Phylogenetic diversity shapes salt tolerance in *Phragmites australis* estuarine populations in East China

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Estuaries are dynamic and selective environments that provide frequent opportunities for the turnover of *Phragmites australis* populations. We studied *Phragmites* genetic diversity patterns in three of the major deltas of China, viz. the Yellow River, the Yangtze and the Liaohe, in relation to *Phragmites* global phylogeography and soil salinity. We found that two distinctly related *P. australis* haplotypes, each with intercontinental distribution, co-occur in these deltas in China. One is European *Phragmites* (Haplotype O) and is related to *P. japonicus*; the other (Haplotype P) has its range in East Asia and Australia and is related to the Asian tropical species *P. karka*. The two haplotypes have differing salt tolerance, with Haplotype O in areas with the highest salinity and Haplotype P in areas with the lowest. Introgressed hybrids of Haplotype P with *P. karka*, and F1 hybrids with Haplotype O, have higher salt tolerance than Haplotype P. Phylogenetic diversity appears as the factor that better explains population structure and salinity tolerance in these estuaries. Future research may explain whether the two *P. australis* haplotypes evolved in East Asia, and East Asia is a center of *Phragmites* diversity, or are introduced and a threat to *P. japonicus* and *P. karka*.

Estuaries are dynamic environments with frequent disturbance and extreme conditions, which provide repeated opportunities for the decline and the establishment of new populations. Propagules can be recruited from nearby populations or be introduced from distant and different environments by migratory birds, or international ship traffic. One of the most common and productive aquatic plants inhabiting the world's estuaries is *Phragmites australis* (Cav.) Trin. ex Steud., a cosmopolitan tall grass with high intraspecific variation and ecological amplitude¹. *Phragmites australis* populations are extremely phylogenetically diverse in the Mississippi River Delta²,³ and in the Danube Delta⁴. Phylogenetic variation, including ploidy variation²–⁴, provides fitness in these dynamic environments due to the enlarged eco-physiological adaptability provided by a gene pool of genotypes of multiple distinct origins²–⁷. Due to their genetic diversity and the strong and variable selection pressure of the environment, estuarine marshlands are evolutionary hotspots of *Phragmites* diversity and can be dangerous gateways for invasions, especially cryptic invasions, in ranges where *P. australis* is native. For example, *P. australis* invasion in North America started at New York Harbor in marshes of the Hudson River estuary, where introduced European *P. australis* displaced the native populations of *Phragmites australis* ssp. *americanus*⁸,⁹.

Salt tolerance is a competitive trait in brackish estuarine environments. As a species, *P. australis* tolerates a wide salinity range¹⁰–¹⁴, but genotypes of different phylogeographic origin differ in salinity tolerance¹⁵ due to their different bioclimatic origins. For example, the invasive Mediterranean *Phragmites* lineage thrives in the brackish Mississippi River Delta thanks to its adaptation to drought evolved in the warm, dry climate of its native range in the Mediterranean and Middle East¹⁵. In the Yellow River Delta in China the genetic diversity of *P. australis* decreases with increasing soil salinity and only specific allelic phenotypes occur in the saltiest patches of the mosaic of saline habitats in the delta¹⁶. Such distinctive genotypes may have evolved locally from native populations as well as from introduced pre-adapted genotypes.

In this study we investigate phylogenetic diversity and its role in salt adaptation in three estuarine *P. australis* populations in China, in the Yangtze, Yellow River and Liaohe deltas. Unlike previous local studies of *P. australis*...
diversity in Chinese coastal marshes, we analysed the genetic diversity patterns of the Chinese populations within the global phylogeographic structure of the genus *Phragmites* in order to resolve their phylogeographic relationships and trace possible introductions. Several *Phragmites* lineages have recently been identified in China and Korea and classified according to the *Phragmites* classification system introduced by Saltonstall based on chloroplast DNA sequences, however a macro-perspective of the evolutionary history of the exceptionally high diversity of *Phragmites* lineages in East Asia is lacking. There are at least three *Phragmites* species in East Asia: *P. japonicus* Steud., *P. karka* (Retz.) Trin. ex Steud. and *P. australis*, with ploidy variation from 2n = 4x to 10x (with 2n = 8x being dominant), and several salt-tolerant ecotypes, Four *P. australis* ecotypes have been phenotyped in the Yellow River Delta based on tissue Na+K+ ratios as an indicator of salt stress. Taka-hashi et al. showed that salt-tolerant reeds from the Yellow River Delta contained low Na+ and high K+ content compared to salt-sensitive plants from a freshwater river in Japan which contained high Na+ and low K+ when exposed to salt. This suggests that salt-tolerant ecotypes in the Yellow River may be able to select K+ over Na+ in saline conditions and maintain ion homeostasis. The *HKT1* gene functions as a K+/Na+ co-transporter in *P. australis* and an insertion in an *HKT1* intron causes alternative splicing of the mRNA into the two transcripts of the salt-tolerant and salt-sensitive reeds. Variation in the genomic sequence of the *HKT1* gene, as documented by and by in wild Korean populations, may therefore distinguish salt-adapted and non-adapted genotypes. Therefore, in addition to (1) the phylogenetic relationships, we investigated also the (2) genetic structure of *Phragmites* populations (inferred by SSR—or microsatellites) in relation to soil salinity, (3) the *HKT1* gene polymorphism and (4) the phylogenetic diversity of chloroplast DNA. Based on previous studies of estuarine *Phragmites* populations elsewhere, we expected high phylogenetic diversity in the study area and a salt-dependent distribution of the phylotypes of different origin.

Results

Phylogenetic relationships of the estuarine populations. Fragment size polymorphism in the *TrnT-TrnL* short region of the cpDNA revealed two haplotype profiles within the Chinese estuarine populations (Fig. 1, Supplementary Information S1). Follow, the sequences matching *Phragmites* Haplotype P and O and their intrahaplotypic microsatellite variants (P1, P2, P3, P4 and O2, O3) or differed from Haplotype P in one to two substitutions (new Haplotypes AS, AT, AU) (GenBank accessions no. KP994324 to KP999334). In total we found 9 different haplotypes of two distantly related phylogeographic groups (Fig. 2) previously defined as the Far East-Australian group (hereafter P-related genotypes) and “European” *Phragmites*, despite its almost cosmopolitan distribution (hereafter O-related genotypes). Compared to the previous phylogenies the inclusion of the new haplotypes from Asia and Australia moved the Asian/Australian group closer to the tropical species *P. mauritianus* and *P. karka* (note the haplotypes of *P. karka* marked blue in the lower part of the PCoA with 71% support, Fig. 2). The populations in the Yangtze River Delta (CML, JDSL, CXL and SJL) were entirely composed of Haplotype P-related genotypes, whereas the population in the Yellow River Delta consisted of O-related genotypes (with the exception of one single P-related genotype). The Tianjin population consisted of O-related genotypes and the Liaohe O population was mixed with O- and P-related genotypes co-occurring in close proximity (Table S1).

The Bayesian analysis of SSR data inferred three ancestral populations for the Chinese estuarine populations (Fig. 3): one for the Haplotype-O related genotypes and *P. japonicus* (Haplotype AM) (orange), one for the Haplotype P-related genotypes (light blue), and one for the hybrids of Haplotype P and *P. karka* (dark blue). This analysis revealed several hybrids in the Yangtze Delta populations between *P. australis* P-related genotypes and a population of *P. karka* in the Mekong Delta in Viet Nam used as a *P. karka* reference in this study (Haplotype I and U and their microsatellite variants), and in the Liaohe Delta population between *P. australis* Haplotype P- and O-related genotypes and between *P. australis* Haplotype P and *P. karka*. Both hybrid types were Haplotype P. Only one hybrid in the Liaohe Delta population was Haplotype O and had admixed ancestry (higher than 20%) with both Haplotype P and *P. karka* (Fig. 3).

Population genetic structure. The SSRs divided the sample set into two main groups, which reflected the haplotype structure of the populations better than their geographic distribution (Fig. 4a,b), and the structure of *HKT1* homo-and heterozygotes (Fig. 4c,d).

Significant genetic divergence among the seven populations (Supplementary Information S1) was detected by the AMOVA of SSR data (Table 1a). The extent of divergence increased when the variance was tested between Haplotype O- and P-related genotypes (Table 1b) and disappeared among the populations related to Haplotype O when population structure was tested within haplotypes (Table 1c) and within population (Table 1d). It remained significant among the populations related to Haplotype P, both among populations of different deltas (Table 1e) and among the populations in the Yangtze Delta (CML, CXL and JDSL) (Table 1f). Differences in genetic variance due to SSR alleles were also detected between homozygotes and heterozygotes at the *HKT1*-1 locus of the *HKT1* gene (Table 1g) (but not at the *HKT1*-2 locus, Table 1h), however such a distinction disappeared when the variance was tested between homo- and heterozygotes of Haplotype O (Table 1i,j). It remained significant between the homo- and heterozygotes of the Haplotype P-related genotypes both at the *HKT1*-1 and *HKT1*-2 loci (Table 1k,l).

Three to five alleles per locus were frequent in the SSR profiles of both the Liaohe and Yangtze Haplotype P-related genotypes, whereas Haplotype-O related genotypes showed a diomic inheritance pattern. However, two exceptions of genotypes with 3 alleles at locus Pgt4 were found also in the O-related populations in the Yellow River and Liaohe Deltas. Genetic diversity (I, h and uh) in SSRs was highest within the Haplotype-P related populations due to the higher number of alleles in P-related than in O-related genotypes (Table 2).
Genetic diversity distribution in relation to soil salinity. The factor that best explained the distribution of the genotypes in relation to salt was phylogenetic diversity, considering the four independent phylo-types: Haplotype O- and P-related genotypes and their hybrids (Haplotype P x P. karka and Haplotype P x Haplotype O) (P = 0.01). Genotypes related to Haplotype O were distributed in areas with higher salinity than those related to Haplotype P. Both hybrid types of Haplotype P had a higher variance in salt tolerance than Haplotype O- and P-related genotypes, and occurred in areas of intermediate salinity, compared to the two haplotypes (Fig. 5).

The distribution of HKT1-homozygotes and heterozygotes at both HKT1-1 and HKT1-2 gene loci was not affected by salinity (P = 0.87 for HKT1-1; P = 0.50 for HKT1-2). The HKT1 sequences of the homozygotes showed large variation, especially among the genotypes of Haplotype O. The sequences of the previously documented salt-tolerant genotype from Nanpi in the Yellow River Delta in GenBank matched the sequence of some of our Haplotype O genotypes in the Yellow River Delta. The sequences of the salt-sensitive genotype from Ustonia in Japan (also in GenBank) matched instead the sequences of our P. japonicus references in South Korea and Sakhalin Island (Russia), and was different from the sequences of Haplotype P-related genotypes in the Yangtze River Delta. The sequences of P. karka genotypes were different from those of P. australis Haplotype O and Haplotype P, as well as from those of P. japonicus. We found correlations neither between SSR allelic frequencies and the salinity classes of the Soil Quality layer 5 (SQ5) of the Harmonized World Soil Database, nor with our salinity measurements in the Yangtze Delta, nor between allelic and HKT1 homo/heterozygotes frequencies, nor between HKT1 homo/heterozygotes frequencies and salinity.

Discussion
Phylogenetic relationships of the estuarine populations. Haplotypes of two phylogeographic regions of P. australis co-occur in the coastal populations in East China. The genotypes related to Haplotype O are closely related to the European populations of P. australis and the East Asian endemic species P. japonicus, whereas Haplotypes P, AS, AT, AU are closely related to populations of P. australis in Australia. In this study they appeared closely related also to the tropical species P. karka in Asia and P. mauritianus in Africa. Haplotypes O and P are the dominant haplotypes in the studied populations and both have a wide distribution range. Haplotype O is found across temperate Europe9,18 and Asia19 and its cp-microsatellite variant O2 found in this study is shared by the two continents. Haplotype P has previously been found in East Asia and Australia9,10,15,18,19 and its cp-microsatellite P4 was also found in both continents. The other novel Haplotypes AS, AT and AU appeared to have evolved locally from Haplotype P-related genotypes, given their restricted ranges within the studied populations and the very few base substitutions compared to the sequences of Haplotype P-genotypes analysed in this study. Two populations were identified also by Gao et al.16 to explain the structure of the Yellow River population, and two ancestral populations were discovered for Korean Phragmites20. Gao et al.16 justified the two ancestries as due to two Phragmites species, P. australis (sequenced as Haplotype P in that study) and P. japonicus in Korea. Two branches for Phragmites australis in East Asia were detected also by Yao et al.20 by ITS markers, that were identified as a suitable DNA barcode to capture the high genetic diversity of halo-tolerant Poaceae species in coastal areas.
In our study, Haplotype O-related genotypes occurred in areas with the highest salt impact and Haplotype P-related genotypes in areas with the lowest. As our samples from the Yellow River Delta were all Haplotype O-related genotypes, except for a single Haplotype P, and given our different geographic sampling from that of 16, we cannot relate our results to those of their study directly. However, we also found a salt-dependent distribution of our samples that seems to be explained by genotypes of different phylogenetic origin which, in agreement with 16, have distinct allelic patterns. In agreement with Chu et al. 20, we found two distinct populations for P.

Figure 2. Phylogenetic position of the Chinese populations (purple) in the global phylogeographic structure of the genus *Phragmites*, inferred by cpDNA sequences (*trnT-trnL* and *rbcL-psaI*). The haplotypes previously identified in East Asia and Australia are in red. The coordinates account for 44% of the variation in the data (27% coord. 1 and 17% coord. 2). The circles with the numbers indicate the statistical support from the parsimony analysis. Haplotypes IDs follow the names in GenBank. Haplotypes source: H28, H29, H30, H31, H32 and H33 (An et al. 2012). E4, S2 (corresponding to haplotype P1 in our study) and *Phragmites japonicus* (labeled *Pj*) (Chu et al., 2011). SLJ01 (Hurry et al. 2013). J, O, P, Q, X, Y (Saltonstall 2002). AN (Lambertini et al. unpublished). P1, P2, P3, P4, P5 and O2, O3 have been classified as microsatellites variants of haplotype P and O, as they differ from these in the numbers of repeats at the microsatellite loci. Statistical support is from the parsimony analysis. The PcoA is made with the program GenAlex ver. 6.445.

Figure 3. Population structure of SSR data. Inferred ancestry probability is in Y axis and population ID in X axis. (a) Population structure for K = 3. (b) Evanno’s “deltaK” inferring the most likely number of ancestral populations (K). The graph is made with Structure ver. 2.3.447 and Evanno’s delta K graph is made with Structure Harvester48.
**Figure 4.** Principal coordinate analysis of SSR pairwise genetic distances. The coordinates account for 37% of the variation (14% coord. 1 and 9% coord. 2). (a) The different colours indicate genotypes of populations of different geographic locations. (b) The different colours indicate genotypes related to Haplotype O and Haplotype P inferred by the *trnT* short fragment. (c) The different colours indicate homozygotes and heterozygotes at the *HKT1*-1 gene locus. (d) The different colours indicate homozygotes and heterozygotes at the *HKT1*-2 gene locus. The PcoAs are made with the program GenAlex ver. 6.4.

*Japonicus* and *P. australis* Haplotype P. As in our previous studies, *P. japonicus* is closely related to *P. australis* Haplotype O-related genotypes, both in cpDNA sequences and SSRs alleles. In the present study Haplotype P appeared distantly related to its conspecific Haplotype O and more closely related to *P. karka*, with which it is also introgressed. *Phragmites karka* is known to occur and hybridize with *P. australis* haplotype P in southwestern China. However, in contrast to *P. australis* Haplotype P is the seed donor of the hybrids that we found in the Yangtze and Liaohe deltas. Given the high degree of introgression and the lack of *P. karka* in our samples from the populations where the hybrids occur, *P. karka* introgression appears an inherited trait of Haplotype P rather than a recent local hybridization event.

The polysomic profiles of the SSRs alleles indicate a higher ploidy level for Haplotype P-related genotypes that consistently had more than two alleles, than that of Haplotype O-related genotypes that, instead, had a disomic pattern. Several studies have shown that Asian *P. australis* is dominated by octoploids and to a minor extent by hexaploids and decaploids, and that tetraploids are less frequent. Based on our studies that sequenced the samples previously analysed for genome size by, and our unpublished chromosome counts of Haplotype P and Haplotype O genotypes by "squash and stain", Haplotype P-related genotypes are more likely tetraploids, according to the sample sets that we studied.

The higher genetic diversity of Haplotype P octoploid populations (about the double of that of Haplotype O), and the high degree of introgression, suggests that Haplotype P is an allopolyploid that originated by hybridization between *P. karka* and another still unknown population different from Haplotype O and *P. japonicus*. Haplotype P shares in fact a large part of its genome with *P. karka*, but not with *P. australis* Haplotype O or *P. japonicus*, and the cpDNA is closely related to that of *P. karka*. Ishi and Kadono confirmed that octoploid *P. australis* is fertile in Asia. They found anomalies in neither pollen viability, nor availability, nor barriers to cross-pollination, nor reduced seed set rates in wild octoploid populations in Japan. The high genetic diversity that we found in our Haplotype P populations and the occurrence of hybrids between Haplotype P and Haplotype O in the Liaohe Delta further confirm that Haplotype P-related genotypes can reproduce sexually, and indicate that ploidy level is not a barrier to gene flow between the two lineages. All hybrids between Haplotype P- and Haplotype O-related genotypes, except one, had the cpDNA of Haplotype P, indicating a higher ability of the octoploid genome to be recombined, but also a recent contact between Haplotypes P and O, as obvious hybrids were found only in the Liaohe populations where genotypes of the two lineages coexist. The Liaohe delta is one of the largest reed stands in the world and is managed for pulp and paper production. Haplotype P could have been introduced here, and this could explain the co-dominance of the two distantly related haplotypes only in this delta.

The genetic diversity of Haplotype O-related populations was also relatively high and comparable to that of the European *P. australis* tetraploid population and higher than the diversity of the invasive European populations in North America and in the Gulf Coast. Although this can rule out founder effects due to a recent introduction of Haplotype O to East Asia, the sympatry of the two distantly *P. australis* related lineages and in the same habitat, and their relationships to the two Asian species *P. karka* and *P. japonicus*, pose several questions concerning *P. australis* evolution in East Asia that cannot be explained by natural selection only. These results
Table 1. AMOVA of SSR data testing the structure within and among populations based on (1a) the geographic distribution of the populations, (2b–f) the haplotypic composition of the populations and (2g–l) the HKT1 composition of the populations in homo- and heterozygotes. N sample size, df degrees of freedom, SS Sum of squares, MS mean sum of squares, Est. Var. estimated variance, % percentage of estimated variance, PhiPT fixation index (analogue of Fst), P-value based on 9999 permutations.
have therefore implications also for *Phragmites* taxonomy, systematics and ecology in East Asia and are central for future research.

Population genetic structure. The populations of the two haplotypes also differed in genetic structure. Insignificant population differentiation measures (PhiPTs) confirm gene flow and long-distance dispersal of pollen and seeds among the populations related to Haplotype O, whereas the significant PhiPTs among the populations related to Haplotype P indicate differentiation among populations even at the local scale, as seen among the populations in the Yangtze Delta. Nevertheless, the low PhiPT values (max 0.16) support long-distance gene flow even among the Haplotype P-related populations. This significant structure could be due to the high genetic diversity dispersed by the seeds of an octoploid allopolyploid. Lack of a regional geographic structure is a common feature of *P. australis* populations in Europe\(^2^9\)--\(^3^1\), North America\(^3^2\) and South Africa\(^3^3\). Even in Australia, Haplotype P populations are not genetically distinct\(^1^5\). The significantly different genetic variation pattern of Haplotype P populations in East Asia is therefore unusual for *P. australis*. Previous studies of *P. australis* intraspecific variation in China have found similar patterns and attributed the spatial differentiation to edaphic factors in the Songnen Prairie\(^3^4\)--\(^3^5\), swamp, salt and dune reed ecotypes in the Hexi Corridor in the Gansu province\(^3^6\), and salt tolerance in the Yellow River Delta\(^1^1\),\(^1^6\). None of these studies, however, resolved or considered the phylogenetic diversity of the populations, or realized that population structure and ecology are different within lineages. Resolving phylogenetic relationships appears therefore crucial from this study to address ecology and adaptation in future *Phragmites* studies in East Asia and elsewhere.

Genetic diversity distribution in relation to salinity. The *HKT1* gene encodes one of the main mechanisms of salt tolerance in vascular plants\(^3^7\). It varies in the genomic sequences of *P. australis* in East Asia\(^1^0\),\(^2^3\) and there is allelic variation associated with salt response in wild coastal populations of *Arabidopsis thaliana* in Northern Europe\(^3^8\). In our study salinity did not affect the distribution of homo- and heterozygotes at the *HKT1*-1 and *HKT1*-2 sites of the *HKT1* gene, but the factor that better explained the relationship between salinity and genetic diversity, as well as the *HKT1* variation pattern, was phylogenetic origin. Haplotype O-related genotypes occurred in areas with the highest salinity and Haplotype P-related genotypes in areas with the lowest. Both Haplotype P hybrid types (Haplotype P x *P. karka* and Haplotype P x Haplotype O) occurred in a

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### Table 2. Genetic diversity in haplotype O- and haplotype P-related populations.

| Pop | N         | N SE | Na   | NA SE | Ne   | Ne SE | I     | SE I | h     | h SE | uh   | uh SE |
|-----|-----------|------|------|-------|------|-------|-------|------|-------|------|------|-------|
| YEL O | 21.846    | 0.078 | 0.795 | 0.157 | 1.163 | 0.046 | 0.161 | 0.038 | 0.102 | 0.026 | 0.107 | 0.027 |
| LIA O | 19.795    | 0.075 | 0.795 | 0.157 | 1.149 | 0.039 | 0.159 | 0.036 | 0.099 | 0.024 | 0.104 | 0.025 |
| TIA O | 4.897     | 0.049 | 0.487 | 0.132 | 1.152 | 0.051 | 0.124 | 0.040 | 0.085 | 0.028 | 0.109 | 0.036 |
| CML P | 33.487    | 0.207 | 1.513 | 0.137 | 1.410 | 0.058 | 0.367 | 0.043 | 0.243 | 0.031 | 0.250 | 0.032 |
| CXL P | 31.487    | 0.197 | 1.333 | 0.153 | 1.312 | 0.053 | 0.302 | 0.041 | 0.194 | 0.029 | 0.201 | 0.030 |
| JDSL P | 25.692   | 0.117 | 1.231 | 0.158 | 1.331 | 0.057 | 0.305 | 0.044 | 0.200 | 0.030 | 0.208 | 0.032 |
| LIA P | 17.513    | 0.187 | 1.590 | 0.131 | 1.380 | 0.058 | 0.358 | 0.040 | 0.239 | 0.029 | 0.245 | 0.031 |
| SJL P | 6.000     | 0.000 | 0.744 | 0.150 | 1.245 | 0.058 | 0.203 | 0.047 | 0.140 | 0.033 | 0.168 | 0.039 |

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**Figure 5.** Distribution of phylo-types (X axis) by the salinity classes of the SQ5 layer of excess salt (Y axis). Average salt excess classes values and Bonferroni’s intervals for each phylo-type. (A) (AB) and (B) refer to the significance of the Bonferroni multiple comparison. The graph is produced in R (R core Team).
wider range of salinity than Haplotypes O and P, and had higher average salt tolerance than Haplotype P. The *P. karka* population in the present study is from the Mekong Delta in Viet Nam, another estuarine area subject to increasing salinization.\(^9\). Salt tolerance as a specific trait of *P. karka* remains poorly understood, but our results show that hybridization between estuarine populations of *P. karka*, and Haplotype O, increased salt tolerance in Haplotype P in East China.

Compared to\(^6\) sequences, the pattern of genetic variation of the complete HKT1 gene sequence of the homozygotes appeared more variable and complex than that of two distinct sequences for salt-tolerant and salt-sensitive genotypes. As could be expected, the coding sequence of salt-sensitive Haplotype P was different from that of salt-tolerant *P. karka*. Likewise, the coding sequence of salt-tolerant Haplotype O (matching the salt-tolerant coding sequence of\(^6\)) was distinct from that of its close relative *P. japonicus* (matching the salt-sensitive sequence of\(^6\)), suggesting that salt tolerance has evolved multiple times and independently in East China. The sequences of the hybrids could not be resolved with certainty because of heterozygosity at several loci.

**Conclusions**

As we have shown previously\(^5-7\), phylogenetic relationships play a crucial adaptive role for *Phragmites* populations. Two distantly related *P. australis* haplotypes dominate the estuarine populations in East China. One, Haplotype O, is related to European *Phragmites* sensu Lambertini et al.\(^18\), the other, Haplotype P, is highly introgressed with *P. karka* and has its distribution in East Asia and Australia. The two haplotypes have different salt tolerance, with Haplotype O occurring in the most saline areas in the delta and Haplotype P in the lowest. Hybridization with Haplotype O and introgression with estuarine populations of *P. karka* has increased salt tolerance in Haplotype P and expanded its distribution range to the most saline areas in the Yangtze and Liaohe deltas. Saline tolerance has therefore evolved multiple times and independently in Haplotype P and Haplotype O. Interestingly, salt-sensitive Haplotype P is closely related to salt-tolerant Asian *P. karka*, whereas salt-tolerant Haplotype O is closely related to Asian *P. japonicus*, whose HKT1 sequences matched those of the salt-sensitive genotypes of\(^6\). Future research may address the relationships of the two *P. australis* haplotypes with the two Asian species as well as the evolutionary and ecological significance of the two *P. australis* haplotypes in the range of the Asian species.

The present study has found no evidence of a cryptic invasion in East China, but cannot even resolve conclusively why two *P. australis* haplotypes, each with an intercontinental distribution range, do co-occur in the estuarine populations in East Asia and share the same habitat, i.e. whether East Asia is their center of diversity or they (one or both) have been introduced recently. The high genetic diversity in the populations of both haplotypes suggests an origin in East Asia, whereas the restricted distribution of their viable hybrids only to sympatric areas suggests a recent contact, or recent changes in the latitudinal distribution of the two haplotypes. The polygenic allelic pattern of Haplotype P further suggests a recent polyploidization. A larger sample of Haplotype O populations from across Europe and Asia and of Haplotype P from Australia and the Asian tropical region is needed to solve this enigma. Herbarium specimens may also help reconstruct past distribution ranges and can detect recent changes.

**Methods**

**Sample set.** We collected 174 samples of *P. australis* from 7 populations in East China in the Yangtze, Yellow River and Liaohe deltas and urban populations in the towns of Songjiang and Tianjin (Fig. 1). Accessions from outside of the area of investigation and of other *Phragmites* species were included to trace origins and relationships of the Chinese populations. In specific we used our sequence database\(^5,18\) and produced new sequences for *P. karka* and *P. japonicus* to cover the Asian *Phragmites* species variation. We used a *P. karka* population from the Mekong Delta in Viet Nam (N = 40) and samples of *P. japonicus* (N = 4) from populations in Korea and Sakhalin Island (Russia) (Supplementary Information S1).

**DNA extraction.** DNA was extracted with the E.Z.N.A. tissue DNA kit (Omega Bio-tek Inc.) from apical leaves conserved in silica gel. The samples were ground in a mortar with quartz sand in liquid nitrogen before adding the E.Z.N.A. extraction buffer, following the protocol for dry plant specimens. The samples were treated with RNase, eluted with 100 µl elution buffer, and conserved at -20 °C until amplification. DNA quality and quantity were checked in a Nano Drop Spectrophotometer ND-1000 (Saveen Werner) at 280 nm wavelength. DNA concentration exceeded 50 ng µl⁻¹ in all samples.

**Chloroplast DNA markers.** Two non-coding regions in the chloroplast DNA, the trnT-trnL and the rbcL-psal, have previously been used to classify *Phragmites* diversity worldwide.\(^18,19,20,22\). Of these two sequences, polymorphism in an indel of variable size in the trnT-trnL region can define most *Phragmites* haplotypes.\(^41\). We used the primers of\(^41\) to amplify this variable region, and screened the polymorphism of our sample set. 2 µl DNA were added to 18 µl mastermix consisting of 10 µl 2xMastermix (VWR Amplicon), 10 pmol cy-labeled forward primer (5’-CAT TAC AAA TGC GAT GCT CT - 3’), 10 pmol reverse primer (TrnT-R short: 5’-CGT CCG AGC CAT ATC AAA TT- 3’), and sterile water to reach a final volume of 20 µl. Amplification was run in a Peltier Thermal Cycler PTC-200-MJ Research under the following conditions: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 52 °C for 40 s, 72 °C for 40 s, followed by 72 °C for 7 min. The amplified product (a fragment of about 350 bp) was diluted 20 x and loaded in a 7% acrylamide gel (Repropel-Long REad) in the fragment analyser ALF Express II DNA Analysis System (Amersham Pharmacia Biotech). 5 µl of diluted PCR product were added to 3 µl loading dye (GE Healthcare) previously mixed with 1 µl each of 100 and 300 bp internal sizers (GE Healthcare). DNA was denaturated in the PTC-200 PCR at 94 °C for 5 min prior to loading in the gel. Electrophoresis conditions were 1500 V, 55 °C, 120 min. The first and the last slots of the gel were loaded with 30–500 bp external sizers (GE Healthcare).
All different trnT-trnT' short profiles were subsequently sequenced with the trnT-trnL and rbcL-psaI primers developed by9. Annealing temperatures were 50 °C for both primer sets and the PCR conditions were as for the fragments amplification except that the extension time was increased to 1 min. The PCR product was diluted 20 × and sent to Macrogen Korea for Sanger sequencing with forward and reverse primers in an ABI system.

We downloaded all Phragmites trnT-trnL and rbcL-psaI sequences from GenBank9,25. We aligned our sequences with those downloaded from GenBank with the program BioEdit ver. 7.0 Sequence Alignment Editor30. The initial “clustal” alignment was completed manually. Repeated motifs (minisatellites and microsatellites) and indels were coded as multistate characters. Sequences differing in the number of repeats at microsatellite loci were classified as cp-microsatellite variants following18,22. The final matrix of 134 sequences, 33 of which were from this study, and 1625 base pairs plus 27 multistate characters, was analysed with PAUP ver. 4.0b1032. The parsimony analysis was performed with 37% jackknife deletion, 1000 replicates, jack resampling and stepwise addition41. Individual pairwise genetic distances were also calculated as "total character difference". The matrix of genetic distances was imported in GenAlex ver. 6.444,45 and analysed in a Principal Coordinate Analysis (PCoA).

The PCoA obtained is comparable to that of Fig. 2 of18 and includes all haplotypes so far identified in East Asia.

We followed9 to define Phragmites Haplotypes and their intrahaplotypic microsatellites variants10,22 and17,18 for the names of the phylogeographic units.

**HKT1 gene polymorphism and gene sequences.** We downloaded all Phragmites genomic and mRNA sequences of the PhaHKT1 gene from GenBank20,25. We split the 2000 bp gene sequence into two fragments, HKT1-1 and HKT1-2, of about 1000 bp each. The HKT1-1 fragment contained an indel which was previously found to occur in the Yellow River population (Nanpi and Enchi) but not to have an obvious effect on the ability to tolerate salt, as both mRNA profiles occurred in salt-tolerant reeds25. The HKT1-2 fragment contained two introns, one of which was shown to splice and be translated into proteins of different size in salt-tolerant and salt-sensitive reeds25. We designed a set of primers around the indel in the HKT1-1 part of the gene and another set around the two introns in the HKT1-2 part of the gene, and analysed fragment size polymorphism.

The primers for the fragment containing the two introns in the HKT1-2 part of the gene were HKT1-2F short and HKT1-2R End (Supplementary Information S2) and annealing temperature was 55 °C. The primers for the fragment containing the two introns in the HKT1-2 part of the gene were HKT1-2F short and HKT1-2R End (Supplementary Information S2) and annealing temperature was 52 °C. Mastermix and PCR conditions were as described for the trnT-trnL fragments. The amplified products (between 200 and 300 bp for the HKT1-1 fragments and around 400 bp for the HKT1-2 fragments) were run in a 1.5% agarose gel at 130 V for 2 to 5 h and band profiles scored manually.

We then sequenced all homozygote profiles. The HKT1-1 part of the gene was sequenced with the primers HKT1 full-F2 and HKT1-1 1000R (Supplementary Information S2) and the annealing temperature was 60 °C. The HKT1-2 part of the gene was sequenced with the primers HKT1-2 1000F and HKT1-2R End (Supplementary Information S2) and the annealing temperature was 52 °C. Mastermix and PCR conditions were the same as described for the trnT-trnL and rbcL-psaI sequences. The amplified products were sequenced with the primers HKT1 full-F2, HKT1-2 1000F and HKT1-2R End.

We calculated the genotypic frequencies of homozygotes and heterozygotes at the two loci as the number of individuals sharing the same profile divided by the total number of individuals in the population. HKT1-1 had two alleles and three genotypic classes, whereas HKT1-2 had three alleles and only two genotypic classes (homozygotes and heterozygotes with three alleles; Table S3). We used a two-loci codominant matrix to calculate allelic frequencies with the program GenAlex.

The three sequences obtained for each homozygote genotype (HKT1 Full-F2, HKT1-2 1000F and HKT1-2R End) were assembled with the program Genious ver. 6.1.7 (Biomatters) and the resulting consensus sequences of about 2000 bp were aligned with the same program. We compared our sequences, including those of salt-tolerant and salt-sensitive ecotypes deposited in Gene Bank25 also included in the alignment.

**SSRs.** We studied the allelic variation at loci PAGT4, PAGT8, PAGT9 and PAGT13 previously reported to vary in the Yellow River Delta in relation to soil salinity16 with the primers developed by46. Mastermix, PCR and electrophoresis conditions were the same as described for the trnT-trnL fragment and the resulting structure was investigated in relation to the geographic distribution of the populations, the haplotypic diversity inferred by the trnT short fragment, and the homo- and heterozygotes at the HKT1 gene loci. The significance of each factor (phylogenetic diversity, HKT1 polymorphism) on the SSR genetic structure was tested with AMOVA (using GenAlex) and with the Bayesian clustering software Structure (ver. 2.3.4)57. Statistical significance for the AMOVA was obtained with 9999 permutations. Concerning Structure, we used the admixture model with correlated allelic frequencies to infer the number of ancestral populations, 300,000 burn-in periods, 1,000,000 MCMC reps after burn-in, and 20 iterations. The initial admixture coefficient alpha was set at 1.055 and K was set from 1 to 10. Structure Harvester48 inferred the most likely K according to the Evanno’s method49.
Genetic diversity indices (samples size, number of alleles, number of effective alleles, Shannon Information Index, gene diversity and unbiased gene diversity) were calculated with GenAlex based on the frequency statistics of binary haploid profiles.

**Soil salinity.** We used the Soil Quality layer 5 (SQ5) of the Harmonized World Soil Database v1.250 to extract salinity data at the coordinates of our samples. The SQ5 data file combines soil salinity with soil sodicity and soil phases influencing salt conditions and defines 7 categories of excess salt in the soil. The first five categories range from no to severe excess salt, while categories 6 and 7 include no sods and permafrost areas. We compared the SQ5 layer with published soil salinity maps of the Yellow River Delta16,51–53 and of the Yangtze River Delta54 to ensure updated data and reproducibility of the SQ5 results. As some of our samples in the Yangtze River Delta were collected in areas that were still submerged in the SQ5 layer, we also mined salinity data from our own field work previously conducted at the sampling locations (Xiu Zhen-Li, unpublished data).

We tested differences in excess salt categories among haplotypes and among homo- and heterozygotes at the HKT1-1 and HKT1-2 loci in a multiple factor-ANOVA with Statgraphics Centurion, using General Linear Models and Bonferroni tests at the 95% confidence level. For the Yangtze River Delta populations, we repeated the ANOVA with salinity data measured in the field and also tested Pearson correlations between the SSR allelic frequencies and salinity (as per16), as well as between salinity and HKT1-1 and HKT1-2 allelic frequencies, and homo- and heterozygotes frequencies at the HKT1-1 and HKT1-2 loci, with Statgraphics Centurion.

**Data availability**

GenBank Accessions: KP994327 + KP994334 = Haplotype AS, KP994328 + KP994332 = Haplotype AT, KP994329 + KP994333 = Haplotype AU.

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**Author contributions**

CL wrote the main manuscript text. W.G. explored the Soil Quality layer 5 (SQ5) of the Harmonized World Soil Database and extracted salinity data. W.G. also prepared Figs. 1, 2, 3, 4, 5, S.Y. and X.L. collected the samples and took DNA samples. W.G. also prepared the soil samples for the soil tests. W.G. and S.Y. prepared the salinity data. Y.W. and X.L. collected the samples.
X.L. provided salinity data at the sampling sites in the Yangtze River Delta. H.B. coordinated the project and the sample collections. F.E., X.G., B.K.S. and M.S. assisted with the study. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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