Journal of Biogeography (J. Biogeogr.) (2014) **41**, 600–614

ORIGINAL ARTICLE

Mediterranean origin and Miocene–Holocene Old World diversification of meadow fescues and ryegrasses (*Festuca* subgenus *Schedonorus* and *Lolium*)

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ABSTRACT

Aim The biogeography of the grass genera *Festuca* (subgenus *Schedonorus*) and *Lolium*, which form one of the world main forage groups, is here reconstructed for the first time using nuclear and plastid DNA data. We aimed to test previous hypotheses on the origin of the group ancestor and on the Holocene versus pre-Holocene dispersals of the most recent fodder grasses.

Location The Mediterranean Basin and neighbouring regions: North Africa, Southwest Asia, East and West Africa, and Eurasia.

Methods Sampling included nearly all representatives from the native Old World distribution of this group. We used maximum parsimony, maximum likelihood and Bayesian inference methods to reconstruct phylogenetic relationships. Divergence times were estimated with a Bayesian relaxed clock and secondary calibrations derived from a fossil-dated phylogeny of grasses. Biogeographical scenarios were reconstructed with Bayesian-averaged dispersal–vicariance analysis (Bayes-DIVA) and dispersal–extinction–cladogenesis (DEC), using a stratified palaeogeographical model spanning the last 12 million years.

Results Meadow fescue (*Festuca pratensis*) originated in Eurosiberia, Southwest Asia, 2 million years ago (Ma), whereas ryegrasses (*Lolium*) first diversified in the eastern Mediterranean region around 4.1 Ma, splitting into two autogamous versus allogamous lineages, with Macaronesian *Lolium* embedded within the latter. An alternative scenario suggests, however, an early split of the Macaronesian ryegrasses. Our results support the hybrid origin of the tall fescue (*Festuca arundinacea*).

Main conclusions The ancestor of the fescues and ryegrasses originated in the western Mediterranean in the mid-Miocene. The sister relationship of the tropical African *Festuca simensis* to *Lolium* is a novel finding, suggesting a dispersal of the ancestor of the ryegrasses from Asia to East Africa in the early Pliocene. Our reconstruction rejects the hypothesis of a single Neolithic human-mediated dispersal of *Lolium* species from eastern to western Mediterranean areas, suggesting instead a pre-agricultural distribution of *Lolium* ancestors along the Mediterranean Basin since the Pliocene.

Keywords

Bayes-DIVA, circum-Mediterranean region, dispersal–extinction–cladogenesis, *Festuca* subgenus *Schedonorus*, *Lolium*, Macaronesia, *Micropyropsis*, Neogene–Quaternary diversification, phylogenetic dating.

INTRODUCTION

The Mediterranean Basin and its bordering territories are considered a major hotspot of world plant diversity (Médail & Diadema, 2009). This region acted as a cradle of speciation for many angiosperm groups (Thompson, 2005; Salvo *et al.*, 2010). Two factors have probably contributed to this diversity: a complex palaeogeographical history (Meulenkamp &

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Sissingh, 2003) and the rapid climatic changes that affected this region from the late Tertiary onwards, including the onset of the Mediterranean climate (3.5 Ma; Suc, 1984) and the Pleistocene glaciations (2.0 Ma). A series of plate and microplate tectonic episodes during the Miocene resulted in marine transgressions and regressions that successively isolated and connected the northern and southern sides of the Mediterranean Basin (Krijgsman, 2002; Meulenkamp & Sissingh, 2003), allowing lineage dispersal and concomitant allopatric speciation events in Mediterranean lineages (Sanmartín, 2003; Mico *et al.*, 2009). During the glacial periods of the late Pliocene and Pleistocene, the Mediterranean region acted as climatic refugia, from which species spread and colonized central and northern Europe in post-glacial times (Taberlet *et al.*, 1998), while secondary contacts among species and the potential for hybridization and polyploid speciation arose during the warmest interglacial phases (Barton & Hewitt, 1989). In more recent Holocene times, a series of minor climatic fluctuations and the influence of anthropogenic activity led to both landscape fragmentation and human-mediated seed dispersals, potentially affecting the distributions of circum-Mediterranean plant species (Carrión *et al.*, 2010).

The temperate subtribe Loliinae is a group formed by the large paraphyletic and globally distributed genus *Festuca* L. (9 subgenera; *c.* 500 species) and 10 allied genera (*c.* 40–50 species) nested within (Catalán, 2006; Inda *et al.*, 2008). Recent molecular phylogenetic studies have recovered a basal split of the group into two lineages: the broad-leaved (seven *Festuca* subgenera plus two genera) and the fine-leaved (two *Festuca* subgenera plus eight genera) Loliinae (cf. Catalán, 2006, and references therein). Within the broad-leaved clade, the *Festuca* subgenus *Schedonorus* (P.Beauv.) Peterm. and *Lolium* L. (*Schedonorus*–*Lolium* hereafter) group emerged as a strongly supported and recently evolved (approximately 7.5–4.0 Ma) lineage. This group encompasses representatives of paraphyletic *Festuca* subgenus *Schedonorus* (*Schedonorus* hereafter) plus *F. mairei* St.-Yves (10 taxa classified within 8 species), and the genera *Lolium* (8 to 12 species) and *Micropyropsis* Romero-Zarco & Cabezudo (1 species) embedded within (Inda *et al.*, 2008; Namaganda & Lye, 2008). Inda *et al.* (2008) distinguished two sister clades within the *Schedonorus*–*Lolium* complex, a 'Mahgrebian' and a 'European' group, with a monophyletic *Lolium* embedded within the latter, in agreement with other cytogenetic and molecular studies (Catalán, 2006; Hand *et al.*, 2010). Two main breeding groups have been distinguished within the *Lolium* ryegrasses: an autogamous (e.g. self-pollinated) group containing species such as *L. remotum* Schrank, *L. temulentum* L. and *L. persicum* Boiss. & Hohen., and an allogamous (e.g. cross-pollinated) group including species such as *L. perenne* L., *L. multiflorum* Lam., *L. rigidum* Gaudin and *L. canariense* Steud. (Malik, 1967; Terrell, 1968). However, the closest relative of *Lolium* has not yet been determined.

The *Schedonorus*–*Lolium* complex includes some of the most important forage grasses found in temperate areas across the globe, such as meadow (*F. pratensis* Huds.) and tall (*F. arundinacea* Schreber) fescues and several ryegrasses (*L. perenne*, *L. multiflorum*), which have been extensively used as fodder or for amenity purposes; other species (*L. rigidum*) are aggressive weeds of cereal crops (Terrell, 1968; Catalán, 2006). The group includes both diploid and allopolyploid taxa (Borrill *et al.*, 1977; Catalán, 2006; see Table S1 in Appendix S1 of the Supporting Information). As with many other grasses, allopolyploidization events have probably been recurrent among the closest, most recently diversified taxa, resulting in patterns of reticulate, multifurcate evolution between them (Catalán, 2006).

Despite the large genomic knowledge accumulated for the most intensively cultivated elements of the *Schedonorus*–*Lolium* complex (e.g. Pasakinskiene & Jones, 2005; Hand *et al.*, 2010), very little information exists for their close unexploited relatives (e.g. *F. simensis* Hochst. ex A.Rich., the Macaronesian ryegrasses), some of which have never been studied phylogenetically or biogeographically. Moreover, no biogeographical analysis has been conducted so far on this group, despite the large number of genomic studies focusing on its economically important representatives (Catalán, 2006; Hand *et al.*, 2010). Although meadow and tall fescues and ryegrasses are cultivated today in almost all continents, the circum-Mediterranean region harbours the highest species diversity within the *Schedonorus*–*Lolium* complex, and has been suggested as the ancestral distribution of this group, which dates back to mid-Miocene times (Catalán, 2006; Inda *et al.*, 2008). On the other hand, the current widespread distribution of some fodder ryegrasses, such as Eurosiberian *L. perenne* or Mediterranean *L. rigidum*, has been linked to the spread of agriculture in recent Holocene–Neocene times (Balfourier *et al.*, 1998, 2000).

Here, we used a nearly complete sampling of the *Schedonorus*–*Lolium* complex, including new species and infraspecific taxa, in conjunction with phylogenetic analysis of nuclear and chloroplast DNA sequence data, divergence time estimation and biogeographical reconstruction to: (1) disentangle the early history of diversification of these wild and forage groups of grasses in the circum-Mediterranean region; (2) identify the closest relative of the ryegrasses (*Lolium*) and resolve the relationships within this genus; and (3) evaluate contrasted scenarios of non-anthropogenic pre- and/or post-glacial dispersal versus agriculture-mediated dispersal of ryegrasses.

MATERIALS AND METHODS

Sampling, DNA sequencing and phylogenetic reconstruction

Sampling aimed to include as much infraspecific diversity as possible across most of these taxa in the Old World: the Mediterranean Basin, Macaronesia, Southwest Asia, northern Eurasia and tropical Africa. In all, 10 taxa of *Festuca* (90% of species diversity), 9 of *Lolium* (80%) and 1 of *Micropyropsis*

(100%) were included (Fig. 1, Table S1 in Appendix S1). We used as outgroup taxa two representatives of the closely related broad-leaved *Festuca* section *Subbulbosae* Nyman ex Hack, *F. durandoi* Clauson and *F. paniculata* (L.) Schinz & Thell. (Inda *et al.*, 2008). To this, we added six other less-related outgroup taxa (*Festuca ovina* L., *F. rubra* L., *Poa infirma* Kunth, *Deschampsia cespitosa* (L.) P.Beauv., *Secale cereale* L. and *Brachypodium distachyon* (L.) P.Beauv.), which were used to provide calibration points for the molecular phylogenetic dating of the *Schedonorus–Lolium* complex (Table S1; see 'Divergence time estimation' below).

The nuclear ribosomal intergenic spacer ITS and the plastid *trn*T–L and *trn*L–F regions were amplified and sequenced following protocols in Catalán *et al.* (2004). Phylogenetic inference analyses were conducted on the ITS dataset (72 samples) and the cpDNA (*trn*T–L + *trn*L–F) dataset (59 samples) separately, using maximum parsimony (MP) implemented in PAUP* 4.0 beta10, maximum likelihood (ML) implemented in the online software RAxML 7.2.8, and Bayesian inference (BI) analysis implemented in MrBayes 3.2 (see Appendix S2 for more details). MrModelTest 2.3 was used to select the best substitution model for the two datasets based on the Akaike information criterion (AIC): GTR+Γ+I for ITS and GTR+Γ for chloroplast DNA (cpDNA). Before concatenating the common samples from the nuclear and plastid datasets into a combined analysis, we checked for conflict among the individual topologies. Incongruence was assessed by looking for nodes that were strongly supported (posterior probability support PPS > 0.95) in the Bayesian 50% majority rule consensus tree of the nuclear dataset that were not present in the consensus tree of the cpDNA dataset and vice versa. After removing taxa that were causing conflict (*F. arundinacea*, see Results), we ran a 'partitioned' Bayesian analysis on the concatenated ITS–cpDNA analysis, allowing the substitution model and the overall rate of mutation to differ among the (nuclear versus plastid) partitions. ML and MP analyses were run unpartitioned for the combined dataset.

Divergence time estimation

Divergence times within the *Schedonorus–Lolium* complex were estimated using a Bayesian relaxed-clock approach implemented in BEAST 1.5.2. The combined ITS–cpDNA dataset (without *F. arundinacea*) was run under a partitioned analysis (plastid versus nuclear), with the selected substitution model unlinked across partitions, a Yule tree prior, and an uncorrelated lognormal relaxed molecular clock (see Appendix S2). There are no described fossils of *Festuca* subgenus *Schedonorus*, *Lolium* or *Micropyropsis*. To obtain calibration points for estimating absolute divergence times, we used a secondary calibration approach in which we dated a

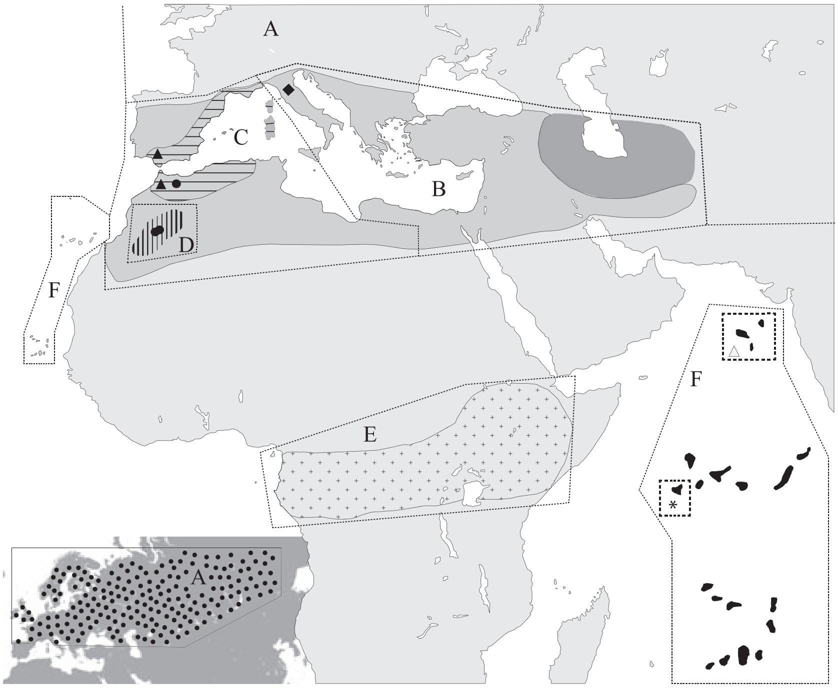

Figure 1 Geographical distribution of *Festuca* subgenus *Schedonorus* and *Lolium*, showing the boundaries (dotted lines) of the operational areas used in the biogeographical analysis. Abbreviations: A, Eurosiberia; B, Eastern Mediterranean + Southwest Asia; C, Western Mediterranean; D, Atlas Mountains; E, Eastern and Western Tropical Africa; F, Macaronesia; dots, Eurosiberian taxa (*F. pratensis, F. arundinacea, F. gigantea, L. perenne, L. multiflorum, L. temulentum, L. retusum*); light grey, *L. rigidum*; dark grey, *L. persicum*; horizontal bars, *F. fenas, F. corsica*; vertical bars, *F. mairei, F. atlantigena, F. letourneuxiana*; crosses, *F. simensis*; black, *L. canariense*; asterisk, *L. edwardii*; white triangle, *L. lowei*; black triangle, *Micropyropsis tuberosa*; black circle, *F. fontqueri*, black diamond, *F. appeninna*.

more inclusive dataset including representatives of broad- and fine-leaved lineages of Loliinae, as well as other more distantly related tribes and subtribes. This larger dataset (58 terminals) was calibrated using the estimated divergence of the Triticeae + Bromeae from the Poeae s.l. (including Aveneae) tribes at 24 ± 1.0 Ma, which was assigned as the prior distribution for the age of the root node; this calibration point was in turn obtained from a large family-wide analysis of grasses based on six fossil calibrations (Vicentini *et al.*, 2008). The more inclusive Loliinae analysis provided us with the stem age of the broad-leaved Loliinae, i.e. the most recent common ancestor (MRCA) of *Festuca* sect. *Subbulbosae* and the *Schedonorus–Lolium* complex (11.4 ± 1.5 Ma), and with the crown age of the *Schedonorus–Lolium* complex (8.97 ± 1.5 Ma).

Biogeographical analysis

We defined six biogeographical areas based on patterns of endemism in the *Schedonorus–Lolium* complex, but also reflecting the geological history of the study region (Fig. 1): A, Eurosiberia; B, Eastern Mediterranean and Southwest Asia; C, Western Mediterranean; D, Atlas (continental north-western Africa); E, Eastern and Western Tropical Africa; and F, Macaronesia. These areas represent major continental plates identified in tectonic reconstructions of the Mediterranean Basin and adjacent regions (Meulenkamp & Sissingh, 2003; Ree & Sanmartín, 2009). The Eurosiberian region included the central and northern Eurasian territories and part of the southern European territories, characterized by a humid temperate-to-cold Eurosiberian climate that was probably established at the onset of the Late Glacial/Holocene (Carrión *et al.*, 2010). The Macaronesian region encompassed the Canarian, Madeiran and Cape Verde archipelagos, where the native Macaronesian *Lolium* species are distributed (Terrell, 1968; Scholz *et al.*, 2000). These islands have been repeatedly reconfigured by volcanic activity from the Miocene (e.g. 20 Ma, Porto Santo, Madeira) to the Pleistocene (e.g. 1.12 Ma, El Hierro, Canary Islands) and even the present (Carracedo, 1999; Geldmacher *et al.*, 2000). Distributions for extant taxa were coded from current botanical records retrieved from our own collections or from Floras and monographs based on studied herbarium specimens.

Ancestral areas and main biogeographical events in the history of the *Schedonorus–Lolium* complex were inferred using two complementary approaches: the maximum likelihood dispersal–extinction–cladogenesis (DEC) model (Ree *et al.*, 2005) implemented in the software Lagrange v. 2.1, and an empirical Bayesian approach to parsimony-based dispersal–vicariance analysis (Bayes-DIVA; Nylander *et al.*, 2008). For the DEC analysis, we used the maximum clade credibility (MCC) tree obtained from beast to infer global extinction and dispersal rates and range inheritance scenarios at each node. For Bayes-DIVA, we used the post-burn-in sample of trees from the beast analysis (9000 trees) to integrate the uncertainty in tree topology and generate marginal probabilities for ancestral areas at each node. Both DEC and Bayes-DIVA analyses were constrained to a maximum number of two areas at ancestral nodes, assuming that ancestors were not more widespread than their extant descendants (Sanmartín, 2003). Two alternative dispersal models were tested in DEC: an unconstrained model (M1) in which dispersal rates were assumed equal among areas, and a stratified model (M2) in which the phylogeny was divided into three time slices, each with a specific matrix of dispersal rates reflecting the palaeogeographical connectivity among the study areas (Buerki *et al.*, 2011; see Table S2 in Appendix S1). The three temporal windows were defined to reflect the main palaeogeographical events in the history of the Mediterranean region that could have affected the evolutionary history of the *Schedonorus–Lolium* taxa (Krijgsman, 2002; Meulenkamp & Sissingh, 2003): time slice I (TSI) – late Miocene (Serravalian to Tortonian, 11–7 Ma): the opening of the Rifian corridor and the Red Sea strait; time slice II (TSII) – latest Miocene to mid-Pliocene (7–3.5 Ma): Messinian salinity crisis, the opening of the Gibraltar Strait, and temporary closure of the Red Sea; and time slice III (TSIII) – mid-Pliocene to present (3.5–0 Ma): geographical configuration of the Mediterranean region very similar to the current one, and the occurrence of the glaciation cycles.

RESULTS

Phylogenetic relationships

The ITS dataset included 654 aligned nucleotide positions, of which 295 (45.1%) were variable and 147 (22.5%) parsimony informative; the cpDNA (*trn*T–L + *trn*L–F) included 1907 aligned positions: 544 (28.5%) variable and 214 (11.2%) parsimony informative. A total of 94 new sequences were generated and deposited in GenBank (see Table S1 in Appendix S1).

Figure 2 shows the results of the Bayesian analysis in MrBayes for the separate nuclear and plastid datasets. MP and ML analyses gave similar topologies (see Figs S1 and S2a in Appendix S3), with all major clades recovered by the three phylogenetic inference methods (Table S3 in Appendix S1). Both ITS and cpDNA markers recovered *Schedonorus–Lolium* as a monophyletic group, although they differed in the basal relationships. The cpDNA tree shows the 'Mahgrebian' *Festuca* lineage diverging first, followed by the '*F. arundinacea* group' ('Western Mediterranean' + 'Eurasian'), whereas in the ITS tree the 'Mahgrebian' and the 'Western Mediterranean' *F. arundinacea* lineages form a strongly supported clade, sister to the remaining species (Fig. 2). The ITS tree was generally better resolved than the plastid tree; the latter shows some *Schedonorus–Lolium* lineages collapsed into a large polytomy, although most intraspecific or geographical groups are congruent with the ITS tree (Fig. 2).

The main incongruence between plastid and nuclear trees – with Bayesian posterior probability > 0.95 – affected the hybrid-allohexaploid 'Eurasian' *Festuca arundinacea* group.

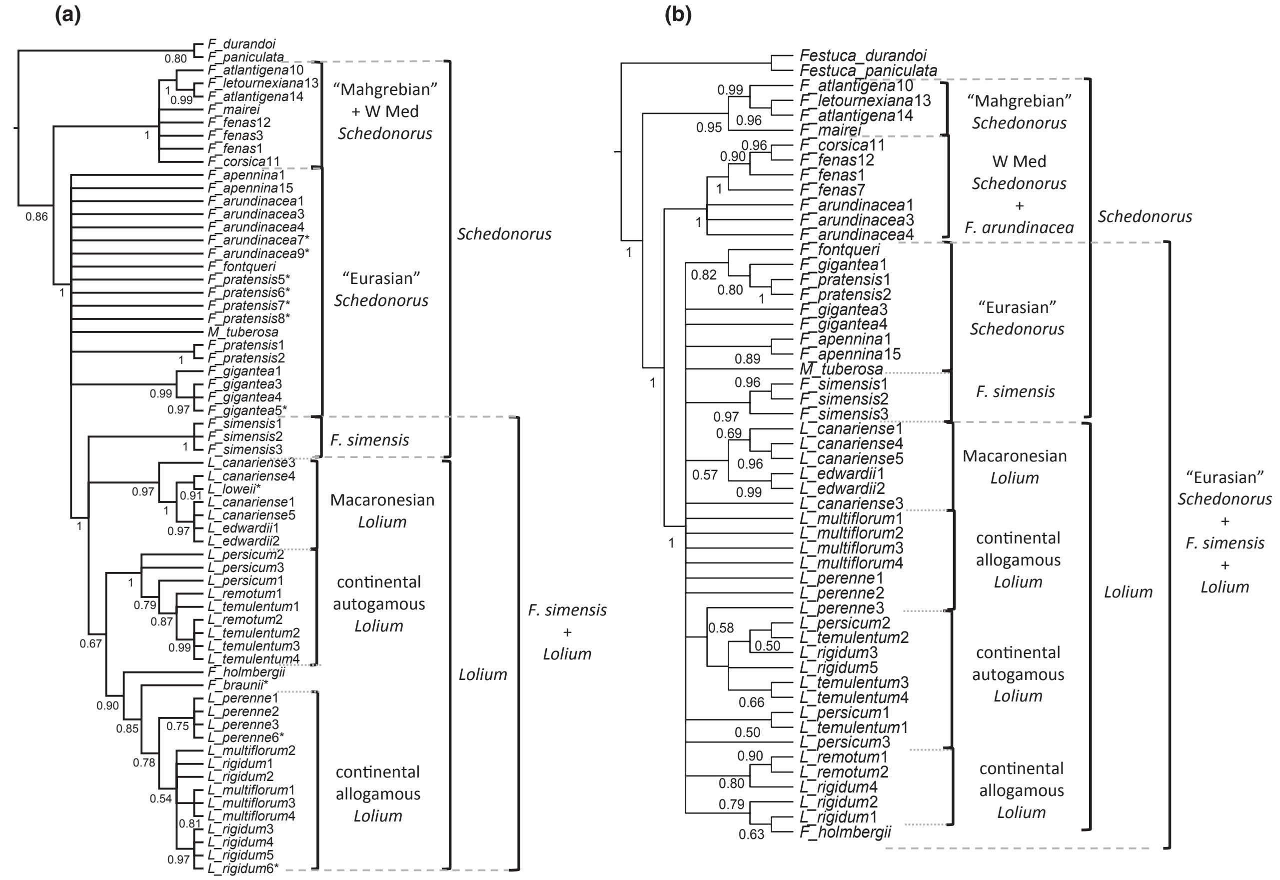

Figure 2 Bayesian (MrBayes) majority rule consensus trees obtained from the analysis of the (a) nuclear (ITS) and (b) plastid (*trn*L–F and *trn*T–F) datasets of the *Schedonorus–Lolium* complex. Numbers above branches are Bayesian posterior probability clade values. Asterisks indicate samples that have been analysed only for one dataset. *F* = *Festuca*, *L* = *Lolium*, *M* = *Micropyropsis*.

Studies based on artificial cross-hybridizations (Chandrasekharan *et al.*, 1972) or genome-mapping data (Humphreys *et al.*, 1995; Pasakinskiene & Jones, 2005) have suggested the allohexaploid origin of *F. arundinacea* from diploid *F. pratensis* and tetraploid *F. fenas* Lag. [syn. *F. arundinacea* var. *glaucescens* Boiss.]. This hybrid nature was also supported by the concatenated ITS + cpDNA tree, which shows *F. arundinacea* in an intermediate position between the Western Mediterranean clade including *F. fenas* and the Eurasian *Festuca* clade with *F. pratensis* (Fig. S3 in Appendix S3). To avoid artefactual resolution of allopolyploid hybrids in combined plastid–nuclear datasets, we excluded this species from all subsequent analyses (see below).

The reduced nuclear–plastid combined dataset (without *F. arundinacea*) resulted in a tree topology (Fig. 3) that was better resolved than the individual analyses and showed stronger support values for the main divergences. There was a strongly supported basal split between a 'Mahgrebian + Western Mediterranean' *Schedonorus* clade and the rest of the taxa, followed by the successive divergence of a 'Eurasian' subclade and the Moroccan *F. fontqueri* St.-Yves. The 'Eurasian' *Micropyropsis*, *F. gigantea* (L.) Villars, and *F. pratensis* + *F. apennina* De Not. collapsed into a polytomy with the *F. simensis*/*Lolium* clade. Within *Lolium*, there was a strongly supported basal split between the Macaronesian and continental ryegrasses, with the latter divided into an autogamous versus allogamous subclade (although this relationship was poorly supported, Fig. 3). Most conspecific specimens grouped into well-supported monophyletic clades (e.g. *F. gigantea*, *F. pratensis*, *F. simensis*, *L. perenne*, *L. rigidum*, *L. remotum*). The main exceptions were the allotetraploid *F. apennina*, which formed a polytomy with its diploid relative *F. pratensis*, and the Macaronesian *L. edwardii* H.Scholz, Stierst. & Gaisberg, which appears embedded within *L. canariense*. *Festuca fenas*, *Lolium persicum*, *L. temulentum* and *L. multiflorum* were also reconstructed as paraphyletic (Fig. 3).

Dating and biogeographical analyses

Figure S3 in Appendix S3 shows the estimated divergence times for the Loliinae dataset used in the secondary calibration analysis. The BEAST analysis of the *Schedonorus–Lolium* ITS + cpDNA dataset without *F. arundinacea* resulted in a tree topology (Fig. 4) that was very similar to the one obtained by MrBayes (Fig. 3). The main exception was the position of the Macaronesian ryegrasses, which were reconstructed as the sister-group of the continental allogamous lineage instead of as the sister-group of all remaining

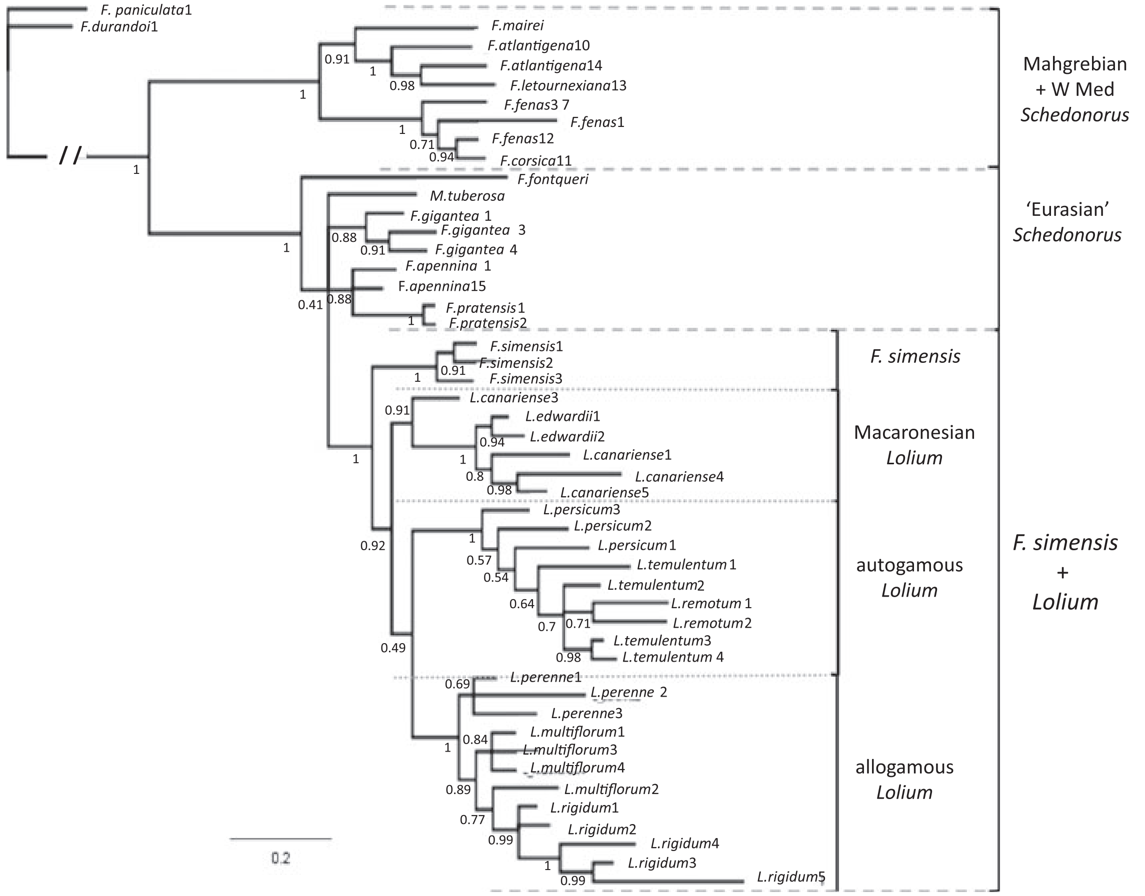

Figure 3 Bayesian (MrBayes) majority rule consensus tree from analysis of the combined nuclear + plastid datasets of the *Schedonorus–Lolium* complex excluding the suspected hybrid *Festuca arundinacea*. Numbers above branches are Bayesian posterior probability values. *F.* = *Festuca*, *L.* = *Lolium*, *M.* = *Micropyropsis*.

Lolium species (Fig. 3). The Eurasian *Festuca* lineages were also recovered as a well-supported clade (Fig. 4). The most recent common ancestor (MRCA) of the *Schedonorus–Lolium* complex was dated as mid-Miocene, with a mean age of 9.2 Ma and a 95% highest posterior density (HPD) confidence interval of 11.4–7.0 Ma. Initial diversification events in the 'Eurasian' and 'Mahgrebian–Western Mediterranean' lineages were reconstructed as occurring during the Messinian (5.8 Ma, 95% HPD: 8.1–3.8 Ma) and early Pliocene (4.9 Ma, 95% HPD: 7.1–2.9 Ma) periods, respectively, while the MRCA of *Lolium* was dated as Pliocene (4.1 Ma, 95% HPD: 5.8–2.7 Ma). Within each of these clades, most diversification events giving rise to extant *Schedonorus–Lolium* taxa were estimated as spanning the late Pliocene (Piacenzian) to the early Pleistocene periods (Fig. 4).

The divergence time estimates can be affected by incomplete taxon sampling (Linder *et al.*, 2005), but also by an uneven sampling effort across taxa or clades, with limited sampling resulting in younger ages and the opposite effect observed for densely sampled infraspecific taxa. To test whether this could have affected age estimates in our analysis, we randomly deleted intraspecific sequences from the original dataset and calculated divergence times for these reduced matrices (30%, 70% and 90% of all sampled taxa) using the original beast settings. Results showed that the mean nodal ages from the new analyses were very similar and fell within the 95% HPD intervals for age estimates in the original dataset, supporting the reliability of our age estimates (Fig. S4 in Appendix S3).

The three different biogeographical analyses (DEC-stratified model M2, Bayes-DIVA, and DEC unconstrained M1) gave overall congruent results (see Figs 5 & 6 and Fig. S5 in Appendix S3, respectively). The main differences were observed in the higher number of terminal dispersal events in Bayes-DIVA (Fig. 6) and in the timing of several dispersal events, which were slightly delayed in Bayes-DIVA with respect to the Lagrange analyses (see Figs 5 & 6 and Fig. S5 in Appendix S3, respectively); for example, the initial dispersal event from the West Mediterranean (area C) to the east (area B). Thus, uncertainty in tree topology (accounted for in Bayes-DIVA but not in Lagrange) did not seem to have a major influence on our biogeographical results. Because the DEC model incorporates the effect of time (calibrated branch lengths) into the biogeographical scenario, we refer in the description below to the Lagrange results. The stratified DEC model showed a better fit to the data than the unconstrained model (−ln likelihood

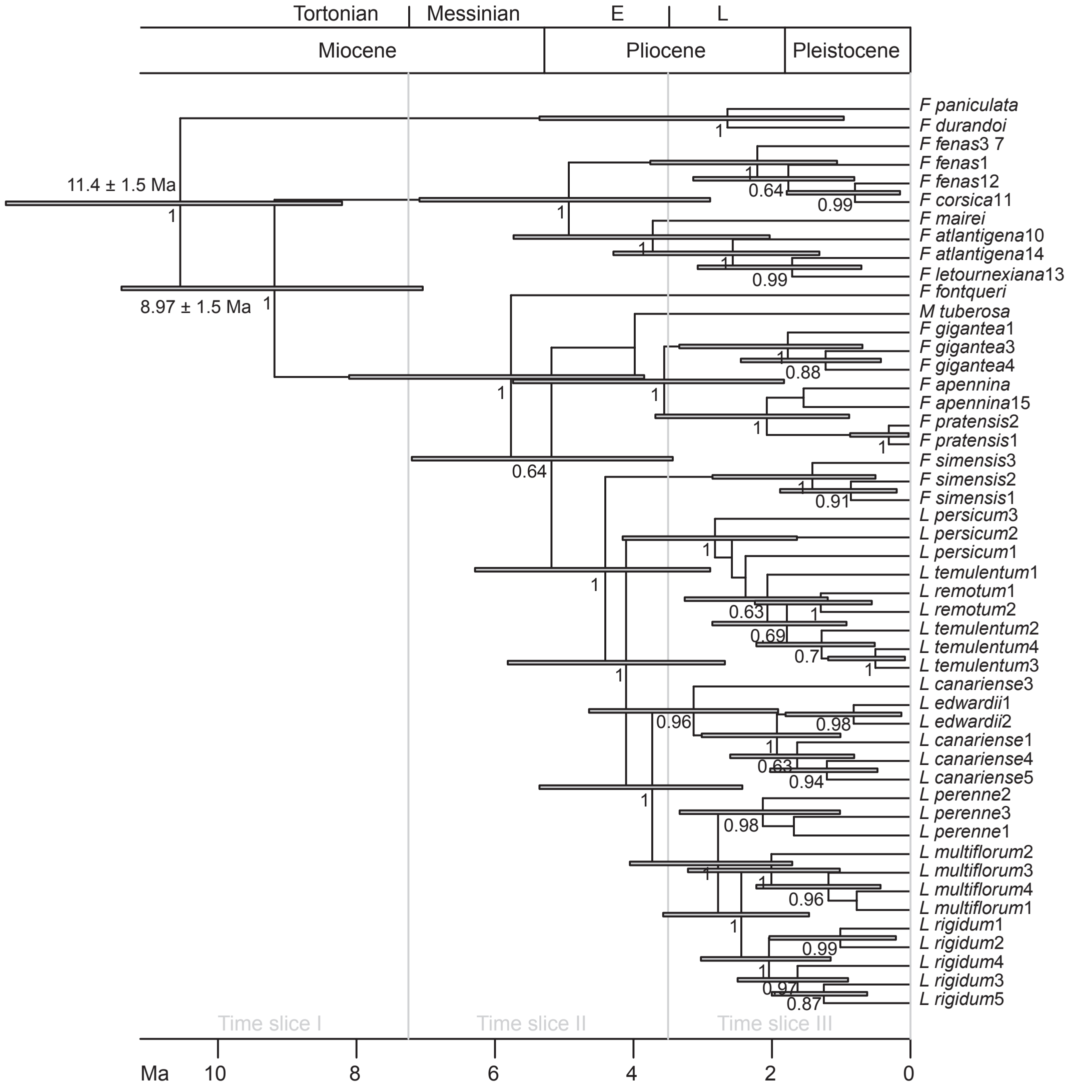

Figure 4 BEAST maximum clade credibility (MCC) tree of the *Schedonorus–Lolium* complex with nodal heights and 95% highest posterior density (HPD) intervals indicated by grey bars. The vertical rectangles show the three time slices (TSI–TSIII) used for the biogeographical stratified analysis. *F* = *Festuca*, *L* = *Lolium*, *M* = *Micropyropsis*.

92.51 vs. 108.2), with an estimated dispersal rate of 0.1246 and a nearly negligible extinction rate (4.28×10^{-9}). This analysis placed the origin of the common ancestor of the *Schedonorus–Lolium* complex in the Western Mediterranean region (area C), with a lower relative probability for a Western Mediterranean + Atlas ancestor (CD) (Fig. 5). A dispersal event from the Western Mediterranean to the Atlas region (C to D), followed by vicariance, is inferred to explain the endemic distribution of the 'Mahgrebian' and 'Western Mediterranean' lineages. The ancestor of *F. fontqueri* and the 'Eurasian' *Schedonorus–Lolium* clade is reconstructed as having dispersed from the Western Mediterranean region to the Eastern Mediterranean region (C to B) in the mid–late Miocene (TI, Fig. 5). Within the 'Eurasian' *Schedonorus* clade, the DEC stratified analysis reconstructs several events of dispersal from the Eastern Mediterranean–Southwest Asian region to northern and central Europe (area A) during the late Pliocene–Pleistocene (Fig. 5). The endemic distribution of *F. simensis* and the Macaronesian ryegrasses are also inferred as the result of dispersal events from the Eastern Mediterranean region (B) to East Africa (E) and Macaronesia (F), respectively, in the early Pliocene. Finally, several allogamous and autogamous *Lolium* lineages are reconstructed as having colonized the Eurosiberian (A) and Western Mediterranean (C) regions from the eastern Mediterranean Basin during the Pleistocene–Holocene period (Fig. 5).

DISCUSSION

A complex phylogenetic evolution

The incongruities found here between the nuclear and plastid trees (Fig. 2a,b) agree well with the highly intricate nature suggested for this group (Catalán, 2006), including potential

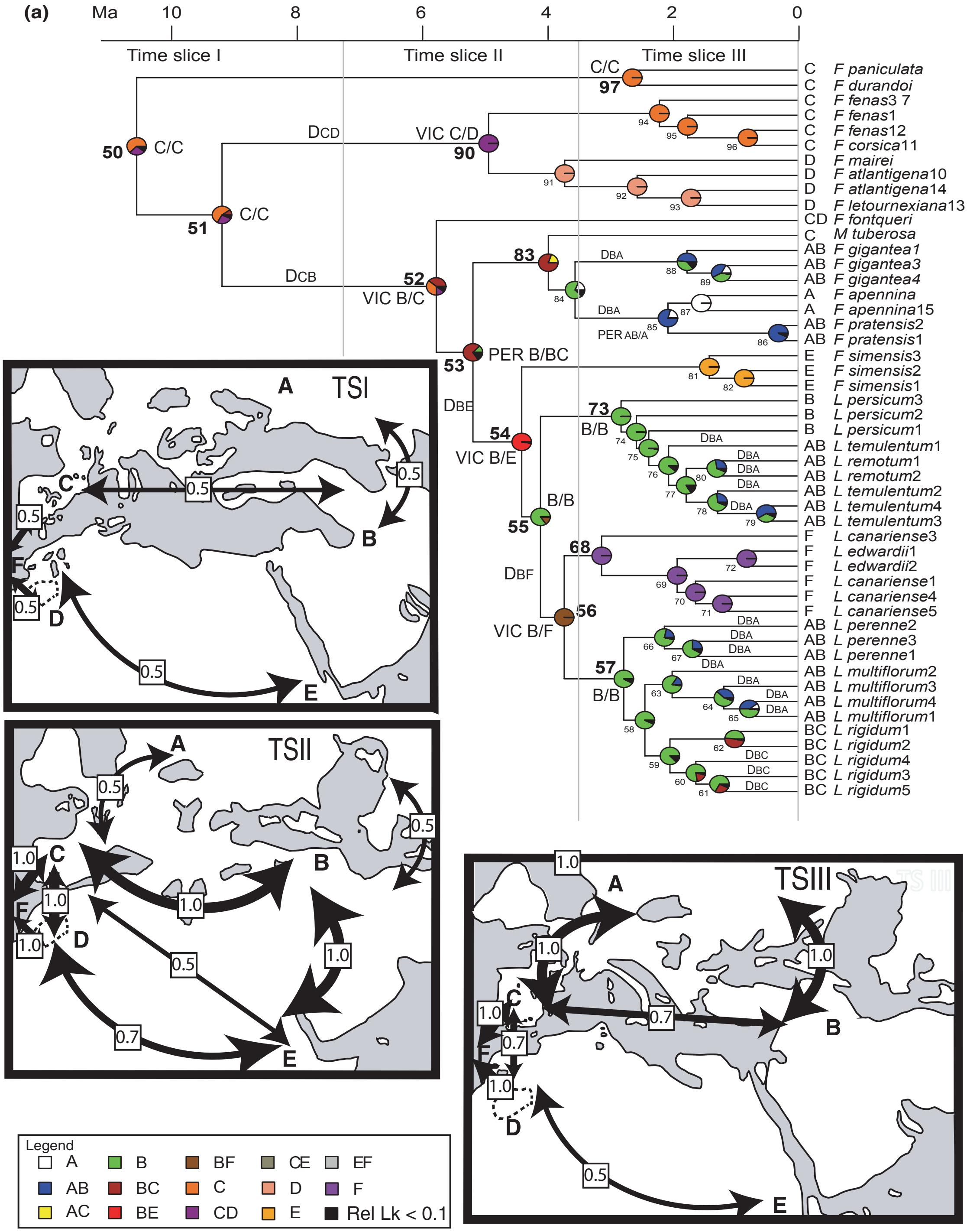

Figure 5 Biogeographical scenarios for the *Schedonorus–Lolium* complex inferred using a dispersal–extinction–cladogenesis (DEC) (LAGRANGE) stratified model, showing the alternative scenarios of a basal split of allogamous versus autogamous (a), or an early split of Macaronesian (b) *Lolium* lineages (see text). The trees correspond to the respective BEAST maximum clade credibility (MCC) chronograms (Figs 4 & 5b), with pie charts at nodes representing the relative (LAGRANGE) probabilities for alternative ancestral ranges. The grey lines represent the three time slices (TSI–TSIII) used in the temporally stratified LAGRANGE model. The maps on the left and below represent the palaeogeographical configuration of the Mediterranean Basin in these three time periods. Arrows and numbers in these maps represent the dispersal rate scalers (dispersal constraints) between areas imposed in the stratified DEC analysis to reflect change in continental connectivity over time (see also Table S2 in Appendix S1). Inferred dispersal events (e.g. DBA = dispersal from B to A) are mapped along the branches, while nodes show the range inheritance scenario (vicariance, VIC, and peripheral isolate speciation, PER) with the highest likelihood. Regions: A, Eurosiberia; B, Eastern Mediterranean + Southwest Asia; C, Western Mediterranean; D, Atlas Mountains; E, Eastern and Western Tropical Africa; F, Macaronesia. *F* = *Festuca*, *L* = *Lolium*, *M* = *Micropyropsis*. Rel Lk < 0.1, ancestral ranges with relative likelihood < 0.1.

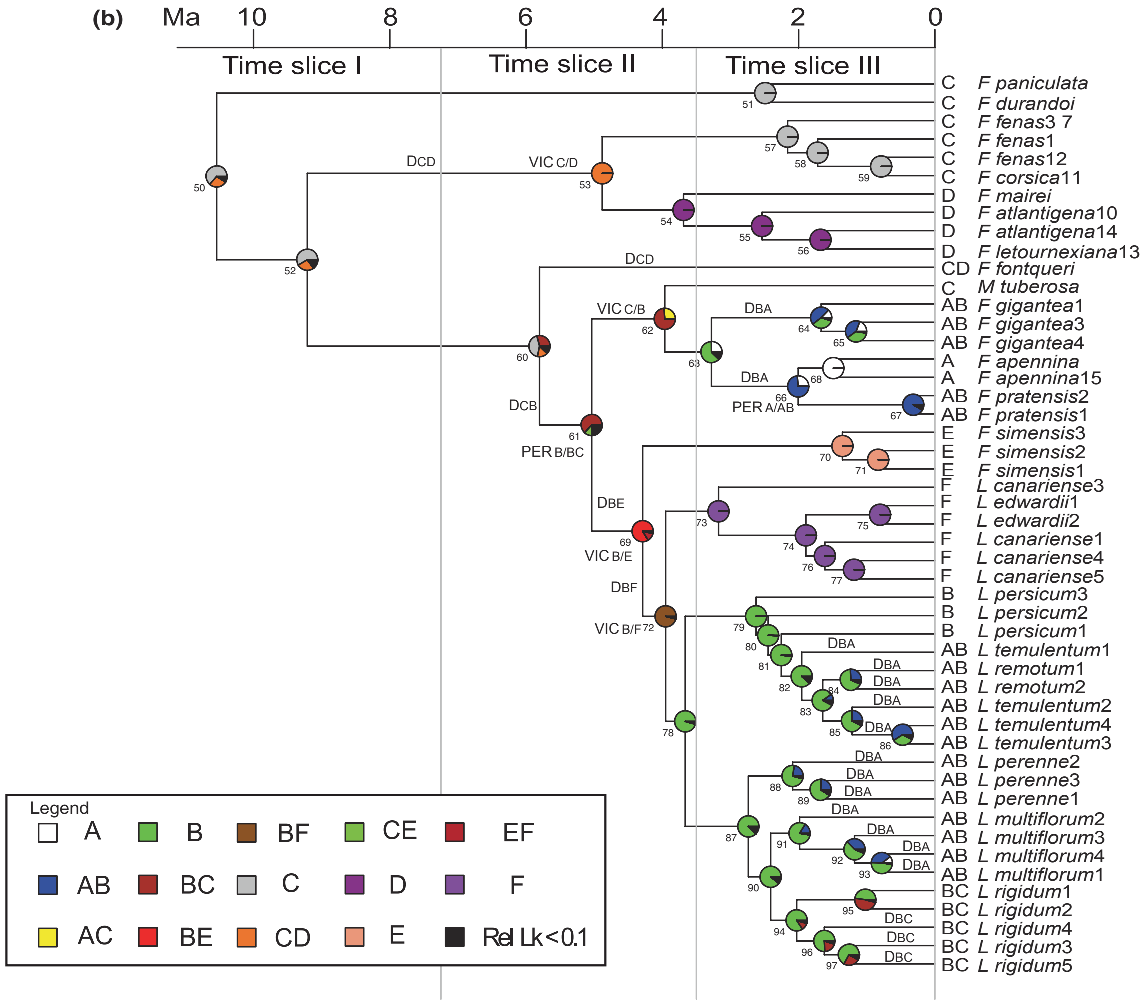

Figure 5 Continued.

hybridization events (e.g. *F. arundinacea*), the existence of polyploid species (e.g. in the Mahgrebian clade), and the possibility of incomplete lineage sorting events among intraspecific or closely related interspecific taxa (Fig. 4; cf. Gaut *et al.*, 2000). Nonetheless, the phylogeny presented here (Figs 4–6) represents the largest sampled and best-resolved hypothesis obtained so far for the *Schedonorus*–*Lolium* complex, and provides confirmation, as well as new findings, on phylogenetic relationships among meadow and tall fescues and the ryegrasses. In agreement with previous studies (Charmet *et al.*, 1997; Catalán *et al.*, 2004; Inda *et al.*, 2008), the 'Mahgrebian + Western Mediterranean' and the 'Eurasian' clades are reconstructed as the earliest diverging lineages within the complex. Our phylogeny also recovers *Lolium* as a monophyletic lineage (Figs 3 & 4), in agreement with previous works (Catalán *et al.*, 2004; Inda *et al.*, 2008; Hand *et al.*, 2010). The strongly supported relationship between the east–west tropical African endemic *F. simensis* and the *Lolium* clade (Figs 3 & 4) is, however, an unexpected finding because the phylogenetic position of *F. simensis* has rarely been addressed in evolutionary studies of this group (Namaganda *et al.*, 2006; Namaganda & Lye, 2008). An amplified fragment length polymorphism (AFLP) study, involving *F. simensis* and other tropical African fescues, identified the former species as a member of the *Schedonorus* core (Namaganda *et al.*, 2006). Other studies suggested that the diploid fescue *F. pratensis* was the closest relative of the (also diploid) *Lolium* ryegrasses (e.g. Jauhar, 1993). However, cytogenetic and other genome analyses have rejected the participation of the *F. pratensis* genome in the *Lolium* genome and vice versa (Pasakinskiene & Jones, 2005). The position of the African wild species *F. simensis* as sister-group to the *Lolium* ryegrasses is therefore a surprising result, which needs to be confirmed with further analysis of additional markers. The phylogenetic position of the endemic (allogamous) Macaronesian ryegrasses, however, remains unresolved, with MrBayes and MP trees (Fig. 3 and Fig. S1 in Appendix S3) supporting a sister-group relationship to the remaining *Lolium* species, while BEAST and ML trees [Fig. 4 and Fig. S2 (in Appendix S3)] recover the Macaronesian clade as sister to a group of continental allogamous ryegrasses. We chose to infer the temporal and spatial evolution of *Lolium* on this last topology because biological evidence supports the differentiation of ryegrasses into an autogamous and an

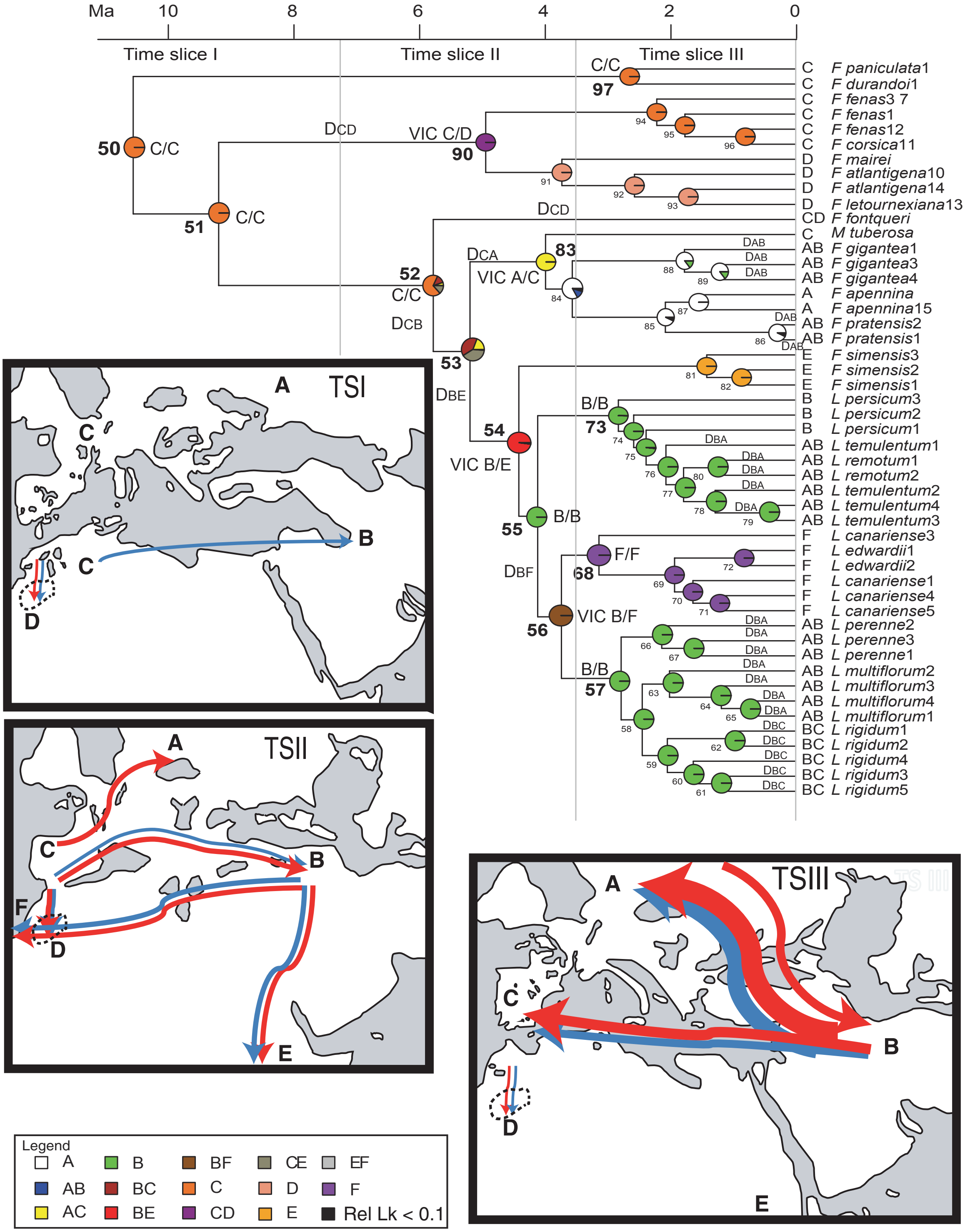

Figure 6 Biogeographical scenarios for the *Schedonorus–Lolium* complex inferred using a Bayes-DIVA model. The tree corresponds to the BEAST maximum clade credibility (MCC) chronogram (Fig. 4), with pie charts at nodes representing the marginal (Bayes-DIVA) probabilities for alternative ancestral ranges. Inferred dispersal events are mapped along the branches, while nodes show the range inheritance scenario (vicariance, VIC) with the highest marginal probability. The maps represent the palaeogeographical configuration of the Mediterranean Basin for the same time slices (TSI–TSIII). Arrows in the inset maps represent the frequency of dispersal events between pairs of areas – mapped along the branches in Bayes-DIVA (red arrows) and the LAGRANGE analysis (Fig. 5, blue arrows); if a dispersal event was inferred along an internode that crosses two time slices, the dispersal frequency was calculated as the proportion of the branch length that fell within each time slice. Regions: A, Eurosiberia; B, Eastern Mediterranean + Southwest Asia; C, Western Mediterranean; D, Atlas Mountains; E, Eastern and Western Tropical Africa; F, Macaronesia. *F* = *Festuca*, *L* = *Lolium*, *M* = *Micropyropsis*.

allogamous lineage (Malik, 1967; Terrell, 1968; Thomas, 1981), with Macaronesian *Lolium* included within the latter (Terrell, 1968). However, new cytogenetic data (Inda & Wolny, 2013), have shown that the Macaronesian species *Lolium canariense* presents intermediate cytogenetic features between *Schedonorus* and the continental *Lolium* lineages, which agrees with the early diverging position of the Macaronesian clade in the *Lolium* lineage supported by MrBayes and the ML tree. Similar to ryegrasses, *L. canariense* shows at least two 25S rDNA sites located in different chromosomes, while it exhibits a karyotypic pattern of distinct chromosomal localization of the 5S and 25S rDNA loci characteristic of *Schedonorus* taxa. To examine the biogeographical implications of this alternative phylogenetic position of the Macaronesian clade, we ran a second Lagrange stratified analysis using the MrBayes topology enforced in beast (Fig. 5b). In the section below, we discuss the differences between these two biogeographical scenarios.

Origin and early diversification of the *Schedonorus–Lolium* complex

In discussing palaeogeographical events below, it is important to note the degree of uncertainty in our date estimates because we used secondary age constraints derived from a more inclusive dated phylogeny, itself calibrated with molecular estimates from a larger fossil-based study. However, an advantage of the Bayesian relaxed-clock approach is the possibility of accounting for this degree of uncertainty by using probability distributions of age estimates. Therefore, we will refer to the entire confidence interval of the posterior distribution, rather than to the mean nodal ages, when attempting to explain biogeographical events.

In their biogeographical analysis of the Loliinae subtribe, Inda *et al.* (2008) postulated that the broad-leaved *Festuca* lineage (including *Schedonorus–Lolium*) migrated from Southwest Asia into East Africa in the early–mid Miocene, using the dispersal route provided by the collision of the Arabian (African) and Anatolian (Eurasian) plates around 16 Ma, later dispersing to the west. Our biogeographical reconstruction suggests that the ancestors of the *Schedonorus–Lolium* complex were already distributed in the Western Mediterranean region around the mid-Miocene (Serravallian–Tortonian, 13–8 Ma; Figs 5 & 6; node N51). The early diversification events that gave rise to the 'Maghrebian + Western Mediterranean' and the 'Eurasian' *Lolium* lineages took place also within this area (Figs 5 & 6; nodes N90 and N52). The configuration of the Western Mediterranean region in this period consisted of a continuous North African platform, which included the oldest chains of the Atlasica region (High and Middle Atlas), and a large and isolated Betic-Rifian range-island, which was separated from the proto-Iberian Peninsula and north-western Africa by the Betic and Rifian marine corridors, respectively (Krijgsman, 2002; Meulenkamp & Sissingh, 2003). The closure of those corridors between the late Tortonian and early Messinian (8.5–6 Ma; Seidenkrantz *et al.*, 2000; García *et al.*, 2003) created a land bridge between north-western Africa and south-western Europe. This could have favoured the dispersal of the African *Schedonorus* ancestor first to the Rifian and Betic ranges and then to the Western Mediterranean region through the Iberian Peninsula, later followed by dispersal to the Atlas (Fig. 5). This land pass has been suggested as one of the main colonization routes of North African biotas to southern European territories at the end of the Tertiary (Sanmartín, 2003). The current distribution in the Atlas and the Rif of *F. fontqueri*, sister-group to the Eurasian *Schedonorus* and *Lolium* clade (Fig. 5, TSII), could also be the result of a colonization of those mountains by Western Mediterranean populations after the closure of the Rifian marine corridor, *c.* 7–6 Ma (Krijgsman, 2002). The palaeogeographical setting of the Western Mediterranean region in the late Miocene (Tortonian) also included the formation of the subcoastal Tell-Atlas range and the present-day configuration of the Balearic and Tyrrhenian islands, which were later connected by land bridges to their closest Iberian and Italian peninsulas during the Messinian salinity crisis, 5.3 Ma (Meulenkamp & Sissingh, 2003). This complex scenario of colliding plates and new land connections could explain the larger number of endemic taxa within *Schedonorus* that currently inhabit the Western Mediterranean Basin, in comparison with other circum-Mediterranean regions.

Land dispersal to the east and Gibraltar vicariance

Biogeographical studies on widespread Mediterranean animals and plants have often suggested the Eastern Mediterranean region as the area of origin of these groups, followed by dispersal to the west in mid-Miocene times (Sanmartín, 2003; Inda *et al.*, 2008; Mico *et al.*, 2009). Our biogeographical reconstruction suggests an alternative scenario, with ancestral lineages reconstructed in the Western Mediterranean region in the mid-Miocene, followed by dispersal to the east in late Miocene times (TSI or TSII, Tortonian–Messinian, Fig. 5). The MRCA of the 'Eurasian' *Schedonorus–Lolium* lineage (N52) is reconstructed as already distributed in the Western and Eastern Mediterranean regions (BC) around the late Messinian (5.5 Ma), suggesting a rapid spread towards the east. This eastward dispersal probably involved a more mesic northern African platform prior to the formation of the Sahara Desert (TSI) or a southern European connection (TSII, Fig. 5) before the onset of the Mediterranean climate at the end of the Messinian–early Pliocene (Thompson, 2005; Salvo *et al.*, 2010).

In contrast to this pattern of widespread dispersal in the 'Eurasian' clade, the 'Mahgrebian + Western Mediterranean' clade represents an example of allopatric speciation driven by tectonic vicariance. The split between the Atlas and Western Mediterranean lineages at the end of the Miocene (4.9 Ma, Fig. 5) is congruent with the opening of the Gibraltar Strait *c.* 5.3 Ma, which broke up terrestrial connections between the African and European (Iberian) platforms.

Diversification of ryegrasses

The MRCA of *Festuca simensis* and *Lolium* is reconstructed as having dispersed from the Eastern Mediterranean and Southwest Asian region into east tropical Africa around the end of the Messinian–early Pliocene (Fig. 5). Biogeographical studies of alpine and montane plants have shown that the Arabian platform acted as the main gateway for Holarctic Asian and Eurasian stocks to enter the East African high mountain ranges at different times during the Pliocene and Pleistocene periods (Koch *et al.*, 2006; Gehrke & Linder, 2009). These repeated colonizations and secondary contact events provided the founders for most of the Afroalpine and Afromontane floras and would explain their present high genetic diversity (Gehrke & Linder, 2009). Uplift of the Eastern African Rift System started in the mid-Tertiary, but reached a climax in the Pliocene (5–2 Ma) with the formation of the Ethiopian Highlands and the rising of the Kenyan and north Tanzanian ranges (Sepulchre *et al.*, 2006). It is possible that the diploid ancestors of tetraploid *F. simensis* arrived to the newly formed volcanic Eastern African mountains from Southwest Asia in the early Pliocene, although intraspecific divergence within this polyploid species – involving further expansion to the western African mountains – seems to have occurred in more recent Pleistocene times (1.4 Ma; Fig. 5).

According to our reconstruction, initial divergence within *Lolium* took place within the Eastern Mediterranean region in the early Pliocene (4.1 Ma; Fig. 5). The autogamous lineage also diverged within this region (2.9 Ma), and later dispersed to the Eurasian region. The ancestor of the allogamous lineage (3.9 Ma) is inferred as widespread in the Eastern Mediterranean and Macaronesia, implying an earlier dispersal event to the west (Fig. 5a). The Atlantic volcanic archipelagos of Canaries, Madeira and Cape Verde were already emerged lands (Carracedo, 1999; Geldmacher *et al.*, 2000) at the time of the estimated crown age of the Macaronesian clade (3.1 Ma; Fig. 5). Different colonization hypotheses have been proposed to explain the distribution of Macaronesian angiosperm lineages (e.g. Carine *et al.*, 2004; Kim *et al.*, 2008). Many of these studies support the geographically close regions of north-western Africa and south-western Europe as the source of colonization events in Macaronesia (Kim *et al.*, 2008). Yet, in some cases, colonization events might have involved more distant source regions such as the East Mediterranean (Carine *et al.*, 2004). The origin of the Macaronesian ryegrasses could be explained by westward dispersal from the Eastern Mediterranean–south-western Asian region across North Africa during one of the humid periods in the Pliocene, as has been postulated for other groups with eastern/western African (Macaronesian) disjunctions (Sanmartín *et al.*, 2010). The alternative biogeographical scenario (Fig. 5b), showing the Macaronesian ryegrasses as a sister-group to the Eastern Mediterranean–Southwest Asian *Lolium* with the African *F. simensis* as their sister-group, also supports an east to west dispersal event. The monophyly of the Macaronesian *Lolium* clade (Fig. 4) suggests a single colonization event in the Canary Islands, probably in Tenerife. This was followed by dispersal to El Hierro and the Madeiran archipelago in recent Pleistocene times, as evidenced by the embedded positions of *L. edwardii* and *L. lowei* Menezes within the widespread and paraphyletic *L. canariense* (Cape Verde populations were not included in this study). This pattern is very similar to what has been proposed for some other Macaronesian endemic plants (Kim *et al.*, 2008).

Eurasian diversification of meadow fescues and ryegrasses preceded the start of agriculture

Multiple dispersal events from the Eastern and Western Mediterranean regions into the northern Eurasian platform were inferred within the 'Eurasian' *Schedonorus* and *Lolium* clade during the late Pliocene and Pleistocene periods (Figs 5 & 6). The ancestor of Eurasian *F. pratensis*, *F. gigantea* and *F. apennina* is reconstructed as coming from the Eastern Mediterranean, with two independent dispersal events into the Eurosiberian region during the Pliocene (Fig. 5). The continental clades in *Lolium* independently colonized the Eurosiberian region in the Pleistocene (Fig. 5), with multiple events of dispersal within each clade. The temporal envelope of these terminal dispersal events spans from early (2.1 Ma) to late (1.7 Ma) Pleistocene times (Fig. 5). By contrast, the colonization pattern inferred for the ancestor of the annual allogamous *L. rigidum* implies a dispersal event from the east to the Western Mediterranean region in the Pleistocene (Fig. 5). The estimated divergence times for the youngest Mediterranean and Eurosiberian ryegrasses *(L. perenne*, *L. multiflorum*, *L. rigidum*), and the giant (*F. gigantea*) and meadow (*F. pratensis*) fescue lineages, pre-date the last glacial and Holocene periods (Figs 4 & 5) (e.g. *F. gigantea* and *L. multiflorum*: Croomerian-Sarian (0.4 Ma); *L. temulentum*: Eemian (0.08 Ma); *F. pratensis*: Würm (0.027 Ma). This scenario of Pleistocene diversification agrees well with the cpDNA haplotype-based study of Fjellheim *et al.* (2006), who proposed repeated expansion and contraction events during the last glacial phases and two parallel northward post-glacial colonization routes across Europe to explain the diversification of *F. pratensis*. Balfourier *et al.* (1998, 2000) argued that the post-glacial colonization of Eurosiberian *L. perenne* and Mediterranean *L. rigidum* was linked to the spread of agriculture in recent Holocene–Neocene times. Our well-sampled, nearly complete phylogeny of the *Schedonorus–Lolium* complex demonstrates that the diversification and migration history of most fescue and ryegrass lineages began long before the introduction of agriculture and the main human migrations. Some potential exceptions are *L. perenne*, commonly used as animal fodder, and *L. rigidum*, a common crop weed, whose divergence times are compatible with a more recent eastern seed-mediated migration (i.e. from the Fertile Crescent to western territories) propagated by early Neolithic farmers (Balfourier *et al.*, 1998, 2000; Catalán, 2006).

ACKNOWLEDGEMENTS

We thank S. Fjellheim (Norway), M. Gaisberg (Germany), M. Namaganda (Uganda), A. Santos (Spain), M. Sequeira (Portugal), and the USDA (USA) and Aberystwyth University (UK) germplasm banks for providing *Festuca* subgenus *Schedonorus*, *Lolium* and ×*Festulolium* seeds, J. Viruel (Spain) for technical assistance and E. Lemonds (Spain) for linguistic assistance. This study was funded by two consecutive Spanish Ministry of Science and Innovation project grants (CGL2006-00319/BOS, CGL2009-12955-C02-01) to P.C. and L.A.I. and by a University of Zaragoza (Spain) grant to L.A.I. In addition, P.C. and L.A.I. were also partially funded by a Bioflora research team grant cofunded by the Spanish Aragon Government and the European Social Fund. I.S. was funded by projects CGL-2009-13322-C03-01 and CGL2012-40129-C02-01 from the Spanish Ministry of Science. S.B. was supported by a postdoctoral grant at RJB from the Swiss National Science Foundation grant (PBNEP3-129903).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary tables (Tables S1–S3).
Appendix S2 Expanded Materials and Methods.
Appendix S3 Supplementary figures (Figs S1–S5).

BIOSKETCH

The research team members work on the systematics and evolution of temperate grasses, with particular interest on the subfamily Pooideae, on the development of new analytical methods in biogeography and on the spatio-temporal evolution of various plant families.

Author contributions: P.C. and I.S. conceived and designed the experiment. L.A.I. performed the experiments. P.C., L.A.I., S.B. and I.S., analysed the data. P.C. and I.S. wrote the paper. The authors declare no conflict of interest.

Editor: Liliana Katinas