

Phenotypic and genotypic variation in Australian native *Sorghum* species along aridity clines

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ABSTRACT

Context. Wild sorghum taxa (*Sorghum* Moench) cover much of the northern Australian landscape and harbour potentially useful traits for crop improvement. Specialised metabolites such as phenolics, silicon and cyanogenic glucosides (which are toxic to grazing animals) are present in *Sorghum* and have been associated with drought tolerance. However, these taxa remain poorly studied, especially regarding intraspecific variation. **Aims.** To investigate the intraspecific diversity of wild sorghums growing along aridity clines, including morphology, biochemistry and genotype. We hypothesised that genotypic and phenotypic diversity would co-vary with niche diversity. **Methods.** Multiple accessions of *Sorghum plumosum*, *S. stipoides* and *S. timorensis* were grown under common conditions for 10 weeks. Concentrations of cyanogenic glucosides, phenolics and silicon were measured alongside five morphological traits. Accessions were genotyped using single-nucleotide polymorphisms. **Key results.** Several traits varied significantly within species, but no trait consistently co-varied with aridity across species. In *S. timorensis*, there appeared to be genotypic differentiation among accessions, but this was not translated into phenotypic differences, which was likely to be due to similarities in climate. **Conclusions.** Our results challenge assumptions that phenotypic and genotypic diversity correlate with niche diversity and support the hypothesis that mature Australian wild sorghums store most of their cyanogenic glucosides in their roots. **Implications.** Wild sorghums harbour advantageous traits for crop improvement, such as niche diversity and low toxicity. Future wild sorghum research must acknowledge intraspecific diversity, and as many populations as possible should be conserved, because genetic diversity is not necessarily discernible from phenotype or provenance alone.

Keywords: Australia, crop wild relatives, cyanogenesis, cyanogenic glucosides, DArTseq, dhurrin, silicon, sorghum.

Introduction

Sorghum (*Sorghum bicolor* [L.] Moench) is one of the world's top five grain crops. Its grain is eaten by approximately 500 million people, and it is also grown for animal feed, forage and biofuel. Therefore, there is a pressing need to increase sorghum's tolerance to drought and heat, disease resistance, and improve its nutritional quality. Sorghum's crop wild relatives, undomesticated species belonging to all subgenera of *Sorghum* Moench, are a potential source of such traits.

Traditional use of wild *Sorghum* taxa for food supply by Aboriginal peoples of Australia points to their long recognition of usefulness (Arndt 1961; Pascoe 2014). Australian *Sorghum* species have attracted interest from researchers and crop breeders as a tertiary gene pool for crop improvement (Ananda *et al.* 2020; Myrans *et al.* 2020). However, little is known about them. Lazarides *et al.* (1991) conducted a comprehensive ecological survey; however, since then, much of the research into wild sorghums has focused on single accessions within species (e.g. Cowan *et al.* 2020; Myrans *et al.* 2021; Cowan *et al.* 2022; Myrans and Gleadow 2022). This is largely due to the difficulty of obtaining seed from the remote areas in which they are found. Whereas the single-accession approach quickly screens traits across a broad range of *Sorghum* species, examination of

intraspecific diversity and local adaptation is needed to give a more complete understanding of the true diversity in the genus and inform research and conservation decisions.

Of the 22 known wild *Sorghum* taxa, 17 are native to Australia, dominating the understorey across large parts of the north of the country. Their populations span diverse climatic regions, but most are adapted to hot, monsoonal areas with poor soil nutrition (Lazarides et al. 1991; Myrans et al. 2020). Water stress and fire frequently threaten many of these populations, likely serving as strong selection pressures for local adaptation (Andrew and Mott 1983; Bowman et al. 2010). Cowan et al. (2020) identified several wild sorghum taxa whose drought tolerance appeared to be greater than that of domesticated sorghum, emphasising the potential value in examining the traits conferring adaptation to aridity in the genus. Knowledge of these traits is also important for conservation efforts in parts of northern Australia affected by climate change and associated increases in aridity (Dai 2013).

One intriguing trait that might co-vary with aridity is cyanogenic glucoside (CNglc) production and regulation. CNgcls are nitrogen-containing secondary metabolites that can be catabolised to release toxic hydrogen cyanide (HCN) during herbivory. A plant's CNglc concentration can be quantified by measuring HCN potential, the total amount of HCN produced by hydrolysing all CNgcls present in the tissue. HCN potentials above $600 \mu\text{g g}^{-1}$ can be considered dangerous to cattle (Boyd et al. 1938; Hunt and Taylor 1976), whereas other animals can be adversely affected by lower HCN potentials (Gleadow and Woodrow 2002; Kumari et al. 2021). Kooyers et al. (2014) reported that the frequency of *Trifolium repens* plants that are able to produce CNgcls is typically higher in warmer locations – a pattern that has emerged independently in different regions. However, such patterns have not yet been tested for in *Sorghum*.

Although CNgcls remain useful in defence against herbivory, the major roles of CNgcls in wild sorghums are unresolved. When mature, wild sorghums typically have very low HCN potential in their leaves ($<10 \mu\text{g g}^{-1}$), and higher HCN potential in their roots ($0\text{--}750 \mu\text{g g}^{-1}$) (Myrans and Gleadow 2022). The storage of CNgcls at such low concentrations, and away from the tissues most vulnerable to herbivore damage, makes wild sorghums attractive sources of genes for improving forage sorghums, which often have toxic leaf HCN potentials (Cowan et al. 2020). It also brings into question the effectiveness of CNgcls as a defence in wild sorghums. It has been hypothesised that CNgcls have multiple roles in plants, including in the storage of nitrogen (Møller 2010; Gleadow and Møller 2014; Bjarnholt et al. 2018). Screening the intraspecific diversity of HCN potential in wild sorghums will give further insight into the roles of CNgcls in wild sorghums and whether these differ with aridity.

Other biochemical traits that can co-vary with aridity are phenolics and silicon (Katz 2019; Carvalho et al. 2022).

Phenolics are organic secondary metabolites containing at least one aromatic ring and hydroxyl group. They can be effective at mitigating stressors such as herbivores, pathogens, reactive oxygen species and extreme temperatures (Lattanzio et al. 2012). Plant silicon is mainly considered to augment physical defence through deposition in structures such as phytoliths and trichomes (Hartley et al. 2015), but may also play roles in more complex chemical-defence pathways (Islam et al. 2022; Thorne et al. 2023), and mitigate other stressors including drought and salinity (Liu et al. 2015; Coskun et al. 2019).

Morphological traits, such as biomass, relative growth rate, specific leaf area, number of tillers and root:shoot ratio, also commonly vary with aridity (Linhart 1974; Bull-Herenu and Arroyo 2009). Typically, relative growth rate, specific leaf area and tillering would be expected to be lower in plants adapted to more arid environments, whereas root:shoot ratio would be expected to be higher. Collectively, these traits confer a more conservative, resource-efficient growth strategy, which is often vital for increasing survival in low-resource arid environments (Coley et al. 1985). Accession genotypes were compared using large numbers of single-nucleotide polymorphisms (SNPs). We expect species with wider distributions and more niche diversity to contain the most intraspecific diversity, both phenotypically and genotypically.

Three Australian wild *Sorghum* species (*Sorghum plumosum* [R.Br.] P.Beauv., *Sorghum stipoides* [Ewart & Jean White] C.A.Gardner & C.E.Hubb. and *Sorghum timorense* [Kunth] Büse) were chosen for this study. *Sorghum plumosum* is perennial, whereas *S. stipoides* and *S. timorense* are annual. According to Lazarides et al. (1991), *S. timorense* is widely distributed but restricted to heavy-textured soils in alluvial floodplains and cracking clay plains, whereas *S. plumosum* and *S. stipoides* can grow on a diverse range of soil types while also being widespread. The estimated extent of occurrence is much larger for *S. plumosum* ($7\,559\,371 \text{ km}^2$) than for *S. timorense* ($4\,475\,649 \text{ km}^2$) or *S. stipoides* ($3\,111\,011 \text{ km}^2$) (Myrans et al. 2020). *Sorghum plumosum*'s perennial life history also means that, unlike the other two species, it is adapted to survive through the entire dry season.

The aim of this study was to quantify phenotypic and genotypic variation within *S. plumosum*, *S. stipoides* and *S. timorense*, and determine which phenotypic traits might confer adaptation to aridity. To do this, we grew 18 accessions of the three study species in a common greenhouse environment, measured biochemical and morphological traits, and screened their genotypic similarity. The accessions were originally collected from provenances of varied aridity levels in northern Australia. This design was chosen because cline studies are a key step in identifying traits and genes associated with local adaptation without the confounding effects of differing growth environments (Bull-Herenu and Arroyo 2009; Carvalho et al. 2022). The following three

hypotheses were tested: (1) within species, HCN potential would increase with aridity of accession provenances; (2) *S. plumosum* would contain the most phenotypic and genotypic variation among accessions; and (3) phenotypes would be more similar within species than among accessions of different species from similar environments.

Materials and methods

Plant material and growth conditions

Six *S. plumosum* accessions, five *S. stipoides* accessions and seven *S. timorensis* accessions were germinated and grown for 10 weeks. Accessions were chosen on the basis of their collection sites occurring along aridity clines in northern Australia (Table 1). Within species, accessions were ordered numerically (P1–P6, S1–S5 and T1–T7) on the basis of increasing aridity of their collection sites (Fig. 1). The *S. plumosum* accessions ranged in aridity index from 0.109 to 0.920, the *S. stipoides* accessions from 0.358 to 0.589, and the *S. timorensis* accessions from 0.128 to 0.296.

Seeds were provided by the Australian Grains Genebank (Horsham, Vic., Australia) and were collected by the Australian Tropical Crops Genetic Resource Centre. All plants ($n = 251$) were grown under controlled greenhouse conditions at Monash University (−37.91, 145.14) between

September and December 2020, with a mean temperature of $31.35 \pm 0.09^\circ\text{C}/21.46 \pm 0.03^\circ\text{C}$, day/night, and a mean photoperiod of 13.7 h. Supplementary light was provided using sodium lamps for 13 h per day (MK-1 Just-a-shade, Ablite, NSW, Australia).

Seeds were germinated in a constant-temperature cabinet, following Cowan *et al.* (2020). Once the coleoptile and radicle had emerged, plants were transferred to the greenhouse and planted in seedling trays containing Debco seed-raising substrate (Evergreen Garden Care, NSW, Australia) and perlite (1:1, v/v ratio) for 1.5 weeks. Seedlings were then transplanted into 1.9 L pots containing Debco seed-raising substrate, perlite and coarse sand (2:2:1, v/v ratio). Plants were grown for a further 8 weeks and watered three times per week, namely twice with 250 mL water, once with 250 mL Thrive all-purpose soluble fertiliser (Yates, Vic., Australia) with an N:P:K ratio of 25:5:8.8 (v/v ratio). Pot positions were randomised and rotated weekly to minimise microenvironment effects. At 10 weeks post-germination, plants were harvested and separated into roots, sheaths and leaves and dried in ovens at 50°C for 72 h.

So as to confirm that stress was not a factor in any phenotypic differences, F_v/F_m , a useful indicator of plant stress, was measured on the youngest fully unfurled leaf using a MINI-PAM Photosynthesis Yield Analyser (Heinz Walz GmbH, Effeltrich, Germany) 7 weeks post-germination

Table 1. Coordinates of the collection sites and corresponding aridity indices for the 18 accessions across the three wild *Sorghum* species examined in this study (*S. plumosum*, *S. stipoides* and *S. timorensis*).

Species	Accession	Accession name	Latitude	Longitude	Temperature ($^\circ\text{C}$) ^A	Annual rainfall (mm) ^A	Aridity index ^B	Soil type
<i>S. plumosum</i>	P1	JC2246	−11.7278	130.8722	26.74	1689	0.920	Sandy loam
	P2	JC2114	−13.5453	131.2220	26.99	1302	0.676	Sandy loam
	P3	JC2053	−14.7922	131.9427	27.31	936	0.454	Clay loam
	P4	JC2010	−15.8483	131.6403	27.29	857	0.315	Sandy loam
	P5	JC2199	−17.6768	135.7017	26.11	593	0.198	Clay loam
	P6	JC2306	−19.2485	134.1803	25.96	411	0.109	N/A
<i>S. stipoides</i>	S1	JC2356	−14.1025	126.7097	27.62	1125	0.589	Sand
	S2	JC2355	−14.3545	126.6492	27.56	1215	0.567	Gravel
	S3	JC2350	−15.6808	126.4032	25.84	986	0.451	Sandy loam
	S4	JC2349	−15.9515	126.3493	25.64	937	0.420	Sandy loam
	S5	JC2347	−16.5725	126.0928	25.70	849	0.358	Gravel
<i>S. timorensis</i>	T1	JC2164	−17.7817	139.4787	26.82	865	0.296	Clay
	T2	JC2159	−18.2123	139.8640	26.85	604	0.242	Clay
	T3	JC2156	−18.7697	140.5072	26.51	626	0.213	Clay
	T4	JC2157	−18.9243	140.4565	26.66	611	0.201	Clay
	T5	JC2209	−19.8732	141.0175	26.27	496	0.160	Clay
	T6	JC2303	−20.6457	141.0265	25.77	418	0.134	Clay
	T7	JC2206	−20.9922	141.8368	25.39	437	0.128	Clay loam

^AMean annual temperature and rainfall according to Fick and Hijmans (2017).

^BMean annual aridity index according to Williams *et al.* (2010).

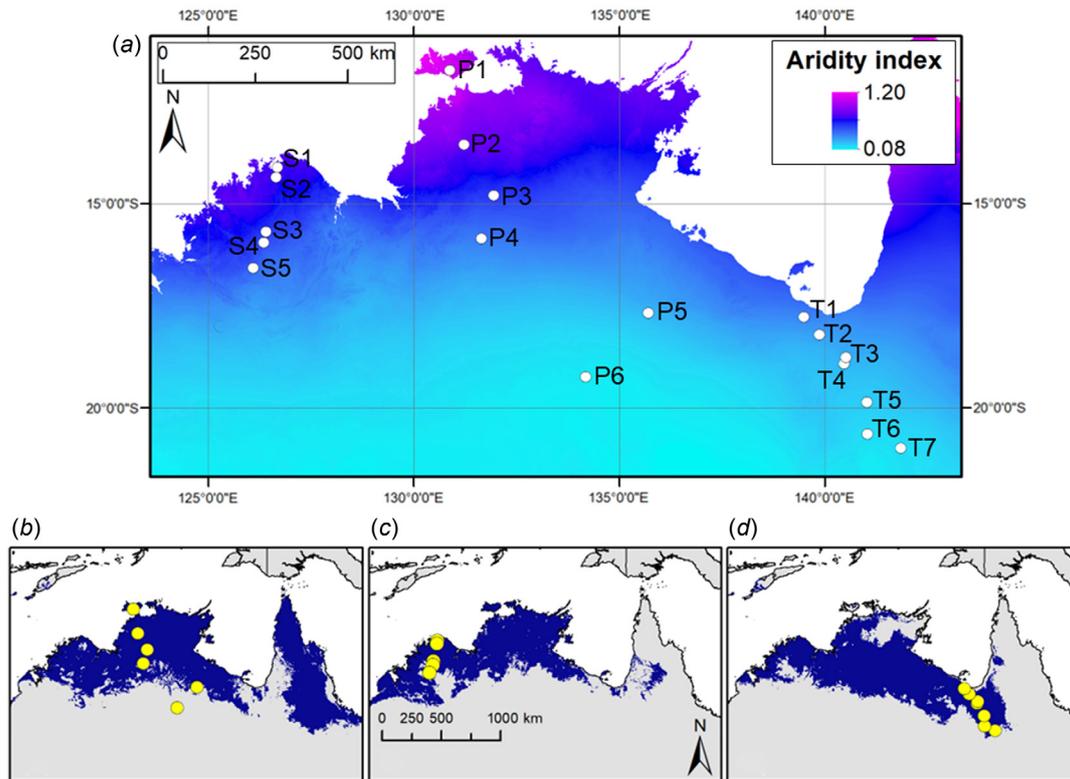


Fig. 1. (a) Mean annual aridity index (Williams et al. 2010) map of northern Australia, with collection sites of the 18 study accessions pinpointed with white circles. Accessions are labelled according to Table 1. High aridity indices (magenta) indicate less arid areas, whereas low aridity indices (cyan) indicate more arid areas. (b–d) Collection sites of the study accessions (yellow circles) are superimposed on the respective modelled distributions (blue) of (b) *S. plumosum*, (c) *S. stipoides* and (d) *S. timorensis* (Myrans et al. 2020).

($n = 6$). Mean F_v/F_m values were similar across all accessions, ranging between 0.78 and 0.82, indicating that all accessions were healthy and approaching the optimal F_v/F_m of 0.83 (Maxwell and Johnson 2000) (Supplementary Table S1).

Morphological parameters

Plant morphology was quantified by counting tillers, measuring total leaf area, and determining the biomass of leaves, sheaths and roots of each plant. Total leaf area was measured using a LI-3000 portable area meter and a LI-3050A belt conveyor (LI-COR Biosciences, NE, USA). Senesced leaves were included in total biomass measurements, but not in leaf-area measurements or chemical analysis. Specific leaf area (SLA; $\text{cm}^2 \text{g}^{-1}$) and root:shoot ratio were calculated using the following equations:

$$\text{SLA} = \frac{\text{total leaf area}}{\text{biomass of green leaves}}$$

$$\text{Root:shoot ratio} = \frac{\text{root biomass}}{(\text{leaf biomass} + \text{sheath biomass})}$$

Relative growth rate (RGR; $\text{g g}^{-1} \text{day}^{-1}$) was calculated using the equation:

$$\text{RGR} = \frac{\ln(W_2) - \ln(W_1)}{t_2 - t_1}$$

where W is whole-plant biomass, and t is time. W_1 was measured at the start of the experiment (t_1), at the seed stage, with the mean W_1 for the accession used in every RGR calculation for that accession. W_2 was obtained by measuring the biomass of the entire plant at the end of the treatment period (t_2).

Chemical analysis

HCN potential was measured through colorimetric assay in 96-well microtiter plates, by using the evolved cyanide method, following Gleadow et al. (2012). Briefly, 10 mg of dried, ground tissue was mixed with 300 μL of β -glucosidase solution to catabolise all its CNgls and release HCN. The HCN reacted with 200 μL of 1 M sodium hydroxide solution to form sodium cyanide, the concentration of which was then measured through mixing 50 μL with 125 μL of 2 M acetic acid, 125 μL of succinimide reagent

and 50 μL of barbituric acid dissolved in pyridine before incubating at room temperature for 15 min. Absorbance was then measured at 590 nm (FLUOstar Galaxy, BMG Labtechnologies, Vic., Australia).

Phenolic concentration was also measured through colorimetric assay in 96-well microtiter plates, by using a Folin–Ciocalteu method adapted from Ainsworth and Gillespie (2007) and Bärlocher and Graça (2020). Phenolics were extracted from 10 mg of dried, ground tissue into 1 mL of 70% acetone solution. 20 μL of sample was then mixed with 20 μL distilled water, 20 μL dilute Folin–Ciocalteu reagent and 200 μL of 2% sodium carbonate in 0.1 M sodium hydroxide solution, before being incubated at room temperature for 2 h. Absorbance was then measured

$$\text{Plant [Si]} = \frac{((\text{leaf [Si]} \times \text{leaf biomass} \div 100) + (\text{sheath [Si]} \times \text{sheath biomass} \div 100) + (\text{root [Si]} \times \text{root biomass} \div 100))}{\text{plant biomass}} \times 100$$

Statistical analysis

Within species, variation in phenotypic traits among accessions was analysed using one-way ANOVAs. Where significant variation was found, *post hoc* comparisons were made using Tukey's tests. Covariance of traits with aridity was analysed using linear regression, by using aridity index as the explanatory variable and the mean trait value for each accession as the response variable. Slope and R^2 were calculated to give the direction and strength of relationships. Linear regression results are presented in Table S2. For all analyses, $P < 0.05$ signified statistical significance. Where raw data were not normally distributed, data were log-transformed to reduce skewness. ANOVA and linear regression analyses were performed using GraphPad Prism ver. 9.0.2 (GraphPad Software, San Diego, CA, USA).

Similarity between suites of phenotypic traits among accessions was analysed using principal-component analysis followed by K-means cluster analysis in R ver. 4.0.0 (R Core Team 2020), using accession means for specific leaf area, herbivory tolerance traits, and the most biologically relevant chemical defence metrics collected (plant silicon concentration, root HCN potential and leaf phenolic concentration). Mean trait values were transformed to z-scores (mean = 0, s.d. = 1) and the silhouette method was used to determine the optimal number of clusters (six) on the basis of having the highest average silhouette width (Kaufman and Rousseeuw 2009).

Genetic analysis

Genotypes were compared using SNPs obtained through DArTseq™ (Jaccoud *et al.* 2001; Kilian *et al.* 2012). Leaf samples were collected from five plants of each study accession and freeze dried. Samples were sent to Diversity Arrays Technology Pty Ltd (Bruce, ACT, Australia) for DNA

at 760 nm (FLUOstar Galaxy, BMG Labtechnologies, Vic., Australia). Gallic acid (91215, Sigma-Aldrich, MO, USA) was used as the standard.

Silicon concentration ([Si]) was measured in leaf, sheath and root tissue by using 80 mg dried, ground tissue through X-ray fluorescence spectrometry (Epsilon-3x, PANalytical, Netherlands), following Reidinger *et al.* (2012) and Hiltbold *et al.* (2016), at the Hawkesbury Institute for the Environment, Western Sydney University (NSW, Australia). Silicon was calibrated against a certified plant reference material of known silicon concentration (NCS ZC73018 *Citrus* leaves, China National Institute for Iron and Steel). Plant silicon concentration (plant [Si], %) was calculated using the following equation:

extraction and genotyping. The raw data comprised 169 228 codominant, genome-wide, biallelic SNPs. Data filtering was performed using the R package 'DARTR' (ver. 2.9.7) (Gruber *et al.* 2018), following a method adapted from Gruber *et al.* (2019). First, we removed loci with less than 99% reproducibility. Then we removed monomorphic loci and loci with call rates below 95%. Last, we removed all but one SNP locus from each sequenced fragment. Our final dataset, which was used for analysis, contained 10 159 SNP loci with 1.64% missing data. We analysed population structure by running a principal-coordinate analysis (PCoA) in DARTR, as well as estimating pairwise F_{ST} (Weir and Cockerham 1984) in the R package 'HIERFSTAT' (Goudet 2005).

Results

Biochemical variation among accessions

In all study accessions, excepting P1, T5, T6 and T7, mean HCN potential was higher in the roots than in the leaves or sheaths. Within accessions, mean HCN potential often varied among different organs across several orders of magnitude. For example, in *S. plumosum* accession P4, mean leaf, sheath and root HCN potentials were 1.20 $\mu\text{g g}^{-1}$, 2.59 $\mu\text{g g}^{-1}$ and 119.91 $\mu\text{g g}^{-1}$ respectively. Mean leaf HCN potential ranged from 0.57 $\mu\text{g g}^{-1}$ in T5 to 5.03 $\mu\text{g g}^{-1}$ in S3; mean sheath HCN potential ranged from 0.48 $\mu\text{g g}^{-1}$ in S1 to 53.23 $\mu\text{g g}^{-1}$ in P1; and mean root HCN potential ranged from 11.96 $\mu\text{g g}^{-1}$ in T5 to 119.91 $\mu\text{g g}^{-1}$ in P4 (Fig. 2). Root HCN potential significantly varied among *S. plumosum* accessions, but there was no significant co-variation between this trait and aridity. There was no significant variation in root HCN potential among accessions of *S. stipoides* or *S. timorensis* (Table S2).

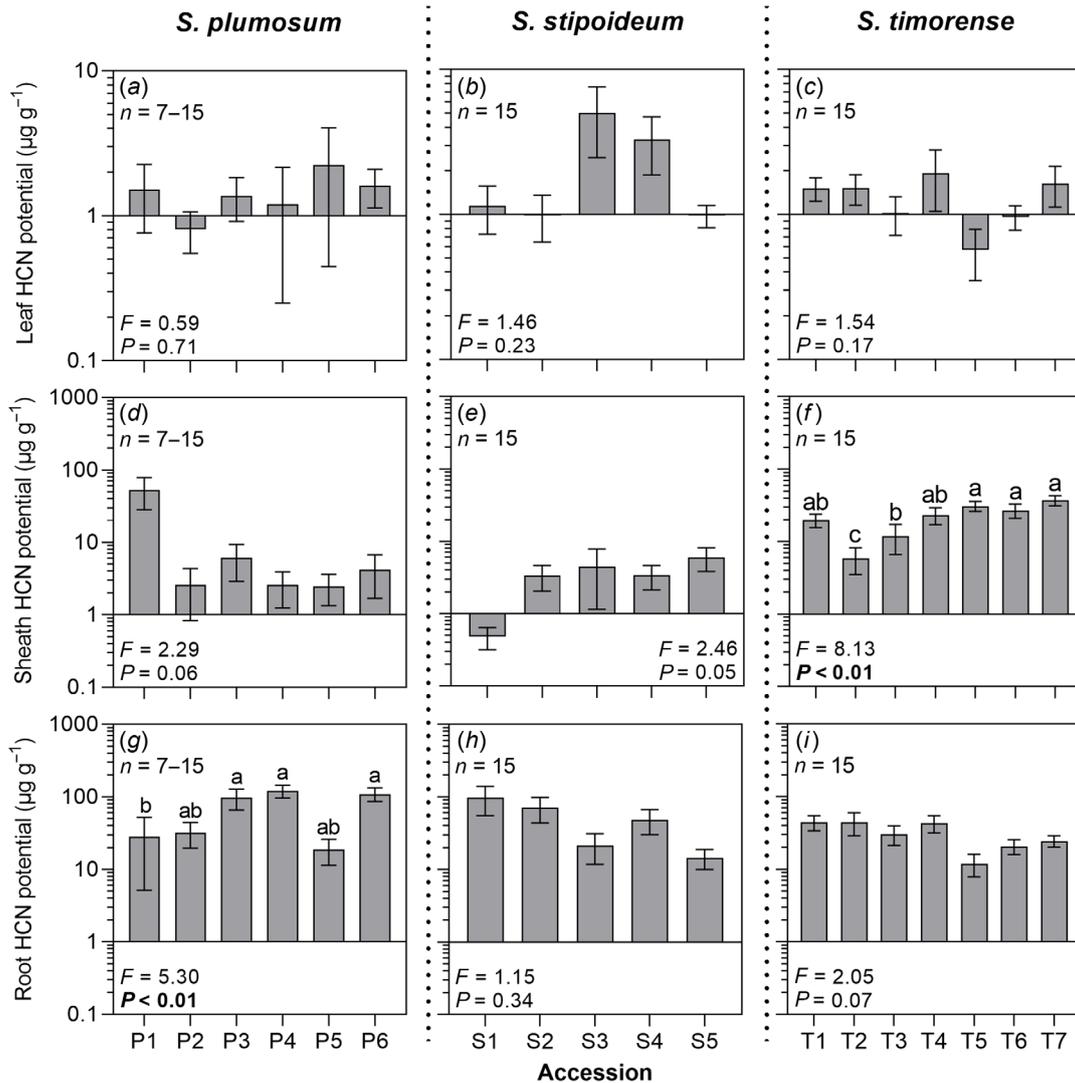


Fig. 2. HCN potential (HCNp) in the (a–c) leaves, (d–f) sheaths, and (g–i) roots in six *S. plumosum* accessions, five *S. stipoides* accessions and seven *S. timorensis* accessions. Plants were grown for 10 weeks in standardised greenhouse conditions. Data were analysed by one-way ANOVA using log-transformed data. *F*- and *P*-values are presented, with significant *P*-values in bold. Columns with different letters are significantly ($P < 0.05$) different. Values are means \pm s.e.

Leaves had higher concentrations of phenolics than did the sheaths or roots in all study accessions, ranging from 13.72 mg g⁻¹ in S1 to 32.38 mg g⁻¹ in P1 (Table S3). Leaf phenolic concentration significantly varied among accessions in all three study species (Fig. 3). However, the relationship between aridity and phenolic concentration was inconsistent across organs and species, with significant positive correlations found in the roots of *S. plumosum* and leaves of *S. stipoides*, and a significant negative correlation found in the sheaths of *S. timorensis* (Table S2).

Mean plant silicon concentration ranged from 0.35% in T6 to 0.64% in P1 (Fig. 3, Table S4). Plant silicon concentration significantly varied among *S. plumosum* accessions. Low-aridity accession P1 had the highest concentration, but

there was no significant variation among the remaining accessions or significant linear correlation with aridity. In *S. stipoides*, plant silicon concentration significantly decreased as aridity increased (Table S2). There was no significant difference in plant silicon concentration among accessions of *S. timorensis*.

Morphological variation among accessions

Mean biomass ranged from 6.5 g in P1 to 18.8 g in P3. Mean relative growth rate ranged from 0.094 g g⁻¹ day⁻¹ in S2 to 0.117 g g⁻¹ day⁻¹ in P4. Both biomass and relative growth rate significantly varied among accessions in *S. plumosum* and *S. stipoides*, but neither significantly co-varied with

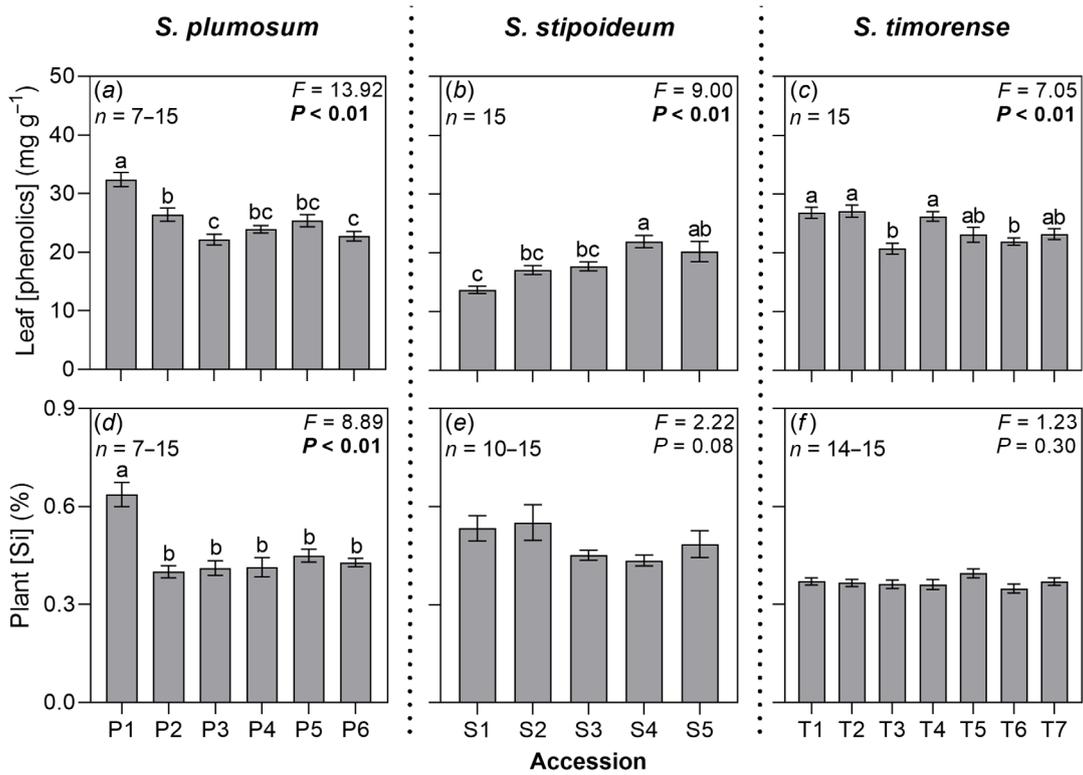


Fig. 3. (a–c) Leaf phenolic concentrations and (d–f) plant silicon concentrations in 18 accessions of three wild *Sorghum* species grown for 10 weeks in a common garden. Accessions are labelled according to Table I. Data were analysed by one-way ANOVA. *F*- and *P*-values are presented, with significant *P*-values in bold. Columns with different letters are significantly (*P* < 0.05) different. Values are means ± s.e.

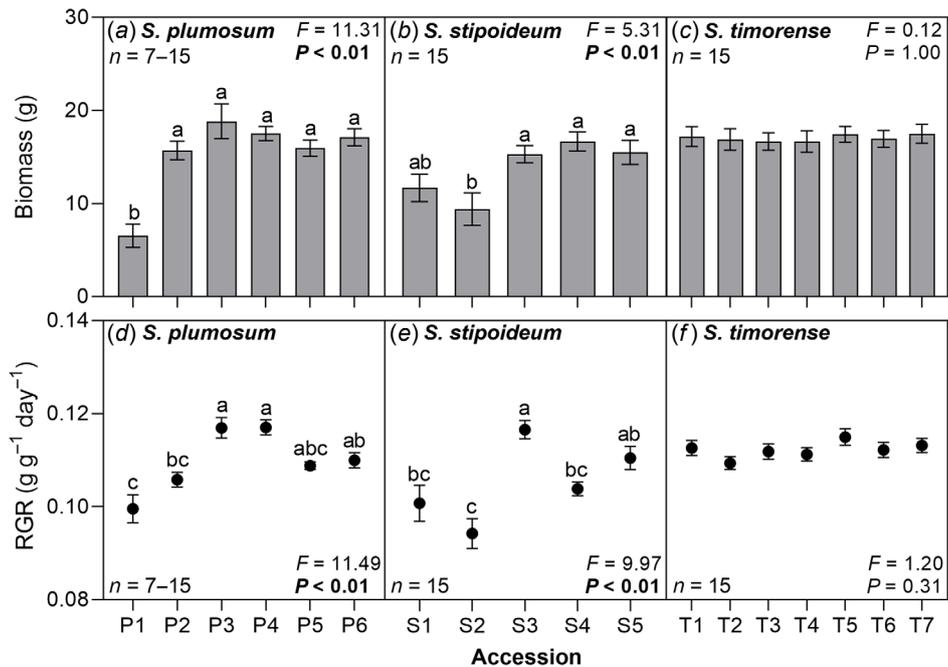


Fig. 4. (a–c) Whole plant biomass and (d–f) relative growth rate (RGR) in 18 accessions of three wild *Sorghum* species grown for 10 weeks in a common garden. Accessions are labelled according to Table I. Data were analysed by one-way ANOVA. *F*- and *P*-values are presented, with significant *P*-values in bold. Columns or points with different letters are significantly (*P* < 0.05) different. Values are means ± s.e.

aridity. No significant variation in either trait was found in *S. timorensis* (Fig. 4).

Mean specific leaf area ranged from 83.5 cm² g⁻¹ P4 to 197.4 cm² g⁻¹ in T7. Specific leaf area significantly varied among *S. plumosum* accessions, but there was no significant co-variation with aridity. There was no significant variation in specific leaf area among accessions of *S. stipoides* or *S. timorensis*. Mean number of tillers was generally high, ranging from 23.6 in P3 to 95.4 in S1. Significant variation in number of tillers was found among accessions in all three study species. However, this trait did not significantly co-vary with aridity in *S. plumosum* or *S. stipoides*. In *S. timorensis*, significant positive correlation was found (Table S2). Mean root:shoot ratio ranged from 0.11 in T7 to 0.32 in P6. Significant variation in root:shoot ratio was found among accessions in *S. plumosum* and *S. timorensis*, but not *S. stipoides*. In *S. plumosum*, root:shoot ratio had a significant positive correlation with aridity, whereas in

S. timorensis a significant negative correlation was found (Table 2, Table S2).

Variation in suites of phenotypic traits among accessions

The K-means cluster analysis separated the 18 study accessions into six clusters according to phenotype. No species had all its accessions grouped into a single cluster, and three clusters contained accessions of multiple species (Fig. 5). Clusters 2 and 5 collectively contained every *S. timorensis* accession and were both characterised by not varying greatly from the mean for any parameter, compared with other clusters. Cluster 6 contained the high-aridity *S. plumosum* accessions P4 and P6 and was characterised by high root HCN potentials and root:shoot ratios, and low specific leaf areas and numbers of tillers. Low-aridity accessions P1 and S2 formed Cluster 1, characterised by high

Table 2. Specific leaf area (SLA), number of tillers and root:shoot ratio in 18 accessions of three wild *Sorghum* species grown for 10 weeks in a common garden. Bold *P*-values indicate significant intraspecific variation among accessions.

Species	Accession	SLA (cm ² g ⁻¹)	Number of tillers	Root:shoot ratio
<i>S. plumosum</i>	P1	150.0 ± 17.2a	52.1 ± 7.7ab	0.14 ± 0.02c
	P2	112.5 ± 12.4ab	56.8 ± 6.6a	0.24 ± 0.01ab
	P3	125.2 ± 8.0ab	23.6 ± 1.7c	0.19 ± 0.02bc
	P4	83.5 ± 8.3b	33.5 ± 3.5bc	0.27 ± 0.01ab
	P5	146.2 ± 11.6a	35.3 ± 1.6bc	0.31 ± 0.03ab
	P6	103.9 ± 8.6ab	32.3 ± 2.2c	0.32 ± 0.03a
	<i>F</i>	4.85	8.22	8.13
	<i>P</i>	<0.01	<0.01	<0.01
<i>S. stipoides</i>	S1	180.4 ± 16.1	95.4 ± 16.9a	0.18 ± 0.02
	S2	134.2 ± 11.9	56.1 ± 11.6ab	0.13 ± 0.01
	S3	164.3 ± 16.1	53.8 ± 7.7ab	0.14 ± 0.01
	S4	146.1 ± 12.3	39.7 ± 5.3b	0.18 ± 0.02
	S5	188.0 ± 28.5	58.2 ± 8.8ab	0.15 ± 0.02
	<i>F</i>	1.84	3.67	2.10
	<i>P</i>	0.12	0.01	0.09
	<i>S. timorensis</i>	T1	174.9 ± 11.4	28.0 ± 2.9d
T2		175.8 ± 16.6	32.5 ± 5.3cd	0.21 ± 0.02a
T3		174.8 ± 22.4	44.7 ± 3.6bcd	0.21 ± 0.02a
T4		178.9 ± 20.5	32.1 ± 4.4cd	0.18 ± 0.02a
T5		187.0 ± 22.9	53.5 ± 6.2ab	0.14 ± 0.01ab
T6		170.6 ± 15.2	48.0 ± 4.6abc	0.17 ± 0.02ab
T7		197.4 ± 14.8	67.1 ± 3.7a	0.11 ± 0.01b
<i>F</i>		0.26	9.58	7.36*
<i>P</i>	0.95	<0.01	<0.01	

Accessions are labelled according to Table 1. Values are means ± s.e.

For all accessions, *n* = 10–15, except for P1 (*n* = 9), P2 (*n* = 11–12), P3 (*n* = 11–13) and P5 (*n* = 7). Asterisks after *F*-values depict ANOVAs for which data were log-transformed.

Values within a column and within a species followed by the same letter are not significantly different at *P* = 0.05.

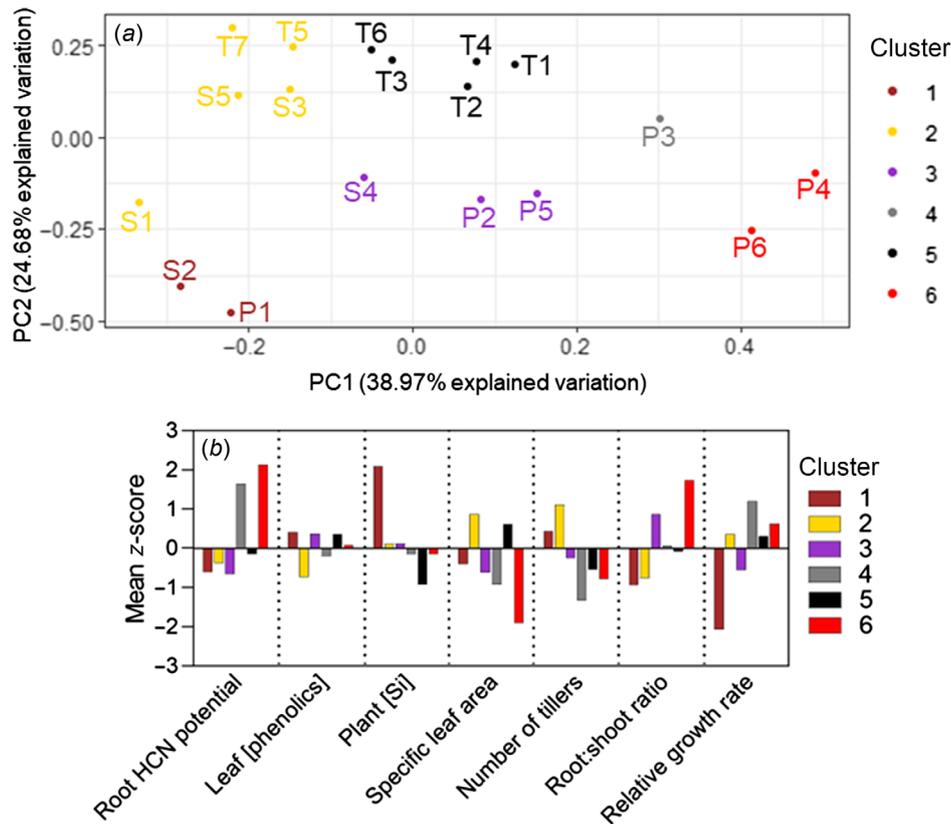


Fig. 5. K-means cluster analysis of seven phenotypic traits from 18 accessions of three wild *Sorghum* species grown for 10 weeks in a common garden. (a) Cluster analysis results plotted on the first two principal-component (PC) axes and coloured according to cluster. Accessions are labelled according to Table 1. (b) Bar graph showing clusters' mean z-scores for each trait.

silicon and low relative growth rates. Cluster 4 contained only accession P3, characterised by a high root HCN potential, and a low number of tillers and specific leaf area. Cluster 3 contained the remainder of the *S. plumosum* and *S. stipoides* accessions. Like in Clusters 2 and 5, the mean z-scores in Cluster 3 did not vary greatly from the overall mean for any parameter (Fig. 5).

Genotypic variation among accessions

The genotype PCoA separated the 18 accessions into four major clusters across the first two principal components; all *S. stipoides* accessions formed one cluster; all *S. plumosum* accessions formed one cluster; accessions T1–4 formed a cluster; and accessions T5–7 formed the final cluster (Fig. 6a). Across the third and fourth principal components, the *S. timorensis* accessions split into the same clusters, but S1 and S2 formed a separate cluster from the other *S. stipoides* accessions, and P1 separated from the *S. plumosum* cluster (Supplementary Fig. S1). The pairwise F_{ST} comparisons showed similar patterns. Every *S. plumosum* accession's highest F_{ST} comparison arose when compared with P1 (Fig. 6b). Similarly, all *S. stipoides* accessions other

than S1 gave their highest F_{ST} value when compared with S2 (Fig. 6c). In *S. timorensis*, there were low F_{ST} values when comparing any two accessions within the T1–4 and T5–7 clusters, and high F_{ST} values when comparing any two accessions from different clusters (Fig. 6d).

Discussion

Morphological variation

Several morphological traits varied intraspecifically, but it was difficult to identify consistent trends between these traits and aridity. In general, plants had large numbers of tillers (ranging from 23.6 to 95.4), consistent with the finding of Lazarides *et al.* (1965) where unburnt *S. plumosum* populations had up to 60 tillers per plant in the wild. Number of tillers significantly increased with aridity in *S. timorensis*, but not in the other two species. Root:shoot ratio decreased as aridity increased in *S. timorensis*, but in *S. plumosum* it increased with aridity.

Although morphological variation has been found among accessions from different provenances in other grass species

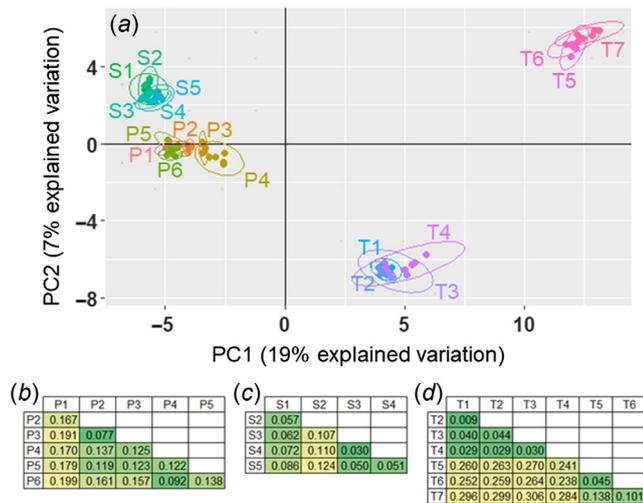


Fig. 6. (a) Principal-coordinates analysis of single nucleotide polymorphisms from 18 accessions of three wild *Sorghum* species, plotted on the first two principal-component (PC) axes. Accessions are colour coded and encompassed by ellipses. (b–d) Pairwise F_{ST} comparisons among study accessions within (b) *S. plumosum*, (c) *S. stipoides* and (d) *S. timorensis*. Numbers are in colour-coded cells, with greener and yellower shades signalling lower and higher F_{ST} values respectively. Accessions are labelled according to Table 1.

(Guo et al. 2017), this variation can become more visible when accessions are grown in a range of environmental conditions. For example, Quiroga et al. (2013) grew two accessions of *Trichloris crinita* in drought and well-watered conditions, finding that morphology did not significantly differ between accessions when grown under well-watered conditions, but did differ when grown under drought conditions. The next steps in quantifying local adaptations in wild sorghums should therefore include assessments of plasticity, such as surveys of wild plants, reciprocal transplant experiments, and growth of accessions in multiple conditions.

Cyanogenic glucoside storage consistent with role in nitrogen metabolism

In 14 of the 18 study accessions, mean HCN potential was higher in roots than in aboveground organs, mirroring patterns found in other wild sorghum species (Cowan et al. 2020; Myrans et al. 2021). CNglcs being stored away from the organs most vulnerable to herbivore attack, and at low concentrations, suggests that defence might not be the primary function of CNglcs in these species, at least when mature. The consistency of low aboveground HCN potentials in Australian wild sorghums, regardless of treatment or provenance (Cowan et al. 2020; Myrans et al. 2021), highlights their potential utility in development of safer forage sorghum suited to a range of environments.

It has been proposed that CNglcs could serve as a store of reduced nitrogen on top of their defensive role (Møller 2010),

and storage of CNglcs in the roots would reduce nitrogen loss in the event of herbivory or fire. The nitrogen stored in these CNglcs could then be remobilised for use in compensatory growth following biomass losses (Selmar et al. 1988; Zagrobelny et al. 2008). It is also possible that root CNglcs could defend these plants against root herbivores such as nematodes (Curto et al. 2012; Moore and Johnson 2017). However, it is unclear whether their HCN potentials are high enough to hinder root herbivores, especially given that many non-ephemeral herbivores in Australia's arid zones are highly specialised and can tolerate the chemical defences of their prey plants (Smith and Morton 1990).

While root HCN potential significantly varied among *S. plumosum* accessions, there was again no clear relationship between this trait and aridity. There was no significant variation in root HCN potential among accessions of *S. stipoides* or *S. timorensis*. These results contrast those of Kooyers et al. (2014), who found that the likelihood of CNglc production in *T. repens* grown in standardised conditions was significantly higher in accessions from more arid provenances. In *T. repens*, CNglc production is a binary trait controlled by one gene, whereas in wild sorghums it is a complex trait controlled by many genes and the environment. HCN potential is therefore a more plastic trait in wild sorghums, making relationships between HCN potential and environmental conditions harder to characterise.

More targeted research into CNglc functions in wild sorghums is needed before we can outline the selection pressures that are likely to be acting on HCN potential (Gleadow et al. 2021). One possibility is to measure HCN potential in plants grown under a range of temperatures or watering regimes so as to quantify the interactive effects of genotype and the environment and assess the plasticity of accessions grown within and outside their optimal environmental conditions.

Concentrations of phenolics and silicon

It was unexpected that the relationship between phenolic concentrations and aridity would vary among species. In the roots of *S. plumosum* and leaves of *S. stipoides*, phenolic concentrations increased with aridity, whereas in the sheaths of *S. timorensis*, phenolic concentration decreased as aridity increased, with no significant relationships detected in other organs. In contrast, Carvalho et al. (2022) found that arid-adapted *Arctotheca calendula* populations had consistently higher leaf phenolic concentrations, and proposed that this could mitigate heat and drought stress. Our result further highlighted the difficulty of predicting plant defence traits on the basis of environmental parameters alone. Like CNglcs, phenolics are thought to play multiple roles in plant metabolism, including defence against herbivores and stress mitigation (Lattanzio et al. 2012). These multiple roles can confound relationships with the environment, making trends difficult to identify.

Plant silicon concentration significantly varied among accessions in *S. plumosum*, with its low-aridity accession, P1, having the highest concentration. In *S. stipoides*, plant silicon concentration significantly decreased with an increasing aridity. High silicon concentrations might be especially beneficial as a defence in tropical, rainy environments, where there are typically higher concentrations of insect herbivores (Ryalls *et al.* 2018). Plant silicon concentrations in this study were comparable to those found previously in both domesticated and wild *Sorghum* taxa (Simpson *et al.* 2017; Myrans *et al.* 2021).

Aridity, phenotype and genotype did not consistently covary

Of the 10 phenotypic traits we tested, *S. plumosum* showed significant intraspecific variation in eight, *S. stipoides* in four, and *S. timorensis* in four, supporting our expectation that *S. plumosum* would display the most phenotypic variation of the study species. This result could have stemmed from *S. plumosum* covering a broader distribution than the other study species (Myrans *et al.* 2020). Despite this, significant linear relationships between traits and aridity were not common in *S. plumosum*, and were no more common than in the other two study species, implying that other abiotic and biotic factors might be more important than aridity for *S. plumosum*'s high phenotypic diversity. Further research into the study accessions would also allow us to better estimate the aridity of provenances during their growing seasons, rather than using an annual average.

No species had all its accessions grouped into a single cluster in the K-means cluster analysis, and the three largest clusters all contained accessions of multiple species. This suggests that not all accessions within species had the same suites of phenotypic traits and could indicate some intraspecific local adaptation. Moreover, there were occasionally more similarities between accessions from different species than within a species. Evidence of phenotypic similarity not necessarily reflecting genetic relatedness has also been found in *Asclepias* L., implying the occurrence of convergent evolution (Agrawal and Fishbein 2006).

Sorghum timorensis was the only study species to be split over two clusters across the first two principal components of the genotype PCoA, and it had the greatest differences in F_{ST} values among accessions. These results suggest that there is greater genotypic variation among study accessions in *S. timorensis* than among those of the other two species, and implies the existence of a partial reproductive barrier between accessions T1–4 and T5–7. The finding was surprising considering that *S. timorensis* is considered the most habitat-specific of the study species (Lazarides *et al.* 1991), and that T6 was separated from T5 and T7 in the phenotype cluster analysis despite their similar genotypes.

The cause of the restricted gene flow between accessions T1–4 and T5–7 is not yet known, with no obvious

geographical barriers between the groups, and no immediately evident differences in soil type (Table 1), geology or hydrology (Boone *et al.* 2022). More extensive genotyping across the species' range may help us better understand its genetic differentiation. The relative phenotypic similarity of T6 to T1–4 could have resulted from the habitat and soil similarity across *S. timorensis*'s range, preventing phenotypic differentiation from being necessary.

The disequilibrium between genotypic and niche diversity in our study accessions challenges broader assumptions often used in conservation, such as that made by Myrans *et al.* (2020), that broader geographic distributions confer greater phenotypic and genotypic diversity in wild species. One potential explanation is that restricted gene flow is crucial for the creation of local adaptation (Kawecki and Ebert 2004), meaning that the largely contiguous distributions of *S. plumosum* and *S. stipoides* might allow sufficient gene flow to prevent sizeable local adaptation, as evidenced by their relative genetic similarity among accessions. Going forward, investigation into phenotypic and genotypic differentiation of *S. plumosum* and *S. timorensis* populations in Indonesian islands, compared with those in mainland Australia, could provide further information on the role of gene flow restriction in differentiation in wild sorghums.

Conclusions

With respect to our hypotheses, we found that (1) there was variation in root HCN potential within *S. plumosum*, but no clear trend between this trait and aridity; (2) *S. plumosum* contained the most phenotypic variation, but *S. timorensis* contained the most genotypic variation; and (3) there was evidence of accessions of different species from similar environments being more phenotypically similar than accessions of the same species. Although two of our hypotheses were partially supported by our results, none was fully supported, demonstrating the complexity of the relationships among phenotype, genotype and the environment. There was evidence of local adaptation, with phenotypic traits varying interspecifically, although aridity did not appear to be the strongest selection pressure on this variation. In the case of *S. timorensis*, there appeared to be genotypic differentiation as a result of underlying geology, but this was not translated into phenotypic differences, possibly as a result of similar soils and climatic conditions at all collection sites.

Our findings represent important progress in determining the roles of, and selection pressures acting on, cyanogenic glucoside production in wild sorghums. They also demonstrated the need to consider intraspecific diversity in all future wild sorghum research, because it adds a fundamental, but often overlooked, complexity to species' adaptation to the environment. Finally, our results highlighted the importance of conserving maximal genetic diversity of wild species, some

of which might not be immediately obvious on the basis of phenotype or provenance alone.

Supplementary material

Supplementary material is available [online](#).

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare no conflicts of interest.

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