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Phylogenetic Relationships in the *Festuca-Lolium* Complex (Loliinae; Poaceae): New Insights from Chloroplast Sequences

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The species within the *Lolium/Festuca* grass complex have dispersed and colonized large areas of temperate global grasslands both naturally and by human intervention. The species within this grass complex represent some of the most important grass species both for amenity and agricultural use worldwide. There has been renewed interest by grass breeders in producing hybrid combinations between these species and several countries now market *Festulolium* varieties as a combination of genes from both genera. The two genera have been differentiated by their inflorescence structure, but controversy has surrounded the taxonomic classification of the *Lolium-Festuca* complex species for several decades. In order to better understand the complexities within the *Lolium/Festuca* complex and their genetic background, the phylogeny of important examplers from the *Lolium-Festuca* complex were reconstructed. In total 40 taxa representing the *Festuca* and *Lolium* species with *Vulpia myuros* and *Brachypodium distachyon* as outgroups were sampled, using two non-coding intergenic spacers (*trnQ-rps16*, *trnH-psbA*) and one coding gene (*rbc*L). Maximum parsimony (MP), Bayesian inference (BI) analyses based on each partition and combined plastid DNA dataset, and median-jointing network analysis were employed. The outcomes strongly suggested that the subgen. *Schedonorus* has a close relationship to *Lolium*, and it is also proposed to move the sect. *Leucopoa* from subgen. *Leucopoa* to Subgen. *Schedonorus* and to separate sect. *Breviaristatae* from the subgen. *Leucopoa*. We found that *F. californica* could be a lineage of hybrid origin because of its intermediate placement between the "broad-leaved" and "fine-leaved" clade.

Keywords: phylogeny, *Festuca*, *Lolium*, *trnQ-rps16*, *trnH-psbA*, *rbc*L

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INTRODUCTION

As one of the largest subtribes from the tribe Poeae (Pooideae, Poaceae), Loliinae encompasses nine genera (*Festuca*, *Lolium*, *Vulpia*, *Nardurus*, *Loliolium* Krecz, and Bobr, *Scleropoa*, *Cutandia* Willk., *Sphenopus*, and *Bellardiochloa* Chiov; Tzvelev, 1982; Soreng and Davis, 2000). Among the genera, *Festuca* is large and complex having more than 600 species with multiple ploidy levels ranging from diploid ($2n = 2x = 14$) up to dodecaploid ($2n = 12x = 84$) whereas *Lolium* is a small genus

with 10 recognized diploid species (Clayton and Renvoize, 1986; Loureiro et al., 2007). The two genera, *Festuca* and *Lolium* include a number of important grasses used as pasture, fodder, and amenity purposes. The *Lolium* genera include the two widely cultivated temperate grass species, *L. multiflorum* (annual or Italian ryegrass) and *L. perenne* (perennial ryegrass) which are characterized by rapid growth and high forage quality. There are more than 3000 cultivars grown around the world with many hybrids naturally or artificially produced (Cai et al., 2011). The *Festuca* genus also includes two agriculturally important forage crop species, tall fescue (*F. arundinacea*) and meadow fescue (*F. pratensis*). They differ from ryegrasses, having larger, deeper root systems and greater water and nutrient-use-efficiency, and generally higher stress tolerance than the ryegrasses. Another important group of *Festuca* species are the fine-leaved fescues which are valued for their forage, turf and ornamental use. Red fescue (*F. rubra* L.) and sheep fescue (*F. ovina* L.) are valued for their narrow leaves which minimize water loss and provide improved drought tolerance (Rognli et al., 2010).

The majority of species within the *Lolium*/*Festuca* grass complex are heterogeneous and largely obligate outbreeders; they are highly diverse in their growth ontogeny, morphology, and their adaptations to onsets of both climatic and edaphic stress. As a consequence they have dispersed and colonized large areas of temperate global grasslands (Humphreys et al., 2006). As a group, the *Festuca-Lolium* complex comprises species that are closely allied and are partially interfertile.

The two genera can be easily differentiated by their inflorescence structure, and the taxonomic classification of some *Lolium* and *Festuca* species are controversial. A number of taxonomic revisions have proposed placing the "broad-leaved" fescues (*Festuca* subgen. *Schedonorus*) into *Lolium* (Darbyshire, 1993) by natural phenomenon and through experimental evidence. This includes the regular occurrence of spontaneous hybridization between species of *Festuca* subgen. *Schedonorus* and chasmogamous species of *Lolium* which on the other hand only rarely occurs between the major "fine-leaved" fescues and *Lolium* (Stace, 1975; Barker and Stace, 1982). Furthermore, analysis of morphological data sets (Stebbins, 1956), DNA restriction site variation (Darbyshire and Warwick, 1992), and seed protein (Buliñska-Radomska and Lester, 1988) suggest placing *Lolium* and *Festuca* subgen. *Schedonorus* together as one lineage. In contrast, others have suggested that the broad leaf fescues be separated into a new genus called *Schedonorus* (Soreng and Terrell, 1997). Aside from the controversial relationship between "broad-leaved" fescues and *Lolium* species, the *Festuca* genus *per se* is also complex with the taxanomic placements of its specific subgenera, sections, and species quite intricate. For instance, sect. *Breviaristatae* was considered as separate from subgen. *Leucopoa* (Tzvelev, 1971; Clayton and Renvoize, 1986), However, Soreng et al. (1990) found that *F. slerophylla* of sect. *Leucopoa* had a close relationship with *F. arundinacea* of subgen. *Schedonorus* based on the chloroplast DNA restriction site variation. By the same method, Darbyshire and Warwick (1992) discovered that examplers of sect. *Breviaristatae* had no phylogenetic affinity with examplers of sect. *Leucopoa*, subgen. *Leucopoa*. Therefore, they suggested that sect. *Leucopoa* from subgen. *Leucopoa* be moved to subgen. *Schedonorus*. *F. mairei* St. Yves, which is one of the key species in the evolution of polyploid fescues (Buliñska-Radomska and Lester, 1988) was first categorized into section Scariosae of subgen. *Festuca* (Stammers et al., 1995), but later was proposed to be reclassified into subgen. *Schedonrus* (Torrecilla and Catalán, 2002).

A better understanding of phylogenetic relationships within the *Festuca-Lolium* complex species would not only be very useful for future species conservation and for improved collection knowledge, but would also greatly assist future forage grass breeding programs. To ensure future grassland resilience and sustainable forage production for livestock agriculture, it has been considered as an increasingly important strategy to hybridize *Lolium* and *Festuca* species in order to gain and combine the complementary attributes of both. As *Lolium* x *Festuca* interspecific species' hybrids grass varieties are marketed under their own category termed *Festulolium* and provide a source of reliable, nutrient-use-efficient, and productive fodder for ruminants (Humphreys et al., 2014). Increased understanding of the phylogenetic relationships between the *Lolium*/*Festuca* species and of how the polyploid fescues and their adaptive benefits have evolved can benefit plant breeders and thereby accelerate the development of *Festulolium* breeding programs to better provide increased forage resilience sufficient to combat climate change.

Analysis of the phylogenetic relationships within the *Festuca-Lolium* complex encompassed the biological technology revolution from macro morphology to micro genetic level. Previous methods include chloroplast DNA (cpDNA) electrophoresis (Lehväslaiho et al., 1987; Soreng et al., 1990; Darbyshire and Warwick, 1992), RAPD (random amplification of polymorphic DNA) technology (Stammers et al., 1995; Wiesner et al., 1995), ITS (internal transcribe spacer) sequences of nuclear rDNA (Charmet et al., 1997; Gaut et al., 2000; Torrecilla and Catalán, 2002), and sequences of chloroplast *trn*L-F region (Catalán et al., 2004; Torrecilla et al., 2004). Despite the large genomic resources available for most intensively cultivated species of the *Festuca-Lolium* complex, conjoined analyses with chloroplast spacers as well as chloroplast genes have not been employed for the analysis of phylogeny among the *Festuca-Lolium* complex. The chloroplast has highly-conserved genes which are elementary to plants and are variable and informative regions over a long time scale. The use of cpDNA can also analyse the maternal source genome donor and has been applied successfully in the phylogenetic analysis of many taxa (Shaw et al., 2007; Sun, 2007; Nock et al., 2011).

In the present study, we sampled 42 taxa, including 28 *Festuca* taxa, 12 *Lolium* taxa and 2 related but out-group species (*Vulpia myuros*, *Brachypodium distachyon*). Chosen taxa have been identified by their morphology, they are representatives of broad-leaved and narrow-leaved species, these species are significant because of their importance in agricultural and amenity use and were therefore deemed the most important for this phylogenetic study. DNA sequence data from chloroplast spacers (*trnQ-rps16*, *trnH-psbA*) and chloroplast gene (*rbc*L) were used to resolve phylogenetic relationships among the *Festuca-Lolium* complex. The main objectives were to: (1) construct the plastid phylogeny

of *Festuca-Lolium* complex using two non-coding intergenic spacers and one coding gene, and compare with the previous analyses; (2) explore the maternal donors of the polyploid species of fescues.

MATERIALS AND METHODS

Taxon Sampling

A total of forty taxa were sampled from the *Lolium-Festuca* complex comprising 28 *Festuca* taxa corresponding to 3 subgenera, 7 sections, and one subsection, and 12 taxa of *Lolium*. *Vulpia myuros* and *Brachypodium distachyon* were included as out-groups based on previous phylogenetic studies of Loliinae (Inda et al., 2008). The taxa names, accessions numbers, ploidy level, origin and abbreviations are listed in **Table 1**. All the seed materials with PI were generously provided by the National Plant Germplasm System of USDA. The seeds were first germinated in petri dishes and then the strong seedlings were transferred to pots. Morphological observation and weeding were regularly undertaken in order to ensure the plant purity. Mitotic analyses of root tips were made to verify the ploidy level of each accession.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from freeze-dried leaf tissue of each accession by the standard CTAB (cetyl-trimethylammonium bromide) method (Doyle and Doyle, 1987) using the TIANcombi DNA PCR Kit (Beijing, China). One individual was sampled for DNA extraction, and five DNA samples were prepared for each taxon. The quality and concentration of the DNA were assessed by NanoVue Plus spectrophotometry produced by General Electric Company and checked on a 1% agarose-gel. The chloroplast *trnH-psbA* gene and *rbc*L were amplified with the universal primers (**Table 2**). The PCR (polymerase chain reaction) was performed in a final volume of 50 μl, containing 4 μl template DNA with the concentration of 10 ng/μl, 4 μl primer with a concentration of 0.01 mmol/μl, 25 μl 2×Premix Taq (TaKaRa) with 0.4 mM dNTPs of each nucleotide, 3 mM $MgCl_2$ buffer, 1.25 U Taq DNA polymerase with pigment included, and addition of ddH_2O to the final volume. The PCR amplification programs started with a 4 min initial denaturation step at 94°C; followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing (55°C for *rbc*L and 50°C for *trnH-psbA*), and 1.5 min extension at 72°C; ending with a final extension step at 72°C for 10 min. The PCR products were checked in a 1% agarose gel and purified with the AxyPrep DNA kit, and then qualified samples were sent to the Majorbio Company (Shanghai, China) for sequencing. Generally, 3–5 PCR products were sent for sequencing from each taxon.

Data Analyses

The returned sequences data were initially spliced by the DNASTAR Seqman (Swindell and Plasterer, 1997) and aligned by the BioEdit ver. 7.0.9 (Hall, 1999). The nucleotide sites' information and the nucleotide frequencies were calculated by MEGA software ver. 5.02 (Tamura et al., 2011). The software package DAMBE ver. 5.5.29 (Xia and Xie, 2001) was used to assess the substitution saturation by plotting pairwise rates of transitions and transversions against a correct genetic distance under F84 model.

In order to evaluate the divergence and relationship among taxa, number of sites (n), number of variable site (s), haplotype diversity (Hd) (Nei and Li, 1979), Tajumas's π (Tajima, 1989), Watterson's θ_w (Watterson, 1975) were calculated, Neutrality test was also performed by the Tajima's and Fu and Li's D statistic (Tajima, 1989; Fu and Li, 1993). All the parameters above were conducted by DnaSP ver. 5.10 (Librado and Rozas, 2009).

Phylogenetic Reconstruction

Phylogenetic analysis of each partition and the combined plastid DNA dataset (*trnQ-rps16*, *trnH-psbA*, *rbc*L) were created by maximum parsimony (MP) and Bayesian inference (BI). Maximum parsimony (MP) analyses were implemented in PAUP* v.4.0b10 (Swofford, 2002). All characters were treated as unweighted and unordered, gaps were treated as "missing." The heuristic search option using the Tree Bisection-Reconnection (TBR) branch swapping and MUL-Tree option on, 10 replicates of random addition sequence with the stepwise addition option was employed to obtain the most parsimonious trees. The consensus tree option was set as "retain groups with frequency>50%". Topological robustness MP analysis was evaluated by bootstrap analysis using a full heuristic search with 1000 replicates (Felsenstein, 1985) each with simple addition sequence.

Bayesian inference was carried out in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). It performs Bayesian phylogenetic analysis of information from different data partition or the combined dataset. The optimal evolutionary model used for different data matrixes were estimated by jModelTest v.2.1.7 (Posada, 2008; Darriba et al., 2012) was used to determine the using the Akaike information criterion (AIC). The best-fit model was TPMluf+G for *trnQ-rps16* data, TIM1+I+G for *trnH-psbA* data, TIM2+G for *rbc*L, and TPMluf+I+G was chosen as the most appropriate for combined data analysis. Four MCMC (Markov Chain Monte Carlo) chain (one cold and three heated) were run for 200,000 generations for *trnQ-rps16* data, 600,000 generations for *trnH-psbA* data, and 120,000 generations for *rbc*L and combined data, each sampling every 10 generations. The analysis was continued until the standard deviation of split frequencies below 0.01. The first 5000, 15,000, and 3000 trees were stationary discarded as "burn-in" for *trnQ-rps16* data, *trnH-psbA* data and *rbc*L or combined data, respectively (determined empirically from the log-likelihood values using Tracer V1.4; Rambaut and Drummond, 2013). The remaining trees were employed to construct the 50%-majority rule consensus trees and frequencies of clades were evaluated by posterior probabilities (PP).

A Network representation may be more appropriate than the tree presentation when the existence of reticulate evolution such as gene transfer, hybridization, and recombination. The median-jointing (MJ) network analysis can reveal the relationships between ancestral and derived haplotypes which was first employed to discuss the human mtDNA variation (Bandelt et al., 2000). Compared with other graph construction approaches

TABLE 1 | List of *Lolium*/*Festuca* examplers and the outgroup used in this study.

Taxon	Ploidy	PI	Origin	Abbr.
***Festuca* L.**				
Subgen. *Festuca*				
Sect. *Festuca*				
Subsect. *Festuca* ("*F. ovina* complex")				
Festuca hystrix	2x	PI 302896	Spain	FHYS
Festuca idahoensis	4x	PI 601053	USA	FIDA
Festuca ovina	2x	PI 235218	France	FOVI
Festuca valesiaca	2x	PI 634225	Ukraine	FVAL
Festuca brachyphylla	6x	W6 25548	Greenland	FBRA
Festuca lemanii	6x	PI 286207	Czech	FLEM
Festuca pseudovina	2x	PI 374046	Hungary	FPSE
Sect. *Aulaxyper* Dumort ("*F. rubra* complex")				
Festuca ampla	4x	PI 283275	Portugal	FAMP
Festuca heterophylla	4x	PI 249742	Greece	FHET
Festuca rubra	6x	PI 595056	Norway	FRUB1
Festuca rubra	6x	PI 318993	Spain	FRUB2
Festuca rubra subsp. Arctica	6x,8x	PI 659648	Iceland	FARC
Sect. *Amphigenes* (Janka) Tzvel				
Festuca pulchella	2x	PI 287542	Poland	FPUL
Subgen. *Schedonorus* (P. Beauv.) Peterm.				
Sect. *Plantynia* (Dum.) Tzvelev				
Festuca gigantean	6x	PI 206646	Turkey	FGIG
Sect. *Schedonorus* (P. Beauv.) Koch				
Festuca arundinacea	6x	PI 634240	France	FARU
Festuca arundinacea subsp. atlantigena	8x	PI 577096	UK	FATL
Festuca arundinacea subsp. fenas	4x	PI 595048	France	FFEN
Festuca arundinacea subsp. orientalis	6x	PI 634282	Ukraine	FORI
Festuca pratensis subsp. pratensis	2x	PI 234777	Germany	FPRA
Festuca pratensis subsp. apennina	4x	PI 610808	Switzerland	FAPE
Festuca mairei	4x	PI 610941	Morocco	FMAI1
Festuca mairei	4x	PI 283312	Sweden	FMAI2
Subgen. *Leucopoa* (Grised.) Hack				
Sect. *Breviaristatae*				
Festuca altaica	4x	PI 639774	Mongolia	FALT1
Festuca altaica	4x	PI 236847	Canada	FALT2
Festuca californica	4x,8x	W6 26789	USA	FCAL
Sect. *Leucopoa*				
Festuca spectabilis	6x	PI 383658	Turkey	FSPE1
Festuca spectabilis	6x	PI 384871	Iran	FSPE2
Festuca kingii	8x	PI 232305	USA	FKIN
***Lolium.* L**				
Lolium multiflorum	2x	PI 577241	Italy	LMUL1
Lolium multiflorum	2x	PI 545668	Turkey	LMUL2
Lolium perenne	2x	PI 547390	Iran	LPER1
Lolium perenne	2x	PI 598510	Turkey	LPER2
Lolium rigidum	2x	PI 254899	Iraq	LRIG1
Lolium rigidum	2x	PI 516608	Morocco	LRIG2
Lolium temulentum	2x	PI 422589	Morocco	LTEM1
Lolium temulentum	2x	PI 298417	Turkey	LTEM2
Lolium persicum	2x	PI 229764	Iran	LPERS1

(Continued)

TABLE 1 | Continued

Taxon	Ploidy	PI	Origin	Abbr.
Lolium persicum	2x	PI 545637	Turkey	LPERS2
Lolium subulatum	2x	PI 197310	Argentina	LSUB
Lolium canariense	2x	PI 320544	Spain	LCAN
Out-group				
Vulpia myuros	6x	PI 204448	Turkey	VMYU
Brachypodium distachyon	2x	W6 39443	Turkey	BDIS

TABLE 2 | Details of primer pairs used to amplify the *trnQ-rps16*, *trnH-psbA,* and *rbc*L gene.

Region	Primer name	Sequence (5′-3′)	References
trnQ-rps16	trnQ	GCG TGG CCA AGY GGT AAG GC	Shaw et al., 2007
	rps16	GTT GCT TTY TAC CAC ATC GTT T	
*rbc*L	1F	ATG TCA CCA CAA ACA GAA AC	Kress and Erickson, 2007
	724R	TCG CAT GTA CCT GCA GTA GC	
trnH-psbA	psbAF	GTT ATG CAT GAA CGT AAT GCT C	Sang et al., 1997

(minimum-spanning network and statistical parsimony), the MJ network using the maximum parsimony (MP) method can provide the best measure of the true genealogy (Cassens et al., 2005). The MJ network was constructed by the Network 4.6.1.3 program (Fluxus Technology Ltd), the test of recombination was performed by the GARD Processor method with the HyPhy package (Pond and Muse, 2005). Based on the result of the test, the combination of the three plastid DNA datasets was used to generate the MJ network because of the absence of recombination signal in alignment (Log Likelihood = −4126.04; AIC = 8430.02).

RESULTS

Sequences Analyses

The length of *trnQ-rps16*, *trnH-psbA* and *rbc*L sequences were 700, 579, and 632 bp respectively, in the final aligned sequences of 40 taxa excluding the out-groups. The specific sites' information and nucleotide frequencies were shown in **Table 3**. Both the tests of substitution saturation for *trnQ-rps16*, *trnH-psbA* spacers and *rbc*L gene under the F84 model showed a basically linear regression which demonstrated no saturation effects among the mutation of different sequences (see Supplementary Figures 1–3).

The nucleotide diversity information containing the number of sites (*n*), number of variable site (*s*), haplotype diversity (Hd), the average pairwise diversity (π), and the diversity based on the number of segregating sites (θ_w) of *trnQ-rps16*, *trnH-psbA* spacers, and *rbc*L gene were calculated. The neutrality test results showed negative for the three sequences which might be because of the genetic bottleneck. Above values were displayed in **Table 4**.

All 126 sequences have been submitted to the database of NCBI (National Center for Biotechnology Information), the accession numbers are from KT43895 to KT439068 (see Supplementary Excel, Datasheet).

TABLE 3 | Features of the matched data matrix for *trnQ-rps16, trnH-psbA,* and *rbc*L gene sequences.

Gene	C	V	Pi	S	ii	si	sv
TrnQ-rps16	557	106	68	38	540	12	13
trnH-psbA	524	34	26	8	540	4	6
*rbc*L	606	26	16	10	625	5	1

C, conserved sites; V, variable sites; Pi, parsimony-informative sites; S, singleton sites; ii, identical pairs; si, transitional pairs; sv, transversional pairs.

Phylogenetic Analyses

Chloroplast Spacer *trnQ-rps16* Data

The aligned *trnQ-rps16* sequences produced a total of 697 characters, of which 109 were variable and 75 were parsimony-informative. The parsimony analysis for *trnQ-rps16* sequences resulted in 215 most parsimonious trees (tree length = 215; consistency index = 0.9395; retention index = 0.9791; rescale consistency index = 0.9199). The 50% MP majority-rule consensus tree was identical to the tree obtained from BI except for some nodes presenting different statistical support. The tree shown in **Figure 1** was MP tree with bootstrap support (BS) above the branches and posterior probabilities (PP) of BI tree below the branches. According to the tree, the two major clades had been strongly supported, mainly corresponding to the width of blades. The first clade included the narrow-leaved fescues and *Vulpia myuros*, the second clade contained all the *Lolium* samples and the broad-leaved *Festuca* taxa.

Chloroplast Spacer *trnH-psbA* Data

The total character of the aligned *trnH-psbA* sequences was 585, of which 27 characters were variable and parsimony-informative. The parsimony analysis for *trnH-psbA* sequences resulted in 91 most parsimonious trees (tree length = 190; consistency index = 0.3000; retention index = 0.5994; rescale consistency index =

TABLE 4 | Estimates of nucleotide diversity and test statistics for *trnH-psbA*, *trnQ-rps16,* and *rbc*L gene sequences data sets.

Gene	*n*	*s*	Π	Hd	θ_W	Fu and Li's D	Tajima's D
trnQ-rps16	700	52	0.03736	0.871	0.03071	−0.14073 ($p > 0.10$)	0.77642 ($p > 0.10$)
trnH-psbA	579	31	0.01723	0.940	0.01337	−0.12620 ($P > 0.10$)	0.99644 ($P > 0.10$)
*rbc*L	632	26	0.01071	0.867	0.00967	−1.03905 ($p > 0.10$)	0.36442 ($p > 0.10$)

n, total number of sites; s, number of polymorphic sites; π, nucleotide diversity per site; Hd, haplotype diversity; θ_W, diversity based on the number of segregating sites.

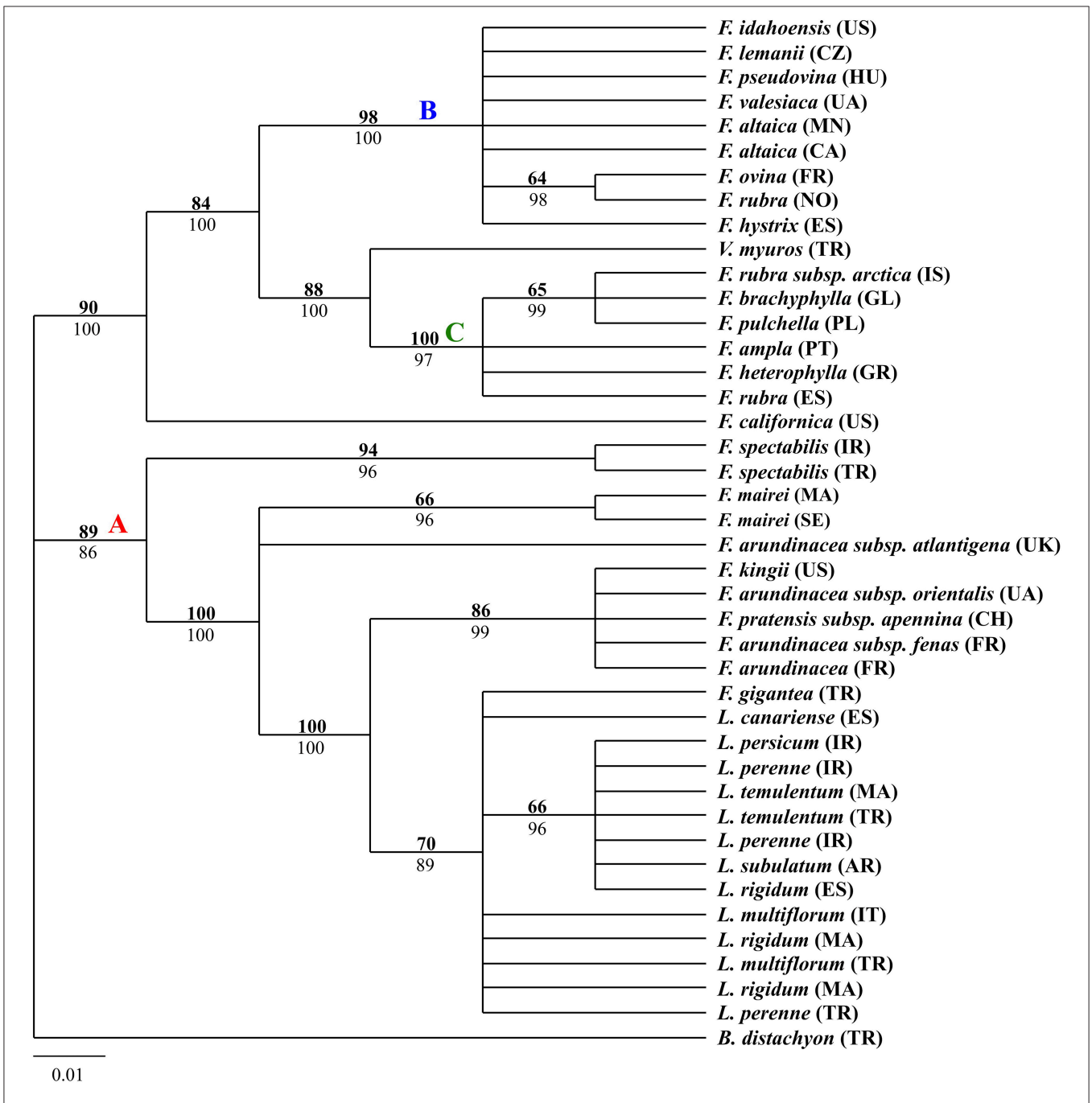

FIGURE 1 | Fifty-percent majority-rule BI tree inferred from the chloroplast non-coding intergenic spacers *trnQ-rps16* sequences of *Festuca/Lolium* examplers. The number above and below the braches indicate boot strap values ≥50% and Bayesian posterior probability values ≥90%.

0.1798). The 50% MP majority–rule consensus tree and the tree from BI were different but poorly resolved because of the limited difference among the sequences. The trees are shown in Supplementary Figure 4.

Chloroplast Gene *rbc*L Data

The aligned *rbc*L sequences yielded a total of 632 characters with 26 variable characters and 16 informative characters among which the parsimony analysis for *trnH-psbA* sequences resulted in 48 most parsimonious trees (tree length = 51; consistency index = 0.8235; retention index = 0.9511; rescale consistency index = 0.7832). The 50% MP majority-rule consensus tree was highly congruent to the tree obtained from BI except for some nodes presenting different statistical support. The tree showed in **Figure 2** was MP tree with bootstrap support (BS) above the branches and posterior probabilities (PP) of BI tree below the branches. The tree outline was different form the other two trees. There were two major clades which had been well supported, one large clade including most of the taxa and one small clade including *F. ampla*, *F. rubra* subsp. *arctica*, *F. brachyphylla*, *F. heterophylla*, *F. pulchella*, and *F. rubra*.

The Combined Dataset

Of 1926 total characters within the combined data set of the three plastid DNA regions, 135 characters were variable, and 128 characters were informative. The cladistics parsimony search yielded 363 most parsimonious trees with the tree length of 368 steps, a consistency index (CI) of 0.7853, retention index (RI) of 0.9349, and rescale consistency index (RC) of 0.7342. The 50% MP majority-rule consensus tree was largely incongruent to the tree obtained from BI analyses (**Figure 3**). According to the classification, the 50% MP tree was mainly influenced by the sequences of the *rbc*L and the BI tree was largely affected by the *trnQ-rps16* sequences data. The two major clades were highly supported in the BI tree rather than in the MP tree.

Network Analysis

In order to get better insights into the number of haplotypes of the combined sequences and their relations, a median-joint network was employed. Each circular network node represents a single sequence haplotype, with node size being proportional to the number of isolates with the haplotype. Mv (median vectors representing missing intermediates) reveals unsampled nodes inferred by MJ network analysis, and the number along the branches shows the number of mutations. 33 haplotypes were derived from 40 taxa which revealed higher levels of haplotype diversity of the combined sequence data (**Figure 4**). MJ analysis generally grouped according to the clades shown by the phylogenies of the combined data. The taxa were grouped into three clades, and the clade I and clade II could be considered as one group, and clade I and II were 46 and 35 mutational steps from clade III, respectively. All the *Lolium* taxa were nested with all the broad-leaved *Festuca* taxa.

DISCUSSION

From the combined analyses of the chloroplast spacers *trnQ-rps16, trnH-psbA*, the chloroplast gene *rbc*L, and the combined data set and the MJ network of the combined data, the dendrograms achieved all clearly demonstrate three obvious clades. The largest clade (A) contained all the *Lolium* taxa and included the broad-leaved fescues including all the samples of subgenus *Schedonrus* (*F. arundinacea*, *F. pratensis*, *F. mairei* and *F. gigantea*) and samples from sect. *Leucopoa* of Subgenus *Leucopoa* (*F. spectabilis F. kingii*). A smaller clade (B) included all the samples from subsect. *Festuca* including *F. valesiaca*, *F. ovina*, *F. hystrix*, *F. lemanii*, *F. pseudovina*, *F. idahoensis* as well as *F. altaica* and *F. rubra* (NO). The only species of subsect. *Festuca* not included within this clade is *F. brachyphlla*. A smaller third clade (C) included samples from sect. *Aulaxyper* and sect. *Amphigenes* of subgenus *Festuca* (*F. ampla*, *F. heterophylla*, *F. rubra* (ES), *F. rubra* subsp. *arctica*, *F. pulchella*) and *F. brachyphylla*. In the dendrogram of *trnQ-rps16* sequences and BI tree of the combined data set, clade B and C with *F. californica* formed one clade, while in the dendrogram of *rbc*L sequences and MP tree of the combined data set, a new clade was made up by clade A, clade B, and *F. californica*.

As all the *Lolium* species were nested closely with the broad-leaved *Festuca* species in clade A (**Figures 1**–**4**), it could be concluded that the broad-leaved fescues have closer relationship to *Lolium* grass species than to the fined-leaved fescues. There has been debate about the classification of subgen. *Schedonorus.* It was suggested that the subgen. *Schedonorus* (broad-leaved fescues) be included within *Lolium* (Darbyshire, 1993) despite the obvious differences in their inflorescence morphology (raceme for *Festuca* and spica for *Lolium*), whilst others have suggested a split of the subgen. *Schedonorus* into an independent genus, *Schedonorus* (Soreng and Terrell, 1997). According to the result demonstrated within the current study, the former classification seems more reasonable, in other words, subgen. *Schedonorus* has a close relationship to *Lolium*. Among all the examplers of *Lolium*, only *L. canariense*, which is found mainly on poor land in maritime condition (Loos, 1994), has a closer relationship to broad-leaved fescues than to other *Lolium* species. *Two* representatives of sect. *Leucopoa* (*F. kingii* and *F. spectabilis*) were placed in the clade A with representatives from subgen. *Schedonorus* and *Lolium*, while for the representatives of Sect. *Breviaristatae*, *F. altaica* taxa were attached to clade B and *F. california* has developed as an individual group. Similar results were achieved previously where the phylogenetic relationships among the *Festuca-Lolium* complex were described using SRAP markers (Cheng et al., 2015) and in earlier studies (Catalán et al., 2004, 2007; Inda et al., 2008). From the current and previous research, we strongly propose that the sect. *Leucopoa* should be moved from subgen. *Leucopoa* to Subgen. *Schedonorus* or into a separate sect. *Breviaristatae* from the subgen. *Leucopoa*. According to the strict consensus tree and Bayesian 50% MR consensus tree inferred from ITS and *trn*L-F sequences, representatives of sect. *Breviaristatae* (*F. altaica* and *F. californica*) have an intermediate placement between the "broad-leaved" and "fine-leaved" clade (Catalán et al., 2004). In

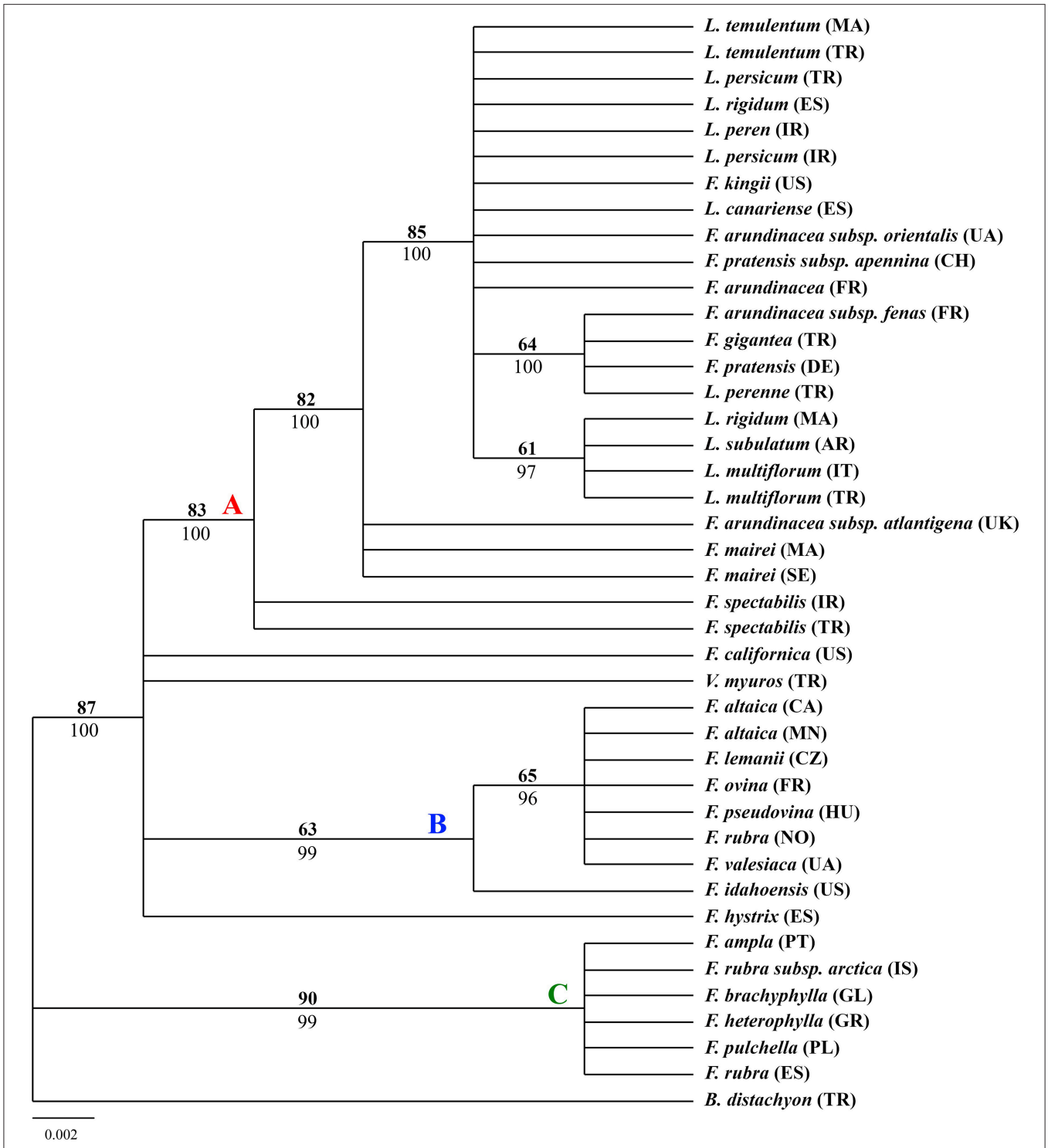

FIGURE 2 | Fifty-percent majority-rule BI tree inferred from the chloroplast gene *rbc*L sequences of *Festuca*/*Lolium* examplers. The number above and below the braches indicate boot strap values ≥50% and Bayesian posterior probability values ≥90%.

the current study, the lineage of *F. californica* could indicate a hybrid origin due to its intermediate placement between the "broad-leaved" (A) and "fine-leaved" clade (B).

Cytological investigations at the Institute for Biological, Environmental, and Rural Sciences (IBERS) using genomic *in situ* hybridization (GISH) and as total genomic DNA probes,

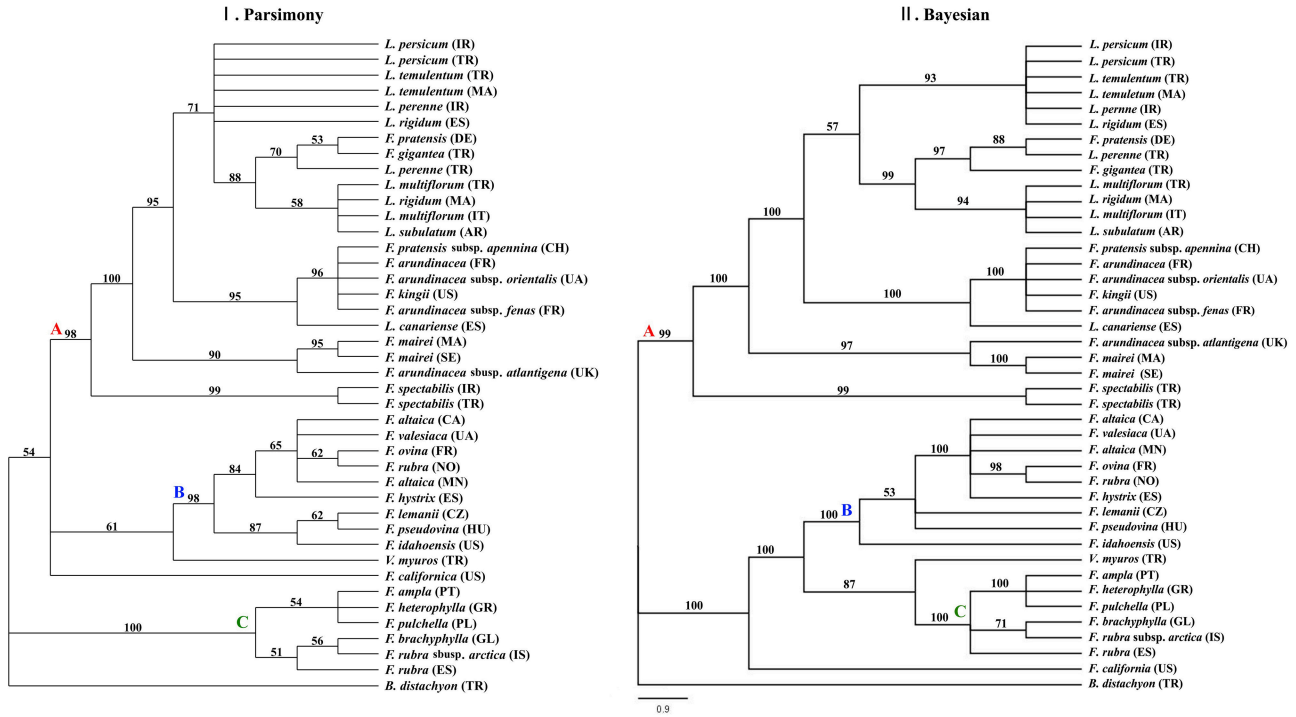

FIGURE 3 | Phylogenies inferred from the combined dataset of *Festuca/Lolium* examplers using maximum parsimony and Bayesian inference. Fifty-percent majority-rule BI tree inferred from the combined dataset of *Festuca/Lolium* examplers. (I) The MP majority rule consensus tree with the numbers at nodes indicating bootstrap values ≥50%. (II) The 50% majority rule consensus tree from the Bayesian inference with the numbers at nodes indicating Bayesian posterior probability values ≥90%.

candidate *Festuca* species have established the close ancestry of *F. pratensis*, *F. arundinacea* var. *glaucescens* (also known as *F. arundinacea* subsp. *fenas*) and *F. arundinacea* (Humphreys et al., 1995). From all the dendrograms in this study, *F. arundinacea*, *F. arundinacea* subsp. *fenas*, *F. pratensis* subsp. *apennina*, *F. arundinacea* subsp. *orientalis*, and *F. kingii* were all gathered closely within one group and thus might share the same ancestry. In the MJ network, *F. pratensis* subsp. *apennina*, *F. arundinacea* subsp. *orientalis*, and *F. kingii* shared the same haplotype, and they are also closely associated to *F. arundinacea*, *F. arundinacea* subsp. *fenas*. The North African fescue species *F. mairei* and *F. arundinacea* subsp. *atlantigena* were classified into one group which showed their close relationship. *F. mairei* was once been placed in sect. *Scariosae* of subgen. *Festuca* using a RAPD analysis (Stammers et al., 1995). However, based on the previous SRAP analysis (Cheng et al., 2015) and the research presented in this paper, it is considered as more accurate to place *F. mairei* within the subgen. *Schedonorus*.

In all clade B studies herein, F. *brachyphylla* was separated from other examplers of the Subsect. *Festuca* of Subgen. *Festuca* and was associated closer to representatives of Sect. *Aulaxyper* and Sect. *Amphigenes*. *F. brachyphylla* used to be considered as an arctic-alpine counterpart to the more temperate-montane *F. ovina*, which both belong to section *Festuca*, but the delimitation of *F. brachyphylla* and *F. ovina* has been considered as controversial with some authors having concluded the taxa of *F. brachyphylla* as a subspecies of *F. ovina* (Cronquist et al., 1977) whilst other authors have included both taxa in a widely defined *F. brachyphylla* (Fjellheim et al., 2001). In the present study, *F. brachyphylla* was differentiated from *F. ovina,* as shown previously by the same authors using SRAPs markers (Cheng et al., 2015) and also from a Bayesian tree of Loliinae which used *trn*TF and ITS data (Inda et al., 2008). It is proposed that the *F. brachyphylla* taxa be considered as a separate entity form Subsect. *Festuca*. In addition, *F. altaica* of sect. *Breviaristatae* was found closely aligned to subsect. *Festuca* known as the "*F. ovina* complex."

In clade C, *F. pulchella* of Sect. *Amphigenes* had a close relationship with sect. *Aulaxyper* known as "*F. rubra* complex." Two examplers of *F. rubra* were clustered within two different groups, it might be because of the large latitude difference. From the MJ network, *F. ampla* shared the same haplotype with *F. heterophylla* which indicated a close relationship between them.

In conclusion, as the two major genera of the grass family, the *Lolium* and *Festuca* taxa can be considered to have expanded to become the predominant temperate grassland of the world. From a taxonomical perspective, it is essential to classify the different species into their correct sections or subsections of subgenera as well as to clarify the relationships of some important species. From an agricultural perspective, it has become increasingly important to hybridize *Lolium* and *Festuca* species in order to gain the attributes of both. Current synthetic *Festulolium* hybrids

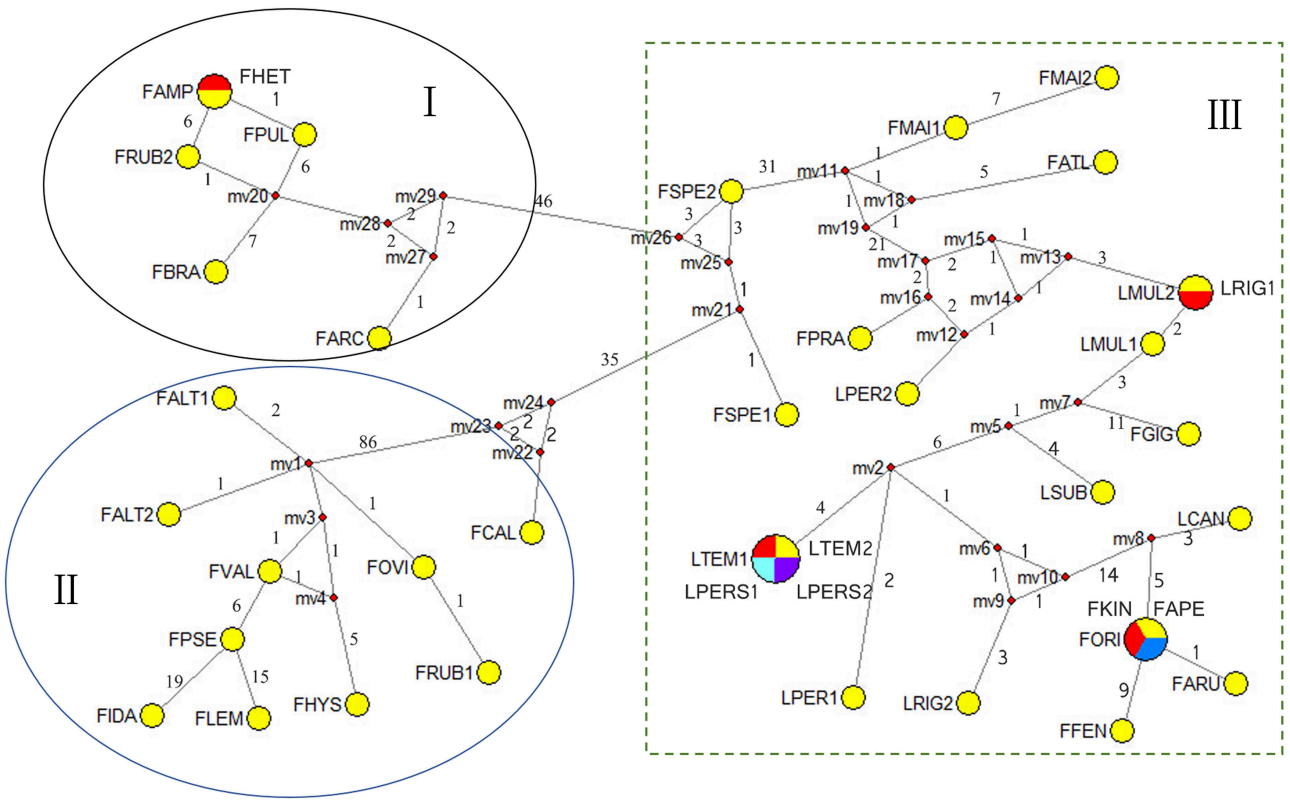

FIGURE 4 | Median-joining networks of the combined data of the *Festuca/Lolium* examplers. Haplotypes are represented by circles. Numbers along branches indicate number of mutational changes between nodes. Abbreviations of species names are listed in **Table 1**.

are frequently genetically unstable, and so it is important to understand how stable polyploids within the *Festuca* taxa have evolved from their progenitor species. In the present work, from the analyses of three plastid DNA data, either from the MP trees or BI trees, from the single or combined data, it is shown that the subgen. *Schedonorus* shares a close relationship with the majority of *Lolium* grasses. The phylogenetic tree can guide the parents chosen for hybrid breeding. It is recommended that *F. mairei* should be included within the subgen. *Schedonorus*. *F. californica* could have a lineage of hybrid origin because of its intermediate placement between the "broad-leaved" and "fine-leaved" clades. Furthermore, it is suggested that *F. brachyphylla* should be treated as a separate entity form the "*F. ovina* complex." The results add more information and understanding into species evolution within the *Lolium*/*Festuca* complex.

AUTHOR CONTRIBUTIONS

YC performed the experiments, analyzed the data and wrote the manuscript. KZ and HY guided the bioinformatics analyses, XZ organized the funding and participated in the samples collecting, XM guided the manuscript writing, JH, and MH provided helpful comments and language editing on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00089

REFERENCES

Bandelt, H. J., Macaulay, V., and Richards, M. (2000). Median networks: speedy construction and greedy reduction, one simulation, and two case studies from human mtDNA. *Mol. Phylogenet. Evol.* 16, 8–28. doi: 10.1006/mpev.2000.0792

Barker, C., and Stace, C. (1982). Hybridisation in the genera *Vulpia* and *Festuca*: the production of artificial F1 plants. *Nord. J. Bot.* 2, 435–444. doi: 10.1111/j.1756-1051.1982.tb01206.x

Buliñska-Radomska, Z., and Lester, R. (1988). Intergeneric relationships of *Lolium*, *Festuca*, and *Vulpia* (Poaceae) and their phylogeny. *Plant Syst. Evol.* 159, 217–227. doi: 10.1007/BF00935973

Cai, H., Stewart, A., Inoue, M., Yuyama, N., and Hirata, M. (2011). "Lolium," in *Wild Crop Relatives: Genomic and Breeding Resources,* ed C. Kole (Berlin; Heidelberg: Springer), 165–173.

Cassens, I., Mardulyn, P., and Milinkovitch, M. C. (2005). Evaluating intraspecific "network" construction methods using simulated sequence data: do existing algorithms outperform the global maximum parsimony approach? *Syst. Biol.* 54, 363–372. doi: 10.1080/10635150590945377

Catalán, P., Torrecilla, P., López-Rodríguez, J. A., Müller, J., and Stace, C. A. (2007). A systematic approach to subtribe Loliinae (Poaceae: Pooideae) based on phylogenetic evidence. *Aliso J. Syst. Evol. Bot.* 23, 380–405. doi: 10.5642/aliso.20072301.31

Catalán, P., Torrecilla, P., Rodrìguez, J. Á. L., and Olmstead, R. G. (2004). Phylogeny of the festucoid grasses of subtribe Loliinae and allies (Poeae, Pooideae) inferred from ITS and trnL–F sequences. *Mol. Phylogenet. Evol.* 31, 517–541. doi: 10.1016/j.ympev.2003.08.025

Charmet, G., Ravel, C., and Balfourier, F. (1997). Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers and ITS rDNA. *Theor. Appl. Genet.* 94, 1038–1046. doi: 10.1007/s001220050512

Cheng, Y., Ma, X., Zhou, K., Humphreys, M. W., and Zhang, X. Q. (2015). Phylogenetic analysis of Festuca–Lolium complex using SRAP markers. *Genet. Resour. Crop Evol.* 63, 7–18. doi: 10.1007/s10722-015-0324-5

Clayton, W. D., and Renvoize, S. A. (1986). *Genera Graminum. Grasses of the World*. Kew Bulletin Additional Series, 13. London: Her Majesty's Stationery Office.

Cronquist, A., Holmgren, A. H., Holmgren, N. H., Reveal, J. L., and Holmgren, P. K. (1977). Intermountain flora. *Vascular plants of the Intermountain West, USA Vol. 6. The monocotyledons*. New York, NY: Columbia University.

Darbyshire, S. J. (1993). Realignment of *Festuca* subgenus *Schedonorus* with the genus *Lolium* (Poaceae). *Novon* 3, 239–243. doi: 10.2307/3391460

Darbyshire, S. J., and Warwick, S. I. (1992). Phylogeny of North American *Festuca* (Poaceae) and related genera using chloroplast DNA restriction site variation. *Can. J. Bot.* 70, 2415–2429. doi: 10.1139/b92-300

Darriba, D., Taboada, G. L., Doallo, R., and Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9, 772–772. doi: 10.1038/nmeth.2109

Doyle, J. J., and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19, 11–15.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.2307/2408678

Fjellheim, S., Elven, R., and Brochmann, C. (2001). Molecules and morphology in concert. II. The Festuca brachyphylla complex (Poaceae) in Svalbard. *Am. J. Bot.* 88, 869–882. doi: 10.2307/2657039

Fu, Y.-X., and Li, W.-H. (1993). Statistical tests of neutrality of mutations. *Genetics* 133, 693–709.

Gaut, B., Tredway, L., Kubik, C., Gaut, R., and Meyer, W. (2000). Phylogenetic relationships and genetic diversity among members of the *Festuca-Lolium* complex (Poaceae) based on ITS sequence data. *Plant Syst. Evol.* 224, 33–53. doi: 10.1007/BF00985265

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95–98.

Huelsenbeck, J. P., and Ronquist, F. (2001). MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755. doi: 10.1093/bioinformatics/17.8.754

Humphreys, M., Thomas, H., Morgan, W., Meredith, M., Harper, J., Thomas, H., et al. (1995). Discriminating the ancestral progenitors of hexaploid Festuca arundinacea using genomic *in situ* hybridization. *Heredity* 75, 171–174. doi: 10.1038/hdy.1995.120

Humphreys, M. W., O'Donovan, S. A., Farrell, M. S., Gay, A. P., and Kingston−Smith, A. H. (2014). The potential of novel Festulolium (2n= 4x= 28) hybrids as productive, nutrient−use−efficient fodder for ruminants. *Food Energy Secur.* 3, 98–110. doi: 10.1002/fes3.50

Humphreys, M. W., Yadav, R. S., Cairns, A. J., Turner, L. B., Humphreys, J., and Skøt, L. (2006). A changing climate for grassland research. *New Phytologist* 169, 9–26. doi: 10.1111/j.1469-8137.2005.01549.x

Inda, L. A., Segarra-Moragues, J. G., Müller, J., Peterson, P. M., and Catalán, P. (2008). Dated historical biogeography of the temperate Loliinae (Poaceae, Pooideae) grasses in the northern and southern hemispheres. *Mol. Phylogenet. Evol.* 46, 932–957. doi: 10.1016/j.ympev.2007.11.022

Kress, W. J., and Erickson, D. L. (2007). A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. *PLoS ONE* 2:e508. doi: 10.1371/journal.pone.0000508

Lehväslaiho, H., Saura, A., and Lokki, J. (1987). Chloroplast DNA variation in the grass tribe Festuceae. *Theor. Appl. Genet.* 74, 298–302. doi: 10.1007/BF00274710

Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452. doi: 10.1093/bioinformatics/btp187

Loos, B. P. (1994). *The Genus Lolium: Taxonomy and Genetic Resources*. Wageningen: Centrum voor Plantenveredelings-en Reproduktieonderzoek DLO.

Loureiro, J., Kopecký, D., Castro, S., Santos, C., and Silveira, P. (2007). Flow cytometric and cytogenetic analyses of Iberian Peninsula *Festuca* spp. *Plant Syst. Evol.* 269, 89–105. doi: 10.1007/s00606-007-0564-8

Nei, M., and Li, W.-H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A.* 76, 5269–5273. doi: 10.1073/pnas.76.10.5269

Nock, C. J., Waters, D. L., Edwards, M. A., Bowen, S. G., Rice, N., Cordeiro, G. M., et al. (2011). Chloroplast genome sequences from total DNA for plant identification. *Plant Biotechnol. J.* 9, 328–333. doi: 10.1111/j.1467-7652.2010.00558.x

Pond, S. L. K., and Muse, S. V. (2005). "HyPhy: hypothesis testing using phylogenies," in *Statistical Methods in Molecular Evolution,* ed R. Nielsen (New York, NY: Springer), 125–181.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.* 25, 1253–1256. doi: 10.1093/molbev/msn083

Rambaut, A., and Drummond, A. J. (2013). *Tracer v1.6*. Available online at: http://beast.bio.ed.ac.uk/Tracer

Rognli, O. A., Saha, M. C., Bhamidimarri, S., and van der Heijden, S. (2010). "Fescues," in *Fodder Crops and Amenity Grasses,* eds B. Boller, U. K. Posselt, and F. Veronesi (New York, NY: Springer), 261–292.

Sang, T., Crawford, D., and Stuessy, T. (1997). Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). *Am. J. Bot.* 84, 1120–1120. doi: 10.2307/2446155

Shaw, J., Lickey, E. B., Schilling, E. E., and Small, R. L. (2007). Comparison of whole chloroplast genome sequences to choose non-coding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *Am. J. Bot.* 94, 275–288. doi: 10.3732/ajb.94.3.275

Soreng, R., and Davis, J. (2000). "Phylogenetic structure in Poaceae subfamily Pooideae as inferred from molecular and morphological characters: misclassification versus reticulation," in *Grasses: Systematics and Evolution*, eds S. W. Jacobs and J. Everett (Melbourne: CSIRO Publishing), 61–74.

Soreng, R., Davis, J., and Doyle, J. (1990). A phylogenetic analysis of chloroplast DNA restriction site variation in Poaceae subfam. Pooideae. *Plant Syst. Evol.* 172, 83–97. doi: 10.1007/BF00937800

Soreng, R., and Terrell, E. (1997). Taxonomic notes on *Schedonorus*, a segregate genus from *Festuca* or *Lolium*, with a new nothogenus, x Schedololium, and new combinations. *Phytologia* 83, 85–88.

Stace, C. A. (1975). *Hybridization and the Flora of the British Isles.* London; New York, NY; San Francisco, CA: Academic Press.

Stammers, M., Harris, J., Evans, G., Hayward, M., and Forster, J. (1995). Use of random PCR (RAPD) technology to analyse phylogenetic relationships in

the *Lolium*/*Festuca* complex. *Heredity (Edinb).* 74, 19–27. doi: 10.1038/hdy.1995.3

Stebbins, G. L. (1956). Taxonomy and the evolution of genera, with special reference to the family Gramineae. *Evolution* 10, 235–245. doi: 10.2307/2406009

Sun, G. (2007). Genetic diversity of rbcL gene in Elymus trachycaulus complex and their phylogenetic relationships to several Triticeae species. *Genet. Resour. Crop Evol.* 54, 1737–1746. doi: 10.1007/s10722-006-9183-4

Swindell, S. R., and Plasterer, T. N. (1997). "Seqman," in *Sequence Data Analysis Guidebook,* ed S. Swindell (Totowa, NJ: Humana Press), 75–89.

Swofford, D. L. (2002). *PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4beta 10*. Sunderland, MA: Sinauer Associates.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123, 585–595.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Torrecilla, P., and Catalán, P. (2002). Phylogeny of broad-leaved and fine-leaved *Festuca* lineages (Poaceae) based on nuclear ITS sequences. *Syst. Bot.* 27, 241–251. doi: 10.1043/0363-6445-27.2.241

Torrecilla, P., José-,Angel, López-Rodríguez, and Catalán, P. (2004). Phylogenetic relationships of *Vulpia* and related genera (Poeae, Poaceae) based on analysis of ITS and trnL-F sequences. *Ann. Missouri Bot. Gard.* 91, 124–158. Available online at: http://www.jstor.org/stable/3298573

Tzvelev, N. (1971). On the taxonomy and phylogeny of genus *Festuca* L. of the USSR Flora. I. The system of the genus and the main trends of evolution. *Bot. Zhurn* 56, 1252–1262.

Tzvelev, N. (1982). Poryadok zlaki (Poales). Semeystvo zlaki (Poaceae, ili Gramineae). *Zhizn Rasteniy v Shesti Tomakh* 6, 341–378.

Watterson, G. (1975). On the number of segregating sites in genetical models without recombination. *Theor. Popul. Biol.* 7, 256–276. doi: 10.1016/0040-5809(75)90020-9

Wiesner, I., Samec, P., and Našinec, V. (1995). Identification and relationships of cultivated accessions from *Lolium-Festuca* complex based on RAPD fingerprinting. *Biol. Plant.* 37, 185–195. doi: 10.1007/BF02913208

Xia, X., and Xie, Z. (2001). DAMBE: software package for data analysis in molecular biology and evolution. *J. Heredity* 92, 371–373. doi: 10.1093/jhered/92.4.371

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