Selection of reference genes suitable for normalization of qPCR data under abiotic stresses in bioenergy crop *Arundo donax* L.

Michele Poli$^{1,2}$, Silvio Salvi$^2$, Mingai Li$^1$ & Claudio Varotto$^1$

Suitable reference gene selection in qRT-PCR is a key pre-requisite to produce reliable data in gene expression analyses. In this study, novel primers for six commonly used reference genes (AC1, TLF, Act2, TUB α, EF-1α and GAPDH) plus two new candidates (pDUF221 and RPN6) were designed and comparatively tested for expression stability under abiotic stresses (osmotic, heavy metal and heat shock) in shoot, root and their combination of *Arundo donax* L., a rising non-food energy crop. Expression stability rankings from the most to the least stable gene in each condition and in two tissues (young shoots and roots) were generated with geNorm, NormFinder and BestKeeper programs. All programs provided similar rankings and, strikingly, in most cases identified one of the new candidates, RPN6, as the most suitable reference gene. This novel set of reliable references allows to choose either the best combination of reference genes across multiple stress/organ conditions or to select condition-specific genes that can improve the quality of qRT-PCR analysis. This work provides a solid basis for the functional characterization of *A. donax*, by enabling accurate quantification of the transcriptional responsiveness under a series of common stress conditions of any gene of interest in this promising biomass/bioenergy species.

Reverse transcription - quantitative polymerase chain reaction (RT-qPCR) is a well-established and widely used technique for gene expression analyses in many biological fields. Its precision and sensitivity allow to accurately measure the transcriptional variations of a gene among different samples, providing basic information on its function through the characterization of its tissue- or stress-specific expression pattern. Two methods for quantification are commonly used in qRT-PCR experiments: absolute and relative quantification. The absolute quantification identifies the exact copy number of transcribed RNA in a given sample. This method relies on a pre-built calibration curve that associates the known concentrations of cDNA template standards with the corresponding fluorescence data produced during RT-PCR amplification. Fitting the real-time PCR data from unknown samples to the calibration curve, thus, provides the extrapolated absolute number of copies of target gene. In addition, by normalizing the copy numbers of target gene with that of reference gene, it could also provide relative quantification comparisons between samples. Relative quantification compares the gene of interest with an internal reference gene to obtain the expression variation. This method requires a gene (or multiple genes) with stable expression levels as calibrator to be compared with the gene of interest in order to eliminate possible sources of errors/differences in sample preparation (e.g. RNA extraction, quantification, reverse transcription). Between these two methods, relative quantification has broader applications. It is not only used to assess differential expression of target genes in different tissues/cells, in test conditions with respect to a control condition and in allelic discrimination, but it is also widely used to confirm microarray and transcriptomic data. Despite many advantages and applications, the most critical aspect of relative quantification is the choice of the internal control, that, ideally, should be expressed at constant level in every condition and tissue/cell type. In plants, several widely used reference genes play a role in basic cellular structure and basic metabolism, like 18S rRNA (18S ribosomal RNA), 28S rRNA (28S ribosomal RNA), ACT (Actin), EF-1α (Elongation...
ous study24 was included in this analysis. Finally, one new gene was inversely proportional to the spread of the data30. Based on this criterion, comparison of raw Cq values among candidates without efficiency correction, which in case of variable amplification efficiencies could lead to biased results. Its high biomass production (up to 40 tons/ha) and low input requirements, make it one of the best options as an energy crop, and 92% of sequence similarity to their closest sorghum homologs (Supplementary Table S1). The other candidate nase 2 and 92% of sequence similarity to their closest sorghum homologs which were used for primer design shared a nucleotide similarity greater than 91% to foxtail millet ref-
erences. Published primers for foxtail millet were slightly adapted to giant reed with exception of TLF for which completely new primers were designed (Table 1, Supplementary Table S1). By mining the sorghum transcrip-
tome data is now available for further functional characterization. To date, however, no validated set of reference genes specific for expression studies in A. donax under stressed conditions is available.

To fill this important gap in the functional genomics toolbox of A. donax, in this study eight candidate reference genes with diverse functional categories were selected to assess their expression stabilities in two different organs (shoots and roots) of young seedlings subjected to three different stress treatments: osmotic stress (15% PEG 6000), heavy metal (500 µM CdSO₄) and heat shock (42 °C). Among these candidates, six have been previously used as reference genes in RT-qPCR analyses while the other two were newly selected from transcriptome analyses of giant reed and sorghum, respectively. Finally, to validate the reliability of candidate reference genes, we evaluated their performance under osmotic, heat and heavy metal stress. For the first two stresses we used A. donax DREB2A (Dehydration-Responsive Element Binding Protein 2 A)26, a well-known drought- and heat-responsive gene. In addition, we also confirmed with the two most stable and the two least stable among our new reference genes the expression pattern of ḭsp 5 (isoprene synthase), a hemiterpene synthase which is transcriptionally regulated in response to stress induced by the heavy metal cadmium27.

Results
Gene selection, amplification specificity and efficiency. In this study, we selected eight candidate reference genes from different sources. Four of them, namely TLF (translation factor), Act2 (actin2), Tub α (alpha tubulin) and EF-1 α (elongation factor 1-alpha), have been already used in qRT-PCR experiments in foxtail millet28, the species with a sequenced genome which is most closely related to A. donax21. For all these genes, the A. donax homologs which were used for primer design shared a nucleotide similarity greater than 91% to foxtail millet references. Published primers for foxtail millet were slightly adapted to giant reed with exception of TLF for which completely new primers were designed (Table 1, Supplementary Table S1). By mining the sorghum transcriptome29, we extracted two more candidates: the commonly used GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and RPN6 (26S proteasome non-ATPase regulatory subunit 11). A. donax homologs share respectively 91% and 92% of sequence similarity to their closest sorghum homologs (Supplementary Table S1). The other candidate gene homologous to an Actin gene (AC1) in sorghum (having 89% similarity at nucleotide level) used in the previous study30 was included in this analysis. Finally, one new gene pDUF2221 has also been considered as a candidate based on transcriptomic data analysis in A. donax34. To determine the amplification specificity of each primer pair prior to intensive qPCR analysis, electrophoretic analysis on agarose gel and melting curve assessment were carried out for each amplification; a single amplification band with expected size and length for each primer pair was observed on 2% agarose gels, and a single peak was detected through melting curve analysis for all amplifications and no signal for non-template control (Supplementary Figure S1). Moreover, for all primer pairs, amplification efficiencies were calculated based on standard curve assay generated from amplifications with a series of cDNA dilutions and resulted to be between 92.85% and 104.03% with correlation coefficients (R²) ranging from 0.987 to 0.998 (Table 1, Supplementary Figure S2).

Gene expression profile of candidate reference genes. To assess the transcriptional variation and stability of each candidate reference gene, cycle threshold (Cq) value was obtained using qPCR to estimate the expression level of each gene among all the samples (two A. donax tissues for three stress conditions, each with five time points plus one pre-stress control) (Supplementary Table S2). The highest Cq value was detected for AC1 (lowest expression: 28.58 cycles), while the lowest Cq value was measured for GAPDH (highest expression: 16.16 cycles). Mean expression values per gene varied from 25.28 of GAPDH to 18.84 of GAPDH. To provide a more informative stability index, we calculated also the difference between 75th and 25th percentile (ΔP), which was inversely proportional to the spread of the data30. Based on this criterion, RPN6 (ΔP = 0.82) was the most stable gene, followed by Act2 (1.07), GAPDH (1.01), TUB α (1.27), pDUF2221 (1.31), TLF (1.32), EF-1α (1.48) and AC1 (P = 1.87) (boxes in Fig. 1). This stability ranking among genes is also confirmed by the coefficient
of variation (CV) that ranged from 2.5% of RPN6 to 6.24% of AC1 (Supplementary Table S3). Comparison of the expression profile of each gene from 0 (control) to 24 h after stress application (Supplementary Figure S3) showed that GAPDH was always the most highly expressed gene in every condition/tissue. The lowest expression levels were mainly associated to pDUF221, with the exception of heat shock stress. Only for this stress, in shoot RPN6 had higher Cq at 3 h and 6 h and, in root, RPN6 and AC1 had higher Cq values for the whole time course (Supplementary Figure S3).

Overall expression stability analyses of candidate reference genes under stress treatments. All treatments, divided according to tissue, were included in an overall analysis to understand which candidate gene is the most stable across samples. For shoot tissue, the results were heterogeneous, with a prevalence of

Table 1. Summary of the eight candidate reference genes tested and two A. donax target genes used for their validation. Sequence and amplicon characteristics are provided for each primer pair. Underlined primers/bases are specifically designed for A. donax. E (%) is primer efficiency calculated with standard curves and formula \( E = 10^{- \frac{1}{slope}} \times 100 \); R2 is the correlation coefficient; Tm is the melting curve temperature. Asterisc (*) indicates the target genes used for reference gene validation.

| Gene symbol | Gene Name                  | Accession No. | Primer Pair (5′-3′)                      | Product size (bp) | E (%) | R2  | Tm (°C) | Ref |
|-------------|---------------------------|---------------|----------------------------------------|-------------------|-------|-----|--------|-----|
| AC1         | Actin                     | Unigene036290 | F: TCTTGGCTTGCATTCTGTTGG            | 93                | 100.72| 0.998 | 81.5   | 24  |
|             |                           |               | R: TGGATTGCCAGGGCTTATAC             |                   |       |     |        |     |
| Act2        | Actin 2                   | Unigene057037 | F: CGCATACGTTGGACATTGAC            | 126               | 92.85 | 0.987 | 83.5   | 28  |
|             |                           |               | R: GGCAACTGACCTCTCTTTTC            |                   |       |     |        |     |
| EF-1α       | Elongation factor 1-alpha | Unigene076509 | F: TGACTTGCTGTGCAGTCATCA          | 133               | 97.13 | 0.996 | 83     | 28  |
|             |                           |               | R: GTCGACGAGCATACATCT              |                   |       |     |        |     |
| GAPDH       | Glyceraldehyde 3-phosphate | Unigene069707 | F: TGCAAAGGAAGGCTGCTC           | 167               | 103.83| 0.997 | 82.5   | 29  |
|             |                           |               | R: GAGCAAGGACAGTTTGGTG            |                   |       |     |        |     |
| pDUF221     | Probable membrane protein | Unigene070087 | F: GCAAAAAGTCACGGTGCAGTA         | 91                | 99.94 | 0.998 | 81     | 24  |
|             |                           |               | R: AAAGTGTCCTCGGACTTGAT           |                   |       |     |        |     |
| RPN6        | 26S proteasome non-ATPase | Unigene067565 | F: GACGCCAGCTAGCTTTCAAG           | 78                | 104.03| 0.993 | 80     | 29  |
|             |                           |               | R: TTCACAGGGCAAGGTTG              |                   |       |     |        |     |
| TLF         | Translation Factor        | Unigene076539 | F: GACCTCATCTGGGCTGTTGA          | 110               | 100.32| 0.998 | 80     | 28  |
|             |                           |               | R: TGGTTGGCGGCTGGAACTG            |                   |       |     |        |     |
| TUB α       | Tubulin alpha             | Unigene068813 | F: TACCCGCCACCTCAGTTGT           | 121               | 96.24 | 0.996 | 85     | 28  |
|             |                           |               | R: AGTCAAACCTTGCTGCAATTGCC        |                   |       |     |        |     |
| DREB2A*     | Dehydration-Responsive    | Unigene057213 | F: TCCAGCAGATTATAGTATCC           | 98                | 98.59 | 0.999 | 78     | 24  |
|             |                           |               | R: AGCAAGTGCGTAATAGGCCA          |                   |       |     |        |     |
| IspS*       | Isoprene Synthase         | KX906604      | F: GAGGTCGCTTTGCAATTTGAGR          | 184               | 99.31 | 0.987 | 80.5   | 27  |
|             |                           |               | CAGAGCCAGAACATGTGCTCCAC           |                   |       |     |        |     |

Figure 1. Expression level variability of each candidate reference gene. Boxes extend from the 25th to 75th percentiles, whiskers represent the maximum and minimum values, the line across the box represents the median value of the Cq values for each gene.
EF-1α and RPN6 in the top three genes for all the algorithms (Table 2). In this case, geNorm indicated TLF and TUB α as the most stable genes, while they were ranked as sixth and fourth by NormFinder and as last and fifth by BestKeeper. The least stable genes were instead consistently AC1 and pDUF221 with the only exception of BestKeeper that identified, as mentioned above, TLF as the worst ranking gene. Also, in root EF-1α and RPN6 genes were always in the top three positions (with the exception of Act2 ranked first by BestKeeper), while pDUF221 and AC1 were relegated strictly to the last two ranks by all algorithms. Taking into account both shoot and root organs, RPN6 appeared as the candidate gene with the best scores in the three algorithms followed by EF-1α and AC1 = (first in NormFinder and BestKeeper, second in geNorm) (Supplementary Table S4). Overall intergroup and intragroup analyses were also carried out for all samples with NormFinder. This analysis graphically showed the variation of each gene with respect to four subgroups: control, osmotic, heavy metal and heat shock stresses (Supplementary Figure S4). Overall intergroup and intragroup analyses were also carried out for all samples with NormFinder. This analysis graphically showed the variation of each gene with respect to four subgroups: control, osmotic, heavy metal and heat shock stresses (Supplementary Figure S4). Analysis of the best combination of two genes was consistent with single stability value in root, where EF-1α and RPN6 were the best pair of genes. More surprisingly, in shoot the best pair of genes suggested were RPN6 (second, S = 0.188) and TLF (sixth, S = 0.320) with a combination value of 0.091. It should be noticed that also geNorm algorithm included TLF in the best pair of primers together with TUB α. Considering together shoots and roots, NormFinder suggested the combination between RPN6 and GAPDH with a value of 0.115 (the latter classified at the sixth position as a single gene) (Supplementary Figure S4C). These differences with respect to the results of the gene by gene analyses were due to the fact that NormFinder tended to enhance intergroup stability by balancing over/under expression of the two genes to be as close as possible to zero in each subgroup.

**Expression stability analyses of candidate reference genes under water/osmotic stress treatment.** Water stress was induced by adding 15% of PEG 6000 to the hydroponic solution which decreased the osmotic pressure of the media, consequently inducing osmotic and water limitation stress in the plants without toxic effects in the short term. Reference gene analyses under this simulated drought condition in A. donax shoots showed a high and shared rank for RPN6 (first in NormFinder and BestKeeper, second in geNorm) followed by EF-1α gene (first in geNorm, fourth in NormFinder and third in BestKeeper) (Table 3). In roots, RPN6 was confirmed as the most stable gene followed by GAPDH that had the best value in geNorm (shared with RPN6) and BestKeeper. Using both organ’s samples, RPN6 and GAPDH were the best two genes (Supplementary Table S4), resulting in the top three ranks provided by all programs. The lowest stability values resulted from the AC1 and pDUF221 genes with all algorithms and tissues with exception of BestKeeper that ranked pDUF221 at the fourth position in root and TLF and EF-1α as last in the shoot + root analysis.

### Table 2. Stability ranking of each candidate reference gene using different algorithms in shoot and root. Column "value" corresponds to M in geNorm, S in NormFinder and CV ± SD in BestKeeper.

| Rank | Shoot | Gene | Value | Gene | Value | Gene | Value | Root | Gene | Value | Gene | Value | Gene | Value |
|------|-------|-----|------|-----|------|-----|------|------|-----|------|-----|------|-----|------|
| 1    | TLF   | TUB α | 0.293 | RPN6 | 0.119 | RPN6 | 1.16 ± 0.29 | TLF | 0.137 | TUB α | 1.37 ± 0.33 |
| 2    | EF-1α | RPN6 | 0.345 | TUB α | 0.141 | GAPDH | 1.51 ± 0.30 | TLF | 0.308 | EF-1α | 0.301 | GAPDH | 0.203 | EF-1α | 2.22 ± 0.44 |
| 3    | TUB α | TLF | 0.377 | GAPDH | 0.208 | EF-1α | 1.87 ± 0.40 | TLF | 0.392 | GAPDH | 0.203 | EF-1α | 2.22 ± 0.44 |
| 4    | GAPDH | EF-1α | 0.436 | EF-1α | 0.310 | TUB α | 1.73 ± 0.41 | Act2 | 0.457 | TLF | 0.257 | pDUF221 | 1.87 ± 0.47 |
| 5    | Act2 | GAPDH | 0.485 | GAPDH | 0.359 | TLF | 1.75 ± 0.42 | TUB α | 0.504 | Act2 | 0.261 | Act2 | 2.53 ± 0.56 |
| 6    | AC1 | Act2 | 0.527 | Act2 | 0.362 | Act2 | 2.02 ± 0.47 | pDUF221 | 0.605 | TUB α | 0.261 | TLF | 2.58 ± 0.57 |
| 7    | pDUF221 | AC1 | 0.656 | AC1 | 0.603 | AC1 | 2.50 ± 0.59 | AC1 | 0.705 | AC1 | 0.665 | TUB α | 3.45 ± 0.78 |
| 8    | pDUF221 | pDUF221 | 0.726 | pDUF221 | 0.682 | pDUF221 | 2.61 ± 0.68 | pDUF221 | 0.676 | AC1 | 4.75 ± 1.09 |

### Table 3. Stability ranking of each candidate reference gene using different algorithms under osmotic stress in shoot and root. Column "value" corresponds to M in geNorm, S in NormFinder and CV ± SD in BestKeeper.

| Rank | Shoot | Gene | Value | Gene | Value | Gene | Value | Root | Gene | Value | Gene | Value | Gene | Value |
|------|-------|-----|------|-----|------|-----|------|------|-----|------|-----|------|-----|------|
| 1    | TLF   | TUB α | 0.293 | RPN6 | 0.119 | RPN6 | 1.16 ± 0.29 | TLF | 0.137 | TUB α | 1.37 ± 0.33 |
| 2    | EF-1α | RPN6 | 0.345 | TUB α | 0.141 | GAPDH | 1.51 ± 0.30 | TLF | 0.308 | EF-1α | 0.301 | GAPDH | 0.203 | EF-1α | 2.22 ± 0.44 |
| 3    | TUB α | TLF | 0.377 | GAPDH | 0.208 | EF-1α | 1.87 ± 0.40 | TLF | 0.392 | GAPDH | 0.203 | EF-1α | 2.22 ± 0.44 |
| 4    | GAPDH | EF-1α | 0.436 | EF-1α | 0.310 | TUB α | 1.73 ± 0.41 | Act2 | 0.457 | TLF | 0.257 | pDUF221 | 1.87 ± 0.47 |
| 5    | Act2 | GAPDH | 0.485 | GAPDH | 0.359 | TLF | 1.75 ± 0.42 | TUB α | 0.504 | Act2 | 0.261 | Act2 | 2.53 ± 0.56 |
| 6    | AC1 | Act2 | 0.527 | Act2 | 0.362 | Act2 | 2.02 ± 0.47 | pDUF221 | 0.605 | TUB α | 0.261 | TLF | 2.58 ± 0.57 |
| 7    | pDUF221 | AC1 | 0.656 | AC1 | 0.603 | AC1 | 2.50 ± 0.59 | AC1 | 0.705 | AC1 | 0.665 | TUB α | 3.45 ± 0.78 |
| 8    | pDUF221 | pDUF221 | 0.726 | pDUF221 | 0.682 | pDUF221 | 2.61 ± 0.68 | pDUF221 | 0.676 | AC1 | 4.75 ± 1.09 |
Expression stability analyses of candidate reference genes under heavy metal stress treatment. A. donax is considered a suitable plant for phytoremediation of contaminated soil. Among the heavy metals that can affect soil quality and reduce plant productivity, cadmium (Cd) is one of the most toxic heavy metals. A. donax seems able, however, to cope with it without physiological adaptation, which is an important feature for phytoremediation. When subjected to cadmium toxicity stress, reference gene performances were more heterogeneous with respect to osmotic stress: in shoots, RNA6 and TUB α were ranked in the top three for all the programs, but in roots TLF was among the best three followed by EF-1α (first, second and fourth) (Table 4). On the other hand, the least stable gene in shoot was pDUF221, in root was AC1 and in the full set of heavy metal stressed samples were GADPH and AC1 (Table 4). Noteworthy, BestKeeper indicated as most stable candidate gene as the RPN6 (Table 4). Other general candidate genes to be used in studies encompassing both root and shoot could alternatively be the TLF (ranked third, first and third).

Expression stability analyses of candidate reference genes under heat shock stress treatment. Heat shock is an important factor that affects plant physiology and growth. The capability of A. donax to survive in warm environments is an interesting trait of this plant, which can be relevant to forecast its productivity as heat spells become more frequent. Gene stability in heat condition (42 °C) identified the best number of genes that should be used together in a relative qRT-PCR experiment. Therefore, it has been used in this study to predict the optimal number of reference genes to be used in each stress experiment (osmotic, heavy metal and heat shock). As expected, the number of references depended on the experimental settings:

**Table 4.** Stability ranking of each candidate reference gene using different algorithms under heavy metal treatment in shoot and root. Column “value” corresponds to M in geNorm, S in NormFinder and CV ± SD in BestKeeper.

**Table 5.** Stability ranking of each candidate reference gene using different algorithms under heat shock stress treatment in shoot and root. Column “value” corresponds to M in geNorm, S in NormFinder and CV ± SD in BestKeeper.

**Best reference gene number identification.** geNorm Excel add-in is a useful tool for calculation of the best number of genes that should be used together in a relative qRT-PCR experiment. Therefore, it has been used in this study to predict the optimal number of reference genes to be used in each stress experiment (osmotic, heavy metal and heat shock). As expected, the number of references depended on the experimental settings:
considering shoot and root together geNorm indicated three genes as the most suitable for single stresses and four genes considering all stresses (Fig. 2A). If root and shoot were considered separately, all the values for single stress dropped below the suggested threshold of 0.15 (Fig. 2B, C). With one exception in shoot, by grouping the three stresses, the value was just above the threshold limit when two candidate genes were used while it laid on the threshold with three.

Validation of reference genes by expression pattern analyses of target genes under stress treatments. To demonstrate the reliability of the newly analysed reference genes in *A. donax* for stress treatments, *DREB2A* (*Dehydration-Responsive Element Binding Protein 2A*) which is one of the key genes triggering the response to both drought and heat shock and isoprene synthase gene (*IspS*), which is differentially expressed in heavy metal (Cd) stress treatment in *Arundo donax*, were chosen to validate these reference genes for qPCR internal normalization. We compared the expression profiles of *DREB2A* in shoot under osmotic stress and heat shock treatments using the two most stable genes (*RPN6* and *EF-1α*), their combination (*RPN6* + *EF-1α*) and the least stable genes (*AC1* and *pDUF221*) as reference genes based on stability ranking (Tables 3 and 5, respectively). Fold change of *DREB2A* was calculated with the comparative Ct method. The expression pattern was consistent with a two-fold increase of *DREB2A* expression at 6 h and 11 h and about four-fold increase at 24 h normalized with *RPN6*, *EF-1α* and their combination (Fig. 3A). On the other hand, the pattern obtained using *AC1* as reference displayed an upwards-shifted trend, with a twofold increase since the beginning and a six-fold increase from 6 h on. When *pDUF221* was used as a reference gene, the expression pattern decreased at 3 h, 6 h, and 11 h to finally grow again at 24 h. Under heat shock treatment, the expression levels of *DREB2A* normalized with *RPN6*, *EF-1α* and their combination were extremely consistent at all time points: after a 12-fold increase at 1 h, *DREB2A* expression gradually decreased to four-fold at 3 h and two-fold at 6 h. By contrast, *DREB2A* expression levels were found to be two-fold higher at 1 h, 3 h and 6 h using *AC1* as reference gene and at least two-fold lower at all time points using *pDUF221* compared to when using *RPN6*, *EF-1α* and their combination (Fig. 3B). Furthermore, the expression levels of *IspS* under cadmium treatment were highly consistent at all time points with a 1.8-fold increase at 1 h and a four-fold increase at 3 h using *RPN6*, *TUBα* and their combination as reference genes, respectively. Meanwhile, *IspS* expression levels were shown to decrease by 30% at all time points when normalized with *TLF* compared with *RPN6*, *TUBα* and their combination. When the expression levels of *IspS* were normalization with *pDUF221*, we observed a dramatic increase. A 2.8-fold increased was observed at 1 h, followed by a continuous increase from 6 h till 24 h, instead of the decreased expression at 11 h and the further increase at 24 h observed when normalizing with *RPN6*, *TUBα* and their combination (Fig. 3C).
Discussion

Real Time PCR is a powerful technique for gene expression profiling and functional characterization of genes. Its accuracy, however, is critically dependent from the choice of reference genes whose expression is strictly proportional to the total mRNA amount in the samples to be quantified. Therefore, ideal reference genes should have the same expression levels (measured in terms of Cq value) among different conditions, tissues, developmental stages and crop varieties. In reality, however, gene stability depends greatly on the plant-environment system so that different stress conditions, organs or cultivars can bring undesirable variations in expression of reference genes selected without specific validation, possibly leading also to erroneous quantification. For this reason, a full set of possible reference genes should be developed and systematically tested for each species whenever the experimental conditions change. In this study, eight candidate reference genes were selected for A. donax, an emerging non-food energy crop:

- AC1
- pDUF221
- TLF
- Act2
- TUBα
- EF-1α
- RPN6
- GAPDH

The aim of this study was not only to evaluate the overall performance of these genes as references for qRT-PCR data analysis, but also to provide a detailed indication on which gene is more suitable for specific organ/stress combinations. Identifying stable reference genes is not a trivial task, as it encompasses the choice of the candidates based on literature searches, the identification of the homologs from a non-model species, the design of the primers and their thorough verification in the species of interest, in this case A. donax. Any of these steps can fail, resulting in a considerable waste of time, effort and resources. As long as the conditions used in future studies will be the same as those used here, therefore, the relevance of our results is that they provide for A. donax reliable references without the need for time-consuming testing/optimization. Given its biological features and economic relevance, the three kinds of abiotic stress applied in this study (osmotic, heavy metal and heat shock) are among the most interesting ones to characterize the functional bases of A. donax tolerance to adverse environmental conditions. Three algorithms were applied to the Cq values to measure the stability of the candidate genes: geNorm, NormFinder and BestKeeper. Our results show overall congruence on the stability ranking of the reference genes generated from different algorithms, which indicates the good performance and reliability of the methods. We also divided the analysis into stress and organ subsets to identify for each subset of samples the best performing reference genes. In general, gene RPN6 was always ranked among the top three genes in both organs under osmotic stress, heat shock and overall analysis, which makes it a highly suitable reference (Table 2). Nevertheless, RPN6 showed under heavy metal stress, in Figure 3. Relative expression of target genes in shoot under different durations of stress treatments using different reference genes for normalization. Relative expression of DREB2A under osmotic stress (A) and heat shock treatment (B), relative expression of IspS under heavy metal treatment (C). The two most stable genes, their combination (left of the vertical dotted line) and the least stable genes (right of the vertical dotted line) are used for normalization under each stress condition.
root and with the complete dataset slightly higher variability, especially when the NormFinder and BestKeeper algorithms were used. On the other hand, geNorm classified RPN6 as the best reference gene also in root, making the choice difficult in this context. These results are very interesting, giving the fact that until now, this gene was considered as possible reference gene in quantitative RT-PCR only in Arabidopsis but, to our knowledge, never in monocot species. Its putative orthologue in Arabidopsis (AT1G29150) encodes a protein constituting a lid subunit of the 26S proteasome, which is involved in the ubiquitin-proteasome system (UPS) for degradation of misfolded proteins and for stress response. Other subunits of this large protease complex have been previously suggested as potential source of new and more stable reference genes in Arabidopsis. Worth of notice, in Arabidopsis RPN6 is classified as a cadmium responsive gene, which could explain the variation we observed in response to this stress. We notice, however, that RPN6 still remained more stable than some other commonly used reference gene such as EF-1α or AC1 (Table 4; Supplementary Table S4), indicating a pretty limited transcriptional resposiveness of RPN6 to cadmium in A. donax as compared to A. thaliana. This fact is possibly due to the high resistance of A. donax to cadmium treatment as demonstrated by Papazoglou and colleagues. A useful reference gene across different stresses can be the commonly used EF-1α, which ranked always in the top three positions. From our analysis, this gene often scored better when the organs were considered separately, especially in osmotic and heat shock stresses (Tables 3 and 5), suggesting moderate tissue-specific variability. The EF-1α gene from Setaria italica (L.) P. Beauvios (foxtail millet) was suggested as the best internal control gene under drought and salt condition. Here, we confirm the good stability of gene EF-1α also in A. donax, a close relative of S. italica, but mainly when used in single tissues. Considering both tissues together, the Act2 gene performs better in each stress condition, especially under heavy metal treatment (Supplementary Table S4). GAPDH is, instead, suitable in osmotic stress, where it resulted the best among commonly used reference genes (Table 3). This result is consistent with previous studies showing that GAPDH is one of the best references in plants under drought and salt stress, but not in other conditions. Finally, we suggest to avoid using two of the candidate genes tested due to their instability: pDUF221 and AC1. In the case of pDUF221 this result is likely due to fact that the transcriptomics analysis used for its selection encompassed a single time point only from osmotic stress, while here we tested a 24 h time series and multiple stresses. As for the number of reference genes to be used, geNorm analyses showed some interesting trends based on the different dataset employed. In general, the higher the number of organs/conditions, the higher tended to be the number of genes required for a reliable quantification. In particular, the full dataset (all the treatments + both organs) dropped below the suggested 0.15 threshold only with four reference genes, while in roots it estimated that only two genes were needed (Fig. 2A,C). This is significant because it implies a differential regulation between organs that becomes difficult to quantify in case of studies aimed at the comparison of target genes of aerial and underground tissues. Another interesting point is the marginal decrease of the pairwise variation when considering the combination of all stresses in shoot with two or three reference genes (Fig. 2B). Given the relatively high cost/benefit ratio in the use of an additional reference gene, and considering that 0.15 is an arbitrary threshold, we suggest that use of two internal controls can be a good compromise for both root and shoot when interested in the organ-specific comparison of target expression levels under osmotic, heavy metal and heat stress in A. donax.

DREB2A is a well-known transcription factor, which is associated with drought, salt and heat responses. The DREB2A protein interacts with a cis-acting dehydration-responsive element and activates a downstream cascade of drought and heat-responsive genes, thus providing a better tolerance for plants to these stresses. The consistent expression pattern obtained for DREB2A under osmotic and heat shock treatment normalized with the two best reference genes and highly variable pattern with the two worst ones further confirmed the reliability of the ranking of these reference genes. For the cadmium stress we used the IspS gene, which we recently characterized in A. donax and found to be transcriptionally upregulated by cadmium treatment. The results of the validation indicate that, in the case of heavy metal, the performance of the reference genes for normalization between the best and least stable genes does not differ as much as in the cases of those for PEG and heat shock treatments. Given the relatively low cadmium responsiveness of IspS, however, it is possible that the differences in normalization among reference genes may result bigger for genes with stronger upregulation in response to cadmium.

In summary, this study provides a wide view of the reference genes that can be used or should be avoided in A. donax under specific abiotic stresses and in specific organs, making an important step forward towards the reliable and accurate gene expression quantification in this species. In addition, this study emphasizes further that normalization with reference genes rigorously validated before use for any new experimental design is essential. Moreover, thanks to analysis of related species transcriptomes, a new stable gene (RPN6) has been successfully used for relative quantification, showing that a deeper comparative analysis of plant transcriptomes can unveil additional candidates for a more precise and reliable qRT-PCR analysis. We expect that RPN6 could find application in additional monocotyledonous species.

Methods

Plant materials and stress treatments. Cohorts of A. donax cuttings (Sesto Fiorentino, Florence, Italy 43°49′01.8″N 11°11′57.0″E) were used in this study. The plant growing condition and procedure for stress treatments were the same as those described previously. For stress treatments, plants at the 5-leaf stage were transferred from hydroponic solution to fresh one supplemented with 15% PEG 6000 (osmotic stress), 500 μM CdSO4 (heavy metal stress) or pre-warmed at 42°C (heat stress). The entire shoots and roots (treated and untreated) were independently collected at all different time points (0 h, 1 h 30′, 3 h, 6 h, 11 h and 24 h), immediately frozen in liquid nitrogen, and then stored at −80°C till use. Three biological replicates were applied for all the treatments at every sampling time point.
Candidate reference gene selection, PCR primer design. Among the sequenced genomes deposited in Phytodrome, Sorghum bicolor and foxtail millet (Setaria italica L.) are the two species phylogenetically most closely related to A. donax. A. donax homologs of four common housekeeping genes from foxtail millet TLF (Terminal Flower-like), Act 2 (Actin 2), Tub α (Tubulin alpha), and EF-1 α (Elongation Factor 1 alpha)²⁸ were selected by BLASTN searches against the giant reed reference and water-stress transcriptomes.²³,²⁴ Analogously, two additional genes were identified in the A. donax transcriptomes based on stable expression across tissues (RPN6²⁹) or because already used as a reference gene in sorghum (GAPDH). Finally, the last two genes were selected directly from the giant reed transcriptome (AC1 and pDUF221²⁴) based on their low coefficient of variation (CV) across organs/water stress conditions. The AC1 candidate had been previously chosen (Fu et al. 2016), because among the different A. donax transcripts coding for actin, it was the one with the lowest CV in the PEG-treated transcriptomes. The pDUF221 candidate was chosen exclusively based on the CV from the PEG-treated transcriptomes, where it ranked 5th among the most stable transcripts for this stress (Supplementary Figure S5).

Primers were designed with Primer3Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) using the following parameters: length 18–25 bp (optimum 20), product size 75–200 bp, melting temperature 59–64°C (optimum 60°C); GC content 30–70% (optimum 50%). Primer pairs with free energy (dG) of dimer formation lower than -5 kcal/mol according to the PerlPrimer v1.1.21 software (http://perlprimer.sourceforge.net/) were discarded.

Total RNA isolation and cDNA synthesis. Total RNA was isolated with the Spectrum Plant Total RNA Extraction Kit (Sigma) for shoots and the Rneasy® Plant Mini Kit (Qiagen) for roots, respectively. To assure complete absence of genornal RNA contaminations, total RNA was treated with DNase I (Sigma-Aldrich) and checked on 1% agarose gel for integrity control. Concentration and quality of each sample were measured spectrophotometrically through the OD_{260}/OD_{280} absorption ratio. First strand cDNA was reverse transcribed from 1 μg of total RNA primed with oligo-dT in a total reaction mixture of 20 μL using SuperScript® III Reverse Transcriptase (Life Technologies) according to the manufacturer’s instruction.

PCR amplification specificity and Quantitative Real Time PCR analyses. To assess the amplification specificity of each primer pair prior to qPCR analysis, PCR amplification was performed in a total volume of 10 μL containing 1 μL of 6-fold diluted cDNA (8 ng of starting RNA), 1x PCR Buffer, 100 nM dNTPs, 200 nM of each primer, 0.5 unit of Taq polymerase (Sigma) and 4.9 μL of H₂O; The PCR programme was as follows: 8 min at 95°C, 33 cycles of 40 s at 94°C, 30 s at 60°C and 20 s at 72°C, with 5 min final extension at 72°C. The PCR products were run on 2% agarose gel to check single amplification (Supplementary Figure S1A). The qPCR reaction was conducted by mixing 1 μL of 10-fold diluted cDNA (5 ng of starting RNA), 200 nM of each primer and 6.25 μL of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) in a final volume of 12.5 μL. The programme for qRT-PCR in Bio-Rad C1000 Thermal Cycler was set as: 2 min at 50°C, 2 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 60°C (59°C for target DREB2A gene). The melting curves were recorded after cycle 40 for every gene by constantly raising the temperature from 65°C to 90°C (Supplementary Figure S1B). A standard curve of qPCR reaction was generated from five points (four points for pDUF221gene and target gene DREB2A) of a 6-fold dilution series (10-fold dilution for pDUF221 and TLF). The slope (S) of the standard curve was used to calculate the amplification efficiency (E) of each primer pair as follows: E = 10^{(-S/2.3)} (Supplementary Figure S2). Three technical replicates were used for each sample and every plate contained one No Template Control (NTC) well for each primer pair used. In order to compare different plates in Bio-Rad CFX Manager software the baseline threshold was set at 329.82 and one control sample was used in every plate to check for Cq congruency. For the validation of the reference genes under cadmium stress, as IpsS is under circadian regulation,⁵⁰ at each time point the relative expression was normalized with the untreated control at the same time.

Data analyses with geNorm, NormFinder and BestKeeper. The analyses were conducted on six different datasets that comprise each a single combination of organ (shoots or roots) and independent stress treatment (osmotic, heavy metal or heat shock); in addition, one analysis was performed on the combined dataset comprising both organs in all stress conditions. This is necessary because the reference gene may vary depending on the experimental settings.³⁰ Three different Excel-based algorithms have been applied for data analysis, geNorm version v3.5,⁵⁰ and NormFinder v0.953⁵¹ require relative input data, so the Cq values were converted with the formula 2^{(-ΔΔCt)} where ΔΔCt is the difference of each Cq value minus the lowest Cq value (highest expression level). BestKeeper instead, uses raw Cq values. geNorm calculates stability value (M) based on the average pairwise comparison with a stepwise exclusion of the highest M value (least stable gene). Further, geNorm calculates the number of genes needed for a reliable normalization considering the pairwise variation (Vn/Vn+1) between sequential normalization factors, NF_n and NF_{n+1}. This number is optimal when the addition of one more reference gene does not significantly contribute to the variation of the normalization factor (NF_{n+1}) or, as suggested, the value drops below 0.15. NormFinder uses an ANOVA-based algorithm to estimate intra- and inter- group variation for a given set of experiments, providing a rank where the most stable gene is the one with lowest stability (S) value. Moreover, NormFinder provides the best gene pair combination that minimizes the expression differences among subgroups, if subgroups are set. BestKeeper, differently from the other algorithms, does not provide a direct rank list but calculates standard deviation (SD) of Cq and coefficient of variation (CV [%Cq]) for each gene. We sorted the Cq values to rank the genes from most stable (lowest CV value) to least stable (highest CV value).

Data Availability All data analysed during this study are included in this article (and its Supplementary Information files).
References

1. Leong, D. T. et al. Absolute quantification of gene expression in biomaterials research using real-time PCR. *Biomaterials* **28**, 203–210 (2007).

2. Ginzinger, D. G. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp. Hematol.* **30**, 503–12 (2002).

3. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* **29**, e45 (2001).

4. Cubero, J. & Graham, J. H. Quantitative real-time polymerase chain reaction in drosophilid flies and allelic discrimination to differentiate xanthomonas strains on citrus. *Phytopathology* **95**, 1333–1340 (2005).

5. Sun, H. et al. DNA microarray revealed and RNAi plants confirmed key genes conferring low Cd accumulation in barley grains. *BMC Plant Biol.* **15**, 259 (2015).

6. Khalil-Ur-Rehman, M. et al. Comparative RNA-seq based transcriptomic analysis of bud dormancy in grape. *BMC Plant Biol.* **17**, 18 (2017).

7. Bustin, S. A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622 (2009).

8. Kozera, B. & Rapacz, M. Reference genes in real-time PCR. *J. Appl. Genet.* **54**, 391–406 (2013).

9. Yim, A. K. et al. Using RNA-Seq data to evaluate reference genes suitable for gene expression studies in soybean. *PLoS One* **10**, e0136343 (2015).

10. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034 (2002).

11. Andersen, C. L., Ledet-Jensen, J. & Orntoft, T. Normalization of real-time quantitative RT-PCR data: a mode-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250 (2004).

12. Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**, 509–515 (2004).

13. Xie, F., Xiao, P., Chen, D., Xu, L. & Zhang, B. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol. Biol.* **80**, 75–84 (2012).

14. Silver, N., Best, S., Jiang, J. & Thein, S. L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol.* **7**, 33 (2006).

15. De Spiegelaere, W. et al. Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One* **10**, 1–13 (2015).

16. Hardion, L., Verlaque, R., Saltonstall, K., Leriche, A. & Vila, B. Origin of the invasive Arundo donax (Poaceae): a trans-Asian expedition in herbaria. *Ann. Bot.* **114**, 455–462 (2014).

17. Dragoni, F. et al. Giant reed (Arundo donax L.) for biogas production: land use saving and nitrogen utilisation efficiency compared with arable crops. *Ital. J. Agron.* **10**, 192–201 (2015).

18. Mann, J. J., Barney, J. N., Kyser, G. B. & Di Tomas, J. M. Miscanthus x giganteus and Arundo donax shoot and rhizome tolerance of extreme moisture stress. *GCB Bioenergy* **5**, 693–700 (2013).

19. Nassi o Di Nasso, N., Angelini, L. G. & Bonari, E. Influence of fertilisation and harvest time on fuel quality of giant reed (Arundo donax L.) in central Italy. *Eur. J. Agron.* **32**, 219–227 (2010).

20. Martinoia, M., D’Agosta, G. M., Copani, V., Patané, C. & Cosentino, S. L. Biomass yield and energy balance of three perennial crops for energy use in the semi-arid Mediterranean environment. *F. Crop. Res.* **114**, 204–213 (2009).

21. Di Nasso, N. O., Roncucci, N. & Bonari, E. Seasonal Dynamics of Aboveground and Belowground Biomass and Nutrient Accumulation and Remobilization in Giant Reed (Arundo donax L.): A Three-Year Study on Marginal Land. *BIOENERGY Res.* **6**, 725–736 (2013).

22. Papazoglou, E. G., Karantounia, G., Vemmos, S. N. & Bouranis, D. L. Photosynthesis and growth responses of giant reed (Arundo donax L.) to the heavy metals Cd and Ni. *Environ. Int.* **31**, 243–9 (2005).

23. Sablok, G. et al. Fuelling genetic and metabolic exploration of C3 bioenergy crops through the first reference transcriptome of Arundo donax L. *Plant Biotechnol. J.* **12**, 554–567 (2014).

24. Fu, Y. et al. Dissection of early transcriptional responses to water stress in Arundo donax L. by unigene-based RNA-seq. *Biotechnol. Biofuels* **9**, 84 (2016).

25. Barrero, R. A. et al. Shoot transcriptome of the giant reed, Arundo donax. *Data Br.* **3**, 1–6 (2015).

26. Sakuma, Y. Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell* **18**, 1292–1309 (2006).

27. Li, M. et al. In Planta Recapitulation of Isoprene Synthase Evolution from Ocimene Synthases. *Mol. Biol. Evol.* 1–17 doi:10.1093/molbev/msx178 (2017).

28. Kumar, K., Muthamilarasan, M. & Prasad, M. Reference genes for quantitative real-time PCR analysis in the model plant foxtail millet (Setaria italica L.) subjected to abiotic stress conditions. *Plant Cell. Tissue Organ Cult.* **115**, 13–22 (2015).

29. Shakoorn, N. et al. A Sorghum bicolor expression atlas reveals dynamic genotype-specific expression profiles for vegetative tissues of grain, sweet and bioenergy sorghums. *BMC Plant Biol.* **14**, 35 (2014).

30. Mar, J. C. et al. Data-driven normalization strategies for high-throughput quantitative RT-PCR. *BMC Bioinformatics* **10**, 1–10 (2009).

31. Money, N. P. Osmotic pressure of aqueous polyethylene glycols: relationship between molecular weight and vapor pressure deficit. *Plant Physiol.* **91**, 766–9 (1989).

32. Lawlor, D. W. Absorption Of Polyethylene Glycols By Plants And Their Effects On Plant Growth.pdf. *New Phytol.* **69**, 501–513 (1970).

33. Benavides, M. P., Gallego, S. M. & Tomaro, M. L. Cadmium toxicity in plants. *Brazilian J. Plant Physiol.* **17**, 21–34 (2005).

34. Papazoglou, E. G., Serelis, K. G. & Bouranis, D. L. Impact of high cadmium and nickel soil concentration on selected physiological parameters of Arundo donax L. *Eur. J. Soil Biol.* **43**, 207–215 (2007).

35. Bita, C. E. & Gerats, T. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Front. Plant Sci.* **4**, 1–18 (2013).

36. Brulle, F., Bernard, F., Vandenbulcke, F., Cuny, D. & Dumez, S. Identification of suitable qPCR reference genes in leaves of Brassica oleracea under abiotic stresses. *Ecotoxicology* **23**, 459–471 (2014).

37. Matsukura, S. et al. Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes. *Mol. Genet. Genomics* **283**, 183–196 (2010).

38. Guénin, S. et al. Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. *J. Exp. Bot.* **60**, 487–493 (2009).

39. Angelini, L. G. & Ceccarini, L. Nanni Di Nasso, N. & Bonari, E. Comparison of Arundo donax L. and Miscanthus x giganteus in a long-term field experiment in Central Italy: Analysis of productive characteristics and energy balance. *Biomass and Bioenergy* **33**, 635–643 (2009).

40. Papazoglou, E. G. Arundo donax L. stress tolerance under irrigation with heavy metal aqueous solutions. *DESALINATION* **211**, 304–313 (2007).
41. Lee, J. H., Terraghi, W. & Deng, X. W. DWA3, an Arabidopsis DWD protein, acts as a negative regulator in ABA signal transduction. Plant Sci. 180, 352–357 (2011).
42. Lee, J.-H. et al. Characterization of Arabidopsis and rice DWD proteins and their roles as substrate receptors for CUL4-RING E3 ubiquitin ligases. Plant Cell 20, 152–67 (2008).
43. Kim, S. et al. Characterization of a novel DWD protein that participates in heat stress response in Arabidopsis. Mol. Cells 37, 833–40 (2014).
44. Stone, S. L. The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling. Front. Plant Sci. 5, 135 (2014).
45. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K. & Scheible, W.-R. Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization. Plant Physiol. 139, 5–17 (2005).
46. Sarry, J. E. et al. The early responses of Arabidopsis thaliana cells to cadmium exposure explored by protein and metabolite profiling analyses. Proteomics 6, 2180–2198 (2006).
47. Guo, J., Ling, H., Wu, Q., Xu, L. & Que, Y. The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses. Sci. Rep. 4, 7042 (2014).
48. Lin, Y. et al. Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. PLoS One 9 (2014).
49. Yang, Z., Chen, Y., Hu, R., Tan, Z. & Huang, B. Identification and validation of reference genes for quantification of target gene expression with quantitative real-time PCR for tall fescue under four abiotic stresses. PLoS One 10, 1–15 (2015).
50. Loivamäki, M. et al. Circadian rhythms of isoprene biosynthesis in grey poplar leaves. Plant Physiol. 143, 540–51 (2007).

Acknowledgements
The authors wish to thank Enrico Barbaro for help in plant collection and PCR analysis, Violeta Velikova and Francesco Loreto for discussion. This work was supported by the Autonomous Province of Trento (Italy) through core funding of the Ecogenomics group (ML and CV), the NIDRA fellowship of Fondazione Mach FIRST PhD school (MP) and by the MAN-VIP project (Team 2011 Call approved with provincial government resolution no. 2902 on 14 December 2010).

Author Contributions
M.L. and C.V. conceived the study and supervised it with the help of S.S. M.P. did the experimental work and drafted the manuscript. All authors reviewed the manuscript and finalized it.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-11019-0

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017