**Miscanthus**: a case study for the utilization of natural genetic variation

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Abstract
Cultivars of *Miscanthus* used as bioenergy crops or tested in trials are largely clonally propagated, wild sourced genotypes or clonally propagated F1 hybrids. One of the most productive taxa is the sterile triploid *M. × giganteus*. Little domestication or breeding has been undertaken and there is huge potential to utilize the extensive genetic resources of the genus for crop improvement. The challenge is to generate new highly adapted genotypes suitable for a range of environments. Production on marginal land, not used for food crops, is particularly desirable, but presents many barriers to crop breeders, as these are largely unproductive and/or stressful environments. This article outlines progress made in characterizing natural genetic variation in *Miscanthus* including next-generation single-nucleotide polymorphism genotyping, quantitative trait locus analysis and association mapping. It also explains how this knowledge is being used to develop novel genotypes suited for growth in a broad range of agricultural and marginal lands by defining breeding pools, generating novel crosses, manipulating polyploidy and applying genomic selection approaches.

Keywords: adaptive variation; association mapping; genome size; genomic selection; *Miscanthus*; phylogeny; ploidy

**Introduction**

*Miscanthus* is a perennial rhizomatous grass genus that is currently under intense development as a bioenergy crop. It has, since the late 1970s, come to the attention of the plant breeding community for energy and fibre (1970s onwards; Jones and Walsh, 2001) and is hence considered undomesticated (Yan et al., 2012; Slavov et al., 2014). Cultivars of *Miscanthus* used as crops or tested in trials are largely clonally propagated (single-genotype), wild sourced material or clonally propagated F1 hybrids (Hodkinson et al., 2002c; Glowacka et al., 2014a, b). There is a need to generate new broadly adapted genotypes suitable for a range of environments including both agricultural and marginal lands (Clifton-Brown et al., 2008; Chou, 2009; Jorgensen, 2011; Qin et al., 2011; Jing et al., 2012; Nijsen et al., 2012). There is a movement towards developing crops suited for marginal land so that fertile land is not taken away from food production (Cai et al., 2011; Donnelly et al., 2011; Gopalakrishnan et al., 2013). For example, the EU FP7 project GrassMargins aims to develop genotypes suitable for growth on European marginal land (http://www.grassmargins.com). Furthermore, China possesses 100 million hectares of marginal and degraded land, especially in the northern and western regions, that has the potential to produce approximately 1 billion tons of *Miscanthus* feedstock (Sang, 2011; Sang and Zhu, 2011). To achieve this potential, many plant traits will need to be optimized including yield, flowering, drought tolerance, frost and cold tolerance, and biomass chemical composition (reviewed in Jones et al. (2014)).

*Miscanthus* breeding is a case study of genetic resource utilization for novel crop development.
The genus has a wealth of genetic resources, and the progress made in characterizing and utilizing this diversity is outlined in this review. We do not consider reverse-genetic studies and genetic engineering approaches for crop development here. Such details can be found elsewhere (Wang et al., 2011; Xie and Peng, 2011; Feltus and Vandenbergbrink (2012); Perera et al., 2013). We instead focus on the problems and prospects of using natural genetic variation for Miscanthus crop production. Much progress has been made on the fundamental characterization of Miscanthus species such as on their taxonomy and phylogenetics. Furthermore, several studies have outlined population genetic variation and examined adaptive variation of a range of genotypes. The ongoing challenge is to combine the genotypic and phenotypic knowledge for crop development and to better incorporate natural genetic diversity into breeding programmes. Next-generation sequencing and breeding technologies utilizing association studies and genomic selection (GS) offer considerable potential in this respect. Although many genetic resource collections of Miscanthus exist in Europe and the Americas outside of Asia, there is neither a directory of Miscanthus collections nor a coordinated programme for the conservation of its genetic resources.

**Taxonomy, phylogeny and distribution**

*Miscanthus sensu lato* (s.l., in the broad sense) includes about 20 species depending on the author (Clayton and Renvoize, 1986; Scally et al., 2001a, b; Clayton et al., 2006 onwards). However, its generic limits have been revised based on molecular phylogenetics (Hodkinson et al., 1997, 2002a; Swaminathan et al., 2010). DNA sequences and fingerprinting data reported by Hodkinson et al. (2002a, b) showed that some species included in Miscanthus s.l. are more closely related to other genera than to Miscanthus. Miscanthus *sensu stricto* (s.s., in the strict sense) includes only those species with a basic chromosome number of 19. Its taxonomic type species is *M. floridulus* (Labill.) Warb. (=*M. japonicus* Anderss; basionym *Saccharum floridulum* Labillardiére described in 1824).

Synonymy is high in the genus. The International Plant Names Index (IPNI, 2014) lists over 60 species, but only 11–12 species can be recognized in *Miscanthus s.s.* (Table 1). Although hybridization is known to occur within the genus, few hybrids have been identified and named despite the lack of breeding barriers and the sympatry of several taxa. *Miscanthus × giganteus* Greef et Deuter ex Hodkinson and Renvoize was described by Hodkinson and Renvoize (2001). They showed that the name *M. × giganteus* Greef et Deuter is illegitimate because neither the type was specified nor a Latin description was provided. They chose to keep the species epithet *× giganteus* to prevent confusion in the literature, but updated the authority names accordingly. New records of natural hybridization between *M. sacchariflorus* and *M. sinensis* have been reported (Nishiwaki et al., 2011). The name *Miscanthus ogiformis* is not correctly applied to *Miscanthus × giganteus* as it does not recognize the hybrid nature of the taxon and

<table>
<thead>
<tr>
<th>Table 1. List of Saccharinae genera and species belonging to Miscanthus s.s.</th>
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<tbody>
<tr>
<td>Saccharinae (Andropogoneae and Panicoideae; Clayton and Renvoize (1986))</td>
</tr>
<tr>
<td><em>Eriochrysis</em> P. Beauv (7 spp.)</td>
</tr>
<tr>
<td><em>Eulalia</em> Kunth (30 spp.)</td>
</tr>
<tr>
<td><em>Eulaliopsis</em> Honda (2 spp.)</td>
</tr>
<tr>
<td><em>Homoegeus</em> Stapf. (5 spp.)</td>
</tr>
<tr>
<td><em>Imperata</em> Cyr. (8 spp.)</td>
</tr>
<tr>
<td><em>Lophopogon</em> Hack. (2 spp.)</td>
</tr>
<tr>
<td><em>Microstegium</em> Nees (15 spp.)</td>
</tr>
<tr>
<td><em>Miscanthus</em> Anderss. (20 spp.)</td>
</tr>
<tr>
<td><em>Pogonatherum</em> P. Beauv. (3 spp.)</td>
</tr>
<tr>
<td><em>Saccharum</em> L. (35–40 spp; here including <em>Erianthus</em>)</td>
</tr>
<tr>
<td><em>Spodiopogon</em> Trin. (9 spp.)</td>
</tr>
<tr>
<td><em>Polytris</em> Hack. (1 sp.)</td>
</tr>
<tr>
<td><em>Polianiopsis</em> (1 sp.)</td>
</tr>
<tr>
<td><em>Diandranthus</em></td>
</tr>
<tr>
<td><em>Sclerostachya</em></td>
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spp., species.

*Likely to be an infraspecific taxon of *M. sacchariflorus*. |
cannot be linked to the type (Ibaragi et al., 2013). Miscanthus floridulus and M. sinensis also have sympatric distributions and similar morphology. Phenotypic evaluation of these show that the two species intergrade in their morphology and that hybrids are potentially common (Scally et al., 2001a). There is clearly a need for more research on natural hybrids and hybrid zones in Miscanthus and the taxonomic treatment of these taxa.

Miscanthus is classified in the predominantly tropical grass tribe Andropogoneae and subtribe Saccharinae (Clayton and Renvoize, 1986; Clayton et al., 2006 onwards; Bouchenak-Khelladi et al., 2008; Teerawattananon et al., 2011; Kellogg, 2013). Saccharinae includes the sugarcane genus Saccharum L. s.l. and several less well-known genera (Table 1). The term 'Saccharum complex' has been used to describe a taxonomically difficult subset of Saccharinae (Erianthus, Miscanthus, Narenga, Saccharum and Sclerostachya) implicated in the origin of sugarcane (Daniels and Roach, 1987).

Miscanthus species are unusual among Andropogoneae because they have bisexual paired spikelets, both with hermaphrodite flowers (Fig. 1). Other Andropogoneae have paired spikelets, but with the exception of a few genera such as Ischaemum L. and Schizachyrium Nees, one of these is usually male or sterile (Clayton and Renvoize, 1986).

Morphological descriptions of Miscanthus are included in several floras including Chen and Renvoize (2006) for China, Koyama (1987) and Osada (1993) for Japan, Cope (1982) for Pakistan, Gilliand (1971) for Malaya and Hodkinson (submitted) for Thailand. Miscanthus species are perennial and rhizomatous (Fig. 1) with erect cane-like stems growing up to 7 m tall (in M. lutarioriparius = M. sacchariflorus). They are sometimes tufted with short plumeose racemes. Its spikelets are pedicellate and paired (one with a short pedicel and another with a long pedicel). The inflorescence axis may be long and have relatively short racemes as in M. floridulus or may be

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**Fig. 1.** Line drawings of (a) Miscanthus sinensis and (b) M. sacchariflorus (from Sun et al. (2010), with permission). (a) A, Panicle and leaf; B, paired spikelets; C, back of a lower glume; D, ventral side of an upper glume; E, ventral side of an upper lemma with awn; and F, stamens and gynoecium. (b) A, Rhizome and culm; B, panicle and leaf; C, paired spikelets; D, back and ventral sides of a lower glume; E, ventral side of an upper glume; F, back of a lower lemma; G, back of an upper lemma without awn; and H, lodicule.
short with long racemes (subdigitate inflorescence, as in most *M. sinensis* and *M. sacchariflorus*; Fig. 1).

Some comparative morphological and anatomical studies have been published on *Miscanthus* (Lee 1964a, b, c, d; Scally *et al.*, 2001a, b; Sun *et al.*, 2010). These studies helped define species boundaries, improved infrageneric classification and quantified morphological variation. Important diagnostic characteristics are found in inflorescence axis length, raceme length and number, spikelet size, spikelet callus hair length, glume and lemma size, nerves on glumes, dorsal hairs of glume, and presence or absence of awns (Lee 1964a, b, c, d; Scally *et al.*, 2001a, b; Chen and Renvoize, 2006). For example, Scally *et al.* (2001a, b) used 31 morphological characteristics predominantly from spikelets and the inflorescence to study variation in *Miscanthus* species using principal component analysis and detrended correspondence analysis. *Miscanthus sacchariflorus* and *M. sinensis* were clearly differentiated with these methods, but the other species clustered with the *M. sinensis* group. There is huge morphological variation present in *M. sinensis*. A standardized list of morphological descriptors has not yet been published, but would be of high value for phenotyping studies (Scally, 2001; De Cesare, 2012).

Groups of species at sectional rank within *Miscanthus* have been described and keys to *Miscanthus* species provided by Hodkinson *et al.* (1997) and Chen and Renvoize (2006). The most comprehensive effort to taxonomically subdivide the genus was made by Lee (1964a, b, c, d), who separated the genus into four sections. Three can be assigned to *Miscanthus s.s.* (sections *Kariyasua*, *Miscanthus* and *Triarrbena*) and one (section *Dianandra*) is not part of *Miscanthus s.s.* because of DNA sequence evidence (Hodkinson *et al.*, 2002a) and chromosome number (Fig. 4; Table 2). Section *Dianandra* species also have two anthers compared with three anthers in *Miscanthus s.s.* Other species assigned to *Miscanthus s.l.* are better included in Miscanthidium (an African taxon; *M. ecklonii*, *M. junceus* and *M. sorgbium*, *M. violaceus*, Sclerostachya fusca and Diantranschma (various combinations including *M. nepalensis* and *M. nudipes*) (Hodkinson *et al.*, 2002a).

*Miscanthus s.s.* is native to Eastern Asia, Southeastern Asia and the South Pacific (Fig. 2), with the highest species diversity being recorded in Eastern Asia, especially in China and Japan (Chen and Renvoize, 2006; Sun *et al.*, 2010). Its native latitudinal range extends from temperate Southeast Russia at 50°N to tropical Polynesia at 22°S. Its native longitudinal distribution extends from Burma and Andaman and Nicobar Islands at 92°E to Fiji at 179°W. Its species have radiated to occupy a wide range of biomes and

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**Table 2.** List of chromosome studies carried out on the x = 19 *Miscanthus* s.s. taxa

<table>
<thead>
<tr>
<th>Taxons</th>
<th>2n and ploidy</th>
<th>Published ploidy counts</th>
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<tbody>
<tr>
<td><em>M. floridulus</em></td>
<td>2n = 2x = 38</td>
<td>Bremer (1934); Li <em>et al.</em> (1948); Li and Ma (1951); Adati and Mitsuishi (1956); Adati (1958); Chen and Hsu (1962); Price (1963a, b); Price and Daniels (1968); Hodkinson <em>et al.</em> (2001, 2002c)</td>
</tr>
<tr>
<td><em>M. × giganteus</em></td>
<td>2n = 3x = 57, 58b</td>
<td>Adati and Mitsuishi (1956); Adati (1958); Linde-Laursen (1993); Lafferty and Lelley (1994); Hodkinson <em>et al.</em> (2001, 2002c)</td>
</tr>
<tr>
<td><em>M. intermedium</em></td>
<td>2n = 6x = 114</td>
<td>Adati and Mitsuishi (1956); Adati (1958)</td>
</tr>
<tr>
<td><em>M. sinensis</em></td>
<td>2n = 2x = 38</td>
<td>Adati and Mitsuishi (1956); Cevalier (1956); Adati (1958); Hirayoshi <em>et al.</em> (1959); Linde-Laursen (1993); Lafferty and Lelley (1994); Hodkinson <em>et al.</em> (2001, 2002a, b, c)</td>
</tr>
<tr>
<td><em>M. sinensis</em> ssp. condensatus</td>
<td>2n = 3x = 57</td>
<td>Adati and Mitsuishi (1956); Adati (1958); Hirayoshi <em>et al.</em> (1959)</td>
</tr>
<tr>
<td><em>M. lutariroparparis</em></td>
<td>2n = 4x = 76</td>
<td>Li <em>et al.</em> (2013)</td>
</tr>
<tr>
<td><em>M. oligostachybus</em></td>
<td>2n = 2x = 38</td>
<td>Adati and Mitsuishi (1956); Adati (1958)</td>
</tr>
<tr>
<td><em>M. sacchariflorus</em></td>
<td>2n = 2x = 38</td>
<td>Adati and Mitsuishi (1956); Adati (1958); Lafferty and Lelley (1994); Hodkinson <em>et al.</em> (2001, 2002c)</td>
</tr>
<tr>
<td><em>M. tinctorius</em></td>
<td>2n = 2x = 38</td>
<td>Adati and Mitsuishi (1956); Adati (1958); Hirayoshi <em>et al.</em> (1959)</td>
</tr>
</tbody>
</table>

*a Several other taxa classified as *Miscanthus* s.l. do not share a basic chromosome number of 19; they are more commonly based on 10 or 15 such as *M. fuscus*, n = 15 (Li, 1959); *M. nepalensis*, n = 20 (Mehra *et al.*, 1968); *M. nudipes*, n = 20 (Mehra *et al.*, 1968); Miscanthidium violaceum, n = 14 (Brett, 1954); and Narenga porphyrocoma, n = 15 (Burner *et al.*, 1991). b Linde-Laursen recorded mostly 58 chromosomes with some at 57. c Based on flow cytometry.
climatic zones. Some species such as *M. floridulus* generally grow at sea level or in warm tropical climates, but others such as *M. paniculatus* can tolerate high altitudes of up to 3100 m on dry mountain slopes of Guizhou, Sichuan and Yunnan in China (Chen and Renvoize, 2006).

Given such a wide native distribution, it is not surprising that *Miscanthus* has also become naturalized following human introduction in many regions of the world including Eurasia, North and South America, and New Zealand (Meyer et al., 2010; Quinn et al., 2010, 2011, 2012; Barney et al., 2012; Matlaga et al., 2012; Clark et al., 2014). Clark et al. (2014) used high-density single-nucleotide polymorphism (SNP) markers to show that naturalized populations of *M. sinensis* were derived from a subset of ornamental cultivars that were themselves derived from Southern Japan.

**Chromosome variation**

The chromosome numbers of *Miscanthus* s.s. are relatively small, generally 25 μm in metaphase of mitosis (Adati, 1958; Burner, 1991; Linde-Laursen, 1993; Hodkinson et al., 2001; Chromiec-Gła ˛bik et al., 2012), compared with those of some grasses, but are not unusual in Panicoideae (Celarier and Paliwal, 1957; Sede et al., 2010). Early studies on *M. floridulus* and *M. sinensis* failed to reach a consensus on the basic (monoploid) chromosome number of the genus (Avdulov, 1928, 1931; Church, 1929; Hunter, 1930). However, subsequent meiotic and mitotic counts of *M. floridulus*, *M. × giganteus*, *M. intermedius*, *M. oligostachyus*, *M. sacchariflorus*, *M. sinensis* and *M. tinctorius* (Table 2 and Fig. 3) established the basic number at *x* = 19 (Bremer, 1934; Li et al., 1948; Li and Ma, 1951; Adati and Mitsuishi, 1956; Adati, 1958). Regular meiotic behaviour with 19 bivalents has been observed in all *Miscanthus* s.s. with 2*n* = 38 chromosomes. Further evidence for *x* = 19 comes from the examination of chromosome numbers in polyploids, ranging from diploids to hexaploids (Table 2) that are represented by multiples of 19 (Adati and Shiotani, 1962). Karyotypes have been described by Adati (1958) for *M. floridulus*, *M. intermedius*, *M. oligostachyus*, *M. sacchariflorus*, *M. sinensis* and *M. tinctorius*, by Lafferty and Lelley (1994) for *M. × giganteus*, and by Chromiec-Gła ˛bik et al. (2012) for *M. × giganteus*, *M. sacchariflorus* and *M. sinensis*.

Adati and Shiotani (1962) proposed that the *x* = 19 basic chromosome number of *Miscanthus* is of allopolyploid origin from two parental lineages with *x* = 10 and *x* = 9, but this hypothesis remains to be rigorously tested. Recent mapping studies have shown a high similarity of the *Miscanthus* genome to the *Sorghum* genome and indicated whole-genome duplication in *Miscanthus* relative to *Sorghum* (Kim et al., 2012; Ma et al., 2012; Swaminathan et al., 2012). Ma et al. (2012) used genotyping by sequencing (GBS) of diploid *x* = 19 *M. sinensis* to demonstrate that *Miscanthus* is an ancient polyploid relative to *Sorghum bicolor* consisting of two subgenomes. Each pair of the 19 *M. sinensis* linkages aligned to one sorghum chromosome, except one that mapped to two sorghum chromosomes. Swaminathan et al. (2012) used RNA sequencing (RNA-Seq)-based markers to also determine 19 linkage groups and showed the genome-wide duplication in *Miscanthus* relative to *Sorghum* with subsequent insertional fusion of a pair of chromosomes. Whether this ancient duplication in the *Miscanthus* genome involved allopolyploidy or auto-polyplody remains to be determined (Ma et al., 2012; Swaminathan et al., 2010, 2012).

The basic chromosome number of 19 in *Miscanthus* s.s. does not correspond to some other *Miscanthus* (sensu Clayton and Renvoize, 1986) species including Asian *M. fuscus*, *M. nepalensis* and *M. nudipes* and African *M. ecklonii*, *M. junceus*, *M. sorgbumb* and *M. violaceus* that generally have a basic chromosome number of 10 or 15 (Table 2; footnote). These taxa are better treated in genera separate from *Miscanthus* (Hodkinson et al., 2002a; as described above).

**Genome size variation**

Genome size has been studied by flow cytometry in *Miscanthus* and found to exhibit considerable variation among species (Table S1, available online). Rayburn
et al. (2009), using three accessions of each species, showed that diploid *M. sinensis* had a 1C nuclear DNA content of 2.75 pg and diploid *M. sacchariflorus* 2.25 pg. Therefore, they estimated the genome size of diploid *M. sinensis* to be approximately 20% greater than that of diploid *M. sacchariflorus*.

Li et al. (2013) examined nuclear DNA content variation in *M. lutariarioparius*, *M. sacchariflorus* and *M. sinensis* collected from a range of habitats, altitudes and latitudes in China. They found little variation among the species at the diploid level, suggesting that genome size was stable within the species (among populations). However, in accordance with the results reported by Rayburn et al. (2009) and De Cesare (2012), their results indicated a large difference among diploid species (1C = 2.69 pg in *M. sinensis* compared with 2.19 pg in *M. sacchariflorus* and *M. lutariarioparius*). Li et al. (2013) also estimated the genome sizes of diploid *M. sinensis* and *M. lutariarioparius* and found that they had smaller genomes than expected when compared with the genome sizes of their diploid progenitors (1C = 4.27 pg and 4.28 pg compared with the expected value of 4.37 pg). This could indicate genome downsizing after polyploidization (Leitch et al., 2008; Bento et al., 2011).

Li et al. (2013) did not include *M. × giganteus* in their studies, but Rayburn et al. (2009) showed that triploid *M. × giganteus* had a total nuclear content of 7.0 pg, diploid *M. sacchariflorus* had a 1C content of 2.25 pg and diploid *M. sinensis* had a 1C content of 2.75 pg (Table S1, available online). Rayburn et al. (2009) therefore, by simple deduction from predicted genome sizes, provided evidence that *M. × giganteus* is more likely the result of a combination of a 2× *M. sacchariflorus* gamete and a 1× *M. sinensis* gamete (sum 4.5 + 2.75 = 7.25 pg) than that of a 2× *M. sinensis* gamete and a 1× *M. sacchariflorus* gamete (5.5 + 2.25 = 7.75 pg).

From these values, it is possible to estimate genome size in base pairs (bp) for the following three species: *M. × giganteus*, *M. sacchariflorus* and *M. sinensis* (diploids to tetraploids; higher-ploidy plants excluded). The genomes (Table S1, available online), ranging in estimated size from 2.1 Gbp (diploids) to 5.62 Gbp (tetraploids), are large in comparison with those of *Arabidopsis* (125 Mbp), similar in size to those of maize (2.3 Gbp), small in comparison with those of bread wheat (17 Gbp) and tiny in comparison with the largest genome measured thus far, *Paris japonica* (Pellicer et al., 2010), of 150 Gbp.

Swaminathan et al. (2010) used genomic and small RNA-Seq to characterize the genome of *M. × giganteus*. Coding regions were found to show a high sequence similarity to those in other grasses, but 95% of the genome was found to fall within 12 repeat classes of DNA related to transposons or centromeric DNA. The major repeats actively produce small RNAs. Most small RNAs (sRNAs) are in the 24-nucleotide size range (probably small interfering RNA (siRNAs)). Retrotransposons (class 1 transposons) are the most common sRNA (32%), followed by DNA transposons (class 2 transposons). Thus, siRNAs were suggested to represent...
a large component of the small-RNA transcriptome of Miscanthus (Swaminathan et al., 2010).

Origin of Miscanthus giganteus and polyploid M. sacchariflorus taxa

The allopolyploid origin of M. × giganteus has been established via morphological, geographical, cytogenetic, molecular genetic and pollen fertility/seed viability studies. Linde-Laursen (1993) examined meiotic pairing in M. × giganteus and found few trivalents and nearly equal numbers of bivalents and univalents, which indicates that two of the three genomes have high homology and one has low homology to the other two. All pollen grains were sterile with two to five apertures (compared with single-aperture grains in fertile Miscanthus). Meiotic pairing in M. × giganteus contrasts with that in autotriploid M. sinensis ssp. condensatus, which was shown to have a high number of trivalents in pollen mother cells at metaphase 1 (Adati, 1958).

Hodkinson et al. (2002b) used nuclear ribosomal DNA sequences from the internal transcribed spacer (ITS) region to show that both M. sinensis and M. sacchariflorus were the parental genome donors of M. × giganteus. One ITS repeat type in M. × giganteus matched M. sinensis and the other M. sacchariflorus (Fig. 4). AFLP and inter-simple-sequence repeat (ISSR) fingerprinting also confirmed this observation. The molecular cytogenetic techniques such as fluorescent in situ hybridization and genomic in situ hybridization were unable to differentiate among the different parental genomes present in M. × giganteus, indicating that the parental genomes of the triploid are extremely similar at the repetitive DNA level.

Plants classified as M. sacchariflorus also have complex ancestry and are difficult to classify and name because chromosome complements range from diploid to pentaploid (Adati, 1958; Adati and Shiotani, 1962; Fedorov, 1969). Miscanthus sinensis and M. sacchariflorus hybridize and introgression is expected among these taxa to produce monoploid and polyploid taxa (Adati and Shiotani, 1962). The morphological characteristics that differentiate the two species, such as the absence/presence of an awn, length of the callus hairs and that some and another without a satellite chromosome. Two sets are homologous to M. sinensis and two partially homologous. They also argued, on the basis of meiotic and morphological studies, that pentaploid M. sacchariflorus var. latifolius is an allopolyploid combining genomes of M. sacchariflorus and M. sinensis and that M. intermedius is an allopolyploid combining genomes of M. oligostachyus and M. tinctorius.
maternally inherited in grasses, and *M. × giganteus* was shown to have the plastid type of *M. sacchariflorus* in all samples studied. Therefore, the allotriploid *M. × giganteus* inherited its plastid (and by extrapolation mitochondrial DNA) from a *M. sacchariflorus* lineage (Fig. 4).

Some artificial crosses of *M. sinensis* and *M. sacchariflorus* were included in the study carried out by De Cesare (2012). In several of these, the hybrid had the plastid genome of *M. sinensis*, showing that hybridization is possible in both directions (with both species as maternal parent). This is supported by Clark et al. (2014), who determined, in a major SNP study, many US ornamentals labelled as *M. sinensis* to be in fact BC1 or BC2 hybrids of *M. sacchariflorus* and *M. oligostachyus* with *M. sinensis* as the recurrent female parent. There is no reason to believe that the formation of *M. × giganteus* in the wild is unidirectional, but the plastid studies carried out by Hodkinson et al. (2002a) and De Cesare (2012) suggest that this could be nearly the case as all putatively wild sourced *M. × giganteus* accessions have *M. sacchariflorus* plastid DNA. Clark et al. (2014) found the *M. sacchariflorus* plastome in nine of the 11 Chinese interspecific *sacchariflorus × sinensis* hybrids collected from the wild. Triploid seeds have also been found on *M. sacchariflorus* inflorescences in a sympatric zone with *M. sinensis* in Japan (Nishiwaki et al., 2011). Unidirectional hybridization can be caused by several factors including nuclear cytoplasmic DNA incompatibility effects (Anderson and Maan, 1995) or by population factors. For example, if *M. sinensis* was rare and *M. sacchariflorus* common (or if phenological differences created such a pattern), the vast number of seeds set would be from *M. sacchariflorus* ovule donors. However, a small number of *M. sinensis* plants can potentially father a large number of *M. × giganteus* seeds.

Nishiwaki et al. (2011) investigated natural occurrences of triploidy in sympatric populations of tetraploid *M. sacchariflorus* and diploid *M. sinensis* in Japan. The interspecific hybrid, now known as *Miscanthus × giganteus*, was first collected in Yokohama, Japan, by a Danish plant collector (Nielsen, 1990) and subsequently introduced around the world. Japan is therefore a likely source of new natural allotriploid *M. × giganteus*. Nishiwaki et al. (2011) measured seed set of sympatric *M. sinensis* and *M. sacchariflorus* and assessed their DNA content with flow cytometry. Triploid seeds were found on the inflorescences of *M. sacchariflorus*. These plants have great potential as new sources of variation in breeding programmes. However, they originate from the warm moist regions of Southern Japan. The authors speculate that more cold-tolerant *M. × giganteus* would be expected from more northerly and cooler regions of Japan (Nishiwaki et al., 2011).

**Aneuploids and B chromosomes**

Linde-Laursen (1993) reported a hyperploid chromosome number of 58 in *M. × giganteus* (trisomic). Aneuploidy...
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has not otherwise been confirmed in many other cytological studies. However, the occurrence of accessory (B) chromosomes has been reported in some but not all Miscanthus species (Li and Ma, 1951; Price, 1963a, b; Linde-Laursen, 1993). Price (1963a) recorded between 0 and 11 B chromosomes in six clones of M. floridulus, and Linde-Laursen (1993) reported between 0 and 4 B chromosomes approximately 0.7 µm in length in two clones of M. × giganteus. Chramiec-Łąbik et al. (2012) reported one to four B chromosomes in M. × giganteus, two in M. sinensis and four in M. sacchariflorus.

Artificial polyploids and haploids

Chromosome doubling has been used to generate artificial polyploids in Miscanthus and has potential to introduce new genetic diversity into breeding programmes especially for M. × giganteus types by manipulating the ploidy of the parental species, restoring fertility or disrupting the self-incompatibility system (Petersen et al., 2002; Glowacka et al., 2009, 2010a, b; Yu et al., 2009). Another stimulus for artificial polyploid formation has been the desire to generate novel sterile genotypes that lower the risk of invasiveness following introduction as a crop (Petersen et al., 2003; Barney and Ditomaso 2008; Jørgensen, 2011). Petersen et al. (2002, 2003) generated tetraploid M. sinensis from diploid source plants using colchicine or oryzalin treatments during callus induction, during callus proliferation, or on in vitro shoot apices and leaf explants. These tetraploids can be used as parental species in triploid Miscanthus production with M. sacchariflorus. Treatment of shoot apices with colchicine was shown to be the most efficient method for the four genotypes tested.

Triploid M. × giganteus is sterile in a post-zygotic barrier that results from abnormal male and female gametophyte production (Slomka et al., 2012). Hexaploid M. × giganteus has been generated from triploid source material in an attempt to restore its fertility. For example, Yu et al. (2009) treated triploid callus, obtained from immature panicles, with colchicine and oryzalin to generate hexaploids. These were also found to have an increased stomata size (30 µm in the hexaploids compared with 24.3 µm in the triploids), but they did not report any findings for the fertility of the hexaploids. Touchell and Ranney (2012) also used oryzalin for in vitro chromosome doubling of M. × giganteus. Fertility of the resulting hexaploids was shown using pollen viability staining and crossing of the hexaploids with diploid M. sinensis, but in vitro embryo culture was required to obtain viable plantlets.

Haploid plants and double-haploid plants have also been reported (Glowacka et al., 2009; Glowacka et al., 2012) and used in the gene expression studies of Miscanthus (Barling et al., 2013). Glowacka et al. (2012) developed a methodology for haploid formation by anther culture in M. sinensis. Androgenesis has also been attempted in M. × giganteus (Zur et al., 2013), but its efficiency is very low due to cytological chromosome imbalance.

Genotyping: genetic variation and phylogeography

Several multi-locus marker systems have been applied to Miscanthus such as restriction fragment length polymorphism (RFLP; Hernández et al., 2001), randomly amplified polymorphic DNA (RAPD; Chiang et al., 2003), ISSR polymerase chain reaction (ISSR-PCR; Hodkinson et al., 2002c; Zhang et al., 2013a, b) and AFLP (Greef et al., 1997; Hodkinson et al., 2002c). Single-locus co-dominant markers have also been applied including isozymes (Chou et al., 1987; Chou and Chang, 1988; Chou and Ueng, 1992; Von Wühlisch et al., 1994). Many simple-sequence repeat (SSR) markers have been developed for the nuclear genome (Hernández et al., 2001; Hung et al., 2009; Ho et al., 2011; Zhou et al., 2011; Hu et al., 2012; Kim et al., 2012; Yu et al., 2013), but fewer have been developed for the plastid/chloroplast genome (De Cesare et al., 2010; Jiang et al., 2012). Recently, comprehensive SNP surveys have been conducted using next-generation sequencing approaches (Slavov et al., 2014; Clark et al., 2014; Glowacka et al., 2014a, b). A more detailed history of molecular marker development has been given elsewhere (Glowacka, 2011; Ma et al., 2012; Hodkinson et al., 2013).

Studies have demonstrated considerable genetic diversity in breeding collections and wild populations of Miscanthus at the infraspecific level (Greef et al., 1997; Hodkinson et al., 2002c; Glowacka et al., 2014a, b). Greef et al. (1997) and Hodkinson et al. (2002c) showed that AFLP markers could easily differentiate cultivars and infraspecific taxa of Miscanthus. However, they detected very little variation among the accessions of M. × giganteus collections and used the markers to help identify clonal material.

Diversity in M. × giganteus collections is a major cause for concern. Glowacka et al. (2014a, b) used nuclear and chloroplast SSRs in combination with restriction site-associated DNA sequencing to estimate genetic similarity in over 30 M. × giganteus accessions of unknown provenance (legacy cultivars) from collections in North America and Europe and some newly bred M. × giganteus genotypes grown from seed and found that genetic variation in the legacy cultivars was extremely low. A total of 27 of these legacy cultivars were inferred as clones matching the M. × giganteus type specimen.
Population genetics and genetic diversity

Population genetic and adaptive variation data are required to determine gene pools for Miscanthus breeding and to understand physiological adaptations to abiotic stress such as temperature, drought and salinity. These limiting factors are crucial obstacles to overcome for developing crops that are suitable for growth in a wide range of climates and environments including marginal land (Jones et al., 2014). Population genetic information is also important to develop knowledge about the evolution of Miscanthus and the impact of past and future climate on its distribution (Hodkinson (2011); De Souza et al., 2013; Clark et al., 2014).

Several studies have been carried out on genetic variation in Miscanthus, especially in M. sinensis, and the geographical centres of diversity including China, Korea and Japan. For example, Slavov et al. (2014) used SNP and SSR markers to study putatively neutral genetic diversity in a large breeding collection of Miscanthus. They also included 17 phenotypic traits related to biomass, phenology, cell-wall composition and morphology. They used the resulting data to delineate a reduced population of 145 M. sinensis genotypes to be used for association mapping and GS. Their data revealed considerable population genetic differentiation/structure in M. sinensis over the geographical space from Korea to Japan with a longitudinal cline (from 124° to 142° E) accounting for a high proportion of the molecular variation. In contrast, they found that latitude and altitudinal variation best explained variation in the phenotypic traits.

A genetic diversity study was conducted by Zhao et al. (2013a, b) in over 450 M. sinensis accessions collected from a representative range across China using 23 SSR markers. High genetic diversity was detected and clustering of individuals was consistent with geographical distribution. However, within-subpopulation variation was substantially greater (83%) than among-subpopulation variation (17%), which is not unusual given the outbreeding and perennial nature of the species. Miscanthus sinensis also has good dispersal ability via its light feathery spikelets (Fig. 1) that facilitate gene flow.

Mating system has also been shown to contribute to patterns of population diversity and differentiation using RAPD markers and DNA sequence variation in outcrossing M. sinensis (from Japan, China and Taiwan) and inbreeding M. condensatus from Taiwan (Chou et al., 200; Chiang et al., 2003). Chiang et al. (2003) studied sequence variation at the nuclear ADH1 locus and plastid trnL-F spacer regions. Low levels of genetic diversity were detected in M. condensatus that could be explained by bottlenecks caused by selfing in all populations. The ADH1 locus was under positive selection in lineages of M. condensatus that could be explained by pressure to evolve in response to different ecological conditions in saline habitats in which it is distributed (Chiang et al., 2003).

A recent study carried out by Clark et al. (2014) examined a sample of over 600 M. sinensis accessions covering a large proportion of its native range in China, South Korea and Japan using a high-density set of SNP markers and ten plastid microsatellites. The markers detected six genetic clusters from geographically distinct regions. Four clusters were from mainland Asia (Southeast China, Yangtze-Qinling, Sichuan Basin and Korea/North China) and two were from Japan (Southern and Northern). They also included some M. floridulus in their analyses and found them to cluster with M. sinensis, demonstrating their close relationship and questioning their species status. All plastid haplotypes observed in M. floridulus were also common in M. sinensis. This was consistent with the results of the study carried out by Hodkinson et al. (2002a) in which M. floridulus accessions were found to be embedded in a M. sinensis clade and with morphological intergradation of these species (Scally et al., 2001a, b). Only four M. floridulus accessions were included in the study carried out by Clark et al. (2014), and further studies are required to confirm these early observations.

Clark et al. (2014) also provided evidence that Southeast China was the centre of origin for the M. sinensis accessions found in temperate Eastern Asia. Their data were consistent with the hypothesis that Southeast China acted as a refugium during the last glacial maximum. They did not include other more southerly populations of M. sinensis, so it is not clear how important this refugium was in comparison with others that could have existed in former Indo-China, the Philippines, Indonesia and the South Pacific.

Genetic structure has also been detected on finer geographical scales. For example, Iwata et al. (2004) used AFLP fingerprinting and PCR-RFLP to detect three regional subgroups of M. sinensis ssp. condensatus in Miyake Island, Japan. They also detected a rare haplotype most probably transmitted from outside the island. Shimono et al. (2013) investigated variation in Miscanthus sinensis in Japan using chloroplast DNA and detected nine haplotypes from over 600 individuals sampled from 30 populations. Two putative ancestral lineages were detected in the Ryukyu Islands, suggesting that they might have migrated from China via Taiwan or possibly the Korean Peninsula.

Adaptive variation

Field trials and laboratory-based controlled experiments, using a broad range of genotypes, have revealed variation...
in agronomic traits such as yield (Ježowski et al., 2011; Gauder et al., 2012), drought tolerance (Clifton-Brown and Lewandowski, 2002), temperature control of leaf growth (Farrell et al., 2006), frost and cold tolerance (Clifton-Brown and Jones, 1997; Weng and Ueng 1997; Zub et al., 2012; Glowacka et al., 2014a, b), flowering time (Clifton-Brown et al., 2008; Jensen, 2009; Jensen et al., 2011; Zhang et al., 2012), senescence (Robson et al., 2011), chemical composition and morphology (Jørgensen, 2011; Zhang et al., 2012), demonstrated huge phenotypic variation in and among Miscanthus species (Zub and Brancourt-Hulmel, 2010; Jones et al., 2014) that can be utilized in breeding.

Other researchers have set up common garden experiments with different genotypes grown at multiple locations to provide insights into the natural levels of adaptive variation (Clifton-Brown et al., 1999; Clifton-Brown and Lewandowski, 2000; Yan et al., 2012). Clifton-Brown and Lewandowski (2000) used field trials to examine the overwintering success of newly established Miscanthus genotypes from different sources in Asia. They planted these at four sites across a temperature gradient in Europe (Sweden, Denmark, Germany and England) and found considerable variation among the limited number of genotypes that they tested. Yan et al. (2012) also used common garden experiments, but for a much larger sample of Miscanthus (93 genotypes) collected across their natural geographical range in China. They grew these in three locations representing temperate grassland with cold winter, semi-arid Loess Plateau and relatively warm and wet Central China and detected high variation in growth traits and significant levels of site × population interactions for most traits. Genotypes with high levels of plasticity that can produce good yields, in a broad range of habitats, were identified. These physiological experiments, field trials and common garden studies are helping to delineate populations of Miscanthus genotypes suitable for association mapping and GS (Slavov et al., 2014).

**Linking genotype to phenotype**

Some recent studies have used gene expression analysis to understand phenotypic variation in Miscanthus using methods such as RNA-Seq. Chouvarine et al. (2012) used transcriptome sequencing of rhizome samples to generate an exome sequence database for Miscanthus complete with gene ontology functional annotations. Their data were used to differentiate closely related Miscanthus cultivars. Barling et al. (2013) also generated a comprehensive expressed sequence tag (EST) catalogue using RNA-Seq that was predicted to represent a high proportion of the Miscanthus transcriptome using comparisons with sorghum gene models. They compared gene expression profiles in different tissues and a range of developmental stages. They also analysed expression profiles in rhizomes characterized in the spring compared with those characterized in the autumn to reveal biological pathways that exhibit altered regulation. Some candidate gene work has also been undertaken to understand variation in important lignin-related genes. For example, Suman et al. (2011) studied variation in caffeic acid O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR) and ferulate 5-hydroxylase (F5H) genes with target region amplification polymorphism markers and detected sufficient variation to distinguish species of the Saccharum complex. However, they did not include sufficient numbers of genotypes to assess variation within and among the Miscanthus species.

Another study has focused on generating genetic linkage maps of Miscanthus that are needed for several applications such as quantitative trait locus (QTL) analysis and marker-assisted selection (MAS). High-resolution maps based on sequence markers allow the use of QTLs accessible from other grass species through alignment based on syntenic relationships (Ma et al., 2012). However, such maps have been produced only recently.

**Mapping**

Some studies have used markers for genetic mapping, but progress has been slow because of the large and heterogeneous genome of Miscanthus. Mapping projects have therefore focused on diploid M. sinensis to facilitate genetic inheritance studies. The first published linkage map for Miscanthus (Atienza et al., 2002) was a breakthrough in the field. This map was generated using 257 PCR fingerprinting markers (RAPD) for offspring cross-mapping using an outbred population of 89 M. sinensis individuals (both parents full sibs). The markers were spread over 28 linkage fragments that spanned a total map length of 1074.5 cM with an average density of 4.2 cM per marker (but half of the fragments contained only two to four markers). Maps based on non-sequence-based markers (RAPD, AFLP and diversity array technology markers) do not provide alignable information for cross-utilization studies (Zhang et al., 2013a, b).

Higher-resolution genetic maps of Miscanthus species based on DNA sequence markers have recently been generated using next-generation sequencing technology (Ma et al., 2012; Swaninathan et al., 2012). This has allowed for data transferability and several comparative genomic analyses. The map of M. sinensis developed...
by Swaminathan et al. (2012) was based on a full-sib (F1) population produced by reciprocally crossing two ornamental clonally propagated *M. sinensis* accessions (Grosse Fontaine × Undine). Their analysis, including 808 segregating SNP and SSR markers, detected 19 linkage groups (consistent with the basic chromosome number *x* = 19). The total length on the new max likelihood map was 1782 cM (estimated total length of 1884 cM accounting for telomeric ends). In an integrated map of Grosse Fontaine and Undine, 97% of the mapped markers lie within 10 cM of another marker.

In the same year, Ma et al. (2012) used an alternative sequencing approach known as GBS to identify the 19 linkage groups and produced a higher-resolution genetic map. It was based on an outcrossing full-sib F1 mapping population (called M X 2). Their composite linkage map combining markers from both parental linkage maps included 3745 SNP markers spanning 2396 cM with an average resolution of 0.64 cM. The mapping population of Ma et al. (2012) segregates for important agronomic traits such as flowering time, biomass yield, stem number, senescence and spring emergence and can be applied for QTL studies and MAS.

**QTLs**

Despite their comparatively low resolution, the early maps (Atienza et al., 2002) were applied to QTL analysis of agronomic and combustion traits (Atienza et al., 2003a, b, c, d). Atienza et al. (2003a) used their genetic map (Atienza et al., 2002) to localize QTLs in *M. sinensis* controlling total height, flag leaf height and basal culm diameter. Field data were collected over two years to investigate developmental and environmental effects. Of the potential 11 reported QTLs, three were considered to be significant including total height, basal culm diameter and flag leaf height. Atienza et al. (2003b) almost simultaneously published a paper using a similar methodology to investigate QTLs of yield components in *M. sinensis*. They detected 20 potential QTLs: six associated with yield, eight with stem yield, two with leaf yield and four with top yield. Atienza et al. (2003c, d) also applied the same mapping population and RAPD markers to investigate QTLs influencing combustion quality traits. Atienza et al. (2003c) detected nine putative QTLs: two for calcium, two for sulphur and five for phosphorus, and Atienza et al. (2003d) detected four for chlorine and two for potassium.

These studies represent significant first steps in QTL detection, but it is not known how stable they are over time (years of trial and age of the plants) and how much they are influenced by the environment (Atienza et al., 2003d). We are currently in a period of considerable progress in QTL mapping in Miscanthus with the application of high-density/resolution genetic maps (Armstead et al., 2009). Because of the advances in DNA sequencing technology, it is likely that the limiting step will be high-quality phenotyping (Myles et al., 2009).

MAS programmes in Miscanthus are underway at several institutions, for example, the University of Illinois, USA, on traits such as yield, stability, flowering time, overwintering ability, low-temperature photosynthesis, leaf extension and drought tolerance (Sacks, pers. commun.). An introgression programme of Saccharum into Miscanthus is ongoing at the same research institute (http://www.energybiosciencesinstitute.org/directory/sacks-erik). Furthermore, a significant MAS Miscanthus breeding programme is being carried out at the Institute of Biological, Environmental and Rural Sciences (IBERS), Wales (http://www.aber.ac.uk/en/ibers/). MAS for salt tolerance is being investigated at Wageningen University, the Netherlands (http://edepot.wur.nl/155120).

**Association mapping and GS**

Association mapping (linkage disequilibrium (LD) mapping) is a method of mapping QTLs that takes advantage of historical LD to link phenotypes to genotypes (Myles et al., 2009). The genome is sampled for markers (such as SNPs) and associations are statistically detected between markers and a particular phenotype. Associations are independently verified to show that they (1) directly contribute to the trait of interest or (2) are linked to (in LD with) a QTL that contributes to the trait of interest. For example, Zhao et al. (2013a, b) found nine SSRs associated with heading date and biomass yield in *M. sinensis* using association analysis between measured traits and 115 SSR marker alleles(628,937),(960,972).
high-density marker association approaches can prove suitable for GS in *Miscanthus* for advances in biomass-related traits such as stem diameter, stem-to-leaf ratio, cell-wall composition, or improved hardiness under adverse climatic or soil conditions.

**Comparative genomics**

Currently, there are few genomic resources available to *Miscanthus* breeders, except for some genomic and EST data (Kim et al., 2014), compared with rich QTL knowledge and physical data aligned with a high-quality reference genome of *Sorghum* (Zhang et al., 2013a, b). However, the genomic resources available to breeders are likely to increase enormously over the next decade and will be utilized together with the resources of other well-characterized grass species such as sorghum, wheat, rice and maize. These resources of other Saccharinae and Sorghinae will prove particularly useful. Comparative genomic resources such as the CSGRqtl database (http://helos.pgml.uga.edu/qtl/) will facilitate the cross-utilization of information among Saccharinae taxa and complement Gramene (http://www.gramene.org), which includes mapping data from a broad diversity of grass taxa. The CSGRqtl database uses sorghum genome sequence as its central reference. It helps facilitate QTL mapping and characterize the function of genes that underlie QTLS. It can facilitate the investigation of genetic control of traits across genomes of divergent taxa and paleoduplicated subgenomes, as is the case in *Miscanthus*. These resources will combine genome data when they become available for *Miscanthus* species.

**Conclusions**

Natural genetic diversity is high in the *Miscanthus* polyploid complex and much progress has already been made in the characterization, evaluation and utilization of these resources so that artificial selection is not restricted by a lack of variation. The natural genetic diversity in *Miscanthus* has been characterized to define gene pools and used to help direct novel crossing work, manipulate ploidy, undertake QTL and association mapping studies, and develop GS selection programmes. *Miscanthus* therefore serves as a model for the use of genetic resources for new crop development. Advances in genetics underlying agronomic traits and the manipulation of these characteristics in breeding programmes will depend on the efficient utilization of existing collections and also on future collections aimed at targeting a maximum natural genetic diversity. There is a need for detailed phenotyping descriptor lists, a network of genetic resource collections and better seed/field bank coordination at the international level.

**Supplementary material**

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S147926211400094X

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