Biomass yields, cytogenetics, fertility, and compositional analyses of novel bioenergy grass hybrids (Tripidium spp.)

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[Correction added on 13 April 2020 after first online publication: the genome values have been updated in the Abstract and Results sections in this version.]

Abstract
High biomass yields have been documented for Tripidium spp. (Erianthus spp., Saccharum spp.), but targeted breeding for bioenergy applications has been limited. Advanced, interspecific hybrids between Tripidium ravennae and T. arundinaceum were planted in replicated field plots in 2016. Comparative feedstock evaluations examined biomass yields, cytogenetics, plant fertility, and compositional analyses relative to Miscanthus × giganteus. Dry biomass yields varied as a function of year and accession and increased each year ranging from 3.4 to 10.6, 8.6 to 37.3, and 23.7 to 60.6 Mg/ha for Tripidium hybrids compared to 2.3, 16.2 and 27.9 Mg/ha for M. × giganteus in 2016, 2017, and 2018, respectively. Cytology and cytometry confirmed that Tripidium hybrids were tetraploid with 2n = 4x = 40 (2C genome size = 5.06 pg) and intermediate between T. ravennae with 2n = 2x = 20 (2C genome size = 2.55 pg) and T. arundinaceum with 2n = 6x = 60 (2C genome size = 7.61 pg). Plant fertility characteristics varied considerably with some accessions producing no viable seeds or fewer than that observed for M. × giganteus. Accessions varied significantly for flowering culm number and height and dates of peak anthesis ranging from 14 September to 2 October. Variations in yield and compositional analyses contributed to variations in theoretical ethanol yields ranging from 10,181 to 27,546 L/ha for Tripidium accessions compared to 13,095 L/ha for M. × giganteus. Relative feed value (RFV) indices for winter-harvested Tripidium accessions varied from 52.8 to 60.0 compared to M. × giganteus with 45.4. RFV for summer-harvested Tripidium accessions varied from 71.6 to 80.5 compared to M. × giganteus with 61.0. These initial findings for Tripidium hybrids, including high biomass yields, cold hardiness, and desirable traits for multiple markets (e.g., forage, bioenergy, bioproducts), are promising and warrant further development of Tripidium as a temperate bioenergy feedstock.

KEYWORDS
bioenergy, bioprocessing, Erianthus, fertility, forage, lignocellulose, Miscanthus, plant breeding, polyploidy, reproductive biology, Saccharum, sterility, Tripidium
INTRODUCTION

The sustainable production of bioenergy feedstocks requires crops to be fast growing with high yields, adapted to regional environments, to have suitable composition for bioprocessing, and to have low production costs (Arnoult & Brancourt-Hulmel, 2015; de Siqueira Ferreira et al., 2013). Members of the Poaceae subtribe Saccharinae, also known as the sugarcane complex, fit many of the parameters of sustainability in dedicated bioenergy feedstock production systems (Alexander, 1985; Arnoult & Brancourt-Hulmel, 2015; Cadoux et al., 2014, Mukherjee, 1957). The Saccharinae are diverse, spanning numerous genera including *Erianthus*, *Miscanthus*, *Narenga*, *Sclerostachya*, *Saccharum*, and *Tripidium* (Daniels & Roach, 1987; Roach & Daniels, 1987; Soreng et al., 2015; Valdés & Scholz, 2006). Species and hybrids in this group can have broad adaptability, pest resistance, high biomass yields, and potential to perennially sequester substantial amounts of carbon with few inputs on marginal lands (Beale & Long, 1997; Boehmel, 2011; Ishida et al., 2008; Kiniry et al., 2012; Mishra, Torn, Huang, 2010; Kalinina et al., 2017; Khanna, Dhungana, Jones, & Clifton-Brown, 2004; Jain, Khanna, Erickson, & Rema Devi, 2011; Augustine, Syamaladevi, Premachandran, & Subramonian, 2014; Berding & Roach, 1987; Cai et al., 2005). Dry biomass yields ranging from 16 to 38 Mg/ha (Palmer et al., 2014). In the past, *T. ravennae* has been utilized on marginal lands and riparian areas for erosion control purposes (Burgess & Hoagland, 2006; Lambert, Dudley, & Saltonstall, 2010; Winston et al., 2014). Furthermore, *T. ravennae* has been reported to grow amenably under unfavorable environmental conditions including acidic and dry soils in warm climates (Hattori, Shiotsu, Doi, & Morita, 2010; Matsuo et al., 2002).

*Tripidium ravennae* (L.) H. Scholz (*Erianthus ravennae, Saccharum ravennae*; ravenna grass; 2n = 2x = 20) is native to eastern Europe, North Africa, and Southwestern Asia, but has since naturalized in many parts of the new world (Chen & Phillips, 1994; Daniels & Roach, 1987; Darke, 2007; eFloras, 2008). *Tripidium ravennae* is cold hardy to USDA Zone 5b, but has relatively low biomass yields ranging from 10 to 14 dry Mg ha⁻¹ year⁻¹ (Palmer et al., 2014). In the past, *T. ravennae* has been utilized on marginal lands and riparian areas for erosion control purposes (Burgess & Hoagland, 2006; Lambert, Dudley, & Saltonstall, 2010; Winston et al., 2014). Furthermore, *T. ravennae* has been reported to grow amenably under unfavorable environmental conditions including acidic and dry soils in warm climates (Hattori, Shiotsu, Doi, & Morita, 2010; Matsuo et al., 2002).

*Tripidium arundinaceum* (*Erianthus arundinaceus, Saccharum arundinaceum*; hardy sugar cane, plume grass, sweetcane; 2n = 3x, 4x, 6x = 30, 40, 60) is native to Southern Asia and is less cold hardy (USDA Zone 7b) than *T. ravennae*, but has dry biomass yields often surpassing *T. ravennae, Miscanthus spp.*, *Saccharum* spp. and have been reported as high as 59 dry Mg ha⁻¹ year⁻¹ (Jackson & Henry, 2011; Mislevy, Martin, Adjei, & Miller, 1997; Palmer et al., 2014). *Tripidium arundinaceum* is characterized by dense vegetative culms, drought tolerance, disease and pest resistance, and low input requirements (Amalraj, Rakkiyappan, & Rema Devi, 2011; Augustine, Syamaladevi, Premachandran, Raviachandran, & Subramonian, 2014; Berding & Roach, 1987; Cai et al., 2005). *Tripidium arundinaceum* is also known for its tillering ability and ratooning performance, making it desirable for introgression with energycane, sugarcane, and bioenergy grass improvement programs (Amalraj et al., 2011; Berding & Roach, 1987; Cai et al., 2005; Sugimoto, 2000).

Bioenergy breeding programs at North Carolina State University have focused on developing *Tripidium* as an alternative bioenergy feedstock. New, advanced (F₂ and F₃) interspecific hybrids of *T. arundinaceum* and *T. ravennae* have demonstrated cold hardiness and overwintering survival in USDA Zone 6b (T. Ranney, personal observations, 2015) with potentially high biomass yields. The objectives of this study were to further evaluate biomass yields, cytogenetics, fertility, and chemical composition for these advanced hybrids to help advance the development of *Tripidium* as a biomass/bioenergy crop.
2 | MATERIALS AND METHODS

2.1 | Field establishment

Field trials were established at Highland Creek Nursery, Hoopers Creek, NC, (35°25′48″N, 82°28′35″W). Based on preliminary biomass yields, 17 F₃ and 1 F₂ Tripidium hybrids (T. arundinaceum × T. ravennae) and 1 M. × giganteus clone (Illinois) were selected for this study. In March 2016, all selections were propagated from divisions and grown in 2.9 L containers in a 100% pine bark media supplemented with 1.04 kg/m³ dolomitic lime and 0.74 kg/m³ micronutrients (Micromax; The Scotts Co.). Field trials were planted in May 2016 with plots arranged in a randomized complete block design with three replicates. Each plot was 2 × 5 m and contained six plants in each of three rows, with plants spaced 1 m apart. Plots were separated by 4 m wide alleys and received drip irrigation, as needed, for the first 3 months of establishment following planting. Plots were treated with S-metolachlor and atrazine (4.7 L/ha, Bicep II Magnum; Syngenta) 2 weeks post-planting and the spot was treated as necessary with glyphosate (12 ml/L; Roundup QuikPRO; Monsanto) or paraquat dichloride (30 ml/L, Gramoxone SL 2.0; Syngenta) to control weeds. Soils were a Comus (colvard) fine sandy loam, coarse-loamy, mixed, active, nonacid, mesic Typic Udifluvents. Soil samples were collected from plots in 2018 and analyzed by the North Carolina Department of Agriculture and Consumer Services Agronomic Division (NCDA&CS) soil testing laboratory (Plank, 1992). Soil pH was 6.2, humic matter was 0.41%, cation exchange capacity (CEC) was 5.1 meq/100 cm³, Ca was 57% of CEC, Mg was 24% of CEC, and indexes for P and K were 71 and 31, respectively, and all within recommended levels based on NCDA&CS recommendations for perennial grasses. Yearly temperature and precipitation data were obtained from KAVL weather station at Asheville Regional Airport (5.6 km west of plots) and compiled by the North Carolina Climate Retrieval and Observations Network of the Southeast database (Table 1; NCCRONOS, 2019). Data for the 30 year temperature and precipitation were obtained from data logged at the KAVL weather station and archived with the National Oceanic and Atmospheric Administration (Table 1; NOAA, 2010).

2.2 | Biomass yield and morphological characteristics

Annual biomass yields were evaluated following plant senescence and harvest was completed by late December or early January. Six plants from the center row of each plot were harvested approximately 10 cm above the ground level and weighed fresh. A 10–15 L sample (~1 kg) of chopped tissue was collected and weighed fresh. Samples were then oven dried for 7 days at 80°C and reweighed to establish dry harvest weights. Data were analyzed using a repeated measures mixed linear model with SAS PROC GLIMMIX (SAS version 9.4; SAS Institute). Means were separated using Scheffe’s multiple comparison

| Table 1 | Monthly precipitation totals and temperature means for each year of trial and 30 year means |
|---------|---------------------------------|
| Month   | Precipitation (mm) | Temperature (°C) |
|         | 2016a               | 2017a | 2018a | 30 year meanb | 2016a | 2017a | 2018a | 30 year meanb |
| January | 83.7                | 94.6  | 102.5 | 93.2 | 1.6 | 6.8 | 0.9 | 2.8 |
| February| 144.7               | 17.9  | 141.4 | 95.5 | 4.4 | 8.7 | 10.2 | 4.6 |
| March   | 39.7                | 99.7  | 78.9  | 97.3 | 11.7 | 9.1 | 7.7  | 8.4 |
| April   | 63.1                | 170.1 | 118.0 | 84.6 | 13.8 | 15.9 | 12.2 | 12.9 |
| May     | 47.5                | 178.9 | 373.0 | 93.0 | 17.6 | 18.2 | 20.7 | 17.3 |
| June    | 64.4                | 69.0  | 65.4  | 118.1 | 23.3 | 21.5 | 23.7 | 21.4 |
| July    | 111.6               | 115.1 | 167.2 | 109.5 | 25.1 | 24.2 | 23.9 | 23.2 |
| August  | 169.1               | 161.4 | 264.5 | 111.8 | 24.5 | 22.7 | 23.3 | 22.7 |
| September| 14.8               | 95.3  | 101.8 | 96.8 | 22.2 | 19.5 | 23.4 | 19.1 |
| October | 13.2                | 246   | 148.7 | 73.9 | 15.9 | 15.2 | 16.0 | 13.6 |
| November| 39.2                | 40.5  | 182.0 | 92.7 | 10.3 | 9.9  | 7.1  | 8.5 |
| December| 58.8                | 62.8  | 276.2 | 91.2 | 5.5  | 4.6  | 5.6  | 4.1 |
| Total (mm) or average (°C) | 849.8 | 1,351.3 | 2,019.6 | 1,157.5 | 14.7 | 14.7 | 14.6 | 13.3 |

aKAVL—Asheville Regional Airport, National Oceanic and Atmospheric Administration (NOAA, 2010). Monthly average daily maximum air temperatures were 31, 30, and 29°C for July (the warmest month) 2016, 2017, and 2018, respectively.

b30 year means were collected from KAVL—Asheville Regional Airport (NCCRONOS, 2019).
test. Plots were observed regularly to identify the flowering period and duration for all accessions in this study. Peak anthesis was determined when ≥50% of inflorescence culms for a given plot were at or past peak receptivity and pollen dehiscence. Inflorescence culm number and height were determined from three randomly selected plants within each plot following peak anthesis. Total culm counts were determined for each plot by counting the number of culms from three randomly selected plants prior to harvest. Biomass yields as a function of plant morphological characters for *Tripidium* accessions were analyzed by multiple regression statistical analysis with SAS PROC REG (SAS version 9.4; SAS Institute).

### 2.3 Cytogenetics

Genome sizes were determined for all taxa in the field study and the hybrid parental species (*T. ravennae* and *T. arundinaceum*) using flow cytometry (Doležel et al., 1998). Interior leaf sheath tissues were sampled from nonflowering culms. Three to six subsamples were run per accession using approximately ≈0.5 cm² of sample tissue. *Tripidium* samples were processed together with ≈0.25 cm² of an internal standard, *Pisum sativum* L. “Cirrad” with a known genome size of 2C DNA content = 8.75 pg (Greilhuber, Temsch, & Loureiro, 2007). *Miscanthus × giganteus* samples were processed together with ≈0.25 cm² of an internal standard, *Magnolia virginiana* “Jim Wilson” with a known genome size of 2C DNA content = 3.75 pg (Parris, Ranney, Knap, & Baird, 2010). Tissues were finely chopped with a razor blade in 0.4 ml of nuclear extraction buffer (CyStain® PI Absolute P Nuclei Extraction Buffer; Sysmex Partec). Tissue extracts were incubated with propidium iodide (PI) stain buffer at room temperature for 5 min prior to filtration (50-mm nylon) followed by a 90 min (±30 min) incubation in the dark at 4°C. Nuclei were processed on a flow cytometer (Partec PA-II; Sysmex Partec) with three samples per plant accession and a minimum of 3,000 nuclei were counted for each individual assay. Holoploid genome sizes (2C) were calculated as a ratio of the mean fluorescence of the sample to the standard multiplied by the genome size of the standard.

Chromosome numbers were determined for the interspecific hybrid (H2012-260-022) and the parental species *T. ravennae* (2006-174) to correlate genome sizes with ploidy. Root tips were excised from actively growing containerized plant materials in mid-July. Approximately 5 cm of the tissue was excised and fixed following Tlaskal (1979). Briefly, roots were incubated for 3 hr at 23°C in a solution containing 0.248 mM cycloheximide and 2 mM 8-hydroxyquinoline and, then moved to 4°C for an additional 3 hr. Roots were rinsed in cold distilled water before overnight fixation at 25°C in ~3 ml of Carnoy’s solution (six parts 95% ethanol: three parts chloroform: one part glacial acetic acid). Fixed roots were rinsed in several exchanges of ethanol before storage at 4°C in a 70% ethanol solution. Cell wall hydrolysis was facilitated in a 3:1 95% ethanol:12 M HCl solution for ~15 min before chromosome staining in a modified carbol fuchsin solution (Kao, 1975; Singh, 2003). Root tips were placed onto a microscope slide with excess carbol fuchsin solution and chromosomes were expressed from cells under a cover slip. Chromosomes were viewed and counted at 1,000× magnification.

### 2.4 Plant fertility

Multiple characteristics of flowering and plant fertility were evaluated. Plant inflorescence numbers were assessed at the onset of flowering. Male fertility was assessed by aceto-carmine staining of pollen (Singh, 2003). Three dehiscent inflorescences were randomly sampled from field plots in the morning and transported to the laboratory. Ten freshly dehiscent, randomly selected, anthers were placed on a microscope slide with one drop of a 1% aceto-carmine solution. Pollen was expressed from anthers with forceps, anthers were then removed, and a coverslip added. Preparations were sealed with valap (1 petroleum jelly:1 lanolin:1 paraffin by weight) and incubated at room temperature (23°C) at least 30 min prior to assessment. Percent pollen viability was calculated as the number of stained, viable pollen grains divided by the total grains counted and multiplied by 100.

Female fertility was assessed by X-ray photography. Three inflorescences were collected from field plots in November 2018. Each inflorescence was sampled at the upper, middle and lower regions of the inflorescence which were trimmed to fit within image capture window (10–15 cm) of the X-ray image system (Faxitron MX-20; Faxitron Biopitics) and mounted between two pieces of 0.5 mm thick polystyrene plastic. Images were processed individually using ImageJ software (Abrámoff, Magalhães, & Ram, 2004). Images were despeckled as necessary and processed to identify maxima by changing the noise tolerance settings. Image processing calibration was confirmed by manual counts. The percentage of seed viability was calculated as a ratio of filled seeds divided by the total number of fertile florets counted and multiplied by 100.

In January 2019, all inflorescence material collected during the X-ray image capture process was surface sown into 1.3 L pots containing a two-part peat to one-part coarse vermiculite media (v/v). Pots were placed in a greenhouse under intermittent mist (10 s every 15 min) for 6 weeks. Germination was monitored weekly and seedlings were removed upon observation. Additional seed germination was determined following 30 days of cold stratification (4°C). After stratification, pots were returned to the greenhouse with intermittent mist and continued observation for an additional 4 weeks. Percent germination was calculated as the total number of seedlings observed (with or without stratification) divided by the number of seeds counted.
in image capture process and multiplied by 100. Overall female fertility of each accession was calculated as the percent seed set multiplied by the percent germinated seedlings divided by 100. Fertility data were subject to analysis of variance with SAS PROC GLM (SAS version 9.4; SAS Institute) and means were separated by Fisher’s least significant difference. The correlation between pollen viability and overall female fertility and between date of peak flowering and seed set, germination, or overall female fertility for the Tripidium accessions was tested using SAS PROC REG (SAS version 9.4; SAS Institute).

2.5 | Compositional analyses

Chemical composition of plant samples (soluble sugars, lignin, cellulose, and hemicellulose) was evaluated using a modified National Renewable Energy Laboratory procedure (Whitfield, Chinn, & Veal, 2016). Approximately 3 L (~1 kg dry weight) of postharvest biomass was collected for continued evaluation and dried at 45°C for 7 days. Samples were ground in a Wiley Mill to pass through a 2 mm mesh screen and stored in sealed polyethylene bags until further processing. Extractable carbohydrates from feedstock materials were quantified by HPLC following Soxhlet extraction, and cellulose and hemicellulose contents were determined by a modified two-stage sulfuric acid hydrolysis protocol following Whitfield et al. (2016). Theoretical maximum ethanol yields were calculated on a dry harvested biomass basis following Kim and Day (2011). A stoichiometric basis of carbohydrate consumption and utilization by Saccharomyces cerevisiae of 51.1% was used in the calculated theoretical ethanol conversion and considered use of both cellulose (C6) and hemicellulose components (C5; Kim & Day, 2011):

\[
L/ha = \frac{[\text{glucose (kg/ha)} + \text{fructose (kg/ha)} + (1.11 \times \text{cellulose (kg/ha)}) + (1.14 \times \text{hemicellulose (kg/ha)})]}{0.7893(\text{EtOH/kg sugar})} \times 0.511(\text{EtOH/kg sugar}).
\]

The utility of the biomass material was further characterized as a forage component for dairy livestock. Green (mid-summer) and dry (winter) harvested material from each replicate was analyzed by North Carolina Department of Agriculture and Consumer Services, Food and Drug Protection Division Laboratory for nutritive qualities. Dry matter (partial and total) were determined following methods of the National Forage Testing Association (Undersander, Mertens, & Thiex, 1993). Samples were ground and prepared in accordance with protocol 922.02 within methods of the Association of Official Analytical Chemists (AOAC; Mertens, 2005). Nitrogen was evaluated following methods of the AOAC (990.03) for total nitrogen or crude protein (AOAC Authors, 2006b). Acid and neutral detergent fiber contents were assessed on the Ankom Technologies A2000 in accordance with AOAC methods

973.18 utilizing the Ankom methods 12 and 13 respectively (Ankom Technology, 2017). Crude fat contents were analyzed by high temperature solvent extraction in accordance with the AOAC standard procedure Am 5-04 (Ankom Technology, 2012). Total ash contents were evaluated by AOAC methods 942.05 (AOAC Authors, 2006a). Data were subject to analysis of variance with SAS PROC GLM (SAS version 9.4; SAS Institute), with cultivar treated as a fixed effect, and means were separated by Fisher’s least significant difference.

3 | RESULTS

Dry biomass yields varied as a function of year, accession, and their interaction (p ≤ .001; Figure 1). At the first harvest (2016), dry matter yields for Tripidium accessions ranged from 3.4 to 10.6 Mg/ha compared to M. × giganteus with 2.3 Mg/ha. Second year dry biomass yields increased for all Tripidium accessions and ranged from 8.6 to 37.3 Mg/ha compared to M. × giganteus with 16.2 Mg/ha. Yields continued to increase for the third year for most accessions and ranged from 23.7 to 60.6 dry Mg/ha for Tripidium compared to 27.9 dry Mg/ha for M. × giganteus. The F₂ Tripidium accession, H2012-260-022, consistently had one of the highest dry yields with 10.6, 37.3, and 60.7 Mg/ha in the first, second, and third seasons, respectively. Yields for Tripidium H2012-260-022 exceed M. × giganteus in each year by 8.3, 21.1, and 44.5 Mg/ha (Figure 1).

Cytology confirmed Tripidium H2012-260-022 to be tetraploid with 2n = 4x = 40 compared with its diploid male parent, T. ravennae, with 2n = 2x = 20 (Figure 2). Testing of genome sizes for all the Tripidium accessions gave a mean of 5.06 ± 0.02 (SEM) pg (Table 2) with no significant difference

FIGURE 1 Dry biomass yields for Miscanthus × giganteus and 18 Tripidium hybrids (T. arundinacea × T. ravennae) over 3 years in Hoopers Creek, NC. Biomass yield is the mean of three replicates ± SEM.
FIGURE 2  Chromosome images of (a) *Tripidium ravennae* (2n = 2x = 20) and (b) *Tripidium* hybrid (*T. arundinaceum* × *T. ravennae*) H2012-260-022 (2n = 4x = 40)

TABLE 2  2C genome size, ploidy, pollen viability, seed set, seed germination, overall fertility, and relative fertility for *Miscanthus × giganteus* and 18 *Tripidium* hybrids (*T. arundinaceum* × *T. ravennae*) determined in 2018

| Accession      | 2C genome size (pg)¹ | Ploidy (x)² | Pollen viability (%)³ | Seed set (%) | Seed germination (%) | Female fertility (%)⁴ |
|----------------|----------------------|------------|-----------------------|--------------|---------------------|-----------------------|
| *Miscanthus × giganteus* | 7.09 ± 0.05  | 3         | 32.3 ± 0.8e           | 1.0 ± 0.1def | 41.3 ± 4.3abc       | 0.3def                |
| H2014-231-002 | 4.91 ± 0.05         | 4         | 73.2 ± 8.4abcd        | 9.3 ± 4.5bc  | 25.9 ± 10.8cdef     | 3.3cdef               |
| H2014-231-003 | 5.03 ± 0.07         | 4         | 78.4 ± 8.3abc         | 19.6 ± 1.3a  | 61.0 ± 4.1ab        | 12.0a                 |
| H2014-231-004 | 5.05 ± 0.05         | 4         | 61.5 ± 9.9bced        | 1.5 ± 0.4cdef| 32.4 ± 15.3bdec     | 0.4cdef               |
| H2014-231-006 | 5.13 ± 0.02         | 4         | 65.0 ± 3.8bced        | 18.3 ± 3.4a  | 42.7 ± 0.4abc       | 7.8b                  |
| H2014-231-008 | 5.20 ± 0.07         | 4         | 82.4 ± 4.0ab          | 5.6 ± 0.3cdef| 55.7 ± 5.5ab        | 3.4cdef               |
| H2014-231-012 | 5.08 ± 0.09         | 4         | 64.3 ± 5.3bced        | 14.3 ± 0.7ab | 62.4 ± 2.0a        | 8.9b                  |
| H2014-258-001 | 4.86 ± 0.07         | 4         | 72.7 ± 0.9bced        | 4.9 ± 1.3cdef| 23.3 ± 3.5cdef      | 1.3cdef               |
| H2014-258-002 | 5.09 ± 0.06         | 4         | 77.9 ± 1.5abc         | 1.9 ± 0.6cdef| 39.0 ± 21.1abc     | 0.5cdef               |
| H2014-258-003 | 5.08 ± 0.12         | 4         | 70.7 ± 14.8abced      | 0.5 ± 0.3ef  | 11.7 ± 11.7cdef     | 0.1cdef               |
| H2014-230-001 | 4.93 ± 0.06         | 4         | 58.9 ± 9.4d           | 0.1 ± 0f    | 0f                  | 0f                    |
| H2014-230-005 | 5.14 ± 0.12         | 4         | 84.3 ± 3.0b           | 0.6 ± 0.1ef  | 56.2 ± 13.6ab       | 0.4cdef               |
| H2014-230-006 | 5.24 ± 0.07         | 4         | 64.1 ± 4.4abcd        | 4.6 ± 4.4cdef| 0f                  | 0f                    |
| H2014-230-008 | 5.15 ± 0.05         | 4         | 35.5 ± 15.1e          | 2.3 ± 0.4cdef| 21.2 ± 2.9cdef      | 0.5cdef               |
| H2014-230-009 | 5.01 ± 0.10         | 4         | 53.2 ± 8.2de          | 0f          | 0f                  | 0f                    |
| H2014-228-003 | 5.02 ± 0.09         | 4         | 5                  | 5            | 5                   | s                     |
| H2014-229-001 | 5.10 ± 0.04         | 4         | 69.5 ± 10.9abcd       | 1.5 ± 0.5cdef| 6.4 ± 6.4ef        | 0.2cdef               |
| H2014-229-002 | 4.98 ± 0.15         | 4         | 85.1 ± 5.8a          | 8.7 ± 3.2bcd | 24.8 ± 19.8cdef     | 0.9cdef               |
| H2012-260-022 | 5.17 ± 0.05         | 4         | 81.1 ± 1.5ab         | 8.1 ± 8.0bde | 14.9 ± 14.9cdef     | 3.6c                  |

Note: Superscript letters indicate statistically significant value *p* ≤ .05.

¹2C DNA values represent the mean value of three to six samples conducted for each taxon.
²Taxa followed by an asterisk were confirmed with cytology.
³Values represent the mean ± SEM. Mean separation within columns by Fisher’s least significant difference at *p* ≤ .05.
⁴Calculated as (seed set × seed germination)/100.
⁵Flowering was not observed within the growing season at the Fletcher, NC evaluation site.
among them, verifying they are all tetraploids. Genome sizes for the *Tripidium* accessions were also intermediate between the original parents *T. arundinaceum* (2n = 6x = 60, 7.61 pg) and *T. ravennae* (2n = 2x = 20, 2.55 pg), further establishing hybridity.

There were significant (p ≤ .01) differences among taxa for pollen viability, seed set, and seed germination. Accession H2014-228-003 never flowered in any year of the study. Pollen viability, an estimate of male fertility, varied from 35.5% to 82.4% among *Tripidium* accessions that flowered, compared to 32.3% for *M. × giganteus*. Seed set varied from 0% to 19.6% for female flowers of *Tripidium* accessions compared to 1% for *M. × giganteus*. Not all of these seeds were viable, and germination rates varied from 0% to 62.4% for *Tripidium* accessions compared to 41.3% for *M. × giganteus*. Overall female fertility, the product of seed set, and seed germination, varied from 0% to 12% for *Tripidium* accessions compared to 0.3% for *M. × giganteus*. There was not a significant correlation between pollen viability and overall female fertility.

There were significant differences (p ≤ .05) among taxa for date of peak anthesis, flowering culm number, total culm number, and flowering culm height (Table 3). Peak anthesis date varied considerably from 9/14 to 10/05 for *Tripidium* hybrids, which generally flowered later than *M. × giganteus* with peak flowering on 9/14. Inflorescence culm number, total culm number, and flowering culm height varied among the *Tripidium* hybrids, which typically had fewer flowering culms, overlapping total culm numbers, and similar or higher flowering culm height than *M. × giganteus*. Multiple regression analysis of the data for *Tripidium* accessions, identified total culm number as the only significant predictor (r = .50; p ≤ .001) of biomass yield among these four traits. Furthermore, there was no significant correlation (p ≤ .05) between date of peak flowering and seed set, germination, or overall female fertility for the *Tripidium* accessions suggesting that variation in female fertility was not a function of length of seed maturation time.

Compositional analyses for cellulose and hemicellulose varied slightly among *Tripidium* accessions. Cellulose

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**TABLE 3** Date of peak anthesis and selected morphological characteristics for *Miscanthus × giganteus* and 18 *Tripidium* hybrids (*T. arundinaceum × T. ravennae*) in Hoopers Creek, NC collected in 2018

| Accession     | Peak anthesis date1,2 | Flowering culm number3 | Total culm number4 | Flowering culm height (m)3 |
|---------------|------------------------|-------------------------|--------------------|---------------------------|
| *Miscanthus × giganteus* | 9/14/2018g            | 120.3 ± 13.1a           | 120.3 ± 13.1abcd   | 3.6 ± 0d                  |
| H2014-231-002  | 9/20/2018defg          | 2.3 ± 0.8g              | 86.4 ± 22.4d       | 4.4 ± 0.1a                |
| H2014-231-003  | 9/19/2018efg           | 34.1 ± 7.6bcd           | 120.1 ± 12.0abcd   | 3.8 ± 0.1bcd              |
| H2014-231-004  | 9/21/2018def           | 37.0 ± 6.4bcd           | 85.9 ± 5.9d        | 4.2 ± 0a                  |
| H2014-231-006  | 9/19/2018eg            | 32.6 ± 3.6cd            | 77.4 ± 18.2d       | 4.2 ± 0.1a                |
| H2014-231-008  | 9/14/2018f             | 10.6 ± 1.6f             | 89.9 ± 22.4d       | 4.4 ± 0.3a                |
| H2014-231-012  | 9/15/2018g             | 24.9 ± 3.3de            | 99.8 ± 17.4bcd     | 3.8 ± 0.1bcd              |
| H2014-258-001  | 9/22/2018de            | 18.6 ± 3.0f             | 122 ± 23.6bcd      | 3.9 ± 0.1b                |
| H2014-258-002  | 9/25/2018ed            | 9.4 ± 1.3g              | 139.4 ± 23.2abcd   | 3.8 ± 0.1bcd              |
| H2014-258-003  | 10/5/2018a             | 3.7 ± 0.9g              | 94.7 ± 27.7d       | 3.8 ± 0.1bcd              |
| H2014-230-001  | 9/17/2018fg            | 3.8 ± 1.0f              | 72.7 ± 6.9d        | 3.6 ± 0.1d                |
| H2014-230-005  | 9/20/2018efg           | 6.6 ± 1.8g              | 96.9 ± 17.7bcd     | 3.8 ± 0.1bcd              |
| H2014-230-006  | 9/25/2018ed            | 4.3 ± 2.2g              | 108.1 ± 18.3abcd   | 4.2 ± 0.1a                |
| H2014-230-008  | 9/17/2018efg           | 24.8 ± 3.4de            | 121.7 ± 13.7abcd   | 3.9 ± 0.1bc               |
| H2014-230-009  | 9/19/2018fg            | 7.4 ± 1.1f              | 147.9 ± 26.3ab     | 4.4 ± 0.1a                |
| H2014-228-003  | 5                      | 0f                      | 116.2 ± 18.3abcd   | 0f                       |
| H2014-259-001  | 9/26/2018bc            | 45.3 ± 2.6bc            | 119.7 ± 16.7abcd   | 3.2 ± 0f                  |
| H2014-259-002  | 10/02/2018ah           | 9.2 ± 3.8ab             | 103.7 ± 16.3bcd    | 3.7 ± 0.1cd               |
| H2012-260-022  | 10/02/2018ah           | 3.0 ± 1.8ab             | 156.3 ± 19.3d      | 3.7 ± 0.22d               |

Note: Superscript letters indicate statistically significant value p ≤ .05.

1Values represent the mean ± SEM. Mean separation within columns by Fisher's least significant difference at p ≤ .05.

2Peak anthesis was determined on the observation of ≥50% of inflorescence culms for a given plot which were at or past peak receptivity and pollen dehiscence.

3Observations were documented at peak anthesis.

4Observations were documented for each severed plant following harvest.

5Flowering was not observed within the growing season at the Fletcher, NC evaluation site.
was significantly lower in *Tripidium* accessions than in *M. × giganteus* and hemicellulose was typically similar to *M. × giganteus* when compared on a unit weight basis (Table 4). However, the higher dry matter yields achieved by several hybrid accessions would result in greater cellulose production per hectare and increase the supply and value of the feedstock. Free glucose and fructose varied significantly (*p* ≤ .05) among *Tripidium* accessions and many were significantly (*p* ≤ .05) higher than *M. × giganteus*. Theoretical ethanol yields calculated on cellulose, hemicellulose, glucose, and fructose ranged from 10,181 to 27,546 L/ha for *Tripidium* accessions compared to 13,095 L/ha for *M. × giganteus* (Table 4).

Forage analysis showed *Tripidium* accessions typically varied among each other and compared to *M. × giganteus* depending on the specific variable (Tables 5 and 6). Crude protein contents ranged from 111 to 138 g/kg for summer-harvested biomass and 53–66 g/kg for winter-harvested *Tripidium* accessions. The protein contents were close to twice that of *M. × giganteus* with 75 g/kg for summer and 27 g/kg for winter-harvested materials. Acid detergent fiber, favorable for rumen digestibility, was lower for *Tripidium* accessions, 375–416 g/kg (summer) and 482–542 g/kg (winter), compared to *M. × giganteus* with 501 g/kg (summer) and 592 g/kg (winter). Neutral detergent fiber was typically higher in *M. × giganteus* in both summer (760 g/kg) and winter (878 g/kg) than in most *Tripidium* accessions (691–750 g/kg in summer and 794–824 g/kg in winter). Nonfiber carbohydrates varied from 103 to 154 g/kg in summer and 89 to 120 g/kg in winter for *Tripidium* accessions with 691–750 g/kg in *M. × giganteus* (winter). Total digestible nutrient content varied from 568 to 685 g/kg in summer and 528 to 576 g/kg in winter-harvested *Tripidium* accessions which were greater than *M. × giganteus* with 568 g/kg (summer) and 487 g/kg (winter). Net energy of lactation was also lower for *M. × giganteus* in summer (0.174 Mcal/kg) and winter (0.11 Mcal/kg) compared to most other *Tripidium* accessions (0.24–0.27 Mcal/kg in summer and 0.14–0.18 Mcal/kg in winter). Overall, relative feed value was greater for all *Tripidium* accessions ranging from 71.6 to 90.5

### Table 4

Dry biomass yield, compositional analysis, and theoretical ethanol yield for *Miscanthus × giganteus* and 18 *Tripidium* hybrids (*T. arundinaceum × T. ravennae*) determined on material harvested in December 2018

| Accession | Biomass yield (Mg/ha) | Acid insoluble lignin (%) | Cellulose (%) | Hemicellulose (%) | Glucose (%) | Fructose (%) | Theoretical ethanol yield (L/ha) |
|-----------|-----------------------|---------------------------|--------------|------------------|-------------|--------------|---------------------------------|
| *Miscanthus × giganteus* | | | | | | | |
| H2014-231-002 | 27.0 ± 2.1 | 19.2 ± 0.1 | 42.5 ± 0.1 | 22.3 ± 0.1 | 0.02 ± 0 | 0.03 ± 0 | 13,095 ± 637 |
| H2014-231-003 | 44.8 ± 10.1 | 18.6 ± 0.5 | 37.3 ± 0.9 | 23.6 ± 0 | 0.10 ± 0.02 | 0.11 ± 0.02 | 11,500 ± 1,073 |
| H2014-231-004 | 35.0 ± 5.8 | 18.2 ± 1.4 | 36.7 ± 0.6 | 23.6 ± 0.2 | 0.10 ± 0.02 | 0.10 ± 0.02 | 13,549 ± 2,564 |
| H2014-231-006 | 29.5 ± 6.5 | 18.5 ± 1.7 | 37.2 ± 0.2 | 23.5 ± 0.2 | 0.09 ± 0.03 | 0.09 ± 0.02 | 13,109 ± 2,902 |
| H2014-231-008 | 27.5 ± 3.6 | 20.0 ± 0.3 | 36.7 ± 0.3 | 23.6 ± 0.4 | 0.05 ± 0.01 | 0.04 ± 0 | 12,028 ± 1,670 |
| H2014-231-012 | 26.2 ± 0.6 | 19.4 ± 0.5 | 36.9 ± 0.3 | 24.0 ± 0.5 | 0.06 ± 0.01 | 0.07 ± 0.01 | 11,608 ± 373 |
| H2014-258-001 | 30.6 ± 4.8 | 19.6 ± 1.0 | 35.7 ± 0.2 | 23.6 ± 0.4 | 0.14 ± 0.02 | 0.11 ± 0.04 | 13,198 ± 1,957 |
| H2014-258-002 | 39.7 ± 2.3 | 18.2 ± 0.7 | 33.7 ± 0.4 | 23.8 ± 0.2 | 0.27 ± 0.1 | 0.29 ± 0.13 | 16,695 ± 951 |
| H2014-258-003 | 30.3 ± 3.8 | 18.7 ± 0.8 | 34.9 ± 0.6 | 23.7 ± 0.2 | 0.21 ± 0.04 | 0.11 ± 0.03 | 12,829 ± 1,633 |
| H2014-230-001 | 23.7 ± 5.3 | 18.2 ± 0.5 | 34.4 ± 0.1 | 24.5 ± 0.4 | 0.12 ± 0.02 | 0.09 ± 0.02 | 10,181 ± 2,343 |
| H2014-230-005 | 35.6 ± 4.3 | 19.4 ± 0.8 | 36.6 ± 1.5 | 24.2 ± 0.3 | 0.13 ± 0.03 | 0.13 ± 0.03 | 15,832 ± 2,189 |
| H2014-230-006 | 41.6 ± 5.0 | 16.6 ± 1.0 | 38.0 ± 4.5 | 25.1 ± 2.4 | 0.20 ± 0.02 | 0.2 ± 0.02 | 18,787 ± 1,464 |
| H2014-230-008 | 36.3 ± 3.6 | 17.7 ± 0.2 | 36.7 ± 0.4 | 24.5 ± 0.3 | 0.08 ± 0.01 | 0.06 ± 0.01 | 16,124 ± 493 |
| H2014-230-009 | 43.7 ± 8.7 | 17.4 ± 0.8 | 35.2 ± 0.5 | 23.9 ± 0.3 | 0.14 ± 0.02 | 0.15 ± 0.07 | 18,739 ± 3,548 |
| H2014-228-003 | 29.4 ± 3.1 | 20.6 ± 0.3 | 33.6 ± 0.5 | 23.3 ± 0.4 | 0.20 ± 0.04 | 0.22 ± 0.06 | 12,241 ± 1,419 |
| H2014-229-001 | 33.7 ± 4.9 | 19.0 ± 0.2 | 33.9 ± 0.2 | 23.8 ± 0.4 | 0.17 ± 0.02 | 0.21 ± 0.01 | 14,200 ± 2,145 |
| H2014-229-002 | 29.8 ± 4.2 | 20.0 ± 0.8 | 34.5 ± 0.5 | 24.2 ± 0.3 | 0.1 ± 0.03 | 0.12 ± 0.07 | 12,728 ± 1,729 |
| H2012-260-022 | 60.7 ± 9.8 | 19.3 ± 0.4 | 37.4 ± 1.3 | 24.5 ± 0.9 | 0.12 ± 0.00 | 0.13 ± 0.00 | 27,546 ± 5,325 |

*Note: Superscript letters indicate statistically significant value p ≤ .05.*

1*Values represent the mean ± SEM. Mean separation within columns by Fisher's least significant difference at p ≤ .05.*

2*A 1.11× (cellulose to glucose) and 1.14× (hemicellulose to xylose) conversion was assumed for the net gain of water during hydrolysis. Ethanol (L) = Glucose, fructose, sucrose, plus xylose (kg/ha) × 0.511 theoretical maximum conversion factor for sugar to ethanol based on stoichiometric biochemistry of yeast and converted to liters using a 0.783 L/kg of ethanol conversion.*
80.5 in summer versus 53 to 60 in winter, which was markedly higher than for *M. × giganteus* at 61 and 45 respectively.

### 4 | DISCUSSION

Prior reports have identified *T. arundinacenum* as a potential biomass and bioenergy feedstock (Dao et al., 2013; Palmer et al., 2014; Wang et al., 2019; Zhang et al., 2013), though cold hardiness is limited to USDA Zone 7b. The new interspecific *Tripidium* hybrids examined in this study demonstrated high biomass yields ranging from 24 to 61 dry Mg/ha for the third growing season within the temperate climes of Western North Carolina, USDA Zone 6b/7a (Figure 1). These biomass yields are similar to those for *S. spontaneum* and other *Saccharum* hybrids (energy canes) that have yielded between 13 and 67 Mg/ha dry weight in the subtropical climes of the southern United States and Caribbean islands (Alexander, 1985; Bischoff et al., 2008; Matsuoka et al., 2014), but these *Saccharum* crops are typically limited to USDA Zones 8 and warmer. Yields of many of the interspecific *Tripidium* hybrids also exceed that of *M. × giganteus*, a hybrid often found to have one of the highest biomass yields of more temperate grasses (Clifton-Brown et al., 2017; Lewandowski et al., 2016). In the third growing season, *Tripidium* H2012-260-022 had more than twice the biomass yield of *M. × giganteus*. Considering that one of the *Tripidium* hybrid’s parents, *T. ravennae*, is cold hardy to USDA Zone 5b, it is likely that further breeding, selection, and evaluation could identify advanced, high-biomass *Tripidium* hybrids suitable for colder regions than North Carolina.

Plant fertility can be important when selecting breeding lines for further crop improvement and/or to avoid reseeding of crops and potential invasiveness. Although all the *Tripidium* hybrids were confirmed to be tetraploids (isoploids), these types of interspecific, interploid hybrids may have reduced or variable fertility. Accession H2014-228-003 did not flower under our environmental conditions. If this trait is stable under other years/environments, it may be desirable to prevent reseeding or prevent gene flow in yet-to-be developed transgenic plants. Similarly,
we were unable to document any female fertility in *Tripidium* accessions H2014-230-001, H2014-230-006, and H2014-230-009 that could be valuable in preventing them from naturalizing. There was no correlation between pollen viability and female fertility, indicating that variations in fertility were probably more complex than just failure of homoeologous chromosomes to pair in meiosis. Surprisingly, we did recover seedlings from an interspecific hybrid (Słomka et al., 2012; Yu, Kim, Rayburn, Widholm, & Juvik, 2010). We completed flow cytometry on 30 seedlings from open pollinated *M. × Widholm, & Juvik, 2010). We completed flow cytometry on 30 seedlings from open pollinated

| Accession     | Crude protein (g/kg) | Acid detergent fiber (g/kg) | Neutral detergent fiber (g/kg) | Nonfiber carbohydrate (g/kg) | Total digestible nutrients (g/kg) | Net energy of lactation (Mcal/kg) | Relative feed value² |
|---------------|----------------------|-----------------------------|--------------------------------|------------------------------|----------------------------------|----------------------------------|----------------------|
| *Miscanthus × giganteus* | 75.2 ± 11.0³ | 500.5 ± 27.1¹ | 760.4 ± 6.0¹ | 150.3 ± 18.7¹ | 568.2 ± 25.5² | 0.174 ± 0.021³ | 61.0 ± 2.2² |
| H2014-231-002 | 124.4 ± 5.9³ | 369.9 ± 4.9³ | 396.4 ± 3.3³ | 583.0 ± 3.3³ | 153.5 ± 3.3³ | 665.9 ± 2.1³ | 0.254 ± 0.003³ |
| H2014-231-003 | 137.6 ± 4.4³ | 374.0 ± 7.3³ | 691.0 ± 11.6³ | 149.2 ± 10.2³ | 684.7 ± 6.8³ | 0.268 ± 0.005³ | 80.5 ± 1.8³ |
| H2014-231-004 | 129.4 ± 9.9³ | 385.5 ± 11.9³ | 715.1 ± 18.8³ | 131.7 ± 12.2³ | 699.1 ± 12.3³ | 0.259 ± 0.009³ | 76.8 ± 3.3³ |
| H2014-231-006 | 118.2 ± 10.0³ | 414.2 ± 7.4³ | 729.3 ± 9.1³ | 134.9 ± 0.9³ | 568.2 ± 25.5³ | 0.240 ± 0.005³ | 72.3 ± 1.6³ |
| H2014-231-008 | 120.5 ± 10.5³ | 416.0 ± 11.5³ | 718.0 ± 11.9³ | 143.8 ± 5.6³ | 665.9 ± 2.1³ | 0.239 ± 0.008³ | 73.3 ± 2.2³ |
| H2014-231-012 | 131.0 ± 2.2³ | 388.8 ± 5.8³ | 727.7 ± 2.9³ | 116.1 ± 0.6³ | 670.0 ± 6.1³ | 0.257 ± 0.004³ | 74.9 ± 0.8³ |
| H2014-258-001 | 121.8 ± 3.2³ | 399.0 ± 2.6³ | 736.4 ± 2.4³ | 133.7 ± 11.1³ | 662.6 ± 1.3³ | 0.251 ± 0.002³ | 73.0 ± 0.1³ |
| H2014-258-002 | 131.6 ± 3.4³ | 399.8 ± 5.9³ | 724.9 ± 0.5³ | 125.7 ± 0.6³ | 663.6 ± 7.5³ | 0.249 ± 0.005³ | 74.1 ± 0.6³ |
| H2014-258-003 | 131.0 ± 7.5³ | 399.7 ± 4.9³ | 728.0 ± 8.6³ | 123.4 ± 8.4³ | 663.8 ± 7.1³ | 0.251 ± 0.004³ | 73.8 ± 1.3³ |
| H2014-230-001 | 112.3 ± 3.5³ | 401.5 ± 5.8³ | 736.9 ± 12.4³ | 131.9 ± 10.7³ | 659.0 ± 4.6³ | 0.248 ± 0.003³ | 72.8 ± 1.8³ |
| H2014-230-005 | 117.5 ± 4.9³ | 408.9 ± 6.6³ | 726.3 ± 19.9³ | 150.7 ± 19.3³ | 660.9 ± 8.7³ | 0.246 ± 0.007³ | 73.2 ± 2.3³ |
| H2014-230-006 | 111.2 ± 9.1³ | 409.3 ± 10.8³ | 739.6 ± 16.1³ | 133.3 ± 10.3³ | 654.9 ± 9.4³ | 0.243 ± 0.008³ | 71.8 ± 2.7³ |
| H2014-230-008 | 120.5 ± 6.5³ | 408.8 ± 9.0³ | 740.4 ± 20.7³ | 118.5 ± 13.0³ | 662.6 ± 8.8³ | 0.249 ± 0.007³ | 72.6 ± 3.0³ |
| H2014-230-009 | 118.0 ± 4.9³ | 410.1 ± 1.9³ | 725.7 ± 7.2³ | 145.8 ± 10.4³ | 656.9 ± 7.3³ | 0.245 ± 0.005³ | 73.0 ± 0.9³ |
| H2014-228-003 | 136.3 ± 7.4³ | 400.2 ± 6.6³ | 728.8 ± 4.8³ | 122.6 ± 3.5³ | 667.1 ± 7.1³ | 0.251 ± 0.005³ | 73.7 ± 1.1³ |
| H2014-239-001 | 126.5 ± 12.4³ | 409.0 ± 14.7³ | 741.0 ± 12.1³ | 111.0 ± 8.0³ | 656.6 ± 14.7³ | 0.246 ± 0.011³ | 72.5 ± 2.4³ |
| H2014-239-002 | 130.4 ± 13.2³ | 381.3 ± 5.8³ | 750.0 ± 19.3³ | 108.3 ± 15.0³ | 682.5 ± 5.8³ | 0.265 ± 0.004³ | 73.5 ± 1.5³ |
| H2012-260-022 | 129.7 ± 4.0³ | 403.0 ± 7.6³ | 747.7 ± 11.9³ | 103.2 ± 14.8³ | 659.7 ± 3.3³ | 0.248 ± 0.003³ | 71.6 ± 0.8³ |

Note: Superscript letters indicate statistically significant value *p* ≤ .05.

¹Values represent the mean ± SE. Mean separation within columns by Fisher's least significant difference at *p* ≤ .05.

²Relative feed values were calculated as digestible dry matter (DDM) × dry matter intake (DMI) divided by 1.29. DDM estimates feed digestibility, which was calculated from acid detergent fiber (ADF) as 88.9 − (0.779 × ADF). DMI estimates potential feed volume consumption in percent of body weight, which is calculated from neutral detergent fiber (NDF) as 120/NDF.

TABLE 6 Forage analysis for *Miscanthus × giganteus* and 18 *Tripidium* hybrids (*T. arundinaceum × T. ravenne*) determined on material harvested in July 2019.
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