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Characterization of phenology, physiology, morphology and biomass traits across a broad Euro-Mediterranean ecotypic panel of the lignocellulosic feedstock *Arundo donax*

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**Abstract**

Giant reed (*Arundo donax* L.) is a perennial rhizomatous grass, which has attracted great attention as a potential lignocellulosic feedstock for bioethanol production due to high biomass yield in marginal land areas, high polysaccharide content and low inhibitor levels in microbial fermentations. However, little is known about the trait variation that is available across a broad ecotypic panel of *A. donax* nor the traits that contribute most significantly to yield and growth in drought prone environments. A collection of 82 ecotypes of *A. donax* sampled across the Mediterranean basin was planted in a common garden experimental field in Savigliano, Italy. We analysed the collection using 367 clumps representing replicate plantings of 82 ecotypes for variation in 21 traits important for biomass accumulation and to identify the particular set of ecotypes with the most promising potential for biomass production. We measured morpho-physiological, phenological and biomass traits and analysed causal relationships between traits and productivity characteristics assessed at leaf and canopy levels. The results identified differences among the 82 ecotypes for all studied traits: those showing the highest level of variability included stomatal resistance, stem density (StN), stem dry mass (StDM) and total biomass production (TotDM). Multiple regression analysis revealed that leaf area index, StDM, StN, number of nodes per stem, stem height and diameter were the most significant predictors of TotDM and the most important early selection criteria for bioenergy production from *A. donax*. These traits were used in a hierarchical cluster analysis to identify groups of similar ecotypes, and a selection was made of promising ecotypes for multiyear and multisite testing for biomass production. Heritability estimates were significant for all traits. The potential of this ecotype collection as a resource for studies of germplasm diversity and for the analysis of traits underpinning high productivity of *A. donax* is highlighted.
1 | INTRODUCTION

Giant reed (Arundo donax L.) is a perennial rhizomatous grass, which has attracted great attention as a potential lignocellulosic biomass due to rapid growth, high apparent productivity and adaptability to marginal land conditions combined with low input requirements (Angelini, Ceccarini, & Bonari, 2005; Angelini, Ceccarini, Nassi o Di Nasso, & Bonari, 2009; Corno, Pilu, & Adani, 2014; Lewandowski, Scurluck, Lindvall, & Christou, 2003; Pilu, Bucci, Badone, & Landoni, 2012). It is distributed throughout the Mediterranean climate zones and has been cultivated mostly in Asia and southern Europe. After establishment and from the second year on, the above-ground biomass can be harvested annually for almost 15–30 years (Ceotto et al., 2013; Pilu, Manca, & Landoni, 2013). A. donax lignocellulosic biomass feedstock can be readily used as solid biofuel in direct combustion for heat generation, or for bioethanol production from alcoholic fermentation of lignocellulose material (Pilu et al., 2012; Silva, Schirmer, Maeda, Barcelos, & Pereira, 2003; Pilu, Bucci, Badone, & Landoni, 2012). Based on a comprehensive utilization indexes obtained from an evaluation system that takes into consideration numerous factors deemed essential for the conversion of lignocellulosic biomass to bioethanol, a recent study revealed that A. donax is a highly promising lignocellulosic feedstock for producing bioethanol compared to corn stalks, switchgrass, pennisetum and silvergrass (Kou, Song, Zhang, & Tan, 2017). Moreover, A. donax has been used as a potential feedstock for thermo-chemical conversion to biochar, bio-oil and gas for energetic purposes (Saikia, Chutia, Kataki, & Pant, 2015). A. donax cropping system is important not only because it provides farmers with an innovative opportunity but also for mitigating greenhouse gas (GHG) emissions, controlling soil erosion and phytoremediating contaminated soils (Cosentino, Copani, Patanè, Mantimeo, & D’Aosta, 2008; Hardion, Verlaque, Saltonstall, Leriche, & Vila, 2012; Mariani et al., 2010; Pilu et al., 2013). Furthermore, A. donax is a resilient and very productive species. It has been shown that the photosynthetic capacity of A. donax in full sunlight is high compared to other C3 species, and comparable to C4 bioenergy grasses (Rossa, Tüffers, Naidoo, & von Willert, 1998). Because of these advantages, A. donax is expected to play a major role in the provision of perennial lignocellulosic biomass across much of Europe. However, successful commercialization of renewable biofuel production depends on the sustainability and biomass yield of lignocellulosic crops.

Field trials of A. donax in central Italy showed an average yield of above-ground biomass of about 40 Mg dry matter (DM) ha$^{-1}$ yr$^{-1}$, with a peak of about 50 Mg DM/ha at the end of the second and third years of growth over a production cycle of 10 years (Angelini et al., 2009). Similarly, in a multi-year study in Alabama, A. donax achieved an average yield of 35.5 Mg DM ha$^{-1}$ yr$^{-1}$ (Ping, Bransby, & van Santen, 2014). However, biomass yield can be doubled at the end of the same growth cycles in northern Italy under fertirrigated conditions (Borin et al., 2013). Biomass yields in these experiments using small plots may be an underestimate of the yields achievable in competitive commercial planting. While increasing yield from the first to the third one-year coppice rotation was often observed in A. donax (Borin et al., 2013; Cosentino, Copani, Patanè, Mantimeo, & D’Aosta, 2008; Pilu et al., 2013). Although the reported biomass yield for A. donax is high under irrigated conditions, the large-scale plantations of this crop remain a major limitation due to the high cost of propagation (Ceotto & Di Candilo, 2010). Biomass yields may also be considerably lower on marginal land. For example, on a sandy loam with limited nutrient availability, A. donax required 3 years to accumulate 20 Mg DM/ha, and build a rhizome mass of 16 Mg DM/ha (Nassi o Di Nasso et al., 2013).

Although A. donax can flower and produce seed under some conditions, as yet there is no evidence that the seeds are viable (Barney & Ditomaso, 2008; Haddadchi, Gross, & Fatemi, 2013; Lewandowski et al., 2003; Mariani et al., 2010; Pilu et al., 2012). Thus, the natural variability in existing populations may occur following spontaneous mutations selected by natural pressure (Cosentino, Copani, D’Aosta, Sanzone, & Mantimeo, 2006). Despite the low genetic diversity revealed in a few studies using molecular markers (Ahmad, Liow, Spencer, & Jasieniuk, 2008; Hardion, Verlaque, Baumel, Juin, & Vila, 2012; Hardion, Verlaque, Saltonstall, Leriche, & Vila, 2014; Mariani et al., 2010; Pilu et al., 2014), inheritable phenotypic differences have been described in growth-related traits (Cosentino et al., 2006; Pilu et al., 2014). For this reason, ecotype selection remains important for genetic improvement of A. donax (Corno et al., 2014; Pilu et al., 2013). Further, it is important that traits which contribute to harvestable biomass yield in grasses such as A. donax are well understood in order to drive genetic gain for accelerated domestication as discussed for other biomass...
crops (Robson et al., 2013). A thorough understanding of the interactions between traits, both positive and negative correlations, is required because selection for one trait can have profound effects on another (Farrar et al., 2012).

Diversity in growth-related traits has been reported using ecotypes collected from regions within the Mediterranean basin (Cosentino et al., 2006; Sánchez et al., 2015) including some studies of ecotypes from a wider range of provenances (Amaducci & Perego, 2015; Pilu et al., 2014); however, a comprehensive study of different parameters that may interact with yield (i.e. phenology, reed morphology, leaf functional traits and physiology) in a large panel of *A. donax* ecotypes collected from diverse environments has not been previously reported.

This study aimed: (a) to characterize a broad Euro-Mediterranean panel of 82 *A. donax* ecotypes by assaying 21 biomass, morphology, physiology and phenology traits in a common garden experiment and to understand how these may correlate to each other; (b) to identify traits that may have contributed to differences in biomass production in field conditions; and (c) to select potential high yielding clusters through a multivariate approach. This study design therefore also serves as a key resource for parametrizing biomass production models of *A. donax*, an emerging lignocellulosic crop that would ultimately be an important contributor to any substantive EU renewable energy strategy that is based on lignocellulosic biomass supply.

## 2 | MATERIALS AND METHODS

### 2.1 | Collection of plant material

From 2006 to 2010, eighty-two Euro-Mediterranean ecotypes of *A. donax* were collected for use in the study. To avoid clonal ramets collection, sites were at least 4 km and at most 474 km apart (mean distance = 75 km) (Figure 1). The local geography of collection sites included riverbanks and riverbeds of the Mediterranean basin, uncultivated fields and marginal areas. At each site rhizomes were collected from a single clump that was considered to be a distinct ecotype.

### 2.2 | Experimental site

In May 2011, rhizomes were planted in a common garden experiment at the experimental field of the “Alasia Franco Vivai,” Savigliano in northern Italy (SAV, 44°36′N, 07°38′E, 340 m above sea level). The soil is alluvial with sandy loam texture. The regional climate is fully humid and warm temperate with warm summers, according to the Köppen-Geiger climate classification system (Kottek, Grieser, Beck, Rudolf, & Rubel, 2006). The average annual precipitation and temperature was 848.2 mm and 10.5°C, respectively. During this study, spring was wetter and colder, and summer was drier than the 5-year seasonal average (Figure 2). During the sampling period and specifically from 19 July to 23 August, the weather was extremely dry and warm, with a daily average maximum temperature of 29.4°C and 12 mm of accumulated rainfall (Figure 2) as assessed by a weather station located 1 km from the common garden experimental field.

### 2.3 | Experimental design

Each ecotype was planted in a 9 m² plot (3 × 3 m) using five homogeneous rhizomes planted in 2 × 2 m spacing with one rhizome in the centre of that space (quincunx system of planting). Plots were harvested at the end of each
growing season. In 2011 and 2012, the site was fertilized and irrigated by furrowing every 20 days while no irrigation water or fertilizer were applied during the 2013 growing season. During the 3 years, no serious incidents of insects or diseases were observed, and no pesticides or fungicides were applied.

2.4 | Phenotypic data collection

Arundo donax ecotypes start the maturity phase by the third one-year coppice rotation (Angelini et al., 2009), making phenotypic comparisons more meaningful from the third growth year onwards. Data were collected from July to November 2013, and traits and their abbreviations are listed in Table 1. For each ecotype, the main plot was divided into four 1.5 x 1.5 m subplots and three of the subplots were randomly selected as biological replicates for all measurements. Empty subplots where the original rhizomes died after establishment were excluded from the selection. No centre rhizome pieces died therefore the contributions to the subplots were always the same. Consequently, at the beginning of the growing season, the mean number of available planted clumps per ecotype was 4.51 (Supporting information Table S1).

2.4.1 | Phenology

Flowering phenological data were recorded in autumn 2013, from 19 September [day of the year (DOY) 262] to 30 October (DOY 303). The timing of five discrete phenological stages was assessed daily by visual inspection following a new scoring system. In detail, Phase 0 (Ph0)—absence of flowers; Phase 1 (Ph1)—onset of flowering with the appearance of the first small inflorescence at the single-stem level; Phase 2 (Ph2)—the first inflorescence had reached at least 30 cm length; Phase 3 (Ph3)—the first inflorescence was completely open; and according to Cosentino et al. (2006), Phase 4 (Ph4)—50% of the reeds showed completely open inflorescences.

2.4.2 | Physiology

Mid-day stomatal resistance (Rs, s/cm) was measured on July 10th (Rs_july) and August 22nd (Rs_august) with a dynamic diffusion porometer (AP4; Delta-T devices, Cambridge, UK). Data were taken from the abaxial mid-leaf position of three healthy, fully mature and fully expanded leaves (5th from the top) from three randomly chosen reeds per ecotype between 11:00 and 13:00 hr. Before each session of measurement, the porometer was calibrated to field temperature and humidity.

The same leaf previously used for stomatal resistance measurement (Rs_august) was collected, oven-dried at 75°C to constant weight, and stored for subsequent carbon isotope discrimination (\(\Delta\), %e) analysis. Leaves were ground into a homogenous fine powder. Samples were combusted in an elemental analyzer (Thermo Flash EA 1112 Series, Bremen, DE); CO\(_2\) and N\(_2\) were separated by chromatography and directly injected into a continuous-flow Isotope Ratio Mass Spectrometer (Thermo-Finnigan Delta XP, Bremen, DE) for isotope analysis. Peach leaf standards (NIST 1547) were run every six samples. \(^{13}\)C/\(^{12}\)C isotope ratio (\(\delta^{13}\)C) was calculated according to the definitions described by Farquhar, Ehleringer, and Hubick (1989):
\[ \delta^{13}C_{\text{sample}}(\%e) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \]

where \( R_{\text{sample}}/R_{\text{standard}} \) were referred to a Pee Dee Belemnite standard.

And \( \Delta \) was calculated according to Farquhar et al. (1989):

\[ \Delta = (\delta^{13}C_{\text{atm}} - \delta^{13}C_{\text{sample}})/[1 + (\delta^{13}C_{\text{sample}}/1000)] \]

where the \( \delta^{13}C_{\text{atm}} \) was assumed \(-8\%e\).

### 2.4.3 Canopy and leaf morphology

Leaf area index (LAI, m\(^2\)/m\(^2\)) was indirectly calculated in each subplot by measuring incoming photosynthetic active radiation (PAR) below the canopy and in an open area using the AccuPAR LP-80 ceptometer (Decagon Devices, Pullman, WA, USA). Three mature leaves of each ecotype were collected from the middle part of the main axis of the longest reed (stem) in each subplot (Hardion et al., 2012). Leaves were immediately transported to the laboratory and photographed using a digital camera. Before image analysis, pictures were corrected for geometric distortions caused by the camera lens using filters available in Adobe Photoshop Elements 12. Leaf length (LfL, cm), leaf width (LfW, cm) and leaf area (LfA, cm\(^2\)) were then measured using the LAMINA software (Bylesjö et al., 2008). The ratio between LfL and LfW (LfL/LfW) was calculated and represents an independent shape variable that has been used extensively in leaf morphometric analysis (Aravanopoulos,
oven ecotype, two for each selected subplot, were weighed and
(c) 20 cm below the stem’s apex (Da, mm); and fresh weight
ground: (a) 20 cm (Db, mm), (b) at half of H (Dm, mm) and
(d) 20 cm below the stem’s apex (Da, mm); and fresh weight
(Cosentino et al., 2006). Six sub-samples of whole stems per
cotype, two for each selected subplot, were weighed and
oven-dried at 75°C until a constant weight was obtained to
determine the stem dry mass (StDM, g DM) and the total
biomass production (TotDM, Kg DM/m).

2.4.4 Growth and biomass production
To assess growth-related traits and biomass production, all
cotypes were harvested at the end of the growing period in
November 2013, by cutting at 5 cm aboveground level. In
each subplot, the weight of the above-ground fresh biomass
was measured and the number of reeds counted to calculate
stem density (StN, n/m). Five stems were randomly selected,
and the following measurements were performed: height of
the stem (H, cm) from the base-node to the top-node without
the inflorescence (if present); number of nodes per stem
(StNd, n); diameter of the stem in three positions above the
ground: (a) 20 cm (Db, mm), (b) at half of H (Dm, mm) and
(c) 20 cm below the stem’s apex (Da, mm); and fresh weight
Poorter, 2010; Sun et al., 2014). All leaves were then
dried at 75°C until a constant weight was obtained to
determine the specific leaf area (SLA, cm²/g) was determined as the ratio
between LfA and leaf dry weight.

2.5 Statistical analyses
Statistical analyses of the data were performed using R soft-
ware (Version 2.15.2, A Language and Environment Copy-
right, 2012). Original values were tested for transformation
using the Box-Cox procedure (Venables & Ripley, 2002) to
ensure homoscedasticity and normality of residuals. Besides
the determination of means and ranges, the coefficient of
variation (CV), calculated as the ratio of the trait’s standard
deviation to its mean value and reported as a percentage (%),
was used to compare the variability of traits independently of
the units of measure. To test differences among ecotypes and
replicates of each ecotype, variables were analysed using the
analysis of variance (ANOVA) model, as follows:

\[ Y_{ijk} = \mu + E_i + R_j + e_{ijk}, \]

where \( Y_{ijk} \) is the individual value, \( \mu \) is the general mean,
\( E_i \) is the effect of the \( i \)th ecotype (random), \( R_j \) is the effect
of the \( j \)th replicate (fixed) and \( e_{ijk} \) is the residual error term.

With regard to the stomatal resistance, measured at two
time-points, the following model was used to test time \((T)\)
and ecotype by time interaction \((E \times T)\) effects:

\[ Y_{ik} = \mu + E_i + T_k + E_i \times T_k + e_{ik}. \]

In this case, \( T \) was the time effect considered as random,
and \( E \times T \) was the interaction effect considered as random.

Because of the absence of a replicated block design, \( R \) could
not be corrected when significant to eliminate micro-environ-
mental variations, and thus, this source of variation was
removed to make the ANOVA models simpler. When the
analysis of variance showed significant \( E \times T \) interaction
\((P \leq 0.05)\), the change in ranking of ecotypes was evaluated by the Spearman rank coefficient \((p)\) on ecotypic means.

Pearson’s correlation coefficient \((r)\) was used to assess
the relationship among all traits, using mean values for
each ecotype. All statistical tests were considered signifi-
cant at \( P \leq 0.05 \).

Multiple linear regression analysis was used to analyse
traits affecting the amount of dry biomass production and
to select a sub-set of variables to be used for cluster analy-
sis. Hence, the analysis was performed taking into consid-
eration TotDM as dependent variable and all other traits
(except flowering stages for which only non-replicated
measurements were obtainable) as independent variables.
As the residuals have to be normally distributed, the Box-
Cox procedure (Venables & Ripley, 2002) was performed
to search for the most appropriate transformation of the
dependent variables of the linear regression model.

Hierarchical cluster analysis was performed to group eco-
types with similar characteristics on a multivariate basis, and
the Euclidean distance was used to determine the difference
between the groups. The analysis was performed using stan-
dardized values of all traits. Ward’s minimum variance
method was used to construct the clusters, and the actual
number of clusters was discerned using “NbClust” package
in R (Charrad, Ghazzali, Boiteau, & Niknafs, 2013), which
provides most of the popular indices for determining the
optimal number of clusters. ANOVA model was used to test
the null hypothesis that clusters (Clu) were equal, as follows:

\[ Y_{ij} = \mu + Clu_i + e_{ij}, \]

where \( Y_{ij} \) is the individual value, \( \mu \) is the general mean,
\( Clu_i \) is the effect of the \( j \)th cluster (random) and \( e_{ij} \) is the
residual error term. LSD post hoc test was used to compare
significant differences among mean cluster values.

To describe ecotypes with their interrelations and at the
same time to create a graphical display of the clusters, the
“CLUSPLOT” algorithm of Pison, Struyf, and Rousseeuw
(1999) was used. Finally, the dimension of the data was
reduced by principal component analysis (PCA), then
“CLUSPLOT” displayed the bivariate plot of the objects
relative to the first two principal components (components
1 and 2) (Pison et al., 1999).

2.6 Broad-sense heritability
Broad-sense trait heritability on an individual basis \((H^2)\)
was estimated as follows:

[Equations and text continue...]

Because of the absence of a replicated block design, \( R \) could
not be corrected when significant to eliminate micro-environ-
mental variations, and thus, this source of variation was
removed to make the ANOVA models simpler. When the
analysis of variance showed significant \( E \times T \) interaction
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relative to the first two principal components (components
1 and 2) (Pison et al., 1999).

2.6 Broad-sense heritability
Broad-sense trait heritability on an individual basis \((H^2)\)
was estimated as follows:
$H^2 = \frac{\sigma^2_G}{(\sigma^2_G + \sigma^2_e)}$,

where $\sigma^2_G$ and $\sigma^2_e$ are the genetic and residual variance components, respectively, which were obtained using the restricted maximum-likelihood (REML) procedure (Holland, Nyquist, & Cervantes-Martinez, 2003). $H^2$ was evaluated for all the studied traits, except for flowering stages for which replicated measurements were not viable.

3 | RESULTS

3.1 | Univariate analysis

A significant level of phenotypic variation among the 82 different ecotypes of *A. donax* was observed in all physiological, canopy, growth and biomass traits measured (Table 2). Flowering phenology of all ecotypes was recorded using a new scoring system developed in this study that covers the onset of flowering (Ph1) to the end of flower development (Ph4). Flowering was evaluated at plot scale, without replication (Table 1); therefore, statistical differences between ecotypes and blocks for flowering phenology trait could not be evaluated (Table 2). Average flowering time of Ph1 and Ph4 at ecotype level were DOY 265 (22 September) and DOY 284 (11 October), respectively, giving an average of 19 days between Ph1 and Ph4. Transition between the first 3 phases was rapid, for example taking an average of 4 days from Ph1 to Ph2 and from Ph2 to Ph3, while an average of 11 days were required to complete the last flowering phase (Figure 3; Table 2).

### TABLE 2 General means, ecotypic range of variation, coefficient of variation (CV, %) and level of significance of ecotype (E) and replicate (R) effects for phenological, morphological, physiological and growth traits in *Arundo donax*. Broad-sense heritability ($H^2$) for physiological, morphological, growth and biomass traits. See Table 1 for trait abbreviations

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean</th>
<th>Range of variation</th>
<th>CV (%)</th>
<th>E</th>
<th>R</th>
<th>$H^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ph1 (DOY)</td>
<td>265.12</td>
<td>262.00–277.00</td>
<td>1.40</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ph2 (DOY)</td>
<td>269.67</td>
<td>262.00–281.00</td>
<td>2.07</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ph3 (DOY)</td>
<td>273.30</td>
<td>262.00–290.00</td>
<td>2.46</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ph4 (DOY)</td>
<td>284.03</td>
<td>262.00–303.00</td>
<td>4.93</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Physiology</strong></td>
<td></td>
<td></td>
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<tr>
<td>$R_{july}$ (s/cm)</td>
<td>0.96</td>
<td>0.57–1.68</td>
<td>28.20</td>
<td>***</td>
<td>***</td>
<td>0.28</td>
</tr>
<tr>
<td>$R_{august}$ (s/cm)</td>
<td>1.50</td>
<td>0.80–2.69</td>
<td>27.76</td>
<td>***</td>
<td>ns</td>
<td>0.62</td>
</tr>
<tr>
<td>$\Delta$ (%)</td>
<td>20.98</td>
<td>19.43–22.23</td>
<td>2.42</td>
<td>***</td>
<td>ns</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Canopy and leaf morphology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAI (m$^2$/m$^2$)</td>
<td>6.11</td>
<td>3.89–8.14</td>
<td>15.23</td>
<td>***</td>
<td>***</td>
<td>0.46</td>
</tr>
<tr>
<td>LfL (cm)</td>
<td>57.32</td>
<td>48.25–65.30</td>
<td>6.27</td>
<td>**</td>
<td>ns</td>
<td>0.17</td>
</tr>
<tr>
<td>LfW (cm)</td>
<td>5.83</td>
<td>4.61–6.73</td>
<td>8.56</td>
<td>***</td>
<td>ns</td>
<td>0.39</td>
</tr>
<tr>
<td>LfL-LfW</td>
<td>9.90</td>
<td>8.59–11.62</td>
<td>7.50</td>
<td>***</td>
<td>ns</td>
<td>0.30</td>
</tr>
<tr>
<td>LfA (cm$^2$)</td>
<td>209.55</td>
<td>146.34–260.93</td>
<td>12.51</td>
<td>***</td>
<td>ns</td>
<td>0.30</td>
</tr>
<tr>
<td>SLA (cm$^2$/g)</td>
<td>133.36</td>
<td>106.72–176.40</td>
<td>10.23</td>
<td>***</td>
<td>ns</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Growth and biomass production</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotDM (kg DM/m$^2$)</td>
<td>9.20</td>
<td>5.26–15.21</td>
<td>23.18</td>
<td>***</td>
<td>***</td>
<td>0.32</td>
</tr>
<tr>
<td>StDM (g DM)</td>
<td>286.40</td>
<td>183.32–467.82</td>
<td>22.16</td>
<td>***</td>
<td>ns</td>
<td>0.39</td>
</tr>
<tr>
<td>StN (m$^2$/m$^2$)</td>
<td>40.44</td>
<td>21.93–80.00</td>
<td>25.30</td>
<td>***</td>
<td>**</td>
<td>0.54</td>
</tr>
<tr>
<td>StNd (n)</td>
<td>35.12</td>
<td>28.47–46.80</td>
<td>10.30</td>
<td>***</td>
<td>ns</td>
<td>0.51</td>
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<tr>
<td>H (cm)</td>
<td>412.34</td>
<td>290.80–528.33</td>
<td>10.26</td>
<td>***</td>
<td>ns</td>
<td>0.58</td>
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<tr>
<td>Db (cm)</td>
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<td>16.78–26.01</td>
<td>9.88</td>
<td>***</td>
<td>ns</td>
<td>0.51</td>
</tr>
<tr>
<td>Dm (cm)</td>
<td>16.06</td>
<td>12.25–22.64</td>
<td>11.80</td>
<td>***</td>
<td>ns</td>
<td>0.44</td>
</tr>
<tr>
<td>Da (cm)</td>
<td>11.16</td>
<td>8.52–13.91</td>
<td>9.40</td>
<td>***</td>
<td>ns</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Ns: non-significant; nd: $F$ test and $H^2$ not available for phenology traits. 

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
northern Italy. Ecotypic means (black dots) ± SE for Rs measured in (a) July, 10 (Rs\textsubscript{July}) and in (b) August, 22 (Rs\textsubscript{August}) are shown together with the collection mean (horizontal dashed line). (c) Pairwise trait plot between Rs\textsubscript{July} and Rs\textsubscript{August}/Rs\textsubscript{July} ratio. Unique identifiers of each ecotype are written below the dot, except identifiers (approximately 10% for Db, Dm and Da). Similarly, high ecotype differences were observed for StDM and StN, with a slightly higher variation for the latter (22.16% vs. 25.30%, respectively).

3.2 Phenotypic correlations

The linear relationships among traits were studied using Pearson’s correlation analysis (Figure 6). Significant positive r values among flowering phases showed that when the flowering process began, all the other stages followed through a cascade process. Moreover, the date of the latest flowering stage, Ph4, were moderately related to LAI (r = 0.705, P ≤ 0.001) and inversely related to H (r = -
All flowering phases were significantly and positively correlated with Δ, with Ph4 and Δ ($r = 0.489, P \leq 0.001$) showing the highest correlation.

Regarding stomatal resistance, there were few weak and negative relationships between $R_{S_{\text{July}}}$ and $R_{S_{\text{August}}}$ and other traits, such as flowering time, Δ, LAI, Db and Dm, whereas the two traits positively correlated with each other.

LfL and LfW were positively correlated and interestingly, leaf morphology was strongly related to StN, with a higher correlation with LfW ($r = -0.610, P \leq 0.001$) than LfL ($r = -0.144, P = \text{ns}$).

Among growth traits, as might be expected the strongest correlations were detected between StDM and the diameter of the reed, especially Db ($r = 0.851, P \leq 0.001$). TotDM, on the other hand, did not correlate with diameter of the single stem, but significant correlations were found with StN ($r = 0.526, P \leq 0.001$) and H ($r = 0.500, P \leq 0.001$; Figure 6).
3.3 | Cluster analysis

Hierarchical cluster analysis was used to group ecotypes with similar characteristics on a multivariate basis. To reduce the number of variables to be used in the analysis, a sub-set of informative traits, that is LAI, StDM, StN, StNd, H, Db and Dm, were selected by a multiple regression analysis (adjusted determination coefficient, $R^2 = 0.753$, Table 3).
Our results highlighted significant differences among ecotypes for all phenotypic traits studied. Concerning pheno-

logy, our collection included ecotypes with large
Variation in the late flowering stage. Flowering date is considered one of the most important selection criteria when breeding perennial grasses for cellulosic ethanol (Jakob, Zhou, & Paterson, 2009). In addition, flowering phenology and height growth patterns were shown to be significantly associated with functional traits such as relative growth rate, leaf mass per area and hollow ratio in herbaceous grassland species (Sun & Frelich, 2011). Previously Cosentino et al. (2006) scored flowering in 39 ecotypes collected in southern Italy (35 in Sicily region and 4 in Calabria region) and at Ph4. Surprisingly the variability observed in Ph4 reported by Cosentino et al. (2006), which showed a 41 days difference between the two most divergent ecotypes at the end of the second growing season, perfectly mirrors the variability reported here (Figure 3). Perhaps the most interesting is the finding of phenotypic correlations that highlighted a relationship between flowering time and some morphological traits such as H and SLA. Although we compared within-species variation, a statistically significant and positive relationship among these traits has also been observed in previous studies (Bolmgren & Cowan, 2008; Sun & Frelich, 2011), where taller herbaceous grassland and perennial species flowered earlier than shorter ones. As expected, positive correlations between flowering phenology phases and SLA were observed. Both early flowering time and reduced SLA are associated with plants highly adapted to arid environments (Kooyers, 2015). In A. donax, vegetative and reproductive phases often end at the

**Table 4** Mean cluster values and coefficient of variation (CV, %) of phenological, physiological, morphological, and biomass and growth traits in *Arundo donax*. The number of ecotypes in each cluster (Clu) is enclosed by curly brackets. CV was calculated as the ratio of the standard deviation among ecotypic values in the cluster to the mean cluster value, reported as a percentage within round bracket. For each trait, significant differences among clusters (*F* test) are indicated by asterisks. Different letters among clusters are related to LSD post hoc test (*P* ≤ 0.05). See Table 1 for trait abbreviations

<table>
<thead>
<tr>
<th>Trait</th>
<th>F test</th>
<th>Clu.1 {17}</th>
<th>Clu.2 {6}</th>
<th>Clu.3 {25}</th>
<th>Clu.4 {19}</th>
<th>Clu.5 {15}</th>
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<tr>
<td>Ph1 (DOY)</td>
<td>–</td>
<td>266.71 (1.56)</td>
<td>262.00 (0)</td>
<td>263.45 (0.96)</td>
<td>264.57 (1.04)</td>
<td>266.23 (1.64)</td>
</tr>
<tr>
<td>Ph2 (DOY)</td>
<td>–</td>
<td>272.59 (1.52)</td>
<td>265.20 (0.67)</td>
<td>266.33 (1.86)</td>
<td>268.28 (1.79)</td>
<td>273.00 (2.39)</td>
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<tr>
<td>Ph3 (DOY)</td>
<td>–</td>
<td>278.00 (2.28)</td>
<td>268.33 (0.56)</td>
<td>268.41 (1.80)</td>
<td>271.74 (2.02)</td>
<td>279.00 (1.51)</td>
</tr>
<tr>
<td>Ph4 (DOY)</td>
<td>–</td>
<td>296.35 (3.11)</td>
<td>271.60 (1.29)</td>
<td>273.38 (3.84)</td>
<td>284.58 (5.14)</td>
<td>289.86 (2.97)</td>
</tr>
<tr>
<td>Physiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RsJuly (s/cm)</td>
<td>***</td>
<td>0.76 (17.56)b</td>
<td>0.94 (25.01)ab</td>
<td>0.96 (27.44)a</td>
<td>1.07 (31.76)a</td>
<td>1.06 (20.01)a</td>
</tr>
<tr>
<td>RsAugust (s/cm)</td>
<td>***</td>
<td>1.24 (18.34)c</td>
<td>1.29 (12.97)bc</td>
<td>1.63 (26.20)a</td>
<td>1.62 (29.39)a</td>
<td>1.49 (26.90)ab</td>
</tr>
<tr>
<td>Δ (%e)</td>
<td>***</td>
<td>21.26 (1.94)a</td>
<td>20.97 (2.82)abc</td>
<td>20.72 (2.72)c</td>
<td>21.05 (2.45)ab</td>
<td>20.97 (2.03)bc</td>
</tr>
<tr>
<td>Canopy and leaf morphology</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LAI (m²/m²)</td>
<td>***</td>
<td>7.01 (5.98)a</td>
<td>5.11 (12.34)d</td>
<td>5.71 (15.56)cd</td>
<td>5.99 (15.46)bc</td>
<td>6.25 (10.26)b</td>
</tr>
<tr>
<td>LfL (cm)</td>
<td>**</td>
<td>57.05 (6.99)b</td>
<td>56.37 (3.38)b</td>
<td>59.33 (4.93)a</td>
<td>55.85 (7.01)a</td>
<td>56.52 (5.19)b</td>
</tr>
<tr>
<td>LfW (cm)</td>
<td>***</td>
<td>6.05 (6.81)a</td>
<td>5.97 (6.30)a</td>
<td>5.97 (7.75)a</td>
<td>5.87 (7.49)a</td>
<td>5.30 (8.11)b</td>
</tr>
<tr>
<td>LfL_LfW</td>
<td>***</td>
<td>9.45 (4.00)c</td>
<td>9.52 (6.33)c</td>
<td>10.03 (6.89)b</td>
<td>9.57 (7.14)c</td>
<td>10.72 (4.85)a</td>
</tr>
<tr>
<td>LfA (cm²)</td>
<td>***</td>
<td>213.68 (13.06)ab</td>
<td>213.92 (7.95)ab</td>
<td>222.71 (10.08)a</td>
<td>203.01 (12.50)bc</td>
<td>191.12 (11.93)c</td>
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<tr>
<td>SLA (cm²/g)</td>
<td>**</td>
<td>138.18 (8.36)c</td>
<td>121.41 (5.73)a</td>
<td>129.35 (10.67)bc</td>
<td>134.49 (10.36)bc</td>
<td>136.96 (10.29)c</td>
</tr>
<tr>
<td>Growth and biomass production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotDM (kg DM/m²)</td>
<td>***</td>
<td>8.83 (10.93)b</td>
<td>9.72 (12.25)ab</td>
<td>10.90 (20.68)a</td>
<td>6.83 (14.52)c</td>
<td>9.86 (16.01)ab</td>
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<tr>
<td>StDM (g DM)</td>
<td>***</td>
<td>289.53 (10.39)c</td>
<td>406.21 (8.59)a</td>
<td>331.59 (12.72)b</td>
<td>245.94 (11.54)d</td>
<td>215.84 (10.29)e</td>
</tr>
<tr>
<td>StN (n/m²)</td>
<td>***</td>
<td>38.84 (15.64)b</td>
<td>35.65 (11.60)bc</td>
<td>38.90 (20.46)a</td>
<td>33.65 (14.20)c</td>
<td>55.10 (18.40)a</td>
</tr>
<tr>
<td>StNd (n)</td>
<td>***</td>
<td>34.78 (7.63)b</td>
<td>34.99 (4.87)b</td>
<td>38.95 (8.47)a</td>
<td>33.33 (7.15)bc</td>
<td>32.09 (7.75)c</td>
</tr>
<tr>
<td>H (cm)</td>
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<td>392.86 (3.85)c</td>
<td>493.36 (4.92)a</td>
<td>445.52 (5.91)b</td>
<td>378.01 (8.75)d</td>
<td>395.11 (5.00)c</td>
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<tr>
<td>Db (cm)</td>
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<td>21.59 (4.86)b</td>
<td>24.27 (4.96)a</td>
<td>21.51 (5.64)b</td>
<td>19.47 (5.23)c</td>
<td>18.03 (5.57)d</td>
</tr>
<tr>
<td>Dm (cm)</td>
<td>***</td>
<td>17.03 (5.59)d</td>
<td>20.21 (6.48)e</td>
<td>16.15 (8.32)c</td>
<td>15.42 (6.01)b</td>
<td>13.96 (6.58)a</td>
</tr>
<tr>
<td>Da (cm)</td>
<td>***</td>
<td>11.89 (6.78)a</td>
<td>12.01 (6.85)a</td>
<td>11.02 (9.77)b</td>
<td>11.17 (6.57)b</td>
<td>10.29 (9.27)c</td>
</tr>
</tbody>
</table>

ns: non-significant.

*P* ≤ 0.05, **P** ≤ 0.01, ***P** ≤ 0.001.
same period (from early to late fall; Pilu et al., 2012, 2013); therefore, late flowering together with high SLA may be indicative of improved growth capacity. Likewise, there were strong positive correlations between flowering time and LAI reflecting greater canopy soil coverage for the ecotypes that had longer growing seasons. Ecotypes with high LAI had longer vegetative periods, indicating that late flowering may be advantageous under continuous mild water deficit as it allows stress acclimatization over time, but not under severe drought as it may imply that they could not finish their phenological cycle.

Gradual water reduction in the soil is common in natural ecosystems, and one of the earliest responses to this depletion is by stomata (Bogeat-Triboulot et al., 2007; Chaves, Flexas, & Pinheiro, 2009; Harfouche, Meilan, & Altman, 2014; Plomion et al., 2006). There is also strong evidence that stomatal limitation is preponderant at mild levels of abiotic stresses like drought (reviewed by Cornic & Fresneau, 2002). We have shown a significant variation among ecotypes in mid-day abaxial stomatal resistance and a clear ecotype-dependent response to a period of water shortage as highlighted by the $E \times T$ interaction analysis. Indeed, ecotypes presenting the lowest values of $R_{S\text{july}}$ (i.e. using more water through transpiration) had much higher $R_{S\text{august}}$ (as they might have exhausted the available water) than others, and thus showed a higher $R_{S\text{august}}/R_{S\text{july}}$, for which a negative relationship between both parameters is apparent (Figure 4). However, this response varies among ecotypes. Ideally, the perfect ecotype could be one showing low $R_{S\text{july}}$ (allowing high rate of photosynthesis during the season when there is still some water in the soil) and either having largely increased $R_{S\text{august}}$ (saving water when it is much less available) or keeping low $R_{S\text{august}}$ (meaning that it is still able to uptake water probably due to an efficient root system). It would therefore be interesting in further studies to check drought tolerance of ecotypes positioned at different places along the $R_{S\text{august}}/R_{S\text{july}}$ relationship. The fact that $\Delta$ is only marginally related to stomatal resistance suggests that water use efficiency in *Arundo* ecotypes is more dependent on variability in photosynthetic capacity rather than on stomatal physiology (O’Leary, 1988), for which further studies on the variability and $H^2$ of photosynthetic capacity would be also of interest for selection purposes. We also observed a negative relationship between stomatal activity and LAI, as already seen in various broadleaved and coniferous forest stands under different climates (Granier, Loustau, & Bréda, 2000).

The selection of suitable ecotypes for sustainable lignocellulosic biomass production has to be based on key characteristics, among which carbon isotope composition could be a relevant trait. Mean $\Delta$ observed in this study was lower than the one observed by Nackley, Vogt, and Kim (2014) ($\Delta = -24%e$) in a growth chamber pot experiment. In our study, we observed a difference of 2.8%e between the two most divergent ecotypes in terms of $\Delta$. In a collection of chickpea (*Cicer arietinum*), ranges of variation of 3.1% and 2.7% were observed among genotypes subjected to terminal drought condition (Krishnamurthy et al., 2013). In cocksfoot (*Dactylis glomerata*), tall fescue (*Festuca arundinacea*) and reed canary grass (*Phalaris arundinacea*) grown under water and water-limited conditions, an overall difference of 2.5%e was observed between the two treatments (Mårtensson et al., 2017). Plants of sunflower (*Helianthus annuus*) subjected to two drought treatments (i.e. progressive water-stress and unvaried stressful condition), showed a significant difference between treatments, with a $\Delta$ of 2.86%e and 2.71%e in stressed and no-stressed plants, respectively (Adiredjo et al., 2014). As supported by these papers, the range of variation observed in our study is of high interest and could be the result of the high variability in the ecology of native sites where *A. donax* ecotypes were collected. The small CV observed in $\Delta$, not exceeding 2.5%, was several times smaller than the CV here estimated for other physiological, morphological and growth related traits. However, caution should be taken when interpreting significances of low $\Delta$ variations. Indeed, a supposed low CV value of 2.42% that referred to a general mean value of 20.98% would imply a large variation of $\Delta$ of about 2.8%e. Such a variation is physiologically relevant, suggesting good levels of adaptation for $\Delta$ in our *A. donax* panel. Correlation analysis found weak and negative relationship between $\Delta$ and TotDM; moreover, cluster differentiation highlighted that more productive ecotypes showed lower $\Delta$ values. The consistently negative associations between $\Delta$ and WUE might suggest that crop yield and $\Delta$ might also be negatively related (Condon, Richards, Rebetezke, & Farquhar, 2004). However, large variance in flowering time and reed height, as here observed, could affect the strength and direction of correlation between $\Delta$

<table>
<thead>
<tr>
<th>TABLE 5</th>
<th>Loadings of the sub-set of informative traits in the first two principal components of the PCA, accounting for 69.23% of the ecotype variability in <em>Arundo donax</em>. See Table 1 for trait abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component 1 Coefficients</td>
<td>Component 2 Coefficients</td>
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<td>StDM</td>
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<tr>
<td>Db</td>
<td>0.457</td>
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<tr>
<td>H</td>
<td>0.429</td>
</tr>
<tr>
<td>Dm</td>
<td>0.377</td>
</tr>
<tr>
<td>StNd</td>
<td>0.317</td>
</tr>
<tr>
<td>TotDM</td>
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<tr>
<td>LAI</td>
<td>-0.173</td>
</tr>
<tr>
<td>StN</td>
<td>-0.190</td>
</tr>
</tbody>
</table>

<table>
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<th>Component 2 Coefficients</th>
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<td>-0.268</td>
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</tr>
<tr>
<td>-0.216</td>
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<tr>
<td>-0.601</td>
</tr>
<tr>
<td>0.189</td>
</tr>
<tr>
<td>-0.569</td>
</tr>
</tbody>
</table>
and total yield (Condon et al., 2004). Positive correlations among flowering phases and Δ were found in our work, and this result reflects the antagonist behaviours between drought escape (i.e. early flowering) and avoidance (i.e. higher WUE, i.e. lower Δ) strategies (Kooyers, 2015). Finally, negative correlations between Rs and Δ here observed resulted as expected results, probably due to lower CO₂ inside leaf concentration in response to greater stomatal resistance (Farquhar et al., 1989).

Canopy architecture affects growth performance of tree species used in SRC plantations (Dillen, Marron, Sabatti, Ceulemans, & Bastien, 2009); therefore, LAI was measured to evaluate the variation in canopy density among the large set of ecotypes analysed here. Other leaf traits affecting canopy structure and light capture have also been characterized in the perennial biomass crop genus Miscanthus (Robson et al., 2013). The range of LAI values observed in the current study is consistent with recent findings. The average value of 6.11 m²/m² is similar to that reported by Cosentino, Scordia, Sanzone, Testa, and Copani (2014) in a trial carried out in southern Italy using a local ecotype at the third year of growth under moderate water supply (6.90 m²/m²). Likewise, the maximum value of 8.14 m²/m² is in agreement with the previous recorded LAI of about 9 m²/m² for a local ecotype at its third year of growth in northern Italy (Ceotto et al., 2013). Finally, the value of 3.89 m²/m² measured in the ecotype with the lightest canopy is comparable with the one estimated by Nassi o Di Nasso et al., 2011, 2013. However, the bio-

Correlation analyses in A. donax indicated that high dry biomass yield was largely due to plant apical growth and
stem density (Angelini et al., 2009; Cosentino et al., 2006). The low but statistically significant correlations observed between biomass production and various growth traits (i.e. StDM, StN, StNd and H) highlighted biomass yield as a complex trait made up of multiple simple traits (Clifton-Brown, Robson, & Allison, 2008).

$H^2$ assessments in this study showed that biomass traits had moderate to high $H^2$ estimates, whereas morphology and ecophysiology trait $H^2$ estimates ranged from low to high. Low $H^2$ estimates were observed primarily in physiology traits (Rs, $\Delta$), which is most likely related to some phenotypic plasticity and reflecting the environment in which the trait was sampled. Notably, the wide change of $H^2$ estimates for Rs July ($H^2 = 0.28$) and Rs August ($H^2 = 0.62$) is to be expected because stomatal resistance is an instantaneously measured physiological trait related to leaf gas exchange (Ackerley et al., 2000). Other studies in A. donax have also observed moderate $H^2$ in biomass and morphology traits (Cosentino et al., 2006; Pilu et al., 2014). Individual traits with higher $H^2$ (morphology, biomass) tended to be intercorrelated. Our $H^2$ results for biomass traits ($0.32 \leq H^2 \leq 0.39$) are slightly lower than other perennial grasses such as switchgrass ($0.45 \leq H^2 \leq 0.50$; Rose, Das, & Talaferrro, 2008; Jiang et al., 2014) and miscanthus ($0.34 \leq H^2 \leq 0.55$; Slavov et al., 2013; Gifford, Chae, Swaminathan, Moose, & Juvik, 2015).

Our multivariate analysis demonstrated that biomass and growth traits: StDM, StN, StNd, H, Db and Dm, as well as LAI, were the most indicative of dry biomass production in our panel of 82 ecotypes. These growth-related traits allowed us to identify similar groups and to select promising individual ecotypes. Cluster analysis highlighted clusters 2, 3 and 5 as the most interesting for biomass yield potential and that the high biomass producing ecotypes adopted different growth strategies. For example, ecotypes in Clu.3 deployed heavy and large leaves in a lighter canopy. Ecotypes in the lower side of the biplot were characterized by a higher number of reeds and higher biomass production compared with individuals localized in the upper part (Figure 8). The two most divergent groups, Clu.2 and Clu.5, contained ecotypes that exhibited significant differences in reed diameter and number and, as a consequence, in LAI. High-biomass ecotypes with large and tall stems organized into less-dense and light clumps were grouped in Clu.2. Conversely ecotypes grouped in Clu.5 had small reeds forming dense clumps. Clusters 2, 3 and 5 correspond to 56% of our collection (46 ecotypes); thus an extensive multi-year multi-location examination of phenotypic variation of these 46 ecotypes among many different types of traits could provide considerable insight into key traits favouring the production of biomass yield in different cropping environments. Finally, the most promising ecotypes belong to these three clusters that showed the best combinations of biomass determinants such as H, StN, StNd and LAI. Although $\Delta$ is not a significant biomass determinant, Clusters 2, 3 and 5 showed lower $\Delta$ than the other two, and this underlines its importance. In some growing areas in semi-arid environments, biomass potential may not be achieved due to water shortage, and hence water use efficiency may be a more relevant trait. These traits should be taken into consideration for both clonal selection and systems and synthetic biology approaches. While selection for these traits is warranted, targeted genetic improvement of A. donax bioenergy feedstock in these critical morphological and biomass traits is of great importance. However, to maximize the efficiency of biomass conversion and reduce biomass recalcitrance in A. donax, future breeding efforts should also target cell wall properties optimized for saccharification and fermentation (Scordia, Cosentino, Lee, & Jeffries, 2011, 2012).

Creating a successful bioenergy program from lignocellulosic feedstocks is a major challenge and will require significant research effort to maximize plant biomass production. Our study provides an essential stepping stone towards this goal. The results presented here demonstrate high variation in phenotypic traits among a large collection of A. donax ecotypes spanning most of the species’ natural range. The assembled panel also represents a suitable resource to screen for improved bioconversion efficiency using a similar approach to improve the already favourable comprehensive utilization index for A. donax as discussed by Kou et al. (2017). The study also highlights the availability of different ideotypes (described by Donald (1968) as a biological model which is expected to perform or behave in a predictable manner within a defined environment) of A. donax that achieve high yield through different combinations of traits and it will be interesting to see if these ideotypes differ in their ability to generate high yield across a range of environments. Extensive assessments of traits underscored the consistency observed in phenotypic traits and to our knowledge present the most comprehensive assessments of biomass traits in A. donax to date. Efforts are under way to exploit genomic tools in A. donax to identify loci responsible for the observed trait variation.

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CONFLICT OF INTEREST

The authors declare no competing business interests or relationships that could be viewed as a conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.