) to generate large quantities of blunt-ended PCR product. The final PCR product was subcloned into a pGEM-Script Amp SK(+) cloning vector (Stratagene).

The insert DNA was cycle-sequenced as before. Independent Miscanthus genomic ends were cloned and their sequence determined. These RAGE-PCR-derived sequences matched completely those of the original clones and housed an additional downstream sequence of approximately 300 bp. Primer PoT3 (5'-CCATTAACCACCAGMAACT-3') was then designed based on these two 3' end sequences with primer PoT1, which were used for verifying the amplification of all full-length Miscanthus α-tubulin sequences. Twelve full-length α-tubulin genes were cloned from six Miscanthus taxa. These sequences are deposited in GenBank under accession numbers AJ437637–AJ437644 and AJ431262–AJ431265.

To examine whether α-tubulin genes of Miscanthus are organized in a tandem array, as occurs in maize (Montoliu et al., 1990), PCR amplifications were carried out with a long extension at 72°C for 5 min, annealing at 45°C, using long PCR amplification Taq DNA polymerase (long amplification assistance system, Invitrogen, Carlsbad, California, USA) for 35 cycles using 100 ng Miscanthus genomic DNAs and 100 pmol of primer PoT1 and PoT3. The resulting PCR products were subjected to Southern blot hybridization probed with 32P-labeled full-length α-tubulin genomic clone of M. sinensis var. transmorrisonensis and washed at low stringency as described by Church and Gilbert (1985).

**Sequence alignments and phylogenetic analyses**—Nucleotide sequences were aligned with the Genetics Computer Group (GCG) Wisconsin Package program (Version 10.0, Madison, Wisconsin, USA). Neighbor-joining (NJ) analysis using Kimura's (1980) two-parameter distance was also performed using MEGA2 (Kumar et al., 2001). Confidence of the clades reconstructed was tested by bootstrapping (Felsenstein, 1985) with 1000 replicates using unweighted characters. The nodes with bootstrap values greater than 0.70, as a rule of thumb, are generally supported with ≥95% probability (Hillis and Bull, 1993). Nucleotide diversity was estimated by Tajima's (1989) π and Watterson's (1975) θ statistics. Tests of neutrality and determination of the associated significance as well as the coalescent-based estimations of minimum recombination events (Rm) were done using the program of DnaSP (version 3.51; Rozas and Rozas, 1999). A McDonald and Kreitman's (1991) test of neutrality was used to compare the distribution of synonymous and nonsynonymous (replacement) variation within and between species. Under neutrality, the ratio of replacement to synonymous fixed substitutions (differences) between species should be the same as the ratio of replacement to synonymous polymorphisms within species.

**RESULTS**

**Gene structure of α-tubulin in Miscanthus**—Using the degenerate primer pair PoT1/PoT3, we amplified 12 α-tubulin sequences from Miscanthus taxa. Two different sequences were isolated from each individual of outcrossing M. sinensis complex and M. floridulus, while a single sequence was obtained from an individual of inbreeding M. condensatus. Two individuals of M. sinensis var. formosanus shared a sequence msf3. The derived amino acid sequence of Miscanthus had 97%, 96%, and 94% identity with Zea mays, Oryza sativa, and Arabidopsis thaliana, respectively. Comparisons with homologous sequences of Arabidopsis indicated that there are four introns and five exons in the α-tubulin genes of Miscanthus. All intron splice junctions are in good agreement with the GT (donor site)/AG (acceptor site) rule (Mount, 1982).

Sequences of the α-tubulin genes of Miscanthus genes share high levels of identity, with a range of 96.9–99.8% and 89–98% for exons and introns, respectively, suggesting that they may have arisen by duplication and divergence from a common ancestral gene. Each gene contains four introns that interrupt the nucleotide sequences within codon 38, after codon 346. The highly conserved GT/AG structure commonly noted for introns was seen overall. PCR amplification and Southern blot hybridization with a full-length α-tubulin genomic probe yield only a single hybridization band (∼3000 bp, corresponding to the size of the Miscanthus genomic clone), indicating that tubulin genes of Miscanthus are not organized in tandem.

**Nucleotide sequence polymorphism and tests for genetic recombination**—DNA sequences of the α-tubulin genes were determined for 12 clones. The consensus α-tubulin region sequence of Miscanthus is 3110 bp in length, including 1353 bp of exon sequence and 1757 bp of intron sequence. A high level of polymorphism was detected in exons, with 41 replacements and 57 synonymous changes across all species. Within the M. sinensis complex, 31 nonsynonymous changes and 56 synonymous substitutions were detected (Table 1). The polymorphism within introns was also high, with 296 variable sites.

Within M. sinensis, the distribution of polymorphic sites across nine haplotypes in the entire exon sequences and in partitions of replacement and synonymous sites as well as introns is summarized in Table 2. For the whole sample of Miscanthus, mutations at 58 of the 98 polymorphic sites in the exon sequence region occurred once, nine sites occurred twice, five sites occurred three times, and 17 sites occurred six times (Fig. 2). The distribution of the polymorphic sites in exons was compared to the expectation under a neutral mutation model, based on the methods of Tajima (1989). An excess over the expectation of 34.7 for the classes of unique nucleotide polymorphisms and 10.5 for the sites occurring six times was observed (Fig. 2). Likewise, within M. sinensis, mutations at 49 of the 84 polymorphic sites in the nine exon haplotypes

**TABLE 1.** Estimates of nucleotide diversity, θ, and test statistics of selection for the α-tubulin gene in the Miscanthus sinensis complex, M. floridulus, and other taxa. Syn = number of synonymous substitutions; Rep = number of replacement substitutions.

<table>
<thead>
<tr>
<th>Genetic polymorphism</th>
<th>Miscanthus sinensis</th>
<th>Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
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</tr>
<tr>
<td>θ</td>
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</tr>
<tr>
<td>π</td>
<td>0.0229</td>
<td>0.0088</td>
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<td>23 : 7</td>
</tr>
<tr>
<td>Fu and Li's D^*</td>
<td>-0.7356</td>
<td>-1.3966</td>
</tr>
<tr>
<td>Tajima's D</td>
<td>-0.4578</td>
<td>-1.2184</td>
</tr>
<tr>
<td>Rm</td>
<td>8</td>
<td>2</td>
</tr>
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</table>

*P < 0.05.
occurred once, seven sites occurred twice, nine sites occurred three times, and 19 sites occurred four times. In addition, excessive singletons (165) were detected at α-tubulin introns of Miscanthus sinensis (Table 2).

Within M. sinensis, genetic recombination was detected in exons (Rm = 8) and introns (Rm = 14) (Tables 1, 2). Recombination occurred more frequently at synonymous sites (Rm = 6) than at replacement sites (Rm = 2) (Table 1). Most recombination was involved with a relatively short sequence motif transferring from one allele to another. These were termed “small scale” recombination events (Hughes et al., 1993). On the other hand, genetic recombination seldom occurred in α-tubulin genes of other plants, including Arabidopsis, maize, and rice (Table 1). When the sequence was partitioned, 12 and one recombination events were detected in introns 3 and 1, respectively. In the exon sequence, exon 5 possessed the highest number of recombinations (Rm = 4) (Table 2).

Gene genealogy—A neighbor-joining tree (Fig. 3) of angiosperm α-tubulin genes was reconstructed rooted on algae. Two major groups, I and II, were identified and supported with high bootstrap values. Group I can be divided into three subgroups, A, B, and C, all of which were significantly supported. Group II occurred exclusively in monocots (rice, maize, and goosegrass). Group A occurred exclusively in dicots, including Eucalypta, Pisum, and Arabidopsis, while group C occurred in monocots only. Group C was closely related to group B, a cluster including Arabidopsis and goosegrass.

The α-tubulin genes duplicated in plants frequently. According to the gene tree, duplication occurred at least once in goosegrass, rice, and Arabidopsis, twice in Hordeum, and three times in maize. Nevertheless, six sequences of two clusters (A and B) obtained from Arabidopsis reside in different loci (Kopczak et al., 1992), and six sequences of maize are located in four different loci (Montoliu et al., 1990). In contrast to multiple origins of the α-tubulin genes reported in most monocots, a single monophyletic group of the Miscanthus genes was identified. This group is homologous to the tua5 locus of maize.

Despite the monophyly at the generic level, genealogical analysis resolved two distinct clusters of sequences of Miscanthus, each supported significantly by bootstrap. The two clusters are of nearly equal frequency with six accessions in each group. Individuals of each taxon possessed two sequences belonging to two different clusters, except for the inbreeding M. condensatus. That is, no monophyly of either M. sinensis complex or of M. floridulus was supported, a result consistent with Hodkinson et al. (2002a). When the exon sequence was partitioned into two regions, sites 1–987 and 988–1353, according to recombination analysis using DnaSP software, the systematic position of sequences ms1 and mst2 switched in the phylogeny of sites 1–987 (Fig. 4), indicating a crossover, i.e., a “large-scale” recombination event (cf. Hughes et al., 1993), between the two DNA fragments. In addition, the phylogeny of introns was consistent with the tree of 1–987 sites.

Estimates of nucleotide diversity and statistical tests of neutrality—Nucleotide diversity of the α-tubulin genes was high in Miscanthus, with θ = 0.0216 and π = 0.0240 for exons, and θ = 0.0930 and π = 0.1095 for introns. Within the M. sinensis complex, high levels of genetic diversity were also detected in exons (θ = 0.0220 and π = 0.0229) and introns (θ = 0.0698 and π = 0.0796). The cluster 1 (θ = 0.0178) possessed higher levels of genetic variation than the cluster 2 (θ = 0.0134) of the M. sinensis complex. Within-individual diversity was detected in all outcrossing taxa, M. sinensis, and M. floridulus, while only one single sequence was cloned from the inbreeding M. condensatus.

When the sequence was partitioned into each region, the genetic diversity at synonymous sites in most exons was greater than the diversity of the introns, except for exon 2 and intron 3. In contrast to replacement mutations being more numerous than synonymous changes in tubulin exons of other plants, such as 23 : 92 in rice, the number of nonsynonymous changes was smaller than that of synonymous mutations in M. sinensis and M. floridulus (Table 1). Nevertheless, more nonsynonymous changes than synonymous substitutions occurred in exon 2 of M. sinensis (Table 2).

A number of statistical tests were used to determine signif-

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<th>Intron 1</th>
<th>Exon 2</th>
<th>Intron 2</th>
<th>Exon 3</th>
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<th>Intron (overall)</th>
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<td>0 : 4</td>
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<td>5 : 2</td>
<td>—</td>
<td>7 : 4</td>
<td>—</td>
<td>43 : 20</td>
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<td>0.016</td>
<td>0.042</td>
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<td>0.000</td>
<td>—</td>
<td>0.101</td>
<td>—</td>
<td>0.070</td>
<td>—</td>
<td>0.069</td>
<td>—</td>
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<tr>
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<td>0.045</td>
<td>0.021</td>
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<td>—</td>
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<td>—</td>
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<td>Rm</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>14</td>
<td>—</td>
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<tr>
<td>Fu and Li’s D*</td>
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<td>−1.232*</td>
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<td>−0.146</td>
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<td>−0.887</td>
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<tr>
<td>Tajima’s D</td>
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<td>−1.149*</td>
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<td>−0.009</td>
<td>0.185</td>
<td>−0.568</td>
<td>−0.456</td>
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* P < 0.05.
icant departures from neutrality at the \( \alpha \)-tubulin genes. For the exon sequence data, neither Fu and Li's \( D^* = -0.7356 \) nor Tajima's \( D = -0.4578 \) are significant in \( M. \) sinensis (Table 1). When exon sequence data are partitioned into replacements and synonymous substitutions, a significant deviation from neutrality (Fu and Li's \( D^* = -1.3966 \), Tajima's \( D = -1.2184 \), \( P < 0.05 \), Table 1), from an excess of singletons, was detected in replacement sites of the exon sequence. In addition, all values of \( D^* \) and \( D \) statistics for introns are non-significant. A McDonald and Kreitman test for neutrality was also conducted based on a prediction that the relative levels of intra-allelic replacement and synonymous polymorphisms are correlated with inter-allelic levels of replacement and synonymous substitutions. The comparison between clusters 1 and 2 revealed no fixed replacements and 10 fixed synonymous differences vs. 46 polymorphic synonymous sites and 31 polymorphic replacements (Table 3), indicating an excess of intra-specific replacements (\( P = 0.012 \)). The test demonstrates that the data are not in accord with neutral expectations.

**DISCUSSION**

**Gene structure evolution of \( \alpha \)-tubulin genes**—Although there is a general evolutionary trend of increasing intron number during evolution of \( \alpha \)-tubulin genes: from one in yeast (\( tub1 \) gene) and two in \( Chlamydomonas \) to three in rice and maize (Jeon et al., 2000) and four in others angiosperms, the number of introns do not reflect major plant clades and have much higher levels of genetic diversity. Low genetic variability at the locus in maize is possibly attributed to its long domestication history (Eyre-Walker et al., 1998) and positive selection (Montoliu et al., 1989), which is also indicated by a high number of singletons and the absence of recombination (Table 1). In addition to the high levels of genetic polymorphism recorded in this study, several salient facts were found in \( M. \) sinensis. (1) The \( \alpha \)-tubulin gene is characterized by an excess of intermediate frequency variants. (2) A partition of the \( \alpha \)-tubulin gene sample into two clusters with nearly equal frequency accounts for the excess of intermediate frequency variants. (3) There is a high level of inter-allelic recombination between clusters. (4) Trans-species polymorphism has led to paraphyly of each \( M. \) taxa, as determined from the intron sequence. (5) The Fu and Li \( D^* \) and Tajima's \( D \) statistics indicate a significant deviation from neutrality at the nonsynonymous sites. (6) A higher level of genetic variation exists at synonymous sites of exons than in intron sequences.

Polymorphism retained in populations through speciation has been considered as evidence for balancing selection, a mechanism that maintains long-term genetic variation at a locus with heterogeneity advantage (Klein et al., 1998; May et al., 1999). Nevertheless, retention of ancestral polymorphisms in neutral genes can also be expected if species divergence times have been short and population size is large (cf. Wu et al., 1998). As judged by \( Adh1 \) gene sequences (Chiang et al., 2003), the divergence between the \( M. \) sinensis species com-

<table>
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<tr>
<th>Substitutions</th>
<th>Fixed</th>
<th>Polymorphic</th>
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<tr>
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<td>43</td>
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<tr>
<td>Replacements</td>
<td>0</td>
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</tr>
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</table>

**Table 3.** McDonald and Kreitman statistic between clusters 1 and 2 of the \( \alpha \)-tubulin gene in \( M. \) sinensis (\( P = 0.012 \)).
Complex and *M. condensatus* was estimated to be \(1.17 \times 10^6\) yr before present. And speciation of *M. floridulus* occurred even earlier. Coalescence theory predicts that the coalescence time for pairs of neutral alleles selected at random from a locus in a randomly mating population will be \(2Ne\) generations (Tajima, 1983). Hughes (1999) showed that the mean coalescence time for neutral alleles in a human population with a size of 10,000 would be only 600,000 yr. Thus, neutral polymorphisms are not expected to persist very long for populations. Our previous study based on the *AtTUA* gene and a cpDNA noncoding *atpB-rbcL* spacer revealed that the *M. sinensis* complex had evolved through bottlenecks and undergone subsequent demographic expansion (Chiang et al., 2003). Given such demographic history, neutral alleles would coalesce within species. The wide occurrence of *α*-tubulin trans-species polymorphisms and the approximately equal frequency of each allelic type make it extremely unlikely that *α*-tubulin diversity has been maintained under neutrality. Non-neutral evolution was also supported by the McDonald and Kreitman test. Furthermore, allelic genealogy of partition of two equal-frequency clusters is consistent with balancing selection (Uyenoyama, 1997; Lin et al., 2001).

Apparently, balancing selection resulted not only in the maintenance of large numbers of alleles in populations, but also in greatly enhanced persistence of allelic diversity relative to neutral genetic variation (cf. Richman, 2000). Nevertheless, demographic history certainly plays another key role in determining the level and apportioning of polymorphisms among populations and species. In this study, both frequent genetic recombination and balancing selection seem to have countered the effects of bottlenecks and have recovered the genetic diversity at the *α*-tubulin locus. Nonetheless, the apportionment of polymorphism unveiled the footprint of such population history.

Maruyama and Nei (1981) predicted that balancing selection with heterozygote advantage should enhance the rate of codon substitution. According to their study of an *Mhc* locus, Hughes and Nei (1988) further reasoned that the rate of nonsynonymous substitution per site should be enhanced in genes regulated by balancing selection. Many empirical data (e.g., self-incompatibility loci, Richman and Kohn, 1999; *Mhc* genes, Miller and Withler, 1997; *ospC* gene, Wang et al., 1999, and *het-c* hetrokaryon incompatibility locus, Wu et al., 1998) confirmed this prediction (Hughes, 1999). In this study, however, the occurrence of fewer replacement substitutions in *M. sinensis* seems to have deviated from the balancing selection hypothesis. Nevertheless, as the evolutionary rate at bases of introns is expected to approximate or exceed that of synonymous sites of exons according to a neutrality model, higher levels of polymorphism at synonymous sites of exons than in introns reflect a unique pattern under influences of balancing selection and species/population demography. The most reasonable explanation for the fact that the evolutionary rate in introns of the *α*-tubulin gene is often lower than that in synonymous mutations in exons in *Miscanthus* is that introns are homogenized by frequent interallelic recombination (cf. Garrigan and Edwards, 1999) and subsequent genetic drift (Cereb et al., 1997).

Despite an excess of intermediate frequency polymorphism in the *α*-tubulin sequence, which is considered as evidence for balancing selection, excessive singletons occurred at both introns and exons sequences. Three possible evolutionary forces can result in the excess of low-frequency polymorphisms: hitchhiking (Kaplan et al., 1989), purifying selection (Tajima, 1989), and recent population expansion (Cummings and Clegg, 1998). Given high levels of genetic recombination, both positive and purifying selections as major evolutionary forces would be less likely. Demographic expansion previously illustrated in *M. sinensis* (Chiang et al., 2003) may therefore have contributed to such a partitioning pattern for *α*-tubulin diversity.

In exon 2 of *M. sinensis*, significantly high levels of non-
synonymous changes over silent mutations (4 : 0) indicated that the exon may have functional importance (Table 2). It was demonstrated that the tissue-preferential expression of a rice α-tubulin locus was mediated by the first intron (Jeon et al., 2000). These tightly linked DNA fragments, i.e., exon 2 and intron 1, may also play a critical role in α-tubulin gene expression in Miscanthus.

Dinitroaniline herbicides, possible evolutionary agents, are antimicrotubule drugs that bind to tubulins and inhibit polymerization (Morejohn et al., 1987). Destabilization and inhibition of microtubule assembly are suggested to result from the direct binding of the herbicides to tubule dimers (Zeng and Baird, 1999). Nevertheless, an alteration of the tubulin subunits of microtubules can lead to herbicide resistance in the resistant biotypes, because different allelic products may bind different arrays of peptides (Chernicky, 1985). It has been well demonstrated that a tubulin mutation responding to antimicrotubule herbicides resulted from amino acid substitutions that altered the electrophoretic mobility of α- and β-tubulins (James et al., 1993). Zeng and Baird (1999) showed that dinitroaniline herbicide resistance in goosegrass is inherited with three alleles at a single nuclear gene, the Drp locus, at which a tubulin gene, TUA 1, resides (Yamamoto et al., 1998). Although an RR homozygote had the highest resistance to the herbicides in experiments with medium concentrations of oryzalin, the heterozygote IR and RR had similar drug resistance (cf. Table 1 of Zeng and Baird, 1999).

In this study, a heterozygote advantage could account for the maintenance of extraordinary polymorphism at the α-tubulin locus in Miscanthus sinensis. This finding seems to contradict previous observations in other plants, in which positive selection favored missense point mutations in α-tubulins (My sore and Baird, 1995). However, as stated previously, nonsynonymous substitutions at each allele of the heterozygote under balancing selection were enhanced, agreeing with the positive model except for the trans-species polymorphism and inter-allelic recombination. The positive selection hypothesis should be examined more closely by looking at population genetic structure in other plants; to date, most observations have been made between different loci. Therefore, because balancing selection plays a key role in maintaining genetic polymorphism at the α-tubulin locus in a population exposed to the antimicrotubule herbicides, a heterozygote individual may have an advantage because a heterozygote would be able to present a broader alternative of cellular target sites for the herbicides and thus resist a broader array of herbicides.

Evolution of α-tubulin genes in Miscanthus—Why are there so many tubulins in an organism? At least two different hypotheses have been proposed. Fulton and Simpson (1976) argued that each isotype was involved in a specific microtubule array or function. Novel isotypes usually produce functionally distinct microtubules for specialized cell structures. Post-translational modifications and multiple isoforms of tubulin were confirmed in tobacco cells, supporting this “multitubulin hypothesis” (Smertenko et al., 1997). In contrast, another hypothesis proposed that the polypeptides are functionally equivalent and redundant, but are regulated differentially by specific regulatory signals (cf. Hughes, 1999). Nevertheless, experimental evidence for the presence of tubulin isoforms in individual microtubular structure is ambiguous (cf. Lewis and Cowan, 1990).

In this study, using degenerate primers to compare α-tubulin genes of different families and plants, we detected only one type of α-tubulin gene in Miscanthus. The existence of a single type of α-tubulin gene and the possible loss of other types in Miscanthus suggest that the evolution of multiple gene families is consistent with the redundancy hypothesis. It also explains how the number of gene types could change across plants and how duplication of genes could occur frequently. The bifurcation of the α-tubulin gene in Miscanthus revealed such a phenomenon at the generic level.

LITERATURE CITED


