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ORIGINAL PAPER

Molecular phylogeny of the genus *Asparagus* (Asparagaceae) explains interspecific crossability between the garden asparagus (*A. officinalis*) and other *Asparagus* species

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Abstract The genus *Asparagus* comprises approximately 200 species, some of which are commercially cultivated, such as the garden asparagus (*A. officinalis*). Many *Asparagus* species, including *A. officinalis*, are dioecious and have been grouped into a subgenus distinct from that of hermaphroditic species. Although many interspecific crossings have been attempted to introduce useful traits into *A. officinalis*, only some of the dioecious species were found to be cross-compatible with *A. officinalis*. Here, molecular phylogenetic analyses were conducted to determine whether interspecific crossability is proportional to the genetic distance between the crossing pairs and to further clarify the evolutionary history of the *Asparagus* genus. A clade with all cross-compatible species and no cross-incompatible species was recovered in the phylogenetic tree based on analyses of non-coding cpDNA regions. In addition, a sex-linked marker developed for *A. officinalis* amplified a male-specific region in all cross-compatible species. The phylogenetic analyses also provided some insights about the evolutionary history of *Asparagus*; for example, by indicating that the genus had its origin in southern Africa, subsequently spreading throughout the old world through intensive speciation and dispersal. The results also suggest that dioecious species were derived from a single evolutionary transition from hermaphroditism in *Asparagus*. These findings not only contribute towards the understanding of the evolutionary history of the genus but may also facilitate future interspecific hybridization programs involving *Asparagus* species.

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Introduction

The genus *Asparagus* is a member of the Asparagaceae family (APG III 2009), a large genus containing approximately 200 species distributed throughout the old world (Dahlgren et al. 1985). Although represented by diverse life forms, such as herbaceous perennials, tender woody shrubs, and vines, all *Asparagus* species are characterized by the presence of photosynthetic stems (cladodes) (Obermeyer 1983; Dahlgren et al. 1985; Clifford and Conran 1987). Based on the morphology of its species, the genus is largely divided into three subgenera, namely *Asparagus*, *Protasparagus*, and *Myrsiphyllum* (Clifford and Conran 1987). While all species in the subgenus *Asparagus* are dioecious, those in the latter two subgenera are all hermaphroditic, being distinguished by differences in their floral morphology and the number of ovules (Clifford and Conran 1987). Nowadays, many *Asparagus* species are used as food (e.g., *A. acutifolius*, *A. albus*, *A. maritimus*, and *A. officinalis*) as well as for medical (e.g., *A. adscendens*, *A. racemosus*, and *A. verticillatus*) and ornamental (e.g., *A. asparagoides*, *A. densiflorus*, *A. plumosus*, and *A. virgatus*) purposes within and outside their native range (Kubitzki and Rudall 1998). Moreover, due to their widespread commercial use, some species (e.g., *A. asparagoides*, *A. densiflorus*, and *A. scandens*) have now invaded natural areas outside their geographic range (Lawrie 2006; Williams 2006; Turner et al. 2008).

Although many *Asparagus* species are now widely cultivated for commercial use, the most economically important species is the garden asparagus, *A. officinalis*.

Similarly to other vegetable crops, *A. officinalis* is exposed to many agricultural problems such as drought, crop failure and pestilence (Smith et al. 1990; Ernst and Krug 1998; He et al. 2002; Liu et al. 2010). Still, some wild *Asparagus* species are resistant or tolerant to these factors. For example, *A. acutifolius* not only tolerates drought but it is also resistant to *Puccina asparagi* (rust disease) and *Stemphylium vesicarium* (purple spot disease) infections, which are also common pathogens of *A. officinalis* (Alberti et al. 2004). Therefore, several interspecific crossings were attempted to introduce useful traits into *A. officinalis*, although only a few resulted in the production of viable and fertile hybrids (Kunitake et al. 1996; Marcellán and Camadro 1996; Alberti et al. 2004; Ito et al. 2008; Zhou et al. 2009; Kanno unpublished data). Since fertile hybrids are capable of continuous backcrossing (Alberti et al. 2004; Zhou et al. 2009; Kanno unpublished data), interspecific hybridization would be a valid means to genetically improve *A. officinalis* for commercial production. The results from a series of crossing experiments indicate that all *Myrsiphyllum* and *Protasparagus* species are cross-incompatible with *A. officinalis*, and that cross-compatible species are limited to the subgenus *Asparagus* (Kunitake et al. 1996; Marcellán and Camadro 1996; Alberti et al. 2004; Ito et al. 2008; Zhou et al. 2009; Kanno unpublished data). Since *A. officinalis* is also a member of the subgenus *Asparagus*, the degree of interspecific crossability might be linked to the phylogenetic distance between the species, a phenomenon ubiquitously observed in living organisms (Dehgan 1984; Coyne and Orr 1989, 1996; Ahmad and McNeil 1996). However, not all species of the subgenus *Asparagus* are cross-compatible with *A. officinalis*. Moreover, the geographical distributions of the cross-compatible species do not converge at the Mediterranean region, which is the native origin of *A. officinalis*. This lack of intra-subgeneric compatibility and the observed differences in the geographical distribution of the cross-compatible species weakens the notion of a monophyletic origin of the subgenus *Asparagus*.

Even though *Asparagus* is a highly diverse genus, only a few studies have investigated relationships within the group. Pioneering research on the molecular systematics of *Asparagus* was conducted using restriction fragment length polymorphism (RFLP) analysis of cpDNA (Lee et al. 1997) and nuclear DNA internal transcribed spacer regions (Štajner et al. 2002). The results from both these studies segregated the species of subgenus *Asparagus* from other species of *Protasparagus* and *Myrsiphyllum*. However, neither study conducted statistical tests of the topology of the resulting trees, nor did they include an outgroup taxon, preventing the investigation of the evolutionary relationships between the subgenera. The most recent and comprehensive study on the phylogeny of *Asparagus* was conducted by Fukuda et al. (2005), which analyzed sequence polymorphisms of the non-coding cpDNA regions for 26 taxa, including species of all three subgenera and outgroups. Similarly to the results from the previously mentioned RFLP analyses, the subgenus *Asparagus* was also aggregated in a monophyletic clade in the study by Fukuda et al. (2005). Bootstrap support for the monophyly of this group, however, was less than 50%. The results also showed that one of the two *Myrsiphyllum* species analyzed was a sister species to all other *Asparagus* taxa, and the remaining species (including the second *Myrsiphyllum* species studied) formed part of a large polytomy. Although *Myrsiphyllum* was proposed ancestral, the taxonomic relationships among the subgenera, together with their evolutionary histories, remained unresolved. Considering that an understanding of the interspecific relatedness among the *Asparagus* species should also enable the development and improvement of interspecific hybridization programs of *A. officinalis*, a robust and detailed phylogeny of the genus is therefore needed.

In the present study, an intrageneric phylogenetic tree of *Asparagus* was inferred based on sequence polymorphism of the cpDNA non-coding regions. Since Fukuda et al. (2005) noted the low degree of interspecific variation in the cpDNA regions of *Asparagus* species surveyed (namely *petB* intron, *petD-rpoA* intergenic region, *trnL* intron, and *trnL-trnF* intergenic region), regions with faster substitution rates, as described by Shaw et al. (2007), were used. The resulting phylogeny was used to investigate currently unresolved issues related to the evolutionary history of *Asparagus* as well as interspecific breeding possibilities. First, it was investigated whether species that are cross-compatible with *A. officinalis* would be grouped into a single phylogenetic clade. If crossability simply reflects the degree of genetic relatedness to *A. officinalis*, a detailed intrageneric phylogeny should contribute towards the selection of the most appropriate relatives in future interspecific hybridization attempts. Second, the applicability of a sex-linked molecular marker was investigated for each of the *Asparagus* species examined. The sex determination system in *A. officinalis* is determined by their sex chromosomes, with females being homogametic (XX) and males heterogametic (XY) (Löptien 1979). The sex-linked PCR primers developed by Nakayama et al. (2006) were designed to amplify the male specific region on the Y chromosome of *A. officinalis*. This marker can also distinguish between males and females of *A. schoberioides*, a dioecious wild species within the subgenus *Asparagus* (Nakayama et al. 2006). If a sex-linked marker can be used both to *A. officinalis* and its cross-compatible species, interspecific hybridization programs would benefit from sex identification at an early developmental stage before flowering. Finally, the phylogenetic tree produced was used

to examine the evolutionary history of the genus *Asparagus*. As described above, species of the subgenus *Asparagus* are dioecious, whereas those from the *Protasparagus* and *Myrsiphyllum* subgenera are hermaphroditic (Clifford and Conran 1987). Previous morphological studies have shown that sex differentiation in *A. officinalis* results from the selective abortion of either the gynoecium or androecium of the initially hermaphroditic floral primordia (Bracale et al. 1991). Although it seems likely that dioecy has evolved from a hermaphroditic ancestor, a common evolutionary process in angiosperms (reviewed in Barrett 2002), this theory has not been confirmed in *Asparagus*. A robust and well-resolved phylogenetic tree would resolve this issue and may even determine the number of times dioecy has evolved in this genus. Here, phylogenetic analyses were performed to determine whether dioecism derived from hermaphroditism in *Asparagus* and how frequently in the evolutionary history of the genus this phenomenon occurred. The phylogenetic tree produced was also used to infer the evolutionary history and geographical expansion of the three *Asparagus* subgenera.

Materials and methods

Plant materials

A total of 34 samples from *Asparagus*, including 23 species, were analyzed (Table 1). Some samples from the same species were collected from distinct varieties, cultivars, or populations. *Dracaena sanderiana* and *Cordyline stricta* were selected as closely related outgroups according to the taxonomic classification of APG III (2009). Since the identification of some *Asparagus* species is still ambiguous, only well-defined commercial species (except for the Japanese species) were studied. The taxa sampled were cultivated in the greenhouse of Graduate School of Life Sciences, Tohoku University. The global distribution of the *Asparagus* species was determined from the Germplasm Resources Information Network database (GRIN; http://www.ars-grin.gov/cgi-bin/npgs/html/exsplist.pl), which is an integrated database of the worldwide flora surveys. Of the 60 *Asparagus* species registered in the database, 50 included information of the country where they were recorded. The distribution of each species was plotted on a world map to determine the geographic range of each of the three subgenera.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from the cladodes by a CTAB extraction procedure according to Stewart and Via (1993). Five non-coding regions of cpDNA, *rpl32-trnL*, *trnQ-5′rps16*, *ndhF-rpl32*, *psbD-trnT*, and *3′rps16-5′trnK*, were amplified using primer pairs rpL32-F and trnL$^{(UAG)}$, trnQ$^{(UUG)}$ and rpS16x1, ndhF and rpL32-R, psbD and trnT$^{(GGU)}$-R, and rpS16x2F2 and trnK$^{(UUU)}$x1, respectively, as designed by Shaw et al. (2007). These five non-coding regions have been demonstrated to retain the highest levels of sequence variation among the known cpDNA regions used for interspecific phylogenetic studies (Shaw et al. 2007). Polymerase chain reaction (PCR) was performed in a 20 μL reaction mixture containing 1.0 μL of template DNA, 0.2 mM of each dNTP Mixture, 1× *Ex Taq* Buffer, 0.5 U of *TaKaRa Ex Taq* (TaKaRa), and 0.5 μM of each primer. All PCR amplifications were performed with an S1000 thermal cycler (Bio-Rad) using the "rpl16" cycling program of Shaw et al. (2005). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced directly using a Big Dye terminator ready reaction mix (Applied Biosystems). Both forward and reverse strands of each cpDNA region were sequenced independently and the sequences were determined independently on an ABI 3130 genetic analyzer (Applied Biosystems). The sequence of each cpDNA region will appear in GenBank under the accession numbers AB613827–AB614006 (Table 1).

Phylogenetic analyses

Sequence data of the cpDNA regions were aligned with the ClustalX program (Thompson et al. 1997) and manually corrected to minimize indels. Sequences from all five cpDNA regions were combined into one dataset and indels (including microsatellites) were excluded from subsequent phylogenetic analyses. Phylogenetic trees were constructed based on maximum likelihood (ML), Bayesian, neighbor-joining (NJ), and maximum parsimony (MP) methods. The program jModeltest 0.1 (Posada 2008) was used to search for the best-fit substitution model for our datasets, according to the corrected Akaike Information Criterion (AICc). The selected GTR+G model was used to construct the ML and Bayesian trees. ML analysis was conducted using the PhyML 3.0 software (Guindon et al. 2010) with 1,000 bootstrap replicates to determine the level of statistical support for the branches. Bayesian analysis was conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Four chains of the Metropolis-coupled Markov Chain Monte Carlo (MC3) process were run for 5,000,000 generations, with trees being sampled every 1,000 generations. Default parameters for cold and heated chain were used. Convergence of the runs was confirmed when the average standard deviation of split frequencies was <0.01. The first 1,250 sampled trees (25%) were discarded and the remaining trees were used to construct a majority rule consensus tree. NJ analysis was performed with MEGA4

Table 1 List of *Asparagus* studied, outgroup taxa and GenBank accession numbers of the nucleotide sequence used in the phylogenetic analyses

Taxon name	Variation, cultivar, and population	GenBank accession numbers				
		rpl32-trnL	*trnQ-5′rps16*	*ndhF-rpl32*	*psbD-trnT*	*3′rps16-5′trnK*
A. acutifolius[a]		AB613827	AB613863	AB613899	AB613935	AB613971
A. africanus[b]		AB613828	AB613864	AB613900	AB613936	AB613972
A. albus[b]		AB613829	AB613865	AB613901	AB613937	AB613973
A. asparagoides[c]		AB613830	AB613866	AB613902	AB613938	AB613974
A. cochinchinensis Hepingdao[a]	var. *cochinchinensis*; pop. Hepingdao, Keeling, Taiwan	AB613833	AB613869	AB613905	AB613941	AB613977
A. cochinchinensis Hinase[a,*]	var. *cochinchinensis*; pop. Hinase, Okayama, Japan	AB613831	AB613867	AB613903	AB613939	AB613975
A. cochinchinensis pygmaeus[a]	var. *pygmaeus*	AB613832	AB613868	AB613904	AB613940	AB613976
A. dauricus[a]		AB613834	AB613870	AB613906	AB613942	AB613978
A. declinatus[c]		AB613835	AB613871	AB613907	AB613943	AB613979
A. densiflorus Myers[b]	cv. Myers	AB613836	AB613872	AB613908	AB613944	AB613980
A. densiflorus Sprengeri[b]	cv. Sprengeri	AB613837	AB613873	AB613909	AB613945	AB613981
A. denudatus[b]		AB613838	AB613874	AB613910	AB613946	AB613982
A. falcatus[b]		AB613839	AB613875	AB613911	AB613947	AB613983
A. kiusianus Keyakaigan[a]	pop. Keyakaigan, Fukuoka, Japan	AB613840	AB613876	AB613912	AB613948	AB613984
A. kiusianus Nijinomatsubara[a,*]	pop. Nijinomatsubara, Saga, Japan	AB613841	AB613877	AB613913	AB613949	AB613985
A. kiusianus UminonakamichiSP[a]	pop. UminonakamichiSP, Fukuoka, Japan	AB613842	AB613878	AB613914	AB613950	AB613986
A. macowanii[b]		AB613843	AB613879	AB613915	AB613951	AB613987
A. maritimus[a,*]		AB613844	AB613880	AB613916	AB613952	AB613988
A. officinalis GoldSchatz[a]	cv. Gold Schatz	AB613845	AB613881	AB613917	AB613953	AB613989
A. officinalis MaryWashington500 W[a,*]	cv. Mary Washington 500 W	AB613846	AB613882	AB613918	AB613954	AB613990
A. officinalis Pacific2000[a]	cv. Pacific 2000	AB613847	AB613883	AB613919	AB613955	AB613991
A. officinalis PacificPurple[a]	cv. Pacific Purple	AB613848	AB613884	AB613920	AB613956	AB613992
A. oligoclonos Aso[a]	pop. Aso, Kumamoto, Japan	AB613849	AB613885	AB613921	AB613957	AB613993
A. plumosus Nanus[b]	cv. Nanus	AB613850	AB613886	AB613922	AB613958	AB613994
A. plumosus Pyramidalis[b]	cv. Pyramidalis	AB613851	AB613887	AB613923	AB613959	AB613995
A. pseudoscaber Fukumidori[a]	cv. Fukumidori	AB613852	AB613888	AB613924	AB613960	AB613996
A. pseudoscaber Million[a,*]	cv. Million	AB613853	AB613889	AB613925	AB613961	AB613997
A. racemosus[b]		AB613854	AB613890	AB613926	AB613962	AB613998
A. scandens[c]		AB613855	AB613891	AB613927	AB613963	AB613999
A. schoberioides Hiwadamachi[a,*]	pop. Hiwadamachi, Fukushima, Japan	AB613856	AB613892	AB613928	AB613964	AB614000
A. schoberioides Mt.Takamori[a]	pop. Mt. Takamori, Miyagi, Japan	AB613857	AB613893	AB613929	AB613965	AB614001
A. stipularis[a,*]		AB613858	AB613894	AB613930	AB613966	AB614002
A. verticillatus[a,*]		AB613859	AB613895	AB613931	AB613967	AB614003
A. virgatus[b]		AB613860	AB613896	AB613932	AB613968	AB614004
Cordyline stricta		AB613861	AB613897	AB613933	AB613969	AB614005
Dracaena sanderiana		AB613862	AB613898	AB613934	AB613970	AB614006

[a] Subgenus *Asparagus*

[b] Subgenus *Protasparagus*

[c] Subgenus *Myrsiphyllum*

* Taxa tested for the applicability of the sex-linked marker

(Tamura et al. 2007) using the maximum composite likelihood (MCL) method to estimate genetic distances. MP analysis was also conducted in MEGA4 using the close neighbor interchange (CNI) algorithm with search level 3, in which the initial trees were obtained with the random addition of sequences (10 replicates). One thousand bootstrap replicates were performed both for the NJ and MP analyses.

Application of sex-linked marker

To determine the applicability of a sex-linked marker in each *Asparagus* species studied, PCR was conducted with the sex-linked primer pairs Asp1-T7spf and Asp1-T7spr designed by Nakayama et al. (2006). These primers were originally designed to amplify male-specific regions on the Y chromosome of *A. officinalis*. The marker is physically close to the sex-determination locus (0.2 cM; Jamsari et al. 2004), and a complete linkage between the phenotypic sex has been confirmed (Nakayama et al. 2006). In *A. officinalis* males, a single PCR product of approximately 300 bp is amplified, whereas no signs of amplification are observed for females (Nakayama et al. 2006). Here, it was tested whether this sex-linked marker could be applied to other dioecious species of *Asparagus*. Single males and females from each taxon were tested as shown in Table 1. As a positive control, the 4th intron of the *AODEF* gene, which is a B-class MADS box gene found in *A. officinalis* (Ito et al. 2005), was amplified using the primers AODEFint4fw (5′-GAGAACGGGTGAA GAGCTTGATG-3′) and AODEFint4rv (5′-TGTAGGTAT CTGTCTGCGTGCT-3′). DNA extraction was conducted with the above mentioned CTAB method (Steward and Via 1993). PCR was performed in a 20 μL reaction mixture containing 1.0 μL of template DNA, 0.2 mM of each dNTP Mixture, 1× *Ex Taq* Buffer, 0.5 U of *TaKaRa Ex Taq* (TaKaRa), and 0.5 μM of each primer. PCR conditions were 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C, with a final cycle of 72°C for 10 min in an S1000 thermal cycler (Bio-Rad). Amplified fragments were separated on 2% agarose gels in 1 × TAE buffer and stained with ethidium bromide. Agarose gels were observed and photographed under an UV light illuminator.

Results

The geographical distribution of the three subgenera is shown in a world map (Fig. 1), which represents the distribution of a total of 3, 25, and 22 species of the subgenera *Myrsiphyllum*, *Protasparagus*, and *Asparagus*, respectively. Although the map shows that the genus is widely and continuously distributed throughout the temperate and intertropical zone of the old world, it is absent in some areas of mid-west Africa, probably due to a lack of flora surveys in these regions. Of the three subgenera, *Myrsiphyllum* has the most restricted geographic distribution, with most species limited to Southern Africa. The distribution of the *Protasparagus* species overlapped with that of the *Myrsiphyllum* species, although the former subgenus was more widely distributed in Africa, Western Europe, South to Southeast Asia, and Australia. The species from the *Asparagus* subgenus were found in the Mediterranean Basin and the temperate zone of Eurasia. Those species cross-compatible with *A. officinalis* were distributed across Western Europe and Eastern Asia, far beyond the native region of *A. officinalis* (Mediterranean Basin).

The lengths of the five non-coding cpDNA regions aligned, *rpl32-trnL*, *trnQ-5′rps16*, *ndhF-rpl32*, *psbD-trnT*, and *3′rps16-5′trnK*, were 1,239, 1,632, 1,246, 1,187, and 830 bp, respectively. Of the 572 variable nucleotides in the combined sequence, 241 were parsimony-informative. The majority rule consensus trees obtained from all phylogenetic analyses were essentially homologous in their topologies (Fig. 2). The monophyletic status of the subgenus *Asparagus* was strongly supported (91% ML bootstrap value), with *A. officinalis* at a terminal position in the inferred tree. All species cross-compatible with *A. officinalis* were also clustered into a single subclade, together with *A. officinalis* (79% ML bootstrap value). In contrast, none of the incompatible species was assigned to this subclade. The subgenus *Protasparagus* was divided into two weakly supported clades (57 and 79% in ML tree). Although the number of clades differed among the phylogenetic analyses (ranging from two to five), there was no strong support for a monophyletic origin in this subgenus. In addition, there was no hierarchic relationship between the *Asparagus* and *Protasparagus* subgenera. While a polytomous relationship between the species of these subgenera was observed, the *Myrsiphyllum* species analyzed were basal to both the *Asparagus* and *Protasparagus* species. However, *A. scandens* was not grouped with the two remaining *Myrsiphyllum* species in any of the phylogenetic analyses.

The results from the PCR amplification of the sex-linked marker in the eight dioecious species studied are shown in Fig. 3. A discrete band of the expected size (ca. 300 bp) was observed in *A. officinalis*, *A. maritimus*, *A. pseudoscaber*, *A. kiusianus*, and *A. schoberioides* males, but not in any female. The amplified products were, however, neither observed in males nor females of *A. verticillatus*, *A. stipularis*, and *A. cochinchinensis*. There was also an association between crossability and marker amplification among the *Asparagus* species examined: whereas the sex-linked marker was amplified in all cross-compatible species, amplified products were not observed in any of the cross-incompatible species.

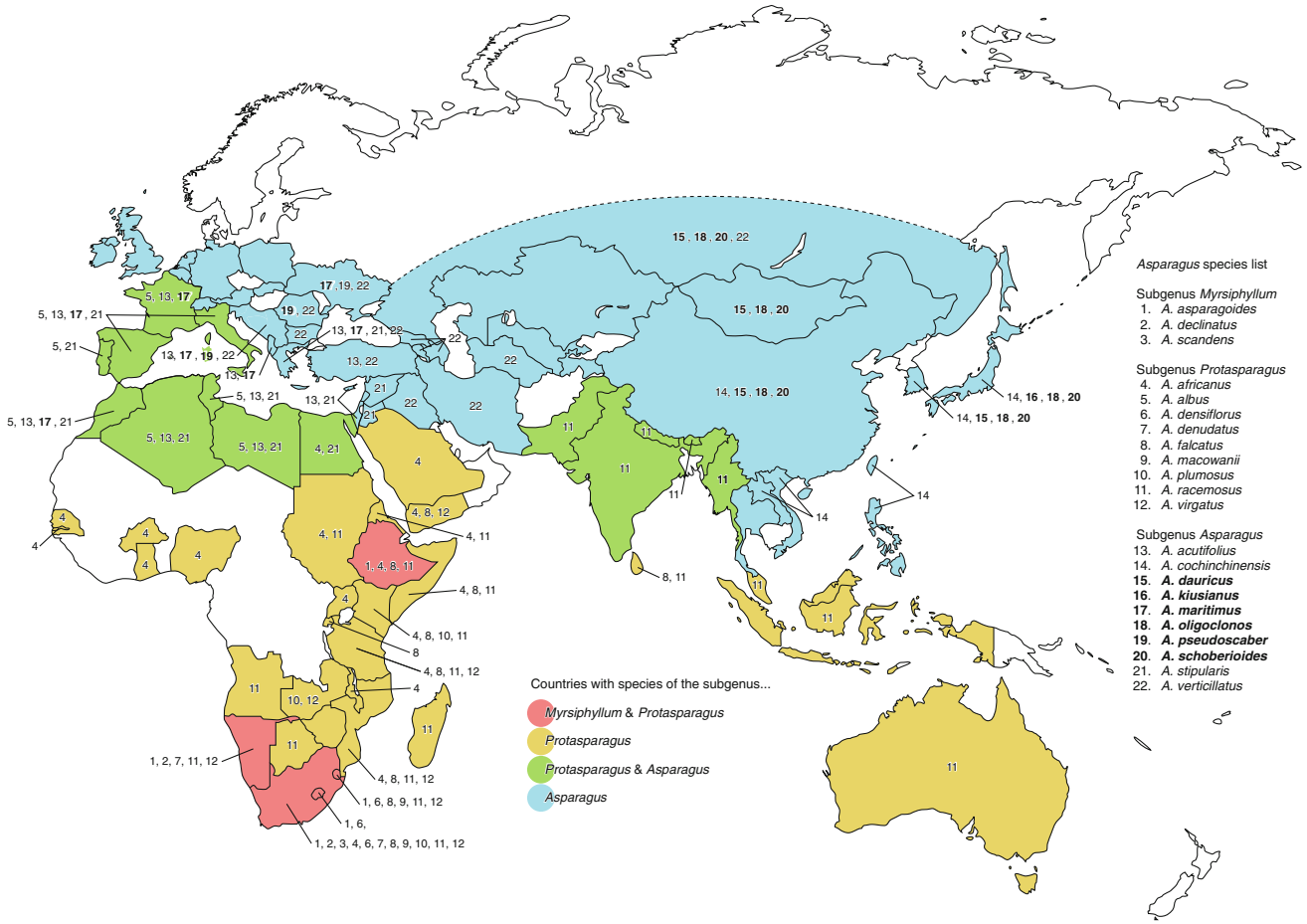

Fig. 1 Distribution of *Asparagus* species. Each country is colored according to the subgenus of the species that it hosts, with *red*, *yellow*, *green* and *blue* corresponding to those countries hosting both *Myrsiphyllum* and *Protasparagus* species, only *Protasparagus*, both *Protasparagus* and *Asparagus*, and only *Asparagus*, respectively. *The numbers within each country* correspond to the species in the list which were used in the phylogenetic analyses. *Numbers in bold* correspond to those species that are cross-compatible with *A. officinalis*

Discussion

Interspecific hybridization is a widely used method for the genetic improvement of many cultivated plants (e.g., Singh 1986; Crow 1998; Chen et al. 2007). In the case of *Asparagus*, a number of attempts to introduce useful agronomic traits from related wild species into *A. officinalis* have been made (Kunitake et al. 1996; Marcellán and Camadro 1996; Alberti et al. 2004; Ito et al. 2008; Zhou et al. 2009; Kanno unpublished data). Still, while some of these species could potentially hybridize with *A. officinalis*, many crossings did not produce fertile hybrids due to pollen-stigma incompatibility or embryo abortion (e.g., Kunitake et al. 1996; Marcellán and Camadro 1996). In addition, it is difficult to predict whether wild species are suitable for interspecific crosses with garden asparagus because compatible species are not always morphologically similar and their geographic distributions do not always overlap with *A. officinalis*. Since interspecific crossing requires laborious and prolonged procedures (e.g., optimization of growth conditions, multi-year growth period), trial and error experiments aimed at detecting cross-compatible species may be inefficient. In the present study, the inferred phylogenetic tree revealed a subclade containing all species within the subgenus *Asparagus* that are cross-compatible with *A. officinalis* (Fig. 2), suggesting that other species (in addition to those presently studied) within this subclade should be capable of hybridization with *A. officinalis*. Considering the large number of wild dioecious species that have not been studied for crossability with *A. officinalis* (e.g., *A. filicinus*, *A. lycopodineus*, and *A. kasakstanicus*, among others), the phylogenetic relationships shown here should contribute towards the selection of appropriate species for future interspecific hybridization attempts.

Previous crossing experiments have found that while both *A. kiusianus* and *A. schoberioides* are cross-compatible with *A. officinalis*, reciprocal crosses with *A. kiusianus* yielded higher fruit set (Kanno unpublished data) than

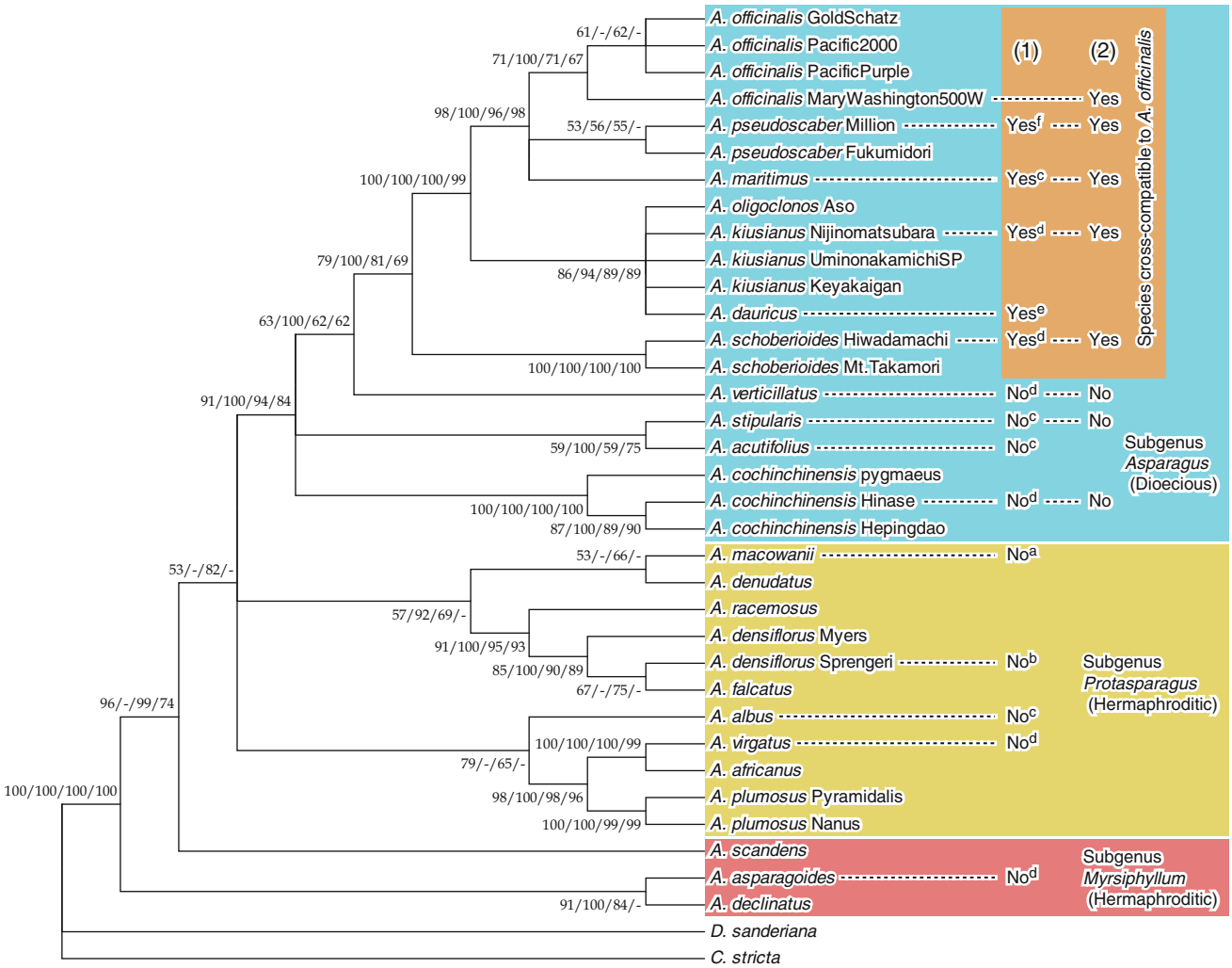

Fig. 2 A 50% majority rule consensus tree of *Asparagus* species based on maximum likelihood analysis of five cpDNA non-coding regions. Node support values indicate maximum likelihood bootstrapping/Bayesian posterior probabilities/neighbor-joining bootstrapping/maximum parsimony bootstrapping, respectively. Comments next to the taxonomic names indicate (1) crossability with *A. officinalis* and (2) applicability of the sex-linked marker. Data determining the crossing compatibility between *A. officinalis* and the other *Asparagus* species were obtained from the crossing experiments of Kunitake et al. (1996) (*a*), Marcellán and Camadro (1996) (*b*), Alberti et al. (2004) (*c*), Ito et al. (2008) (*d*), Zhou et al. (2009) (*e*), and Kanno (unpublished) (*f*)

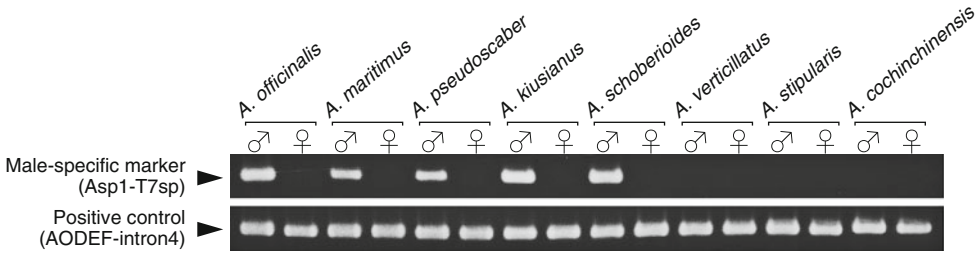

Fig. 3 PCR analysis of eight dioecious *Asparagus* species using Asp1-T7sp primers. The cultivars and populations corresponding to the species tested are shown in Table 1

those with *A. schoberioides* (Ito et al. 2007). Because our phylogenetic tree suggests that *A. kiusianus* is more closely related to *A. officinalis* (Fig. 2), crossability seems to be proportionally associated with the degree of genetic distance between the two species, with hybridization being more likely for those species that are more closely related to *A. officinalis*. In this context, understanding the degree of interspecific relatedness in the genus may facilitate bridge-cross breeding, a commonly used breeding technique to obtain hybrids from distantly related species (e.g., Burk 1967; Khrustaleva and Kik 1998; Jansky and Hamernik 2009). In *Asparagus*, a bridge-cross using

A. maritimus as a bridging species was conducted by Alberti et al. (2004) to overcome the incompatibility between *A. officinalis* and *A. acutifolius*. Hybrids from *A. officinalis* × *A. maritimus* were crossed with *A. acutifolius* and a single seed was obtained. The resulting plant retained fertility and showed morphological and physiological traits that were intermediate with respect to the parents. Since bridging species are preferred to be intermediately related to both crossing species, understanding interspecific relationships within this genus would improve the efficiency of bridge-crosses.

We additionally tested the sex-linked marker designed for *A. officinalis* (Nakayama et al. 2006) in eight dioecious *Asparagus* species. Sex-linked markers have been developed for various species with long vegetative periods, such as kiwifruits (Gill et al. 1998), hops (Jakse et al. 2008), and white poplars (Paolucci et al. 2010), since early sex identification at the seedling stage is important for breeding and cultivation purposes. As shown in Fig. 3, the PCR analysis indicated that the sex-linked marker was able to distinguish males and females in all cross-compatible species. The same results were obtained from additional experiments (a total of 2, 2, 3 and 3 individuals for each sex in *A. maritimus*, *A. pseudoscaber*, *A. kiusianus* and *A. schoberioides*, respectively), indicating that the marker could be readily used for various interspecific crossing programs in *Asparagus*. Nonetheless, more individuals must be tested to provide evidence for correlation of the marker with sex and for homology of the sex determination system among the cross-compatible species. Conversely, the observed amplification failures among males of cross-incompatible species may have resulted from either primer mismatch or from the absence of the homologous male-specific target region. However, because dioecious *Asparagus* species originated from a single evolutionary event (Fig. 2), the sex determination system and genetic components of cross-compatible and incompatible species may not differ to a large extent. Further genomic analyses are necessary to determine the evolutionary history and diversification of the Y chromosome in the dioecious *Asparagus* species.

Since our phylogeny is based on a single locus, the plastid genome, it is possible that nuclear gene histories may differ from the present phylogenetic tree. Nevertheless, our phylogenetic analyses provide a deeper understanding of the evolution of the *Asparagus* species than was previously available. As shown in Fig. 2, the position of *Myrsiphyllum* in the topology suggests that this is the most ancestral *Asparagus* subgenus. In addition, the placement of *A. scandens* as a paraphyletic group inside the two remaining *Myrsiphyllum* species suggests that this species may be more closely related to the ancestors of the *Protasparagus* and *Asparagus* subgenera. However, *Myrsiphyllum* should not be treated as a single evolutionary unit since the species did not form a monophyletic clade. *Protasparagus* species were grouped into two or more clades, showing a polytomous arrangement similar to that observed in a previous study (Fukuda et al. 2005). To account for this polytomy, Fukuda et al. (2005) demonstrated that the pairwise nucleotide divergence of some non-coding cpDNA regions in *Asparagus*, namely the *trnL-trnF* intergenic spacer and *trnL* intron, was much lower than that observed in the same region in other genera. Based on the lower degree of genetic differentiation among the *Asparagus* species, they concluded that the genus had undergone recent and rapid radiation. Here, the polytomy also suggests rapid speciation during the early differentiation of *Protasparagus* via either single or multiple evolutionary origins from *Myrsiphyllum*. It is important to note that the subgenera *Protasparagus* and *Asparagus* are not hierarchically related in the topology of the phylogenetic tree. Considering the number of key characters that the *Asparagus* subgenus shares with *Myrsiphyllum* (e.g., annual aerial parts, two ovules per locule, ovoid seeds; Clifford and Conran 1987), but not with *Protasparagus*, it is possible that the *Asparagus* subgenus derived from a *Myrsiphyllum* species. However, although neither gynodioecious nor androdioecious species have been reported in *Asparagus* (Dahlgren et al. 1985; Clifford and Conran 1987; Kubitzki and Rudall 1998), it is not possible to determine whether dioecism has evolved directly from hermaphroditism in this genus. While some theories consider a direct transition from hermaphroditism to dioecism, it is more likely that the evolutionary shift involved an intermediate step via gyno- or androdioecism (reviewed in Bawa 1980). In fact, multiple transitions from gynodioecism to dioecism have been reported in *Silene*, a genus that also comprises dioecious species with a sex chromosome system (Desfeux et al. 1996). In *A. officinalis*, females have flowers that are all strictly male-sterile, whereas males show a variety of vestigial female organs, from small rudimentary ovaries with no style or stigma to flowers with fertile pistils. Previous analyses have shown that female-sterility correlates with the size of the female organ, that the size of female organs are genetically inherited and that hermaphroditic males possess high selfing rate and severe inbreeding depression (Galli et al. 1993), which are essential for the evolution of dioecism through gynodioecism (Darwin 1877; Lloyd 1975; Charlesworth and Charlesworth 1978). Although it remains uncertain whether an intermediate gynodioecious species existed, our study presents the first robust phylogenetic analysis to demonstrate that dioecy has evolved from hermaphroditism only once in *Asparagus* and there has not been a reversal from dioecy back to hermaphroditism as has been seen in some other monocots (reviewed in Weiblen et al. 2000).

Together with the global distribution of the species (Fig. 1), the phylogenetic analysis presented here offers some insights into the historical biogeography of *Asparagus*. The geographical distribution of *Myrsiphyllum* suggests that the genus *Asparagus* has its origin in Southern Africa, having spread northward subsequently. Since most *Protasparagus* species are found throughout Africa, the subgenus seems to have differentiated from *Myrsiphyllum* in this continent and then spread eastward along the coast of the Indian Ocean. The *Asparagus* subgenus may have differentiated from *Myrsiphyllum* in Northern Africa or around the Mediterranean Basin and then expanded its distribution eastward, along the temperate zone of Asia. Interestingly, the distribution of the *Asparagus* subgenus does not correlate exactly with their phylogeny. For instance, although both *A. kiusianus* and *A. cochinchinensis* have the easternmost distribution within this subgenus, *A. kiusianus* is more closely related to *A. maritimus*, which is found at the westernmost area of the subgenus distribution. Because the observations related to the crossability among species and the results from the sex-linked marker amplification experiments support the validity of the phylogenetic clustering within the *Asparagus* subgenus, these observed discrepancies between the phylogeny and the geographical distribution may result from the presence of differences in the dispersal rate of each species. *Asparagus* species occupy a wide range of environments, such as dry lands, sea coasts and forest floors (Dahlgren et al. 1985). In addition, many species develop reddish or bluish fruits, which often attract birds that act as dispersal vectors (Barnea et al. 1991; Traveset et al. 2001; Nogales et al. 2007). Studies involving invasive *Asparagus* species in Australia have pointed out that the fruits can be dispersed over several kilometers depending on the dispersing bird species (Stansbury 2001; Lawrie 2006). Interspecific differences in the degree of adaptability to different environments and the potential for long-distance dispersal might have therefore shaped the unique distribution of *Asparagus*. In this context, another notable feature within *Asparagus* subgenus is the cross-incompatibility of some Mediterranean species, such as *A. acutifolius*, *A. stipularis* and *A. verticillatus*, with the sympatric *A. officinalis*. While our results from the phylogenetic analysis suggest that these Mediterranean species are not closely related to *A. officinalis*, the loss of cross-compatibility could be a consequence of time since species divergence (i.e., inevitable accumulation of genetic incompatibilities between species) or natural selection for reproductive barriers. Since it is well known that cross-incompatibility can arise rapidly between sympatric species through natural selection (reviewed in Turelli et al. 2001), examination of the premating barriers (temporal and ecological isolation) among the sympatric species is necessary to elucidate the establishment and maintenance of cross-incompatibility. Additional studies of ecological aspects, together with phylogenetic analyses involving a wider range of species, can contribute further to the understanding of the evolutionary history of this group.

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Conflict of interest None.

References

Ahmad M, McNeil DL (1996) Comparison of crossability, RAPD, SDS-PAGE and morphological markers for revealing genetic relationships within and among *Lens* species. Theor Appl Genet 93:788–793

Alberti P, Casali PE, Barbaglio E et al (2004) Interspecific hybridization for *Asparagus* breeding. In: Proceedings of the XLVIII Italian Society of Agricultural Genetics–SIFV-SIGA Joint Meeting. Lecce, Italy

APG III (2009) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Bot J Linn Soc 161:105–121

Barnea A, Yom-Tov Y, Friedman J (1991) Does ingestion by birds affect seed germination? Funct Ecol 5:394–402

Barrett SCH (2002) Evolution of sex: the evolution of plant sexual diversity. Nat Rev Genet 3:274–284

Bawa KS (1980) Evolution of dioecy in flowering plants. Ann Rev Ecol Syst 11:15–39

Bracale M, Caporali E, Galli MG et al (1991) Sex determination and differentiation in *Asparagus officinalis* L. Plant Sci 80:67–77

Burk LG (1967) An interspecific bridge-cross: *Nicotiana repanda* through *N. sylvestris* to *N. tabacum*. J Hered 58:215–218

Charlesworth B, Charlesworth D (1978) A model for the evolution of dioecy and gynodioecy. Am Nat 112:975–997

Chen L, Zhou Z-X, Yang Y-J (2007) Genetic improvement and breeding of tea plant (*Camellia sinensis*) in China: from individual selection to hybridization and molecular breeding. Euphytica 154:239–248

Clifford HT, Conran JG (1987) Asparagaceae. In: George AS (ed) Flora of Australia. Australian Government Publishing Service, Canberra, pp 140–142

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. Evolution 43:362–381

Coyne JA, Orr HA (1996) "Patterns of speciation in *Drosophila*" revised. Evolution 51:295–303

Crow JF (1998) 90 years ago: the beginning of hybrid maize. Genetics 148:923–928

Dahlgren RMT, Clifford HT, Yeo PF (1985) The families of the monocotyledons. Springer, Heidelberg

Darwin C (1877) Different forms of flowers on plants of the same species. John Murray, London

Dehgan B (1984) Phylogenetic significance of interspecific hybridization in *Jatropha* (Euphorbiaceae). Syst Bot 9:467–478

Desfeux C, Maurice S, Henry J-P et al (1996) Evolution of reproductive systems in the genus *Silene*. Proc R Soc Lond B 263:409–414

Ernst M, Krug H (1998) Seasonal growth and development of asparagus (*Asparagus officinalis* L.). III. The effect of temperature and water stress on carbohydrate content in storage roots and rhizome buds. Gartenbauwissenschaft 63:202–208

Fukuda T, Ashizawa H, Suzuki R et al (2005) Molecular phylogeny of the genus *Asparagus* (Asparagaceae) inferred from plastic *petB* intron and *petD-rpoA* intergenic spacer sequences. Plant Species Biol 20:121–132

Galli MG, Bracale M, Falavigna A et al (1993) Different kinds of male flowers in the dioecious plant *Asparagus officinalis L.* Sex Plant Reprod 6:16–21

Gill GP, Harvey CF, Gardner RC et al (1998) Development of sex-linked PCR markers for gender identification in *Actinidia*. Theor Appl Genet 97:439–445

Guindon S, Dufayard J-F, Lefort V et al (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst Biol 59:307–321

He CY, Hsiang T, Wolyn DJ (2002) Induction of systemic disease resistance and pathogen defence responses in *Asparagus officinalis* inoculated with nonpathogenic strains of *Fusarium oxysporum*. Plant Pathol 51:225–230

Ito T, Suzuki G, Ochiai T et al (2005) Genomic organization of the *AODEF* gene in *Asparagus officinalis* L. Genes Genet Syst 80:95–103

Ito T, Ochiai T, Ashizawa H et al (2007) Production and analysis of reciprocal hybrids between *Asparagus officinalis* L. and *A. schoberioides* Kunth. Genet Resour Crop Evol 54:1063–1071

Ito T, Ochiai T, Fukuda T et al (2008) Potential of interspecific hybrids in Asparagaceae. Acta Hortic 776:279–284

Jakse J, Štajner N, Kozjak P et al (2008) Trinucleotide microsatellite repeat is tightly linked to male sex in hop (*Humulus lupulus* L.). Mol Breed 21:139–148

Jamsari A, Nits I, Reamon-Büttner SM et al (2004) BAC-derived diagnostic markers for sex determination in asparagus. Theor Appl Genet 108:1140–1146

Jansky S, Hamernik A (2009) The introgression of 2× 1EBN *Solanum* species into the cultivated potato using *Solanum verrucosum* as a bridge. Genet Resour Crop Evol 56:1107–1115

Khrustaleva LI, Kik C (1998) Cytogenetical studies in the bridge cross *Allium cepa* × (*A. fistulosum* × *A. roylei*). Theor Appl Genet 96:8–14

Kubitzki K, Rudall PJ (1998) Asparagaceae. In: Kubitzki K (ed) The families and genera of vascular plants, vol 3. Springer, Heidelberg, pp 125–128

Kunitake H, Nakashima T, Mori K et al (1996) Production of interspecific somatic hybrid plants between *Asparagus officinalis* and *A. macowanii* through electrofusion. Plant Sci 116:213–222

Lawrie SL (2006) The ecology of bridal veil (*Asparagus declinatus* L.) in South Australia. Plant Prot Q 21:99–100

Lee Y-O, Kanno A, Kameya T (1997) Phylogenetic relationships in the genus *Asparagus* based on the restriction enzyme analysis of the chloroplast DNA. Breed Sci 47:375–378

Liu Y, Chen H, Zhuang D et al (2010) Characterization of a *DRE*-binding transcription factor from asparagus (*Asparagus officinalis* L.) and its overexpression in *Arabidopsis* resulting in salt- and drought-resistant transgenic plants. Int J Plant Sci 171:12–23

Lloyd DG (1975) The maintenance of gynodioecy and androdioecy in angiosperms. Genetica 45:325–339

Löptien H (1979) Identification of the sex chromosome pair in asparagus (*Asparagus officinalis* L.). Z. Pflanzenzüchtg 82:162–173

Marcellán ON, Camadro EL (1996) Self- and cross-incompatibility in *Asparagus officinalis* and *Asparagus densiflorus* cv. Sprengeri. Can J Bot 74:1621–1625

Nakayama H, Ito T, Hayashi Y et al (2006) Development of sex-linked primers in garden asparagus (*Asparagus officinalis* L.). Breed Sci 56:327–330

Nogales M, Padilla DP, Nieves C et al (2007) Secondary seed dispersal systems, frugivorous lizards and predaroty birds in insular volcanic badlands. J Ecol 95:1394–1403

Obermeyer AA (1983) *Protasparagus* Oberm. nom. nov.: new combinations. S Afr J Bot 2:243–244

Paolucci I, Gaudet M, Jorge V et al (2010) Genetic linkage maps of *Populus alba* L. and comparative mapping analysis of sex determination across *Populus* species. Tree Genet Genomes 6:863–875

Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25:1253–1256

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Shaw J, Lickey EB, Beck JT et al (2005) The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J Bot 92:142–166

Shaw J, Lickey EB, Schilling EE et al (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am J Bot 94:275–288

Singh AK (1986) Utilization of wild relatives in the genetic improvement of *Arachis hypogaea* L. 8. Synthetic amphidiploids and their importance in interspecific breeding. Theor Appl Genet 72:433–439

Smith J, Putnam A, Nair M (1990) In vitro control of *Fusarium* diseases of *Asparagus officinalis* L. with a *Streptomyces* or its polyene antibiotic, Faeriefungin. J Agric Food Chem 38:1729–1733

Štajner N, Bohanec B, Javornik B (2002) Genetic variability of economically important *Asparagus* species as revealed by genome size analysis and rDNA ITS polymorphisms. Plant Sci 162:931–937

Stansbury CD (2001) Dispersal of the environmental weed bridal creeper, *Asparagus asparagoides* by silvereyes, *Zosterops lateralis*, in south-western Australia. Emu 101:39–45

Stewart CN Jr, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques 14:748–750

Tamura K, Dudley J, Nei M et al (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Traveset A, Riera N, Mas RE (2001) Passage through bird guts causes interspecific differences in seed germination characteristics. Funct Ecol 15:669–675

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. Trends Ecol Evol 16:330–343

Turner PJ, Scott JK, Spafford H (2008) The ecological barriers to the recovery of bridal creeper (*Asparagus asparagoides* (L.) Druce) infested sites: Impacts on vegetation and the potential increase in other exotic species. Austral Ecol 33:713–722

Thompson JD, Gibson TJ, Plewniak F et al (1997) The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 24:4876–4882

Weiblen GD, Oyama RK, Donoghue MJ (2000) Phylogenetic analysis of dioecy in Monocotyledons. Am Nat 155:46–58

Williams PA (2006) The role of blackbirds (*Turdus merula*) in weed invasion in New Zealand. N Z J Ecol 30:285–291

Zhou JS, Zhan FX, Tang YP et al (2009) Interspecific hybridization between *A. officinalis* L. and *Asparagus dauricus* Fisch.ex Link. In: XIIth International Asparagus Symposium. Lima, Peru