

RESEARCH ARTICLE

MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *SUAEDA MARITIMA* FROM PARANGIPETTAI COASTAL AREAS, SOUTHEAST COAST OF INDIA

G. Anbarasi* and B. Vishnupriya

Department of Biotechnology, Kongunadu Arts and Science College (Autonomous),
Coimbatore – 641 029. Tamil Nadu, India.

ABSTRACT

Conventional taxonomy is limited with delineating species and controversies arise with DNA barcoding based identifications. Hence, an alternative supporting approach is very much needed to identify species and differentiate them within the species based on the genetic material. 18S rRNA genes have been particularly helpful in analyzing phylogeny at the species level. In addition, bioinformatics which represents a new, growing area of science uses computational approaches to answer biological questions. Salt tolerant coastal salt marsh plant of *Suaeda maritima* was selected for 18s rRNA sequencing to solve the ambiguity in its species level identification. Similarity search of study species shared 99% similarity with 5 species of *Atriplex canescens* clone s128, *Atriplex torreyi* var. *griffithsii* clone p508, *Spinacia oleracea*, *Oenothera laciniata* clone, *Beta vulgaris*. Phylogenetic tree infer that *S.maritima* is closely related to *Spinacia oleracea* and *Oenothera laciniata*. *Atriplex canescens* (fourwing saltbush), *Atriplex torreyi* and *Phaulothamnus spinescens*, *Celosia argentea* found to be closely related and are in one group. Hence, this study result clearly shows thus study species evaluated from angiosperm and provides key step in understanding the evolution of salt tolerance in angiosperm.

Keywords: *Suaeda maritima*, Molecular identification, 18s rRNA sequencing, Phylogenetic tree

1. INTRODUCTION

Coastal habitats represent important ecosystems in terms of biodiversity; they can be broadly classified into four distinct types: salt marshes and mangroves, sand dunes, vegetated shingle and rocky cliffs. Of these, salt marshes are particularly important habitats, in terms of their ecosystem value. The plants that grow on coastal marshes must be tolerant to the varying saline conditions. Colonies areas characterized by regular inundation, shifting sedimentation patterns and the hydraulic power of waves such sites are typically found behind sandbars and spits and in estuaries (Little, 2000).

Halophytic plants are generally located in coastal areas and they also exist as in land salt marshes in appropriate geological and climatological settings around the globe. Halophytes represent salt-tolerant species that thrive in the inhospitable habitats of inland and coastal salt marshes, dunes, beaches, deserts and salt flats (Flowers *et al.*, 1986, W.H.O, 1990). Different physiological and anatomical traits of halophytes vary between species to survive in saline habitats. Anatomical structures of plant organs, especially of leaves, thus enabling plant adaptation to its environment. Many leaf traits have been recognized to provide a protection against various environmental conditions and stresses including drought, high air, temperature and high concentration of salt in soil. The morpho-anatomical

alterations of succulent halophytes include increase of cell volume, especially of spongy and water paenchma increase of leaf thickness and decrease in number of stomata (Strogonov, 1973).

Recent advances in sequence analyses methods and softwares have revolutionized the phylogenetic studies. Phylogeny is an evolutionary tree that shows how species are related to each other, and this model has greatly facilitated the discussion and testing the hypotheses also this technique considered as a powerful tool for addressing many different evolutionary questions (Elliott *et al.*, 2006). The identification of one species and its homologs in several species indicates that these genes divergence from their common ancestor. Because of the adaptive characteristics the salt marsh plants have been extensively studied in terms of morphology, pharmacology and cytology.

To date, traditional taxonomy relies mostly on diagnostic morphological characters, requiring expert knowledge to identify specimens. In this regard, DNA sequencing and phylogenetic analysis has proved to be a useful and alternative method for rapid global biodiversity assessment providing an accurate identification system for living organism. But molecular and bioinformatics studies on the vital coastal salt marsh are largely unexplored. Therefore phylogenetic analysis was carried out to study the homologs of study species with other species. This can aid in our understanding of the

evolution of species and could serve to develop model systems for studying the species for future analysis. The main goal of this study was to identify the plant species and to predict the homologs with other species through a phylogenetic analysis.

2. MATERIALS AND METHODS

2.1. Isolation of Genomic DNA

In the present study, samples for PCR analysis (leaf tissue of *Suaeda maritima*) were collected into a sterile eppendorf tube. The tissue was softened for 15s at room temperature using a sterile tip as grinder without any buffer, 500µl of extraction buffer (200mM Tris-HCl (pH 7.5), 250mM NaCl, 25mM EDTA, 0.5% SDS) (Edwards *et al.*, 1991) was added to the tube and mixed for 5s. In order to disrupt plant cells completely, the tube was placed in a water bath at 60°C for 30min. Subsequently, an equal volume of chloroform:iso-amyl alcohol (24:1) was added to the sample, which was then mixed and centrifuged at 15,000g for 5min at 4°C. The aqueous supernatant was transferred to a new eppendorf tube, to which an equal volume of isopropanol was added, mixed and incubated at -20°C for 30min. Following centrifugation at 15,000g for 5min, the pellet was dried and dissolved in 100µl DEPC water. Finally, for precipitation of starch and other insoluble polysaccharides, the tube was placed on ice for 5min and centrifuged at 15000g for 2min; the resulting white pellet was mostly starch (Deshmukh *et al.*, 2007). The supernatant containing the DNA was stable at 4°C.

2.2. Quantitation and Quality Assessment of DNA

The DNA stock samples was quantified using Nanodrop spectrophotometer at 260 and 280nm using the convention that one absorbance unit at 260nm wavelength equals 50µg DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 was considered to be of good purity. Concentration of DNA was estimated using the formula. Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor Quality and purity of DNA were checked by agarose gel electrophoresis. Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer was used for submarine gel electrophoresis. Ethidiumbromide (1%) was added at 10µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl gel loading dye. Electrophoresis was carried out at 80V for 30min at room temperature. DNA was

visualized under UV using UV transilluminator. The DNA was further used for PCR analysis.

2.3. Polymerase Chain Reaction

18SrRNA gene fragment was amplified by PCR from genomic DNA using 18S gene universal primers: 1F:5'CTGGTGCCAGCAGCCGCGGYAA3' and 4R: 5'CKRAGGGCATYACWGACCTGTTAT3'. PCR was carried out in a final reaction volume of 25µl in 200µl capacity thin wall PCR tube in Eppendorf Thermal Cycler. Composition of reaction mixture for PCR is given in Table 1. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given in Table 2.

Table 1. Composition of reaction mixture for PCR

| Components | Quantity | Final |
|-----------------------------------|-----------------|-----------|
| DNase-RNase free water | 7.50 µl | -- |
| 2X PCR master mix (MBI Fermentas) | 12.50 µl | 1X |
| Forward Primer (10 pmole/µl) | 1.00 µl | 10 pmole |
| Reverse Primer (10 pmole/µl) | 1.00 µl | 10 pmole |
| Diluted DNA (30ng/µl) | 3.0 µl | -- |
| GRAND TOTAL | 25.00 µl | -- |

Table 2. Steps and conditions of thermal cycling for PCR

| Steps | Temperature | Time | Cycles |
|----------------------|-------------|--------|--------|
| Initial Denaturation | 95°C | 2 min | 1 |
| Final Denaturation | 94°C | 30 Sec | |
| Annealing | 50°C | 30 Sec | 30 |
| Extension | 72°C | 90 Sec | |
| Final Extension | 72°C | 10 min | 1 |

2.4. Visualization of PCR Product

To confirm the targeted PCR amplification, 5µl of PCR product from each tube was mixed with 1µl of 6X gel loading dye and electrophoresed on 1.2% agarose gel containing ethidium bromide (1 per cent solution at 10µl /100ml) at constant 5V/cm for 30min in 0.5X TAE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (Biorad).

2.5. Purification of PCR product

Amplified PCR product was purified using Qiagen Mini elute Gel extraction kit according to the manufacture's protocol.

2.6. Sequencing of Purified 18S rRNA Gene Segment

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturer's instructions.

2.7. Cycle Sequencing

Cycle sequencing was performed following the instructions supplied along with BigDye® Terminator v3.1 Cycle Sequencing Kit. The reaction was carried out in a final reaction volume of 20µl using 200µl capacity thin wall PCR tube. The cycling protocol (Table 3) was designed for 25 cycles with the thermal ramp rate of 1°C per second. After cycling, the extension products were purified and mixed well in 10µl of Hi-Di formamide. The contents were mixed on shaker for 30min at 300xg. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95°C for 5min, snap chilled and loaded into autosampler of the instrument.

Table 3. Cycling protocol for sequencing reaction

| Step | Temperature | Time |
|--|-------------|--------|
| Denaturation | 96°C | 10 sec |
| Annealing | 50°C | 5 sec |
| Extension | 60°C | 4 min |
| *Repeat step 1 to 3 for 25 cycles | | |

2.8. Electrophoresis and Data Analysis

Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer using appropriate Module, Basecaller, Dyeset /Primer and Matrix files.

2.9. Sequence analysis

The sequence was compared for detecting homologous sequences found in databases using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). BLAST from NCBI was used to compare the query sequence with the database sequence to find its homologues.

2.10. Data analysis

To better present the diversity of the isolates obtained in this study and to assess the genetic relationship of the novel ones here to the known ones, intraspecific phylogenies were reconstructed with the neighbor-joining (NJ) and maximum likelihood (ML) methods in MEGA7.0 program. NJ and ML trees were constructed using

1000 and 100 bootstrap replicates, respectively (Tamura *et al.*, 2011; Nei and Kumar, 2000).

2.11. Wet Lab methodology

Quantitation of DNA was estimated in two different absorbances and the average was taken into consideration. OD values with its average and the concentration of the DNA in µg/µl and ng/µl is shown in Table 4. The isolated genomic DNA of the study species is shown in Fig 1.

Table 4: Quantitation of DNA

| Sample | A ₂₆₀ | A ₂₈₀ | A ₂₆₀ /A ₂₈₀ | Conc. (µg/µl) | Conc. (ng/µl) |
|--------------------|------------------|------------------|------------------------------------|---------------|---------------|
| <i>S. maritima</i> | 0.435 | 0.233 | 1.86 | 2.175 | 2175 |



Fig. 1. Genomic DNA isolation of the study species

2.12. Electrophoresing PCR amplicons

The PCR Products (5µl) was separated by electrophoresis in 1.2% Agarose gels containing ethidium bromide (1 per cent solution at 10µl /100ml). Electropherogram obtained after electrophoresis of the PCR amplicons is shown in Fig 2. The amplified product was visualized as a single compact band of expected size against 100bp DNA ladder (Fermentas make). No overlapping of the band in the case of test organism was observed and so the band was clear.

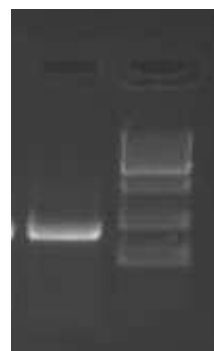


Fig. 2. The electropherogram obtained after electrophoresis of the PCR

2.13. Sequence analysis

The DNA sequence was analyzed with bioinformatic tools and softwares with statistical applications. Simultaneously the DNA sequences

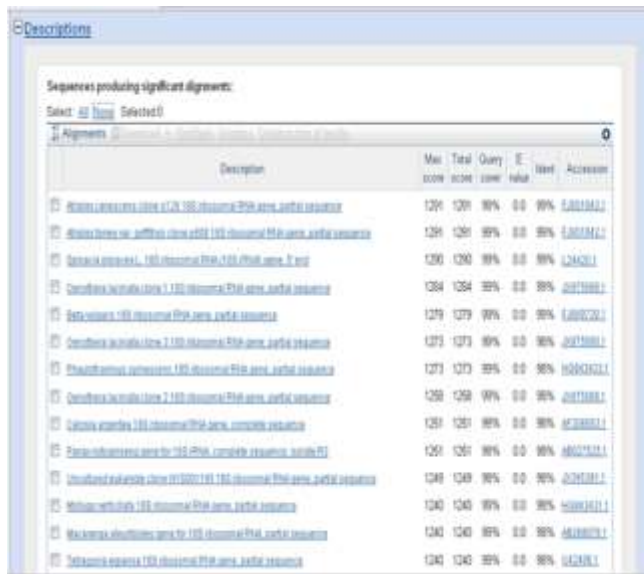
were submitted to NCBI for obtaining the accession number. The DNA sequence was aligned in FASTA format and shown as follows

```
AGGTTGTTTGCAGTTAAAAAGCTCGTAGTTGGACCTTGGGGTGGTACGACCGGTCCGCTCACGGTGTGCA
CCGGTCGTC TCGCCTCTTTCGCCGGCGATGCGCTCCTGGCC TTAAC TGGCCCGGGTCGTGCC TCCGGC ACT
GTTACTTTGAAGAAATTAGAGTGC TCAAAA GCAAGCC TACGC TC TGTATACATTAGCATGGGATAACATTATA
GGATTCCGGTCC TATTGTGTTGGCCTTCGGGATCGGAGTAATGATTAACAGGGACAGTCGGGGGCATTTCGTA
TTTCATAGTCAGAGGTGAAATTC TTGGATTATGAAAGACGAACAAC TCGGAAAGCATTTCGCCAAGGATGT
TTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCCTAGTC TCAACCATAAACG
ATGCCGACCAGGGATCGGCGGATGTTACTTTTAGGACGCCGCCGGCACC TTATGAGAAATCAAAGTTTTTG
GGTCCGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTAG
CCTGC GGCTTAATTTGACTCAACACGGGGAAAC TTACCAGGTCCAGACATAGTAAGGATTGACAGACTGAG
AGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTGTCTGGTTAATTC
CGTTAACGAA
```

2.14. Dry lab methodologies

As a general rule, a top match with a sequence similarity of at least 98% was used as a criterion to designate potential species identifications (Barbuto et al., 2010). The top sequences producing similar and significant alignments was identified for the study species

from BLAST and listed. Maximum identity of 99% with a maximum score of 1291 and the identity varied from 97-99% with the alignments derived from NCBI. Family and their accession numbers of sequences closely related to the test organisms used in the analysis were given in.



| Species | Accession Number | Family |
|--|------------------|----------------|
| <i>Atriplex canescens</i> clone s128 | EF601843.1 | Amaranthaceae |
| <i>Atriplex torreyi</i> var. <i>griffithsii</i> clone p508 | EF601842.1 | Chenopodiaceae |
| <i>Spinacia oleracea</i> | L24420.1 | Amaranthaceae |
| <i>Oenothera laciniata</i> clone I | D2675688.1 | Onagraceae |
| <i>Beta vulgaris</i> | EF669720.1 | Amaranthaceae |
| <i>Phalaenoxanthus spinescens</i> | HQ843433.1 | Achotacaceae |
| <i>Colexia argentea</i> | AF206883.1 | Amaranthaceae |
| <i>Mollugo verticillata</i> | HQ843431.1 | Molluginaceae |
| <i>Halophytum amplexivale</i> | HQ843429.1 | Halophytaceae |

Fig. 3. Sequences Producing Significant Alignments from BLAST

2.15. Multiple Sequence Alignment

The close phylogenetic relationships were found within the samples. This was clear evidence in both Multiple Sequence Alignments and Molecular Phylogenetic analysis by Maximum Likelihood and NJ methods. The alignment was easy and clear because no gaps were found in our sequence. Read lengths were about 724bp long, although in some instances some base calls were uncertain. No insertions, deletions or stop codons were observed (Fig. 4).

2.16. Evolutionary relationships of taxa by Neighborhood joining Method

18srRNA partial sequence of study species shared 99% similarity with 5 species of *Atriplex canescens* clone s128, *Atriplex torreyi* var. *griffithsii* clone p508, *Spinacia oleracea*, *Oenothera laciniata* clone, *Beta vulgaris* in genbank database. In order to strengthen the results, phylogenetic analysis was included in the present study. Phylogram of 18SrRNA gene was built with 9 closely related 18SrRNA sequences were collected from Genbank by referring BLAST results. The

evolutionary relationship was inferred using the neighbor joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to branches (Felsenstein, 1985). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances were computed using the Maximum Likelihood method (Tamura et al., 2004) and in the units of the number of base substitutions per sites. All positions containing gaps and missing data were eliminated from the dataset (complete deletion options). Phylogenetic analysis was conducted in MEGA 5 (Tamura, 2011).



Fig 4. Multiple Sequence Alignment

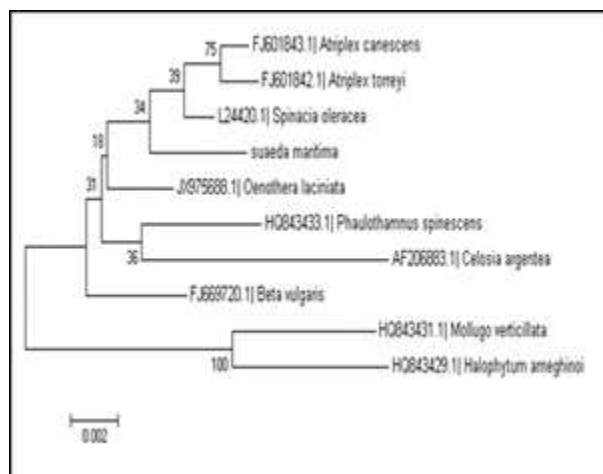


Fig. 5. Evolutionary relationships of taxa by NJ Method

Phylogenetic tree infer that *S.maritima* is closely related to *Spinacia oleracea* and *Oenothera laciniata*. Along with these two species *S.maritima*

forming monophyletic group with *Beta vulgaris* (Sea beet or Sub sp.maritima), *Atriplex canescens* (fourwing saltbush), *Atriplex torreyi*, *Phaulothamnus spinescens* and *Celosia argentea* found to be closely related and were fallen in one group. Phylogram showed distinct clade with *Mollugo verticillata* and *Halophytum ameghinoi* (Succulent annual plant) with strong bootstrap support value of 100%.

3. RESULTS AND DISCUSSION

Halophytes have evolved to cope with a wide variety of climatic conditions, all around the world, and they are found at varying altitudes, although in greatest numbers at sea level. (Khan *et al.*, 2006a). The number of halophytic species is an area of some debate and will remain so while there are no highly specific definitions for levels of salinity or for the mechanisms employed in salt tolerance. Lieth *et al.*, (2008) claimed the occurrence of 1,560 terrestrial halophyte species. However, Khan *et al.*, (2006b) stated that, the number could be as high as 6,000 for 6 terrestrial halophytes (including tidal zones). Flowers *et al.*, (1986) found that about one third of all the angiosperm families contain halophytic genera, but the glycophytes would have lost salt-tolerance through evolutionary processes. It is now accepted that there are approximately 20 significant families containing some 500 halophytic genera. The most widespread and well-studied family is one of the Dicotyledoneae: Chenopodiaceae, which, according to Flowers *et al.*, (1986) includes 44 halophytic genera with 312 halophytic species; these 44 genera represent 44% of the family. Perhaps the most important genera within the research community are *Atriplex* sp, *Salicornia* sp and *Suaeda* sp; this last one is to play a central part in salt tolerance research (Flowers *et al.*, 1986).

Of the *Suaeda* genus, the current research was focused particularly on *Suaeda maritima* (L.) also known as sea blite, which is an annual obligate halophyte that has the distinctive feature in that it thrives in both coastal and inland salt marshes. It is a model plant and its salt-tolerance has been extensively investigated (Flowers *et al.*, 1977; Yeo, 1981; Greenway and Munns, 1983; Hajibagheri *et al.*, 1985; Clipson, 1987). For molecular identification salt marsh plant of *S. maritima* was selected for 18sDNA sequencing to solve the ambiguity in its species level identification.

BLAST algorithm used to compare study sequence with the sequences available in the GenBank database. Maximum identity of 99% with a maximum score of 1291 and the identity varied from 97-99% with the alignments derived from NCBI. On

the contrary, currently there is no 18S sequence data of *S.maritima* deposited in GenBank. Hence, this is the first report of partial 18srRNA sequence of *Suaeda maritima*. According to these intra generic differences between molecular and morphological results, this study extend to sequencing with distinct markers to identify these isolates in future studies. In addition, the absence of sequence data for *Suaeda maritima* (only *Suaeda maritima* isolate 6, 7 and 8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence available in GenBank) made our molecular identification harder. In case of similarity search of study species shared 99% similarity with 5 species of *Atriplex canescens* clone s128, *Atriplex torreyi* var. *griffithsii* clone p508, *Spinacia oleracea*, *Oenothera laciniata* clone, *Beta vulgaris* are belongs to angiosperm.

In order to classify and trace out evolutionary path among related species, it is important to investigate them at the molecular level for better understanding of evolution of plants. 18S gene is one of the useful phylogenetic marker which one can use in understanding phylogenetics. Phylogenetic tree infer that *S.maritima* is closely related to *Spinacia oleracea* and *Oenothera laciniata*. Along with these two species *S.maritima* forming monophyletic group with *Beta vulgaris* (Sea beet or Sub sp.*maritima*). *Atriplex canescens* (fourwing saltbush), *Atriplex torreyi* and *Phaulothamnus spinescens*, *Celosia argentea* found to be closely related and are in one group. Phylogram showed distinct clade with *Mollugo verticillata* and *Halophytum ameghinoi* (Succulent annual plant) with strong bootstrap support value of 100% (Fig: 5). Current study results clearly shows thus study species evaluated from angiosperm. Flowers *et al.*, (1977) and (2010) reported that, halophytes come from a wide range of angiosperm lineages, suggesting that the adaptations involved in salt tolerance have arisen repeatedly during angiosperm evolution. This result provides a key step in understanding the evolution of salt tolerance in angiosperm.

The results of the present study highlight the identification of plant species by investigating them at the molecular level with the use of molecular markers for a better and wide understanding of halophytic plants.

ACKNOWLEDGEMENT

The authors would like to thank CAS Marine Biology for providing facilities and Department of Biotechnology for their financial support of this research.

REFERENCES

1. Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, Z. W. Miller and D.J. Lipman, (1997). Gapped Blast and Psi-Blast: A new generation of protein database search programs. *Nuc. Acids Res.* **25**: 3389-3402.
2. Barbuto, M., A. Galimberti, E. Ferri, M. Labra, R. Malandra, P. Galli and M. Casiraghi, (2010). DNA barcoding reveals fraudulent substitutions in shark seafood products. *Food. Res. Int.* **43**: 376-381.
3. Clipson, N.J., D.R. Lechno and T.J. Flowers, (1988). Salt tolerance in the halophyte *Suaeda maritima* L. Dum: abscisic acid concentrations in response to constant and altered salinity. *J. Exp. Bot.* **39**: 1381-1388.
4. Deshmukh, V.P., P.V. Thakare, U.S. Chaudhari and P.A. Gawande, (2007). A simple method for isolation of genomic DNA from fresh and dry leaves of *Terminalia arjuna* (Roxb). *Wight and Argot. Electron. J. Biotechnol.* **10**: 468-472.
5. Edwards, K., C. Johnstone and C. Thompson, (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nuc. Acids. Res.* **19**: 1349pp.
6. Elliott, M.B., M. David, Irwin, Eleftherios and P. Diamandis, (2006). In silico identification and Bayesian phylogenetic analysis of multiple new mammalian kallikrein gene families. *Genomics.* **88**: 591-599.
7. Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783-791.
8. Flowers, T.J., M.A. Hajibagheri and N.J.W. Clipson, (1986). Halophytes. *Q. Rev. Biol.* **61**: 313-337.
9. Flowers, T.J., P.F. Troke and A.R. Yeo, (1977). The mechanism of salt tolerance in halophytes. *Annu. Rev. Plant. Physiol.* **28**: 89-121.
10. Flowers, T.J., H.K. Galal and L. Bromham, (2010). Evolution of halophytes: multiple origins of salt tolerance in land plants. *Func. Plant Biology* **37**: 604-612.
11. Greenway, H. and R. Munns, (1983). Interactions between growth, uptake of Cl⁻ and Na⁺ and water relations of plants in saline environments. Highly vacuolated cell. *Plant. Cell. Environ.* **6**: 575-589.
12. Hajibagheri, M.A., A.R. Yeo and T.J. Flowers, (1985). Salt tolerance in *Suaeda maritima* (L.) Dum. fine structure and ion concentrations in the apical region of the roots. *New. Phytol.* **99**: 331-343.
13. Khan, M.A., D.J. Weber and B. Gul, (2006a). Halophyte seed germination. In: M. A. Khan and

- D.J. Weber. *Eco-Physiology of High Salinity Tolerant Plants*. Springer, Netherlands, 11-30.
14. Khan, M.A., D.J. Weber and N.P. Yensen, 2006b. Halophyte uses for the twenty-first century. *Ecophysiology of High Salinity Tolerant Plants*. Springer, Netherlands, 67-396.
15. Little, C. (2000). *The Biology of Soft Shores and Estuaries*. Oxford University Press, New York.
16. Nei, M. and S. Kumar, (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press. New York, 333pp.
17. Saitou, N. and M. Nei, (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.
18. Strogonov, B.P. (1973). *Metabolizm rastenij vuslovijah zasolenija* (Metabolism of plants under salinity conditions). Moskva: Izdatelstvo Nauka.
19. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, (2011). Molecular evolutionary genetics analysis: 4using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731-2739.
20. Tamura, K., M. Nei and S. Kumar, (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U.S.A.* **101**: 11030-11035.
21. W.H.O, E. (1990). Ecophysiology of plants in waterlogged and flooded environments. *Aqu. Bot.* **38**: 73-90.
22. Yeo, A.R. (1981). Salt tolerance in the halophyte *Suaeda maritima* (L.)Dum.: intracellular compartmentation of ions. *J. Exp. Bot.* **32**: 487-497.

About The License



The text of this article is licensed under a Creative Commons Attribution 4.0 International License