Independent domestications of cultivated tree peonies from different wild peony species

JUN-HUI YUAN,*† AMANDINE CORNILLE,‡§¶ TATIANA GIRAUD,‡§¶ FANG-YUN CHENG† and YONG-HONG HU*

*Shanghai Chenshan Plant Science Research Center, Chinese Academy of Sciences, Shanghai Chenshan Botanical Garden, 3888 Chennhua Road, Shanghai 201602, China, †Landscape Architecture College, Beijing Forestry University, No. 35 Tsinghua East Road, Haidian District, Beijing 100083, China, ‡Université Paris-Sud, Ecologie, Systématique et Evolution, UMR8079, 91405 Orsay, France, §CNRS, Ecologie, Systématique et Evolution, UMR8079, 91405 Orsay, France, ¶AgroParisTech, Orsay, France

Abstract

An understanding of plant domestication history provides insights into general mechanisms of plant adaptation and diversification and can guide breeding programmes that aim to improve cultivated species. Cultivated tree peonies (genus *Paeonia* L.) are among the most popular ornamental plants in the world; yet, the history of their domestication is still unresolved. Here, we explored whether the domestication in China of historically cultivated peonies, that is, the common and flare cultivated tree peonies, was a single event or whether independent domestications occurred. We used 14 nuclear microsatellite markers and a comprehensive set of 553 tree peonies collected across China, including common tree peonies, flare tree peonies and the wild species or subspecies that are potential contributors to the cultivated tree peonies, that is, *Paeonia rockii* ssp. *rockii*, *P. rockii* ssp. *atava*, *P. jishanensis* and *P. decomposita*. Assignment methods, a principal component analysis and approximate Bayesian computations provided clear evidence for independent domestications of these common tree and flare tree peonies from two distinct and allopatric wild species, *P. jishanensis* and *P. rockii* ssp. *atava*, respectively. This study provides the first example of independent domestications of cultivated trees from distinct species and locations. This work also yields crucial insight into the history of domestication of one of the most popular woody ornamental plants. The cultivated peonies represent an interesting case of parallel and convergent evolution. The information obtained in this study will be valuable both for improving current tree peony breeding strategies and for understanding the mechanisms of domestication, diversification and adaptation in plants.

Keywords: annual, convergence, East Asia, hybridization, ornamental cultivated tree, *Paeonia suffruticosa*

Received 30 April 2013; revision received 14 October 2013; accepted 16 October 2013

Introduction

An understanding of plant domestication can provide insight into the history of human civilization (Diamond 2002) and into general mechanisms of biological adaptation; it can also guide modern breeding programmes aimed at the further improvement of crop species (Gross & Olsen 2010; Miller & Gross 2011). The most fundamental and debated questions regarding the evolution of cultivated plants concern their geographic origins, whether domestication took place once or multiple times for a given crop and whether genetic bottlenecks occurred during their evolution (Diamond 2002; Gross & Olsen 2010).

A single origin of crops from one geographic area and from a single progenitor was initially considered...
the default domestication scenario for most crop species (Glémín & Bataillon 2009). However, for several annual crops, accumulating molecular evidence now suggests that multiple domestications from distinct locations and from wild contributors occurred. Confirmation of multiple independent geographic origins has been made for barley (Morrell & Clegg 2007), Asian rice (Londo et al. 2006) and the common bean (Phaseolus vulgaris; Bitocchi et al. 2012, 2013). Independent domestication from different species was also reported for two species of domesticated cotton (Gossypium hirsutum and G. barbadense L.; Westengen et al. 2005), two domesticated rice species from Asia and Africa (Oryza sativa and O. glaberrima Steud.; Londo et al. 2006) and beans (Phaseolus L.; Chacon et al. 2005). The occurrence of strong genetic bottlenecks during the process of domestication has been documented for most annual propagated seed crops (Tenaillon et al. 2004; Hyten et al. 2006; Liu & Burke 2006; Haudry et al. 2007). In contrast, for perennial tree crops, the investigation of these questions is still in its infancy (e.g. Besnard & Berville 2000; Breton et al. 2006; Myles et al. 2010; Cornille et al. 2012; Besnard et al. 2013).

Trees are fascinating models for studying domestication because their peculiar life history traits permit unique domestication processes (Gross & Olsen 2011; Cornille et al. 2012). Whereas the domestication history of some trees cultivated for food purposes has recently been elucidated (Gross & Olsen 2011; Cornille et al. 2012; Besnard et al. 2013; Delplancke et al. 2013; Roullier et al. 2013; Wincker 2013), the domestication of ornamental plants for aesthetic purposes, such as the rose (Iwata et al. 2000), has not received much attention. Cultivated tree peonies (genus Paeonia L.) are among the most popular ornamental plants worldwide and have been used as ornamental and traditional medicinal plants for over 1600 years in China, their centre of origin (Foster & Yue 1992; Li 2011). Although tree peonies display a rich ethnobotanical history that is deeply embedded in the Chinese culture, the history of the domestication of cultivated tree peonies remains unresolved (Cheng 2007; Li 2006; Zhou 2007; Zhang et al. 2011). The most important traits that have changed during domestication are the number and size of the flowers and the number and colours of the petals. Historically cultivated tree peonies include (i) the common tree peonies (CTPs), Paeonia suffruticosa Andrews, comprising 700–800 local varieties distributed throughout temperate regions in the world (Li 2011) and (ii) the flare tree peonies (FTP), comprising 200–300 varieties that are less extensively commercialized (Wang 1998; Cheng et al. 2005; Li 2005, 2011; Cheng 2007). Flare tree peonies are widely cultivated in the middle Gansu Province and present scattered distributions elsewhere in northwestern China (e.g. Ningxia, Qinghai and Shaanxi) and in some provinces of northern China (Wang 1998; Cheng et al. 2005; Cheng 2007; Li 2011). These two cultivated tree peonies differ in ecology (e.g. the flare tree peonies have greater ecological plasticity and a higher degree of resistance to environmental stress) and in morphology, with all flare tree peonies presenting reddish-purple flares at the bases of the petals that allow for easy identification (Cheng et al. 2005) (Fig. 1). These marked differences led to the as yet untested hypothesis that independent domestications of these two groups of cultivated peonies occurred (Li 1998, 2005; Wang 1998; Cheng 2007).

China is pinpointed as the likely geographic origin of cultivated tree peonies because it hosts the richest tree peony resources in the world; these resources include endemic wild species and greater than 1000 varieties. Tree peonies were introduced to Japan during the Tang dynasty (724–749), and more than 200 varieties of Japanese tree peonies have been developed. Beginning in the 1870s, some common tree peonies were introduced into western countries, such as England and France (Cheng 2007; Li 2011). Almost 110 varieties of tree peonies are currently grown in Europe (Cheng 2007). In the 1880s, a wild tree peony species, P. delavayi, was introduced into France, where nearly 13 cultivars designated P. × lemoinei have been obtained by hybridizing P. delavayi and P. suffruticosa (Wister 1995; Cheng 2007). American tree peony cultivars were developed later by further hybridizing P. × lemoinei with Japanese cultivars (Cheng 2007; Li 2011). The creation of these recent cultivars has been well documented (Cheng 2007); therefore, we focused in this work on the domestication history of the historically cultivated peonies: the common and flare tree peonies in China.

Historical records and morphological comparisons suggest that CTP and FTP cultivars originated independently from different wild species in China. Cultivated CTPs are likely to have originated from the wild species Paeonia jishanensis T. Hong & W.Z. Zhao (Rehder 1920; Li 2005), while one of the two subspecies of P. rockii S.G. Haw & Lauener, T. Hong & J.J. Li ex D.Y. Hong (P. rockii ssp. atava and P. rockii ssp. rockii) growing in the central southern region of Gansu Province (Fig. 1) is the likely progenitor of FTPs (Cheng et al. 2005; Li 2011). Molecular phylogenetic analysis suggests that another wild species, P. decomposita Hand. Mazz., which is distributed in Sichuan Province (Fig. 1), may also have been involved to a lesser extent in the domestication of FTPs (Zhou 2007). This hypothesis remains controversial, however, because P. decomposita displays a restricted distribution in remote mountain areas in northwestern Sichuan province and is therefore unlikely to have been easily transplanted in the past (Li 2011). It
has also been proposed based on morphological features and gene sequences that recent wild-to-crop and crop-to-crop (i.e. FTPs to CTPs and conversely) hybridizations and introgressions have been major forces in the breeding of cultivated tree peonies (Cheng 2007; Zhou 2007; Li 2011). The available phylogenies of cultivated and wild tree peony species are, however, not resolved well enough to provide a basis for elucidation of the domestication history of the CTP and FTP cultivars (Zhou 2007; Zhao et al. 2008; Zhang et al. 2011).

Here, we explored the domestication history of common and flare tree cultivated peonies in their centre of origin using 14 nuclear microsatellite loci and a comprehensive set of 553 trees collected throughout China (Fig. 1). This set included 103 flare tree peony varieties, 72 common tree peony varieties (e.g. *P. suffruticosa*) and the main potential contributors to their evolution, *P. rockii* ssp. *rockii*, *P. rockii* ssp. *atava*, *P. jishanensis* and *P. decomposita* (Fig. 1), that are all diploid (Hong 2010) and allogamous (Cheng et al. 2005; Cheng 2007) and grow in allopatry (Fig. 1). The specific objectives of this study were to test (i) whether the four wild species or subspecies, the FTPs and the CTPs all form distinct genetic clusters; (ii) whether recent wild-to-crop and crop-to-crop hybridizations contributed to the genomes of the FTP and CTP cultivars; (iii) whether the FTP and CTP cultivars have been domesticated independently from distinct wild species or subspecies and (iv) whether footprints of genetic bottlenecks expected during domestication can be detected.

**Materials and methods**

**Sample collection and DNA extraction**

Leaf material was carefully collected from domesticated varieties and wild species to accurately represent the genetic, morphologic and geographic diversity of cultivated and wild tree peonies. The choice of varieties and species was made based on interviews with horticulturalists, botanists and local peony experts, on field surveys and on an examination of the literature.
(Li 1998, 2005; Cheng et al. 2005). Our sampling covered all extant sites of Paeonia rockii ssp. rockii, P. rockii ssp. atava and P. jishanensis according to the records in the literature and specimens available in most herbariums worldwide (Fig. 1; Table S1, Supporting information). We sampled a single population of P. decomposita because this species was not likely to have contributed to the cultivated tree peonies (Li 2011). A minimum distance of 20 m was kept between sampled wild individuals.

Most wild tree peonies, including P. rockii (ssp. rockii and ssp. atava) and P. decomposita, can only reproduce sexually, but P. jishanensis can also propagate asexually by stolons. Cultivated peony varieties are often propagated clonally by grafting, division or layering, although breeders sometimes use seeds. To avoid the inclusion of clones in the data set, we sampled a single tree per variety. A total of 103 FTP varieties of P. rockii propagated commercially, including 49 traditional and 54 modern varieties, all of great ornamental value, were collected (Fig. 1, Table S1, Supporting information). A total of 72 CTPs representing varieties of P. suffruticosa, including 28 traditional and 44 commercial modern varieties, were sampled (Cheng et al. 2005; Li 2005).

DNA was extracted from the collected leaves using an improved cetyltrimethylammonium bromide (CTAB) method (Yuan et al. 2010) and examined by electrophoresis on a 1% agarose gel in TAE buffer. The DNA was then diluted to a concentration of 5–10 ng/μL for subsequent polymerase chain reaction (PCR) amplification.

Microsatellite loci and PCR amplification

We used 14 polymorphic microsatellite loci (Table S2, Supporting information) that were previously shown to be independent and to not belong to the chloroplast genome of Paeonia (Yuan et al. 2012). The PCR conditions were as described by Yuan et al. (2012). Positive and negative controls were systematically run. The PCR products were examined on an ABI PRISM 3730XL DNA analyser with GeneScan 600LIZ size standards (Applied Biosystems, Foster City, CA, USA). Each chromatogram was read using GeneMapper 4.0 (Applied Biosystems). The genotyping results are presented in Table S3 (Supporting information). Samples with uncertain alleles were double checked by repeating the PCR amplifications.

Analysis of genetic diversity within each wild peony species or subspecies and within cultivated tree peonies

Although our sampling was designed to avoid clones within cultivated peonies, footprints of clonality could still be present if some varieties differing by only a few mutations have been propagated by grafting. In wild species, siblings can be collected unintentionally in the field. Because these features could result in spurious genetic structure due to the presence of closely related individuals in the data set, we checked for the existence of clonal genotypes using GENCLONE 2.0 (Arnaud-Haond & Belkhir 2007) and estimated relatedness between pairs of individuals within each species by calculating the rxy of Lynch & Ritland (1999) using RE-RAT (Schwacke & Rosel 2005).

We tested for the occurrence of null alleles with MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). Allelic richness (As) and private allele (Ap) frequencies for a sample size of four were calculated with ADZE (Szpiech et al. 2008). Observed (Ho) and expected (He) heterozygosities and Weir & Cockerham F-statistics were estimated using GenALEX 6.5 (Peakall & Smouse 2012). Inbreeding coefficient (Fis), deviation from the Hardy–Weinberg equilibrium, genotypic linkage disequilibrium and the significance of differences between FST values were assessed using exact tests conducted with GENEPOP 4.0 (Rousset 2008).

We tested for the occurrence of a recent genetic bottleneck in cultivated and wild tree peonies using the method implemented in BOTTLENECK (Cornuet & Luikart 1996; Piry et al. 1999), which is based on the detection of significant heterozygosity excess relative to equilibrium expectations. Heterozygosity excess is an ephemeral pattern that can only be detected within the first several generations after a decline of population size; it initially occurs because allelic diversity is lost faster than heterozygosity, resulting in the appearance of greater heterozygosity than expected based on the number of alleles in the population (Cornuet & Luikart 1996). We used all three available models for microsatellite evolution, that is, infinite allele (IAM), stepwise-mutation (SMM) or two-phase (TPM) models, which yielded different results (Table S4, Supporting information). We report only the results obtained under the TPM model, with the setting values of 10% SMM, 90% IAM and 10% variance, because the TPM model has been reported to be the most conservative and powerful model available (Cornuet & Luikart 1996; Luikart et al. 1998). Models were run for 10,000 replicates. The recent occurrence of a genetic bottleneck was further investigated using the Garza–Williamson index (M, an empirical value of the ratio of number of alleles to range in allele size). This index, analysed here using Arlequin 3.5 (Excoffier & Lischer 2010), is expected to be lower than the critical Mc value of 0.68, a value obtained by simulations based on the empirical data, in bottlenecked populations (Garza & Williamson 2001). The method implemented in BOTTLENECK has low power unless the decline is greater than 90% (Cornuet & Luikart 1996); this approach is most powerful when genetic
bottlenecks are severe and recent (Williamson-Natesan 2005). The Garza–Williamson index method is more likely to correctly detect genetic bottlenecks if the bottleneck lasted several generations or if the population made a rapid demographic recovery (Williamson-Natesan 2005).

Genetic differentiation among wild species and cultivated tree peonies

STRUCTURE 2.3.2 (Pritchard et al. 2000; Falush et al. 2003) was used to infer species delimitation and the contribution of wild species or subspecies to the cultivated peony genome. Analyses were run without prior information. STRUCTURE performs model-based clustering, in which Markov chain Monte Carlo (MCMC) simulations are used to identify K distinct clusters. The algorithm attempts to minimize deviations from Hardy–Weinberg and linkage equilibrium within clusters. Ten independent runs were conducted for each K. Each run was pursued for 2,000,000 MCMC interactions using the following conditions: an initial burn-in of 200,000, an admixture model, correlated allelic frequencies and the same α for all samples. STRUCTURE was run with the full data set (N = 553) and with the pruned data set (N = 323) that excluded clonal and related individuals (i.e. rsy > 0.5). STRUCTURE was run for K ranging from one to eight for species delimitation analyses. The AK statistic, which is designed to identify the number of clusters above which an additional increase in likelihood is weaker, was calculated following the method of Evanno et al. (2005). For analyses of wild species contribution to the cultivated peony genome, STRUCTURE was run at K = 2 on data sets including either CTPs or FTPs and each of the four wild peony species by pairs. The P. rockii ssp. rockii and P. rockii ssp. atava structure had been previously well studied (Yuan et al. 2012); we focus here instead on the species history.

Principal component analysis (PCA) was performed with allele frequencies calculated from the full and pruned data sets using the Multivariate Statistical Package (MVSP) version 3.13b (Kovach Computing Services, Anglesey, Wales, UK).

Approximate Bayesian inference

Using approximate Bayesian computation (ABC), we investigated whether domestication of the cultivated common tree peonies and flare tree peonies was a single event or whether there have been independent domestications. Population structure does not strongly affect ABC results (St Onge et al. 2012); thus, we did not take into account intraspecies structure to avoid useless complex scenarios including higher numbers of parameters (divergence times and effective population sizes). Models were constructed based on an $F_{ST}$ matrix, historical records and previous phylogenetic studies of the domestication history of tree peonies. We excluded P. decomposita from the ABC analyses because it appeared to be the most genetically divergent species based on our microsatellite data and because we had a low sampling size (N = 11). Historical records and previous phylogenetic studies pointed to P. jishanensis as the wild progenitor of CTP cultivars, whereas FTP cultivars were suggested to have originated either from P. rockii ssp. rockii or atava or P. jishanensis. The three models tested here were therefore designed to distinguish between (a) single domestication of CTPs and FTPs from one wild peony species, P. jishanensis (model a, Fig. 2); (b) independent domestications of FTPs from P. rockii ssp. atava and of CTPs from P. jishanensis (model b, Fig. 2) and (c) independent domestications of FTPs from P. rockii ssp. rockii and of CTPs from P. jishanensis (model c, Fig. 2). For all models, we assumed five distinct groups, as indicated by STRUCTURE analyses: the three wild species or subspecies, P. rockii ssp. rockii (PRR), P. rockii ssp. atava (PRA) and P. jishanensis (PJ), and two cultivated peony groups, the CTPs and FTPs. We used ABCtoolbox (Wegmann et al. 2010) with fastsimcoal (Excoffier & Foll 2011) to compare the three scenarios (Fig. 2). Because the juvenile period of Paeonia lasts 4–6 years, we assumed a generation time of 5 years. We estimated the effective size of each group ($N_e$) and the divergence times between groups X and Y ($T_{X-Y}$). We assumed a uniform prior distribution for $N_e$ [600; 4.10$^5$] and $T_{X-Y}$ [1; 5.10$^4$]. Our priors were large because no reliable historical data were available.

For all models, microsatellite data sets were simulated for 14 loci (Pdel02-2, Pdel05, Pdel06, Pdel07, Pdel20, Pdel22, Pdel9b, Pdel33, Pdel35, Jx02-2, Jx05-2, Jx17 and Jx29) that carry perfect repeats (Wang et al. 2008; Yuan et al. 2010, 2012). We generated 10$^6$ genetic data sets from coalescent simulations using model parameters drawn from prior distributions under the three previously specified scenarios. For each simulation, we calculated 31 summary statistics. Within each group, we computed $H$, the mean heterozygosity across loci. We also calculated $F_{ST}$ (Weir & Cockerham 1984) and genetic distances ($\theta$) (Goldstein et al. 1995) between pairs of groups. Following Wegmann et al. (2009, 2010), we used the R package ‘PLS’ (i.e. partial least squares, Mevik & Wehrens 2007) to find the appropriate number of PLS components to use (8, in our case). The same set of 31 summary statistics was also calculated on the observed data set, was PLS-transformed and then was used to calculate the Euclidean distance to each simulation.
We assumed a generalized stepwise model of microsatellite evolution (Estoup et al. 2002). The mutation rate was allowed to vary across loci, with locus-specific mutation rates drawn from a gamma distribution ($\alpha$, $\alpha$/$\mu$), in which $\mu$ is the mutation rate per generation and $\alpha$ is a shape parameter. We assumed a uniform prior distribution for $\mu$ [0.001, 0.01] and a uniform distribution for $\alpha$ [1, 30].

We compared the three models by calculating their Bayes factors (Wegmann et al. 2010) by estimating their relative posterior probabilities based on the 1% of simulated data sets that most closely matched the observed data, that is, 1000 simulated data sets. Once the best model had been chosen, we estimated demographic parameters under this scenario using a general linear model (ABCGLM) post-sampling regression adjustment for the 1000 retained simulations (Leuenberger & Wegmann 2010; Wegmann et al. 2010). We report the mode and 95% confidence interval for each model parameter estimate.

The performance of the method for discriminating between competing historical models was assessed by analysing test data sets (called pseudo-observed data sets) that were simulated with the same number of loci and individuals as the observed data sets. We simulated 1000 such data sets for each competing model using parameter values drawn from the same prior distributions as for the original analyses. We determined the relative posterior probabilities of competing models for each pseudo-observed data set using the model choice procedure described above (Wegmann et al. 2010). Confidence in model choice was then estimated based on the percentage of times that a given scenario did not have the highest posterior probability of the competing scenarios when it was actually the true scenario (type I error) and on the percentage of times that a given scenario had the highest posterior probability when it was not the true scenario (type II error).

Results

Genetic diversity in cultivated common and flare tree peonies

Eight pairs of samples were assigned to clonal groups by GENCLONE: one pair each in CTPs, FTPs and Paeonia jishanensis and five pairs in P. rockii ssp. rockii. The percentage of pairs with pairwise relatedness ($r_{xy}$) greater than 0.5 (i.e. full-siblings) was 1.1% in CTPs ($N$ = 27 pairs), 0.72% in FTPs ($N$ = 38), 13.55% in P. jishanensis ($N$ = 553) and 13.55% in P. rockii ssp. rockii ($N$ = 1075). To avoid biases, STRUCTURE analyses were then conducted on the full ($N$ = 553) and pruned data set ($N$ = 323), that is, keeping a single individual per pair of related individuals and per pair of clone mates.

Tests for linkage disequilibrium with the Bonferroni correction showed no significant linkage disequilibrium. No locus pair deviated significantly from equilibrium in more than six or seven of 30 total sampled sites, suggesting that all loci were physically unlinked.

Moderate genetic diversity was found in the cultivated common ($H_O = 0.61$) and flare ($H_O = 0.58$) tree peonies (Table 1). The four wild species or subspecies showed significantly lower genetic diversity compared to FTPs and CTPs (Wilcoxon-sign-rank [WSR] tests on $H_O$ between FTPs/CTPs and each wild species or subspecies,
Table 1 Genetic polymorphism within the cultivated common and flare tree peonies and the four wild peony species or subspecies

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>HO</th>
<th>HE</th>
<th>Fst</th>
<th>AR</th>
<th>AP</th>
<th>PNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paeonia rockii</td>
<td>31</td>
<td>0.40</td>
<td>0.52</td>
<td>0.27***</td>
<td>2.19</td>
<td>0.74</td>
<td>0.07</td>
</tr>
<tr>
<td>jishanensis</td>
<td>11</td>
<td>0.36</td>
<td>0.33</td>
<td>-0.12***</td>
<td>1.57</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td>Paeonia decomposita</td>
<td>275</td>
<td>0.50</td>
<td>0.72</td>
<td>0.31***</td>
<td>2.51</td>
<td>1.03</td>
<td>0.13</td>
</tr>
<tr>
<td>Paeonia rockii ssp. rockii</td>
<td>61</td>
<td>0.45</td>
<td>0.58</td>
<td>0.24***</td>
<td>2.31</td>
<td>0.46</td>
<td>0.08</td>
</tr>
<tr>
<td>FTPs</td>
<td>103</td>
<td>0.58</td>
<td>0.66</td>
<td>0.14***</td>
<td>2.53</td>
<td>0.44</td>
<td>0.05</td>
</tr>
<tr>
<td>CTPs</td>
<td>72</td>
<td>0.61</td>
<td>0.66</td>
<td>0.08***</td>
<td>2.54</td>
<td>0.60</td>
<td>0.03</td>
</tr>
</tbody>
</table>

N: sample size; HO and HE: observed and expected heterozygosities, respectively; Fst: inbreeding coefficient; AR and AP: allelic richness and private allelic richness, respectively, averaged across loci estimated by rarefaction using a standardized sample size of four; PNA: proportion of null alleles; FTP: flare tree peony; CTP: common tree peony. ***P-value < 0.0001.

all P < 0.05 (Table 1). Allelic richness (ARs) was found to be significantly higher in cultivated CTPs and FTPs than in wild species or subspecies (Table 1; CTPs: AR = 2.54, FTPs: AR = 2.53, WSR, P ≤ 0.003; FTPs: AR = 2.53, WSR, P ≤ 0.002). FTPs, CTPs and wild peonies all displayed significant Fst (Tables 1 and S4, Supporting information), indicating heterozygote deficiency. Null alleles were identified in some populations of wild and cultivated common and flare tree peonies. The presence of these alleles and a Whalund effect may explain the observed deviations from Hardy–Weinberg equilibrium (Tables 1 and S4, Supporting information).

The BOTTLENECK analysis showed no significant heterozygosity excess in the cultivated common and flare tree peonies but revealed significant heterozygosity excess in some populations of wild peonies, namely P. jishanensis, P. rockii ssp. atava and P. rockii ssp. rockii (Table S4, Supporting information). The Garza–Williamson index was lower than the threshold value (i.e. Mc = 0.68) in cultivated peonies (CTPs: Mc = 0.28 ± 0.12, FTPs: Mc = 0.26 ± 0.26) and in all populations of wild tree peonies (Table S4, Supporting information). Together, these results suggest that the wild tree peonies P. jishanensis, P. rockii ssp. atava and P. rockii ssp. rockii have suffered from demographic bottlenecks, while cultivated common and flare tree peonies have not.

Common and flare tree peonies form well-separated genetic clusters

The STRUCTURE results obtained with the full data set (N = 553) (Fig. 3 and S1, Supporting information) and those obtained with the pruned data set (N = 323) (Fig. S2, Supporting information) were highly similar; we therefore used the full data set for further analyses. The ΔK value was the greatest at K = 4 (Fig. S3, Supporting information; ΔK = 918, Prin L. = -13923). However, at K = 4, the CTPs remained clustered with P. jishanensis (Fig. 3 and S1, Supporting information). At K = 5, most taxonomic species or subspecies (e.g. P. jishanensis, P. rockii ssp. rockii and P. rockii ssp. atava), except P. decomposita, which mingled within P. rockii ssp. rockii, were assigned to distinct clusters in the main mode (i.e. the clustering solution found in 90% of the simulation replicates). In addition, a genetic structure appeared within P. rockii ssp. rockii. No additional well-delimited clusters corresponding to subdivisions of previous clusters were detected beyond K = 5, except for a structure within P. rockii ssp. atava and P. rockii ssp. rockii (Fig. 3 and Fig. S1, Supporting information). Albeit intraspecies structure was found, K = 5 appeared to be the clustering solution that was most relevant to our questions aimed at determining the genetic differentiation of tree peony species.

At K ≥ 3, the cultivated CTPs and FTPs were clearly assigned to different clusters, while the cultivated FTPs and wild P. rockii ssp. atava were clustered together (Fig. 3 and Fig. S1, Supporting information). At K = 4, the CTPs and P. jishanensis remained assigned to the same cluster. This result is in agreement with the PCA results (Fig. 4), which show that the CTPs were distinct from the FTPs, with the CTPs lying very close to P. jishanensis and the FTPs appearing mingled within P. rockii ssp. atava (Fig. 3). These results support the hypothesis of independent origins of the two groups of cultivars from distinct wild peony species.

Genetic differentiation (FST) between each cultivated and each wild peony species or subspecies was significant (P < 0.001). Paeonia decomposita appeared to be the most differentiated from the other species (Table 2). The lowest differentiation was found between the cultivated FTPs and wild P. rockii ssp. atava (FST = 0.05), in agreement with the STRUCTURE and PCA plots (Fig. 3,4 and S1; Supporting information). The CTPs appeared closer to the FTPs (FST = 0.07), P. rockii ssp. rockii (FST = 0.09) and P. rockii ssp. atava (FST = 0.11) than to P. jishanensis (FST = 0.12) (Table 2).

Rare wild-to-crop and crop-to-crop hybridizations during recent cultivated tree peony history

The recent contribution of each wild tree peony species to common or flare tree peonies was further investigated by running STRUCTURE at K = 2 by pairs of species, including CTPs or FTPs and each wild species or subspecies (Fig. S4 and Tables S4, S5 and S6, Supporting information).
At $K = 2$, all pairs of species were separated into two well-delimited clusters, except the pair that included the cultivated FTPs and $P$. rockii ssp. atava (Fig. S4b, Supporting information); this pair showed a substructure within $P$. rockii ssp. atava. Further increasing $K$ failed to separate the FTPs and $P$. rockii ssp. atava (Fig. S4b, Supporting information).

Some rare footprints of introgression were detected between cultivated and wild species (admixed individuals in Fig. S4a and S4b and Tables S5 and S6, Supporting information).
information) and also between the two cultivated groups, CTPs and FTPs (admixed individuals in Fig. S4c and Tables S5 and S6, Supporting information).

Modelling the domestication history of common and flare tree peonies

We used ABC to statistically determine which of the three models (Fig. 2) assuming alternative domestication histories of the cultivated CTPs and FTPs was the most likely. The relative posterior probabilities ($P$) calculated for each model provided the strongest statistical support for model $b$, suggesting that FTPs and CTPs were domesticated independently (Bayes factor for model $b = 18.4$, Table 3). The model that assumed a single domestication event of FTPs/CTPs from *P. jishanensis* had the lowest relative posterior probability (Table 3). For model $b$, we obtained estimates of effective population sizes of 16,737 [95% confidence interval: 608–242,674] for $N_{Pj}$, 53,047 [4639–252,751] for $N_{Prr}$, 44,977 [4638–254,751] for $N_{Pra}$, 53,046 [8,654–363,703] for $N_{ftp}$ and 32,874 [4639–367,723] for $N_{ctp}$. Using a generation time of 5 years, *P. jishanensis* and *P. rockii* ssp. rockii diverged 220,297 years ago ($T_{pffpp}$, [96,538–247,518]), *P. jishanensis* and *P. rockii* ssp. atava diverged 139,574 years ago ($T_{pffpra}$, [39,300–224,806]) and CTPs and FTPs diverged independently from *P. jishanensis* and *P. rockii* ssp. atava 74,931 and 35,168 years ago, respectively ($T_{pffctp}$/$T_{pffpra}$, [14,978–177,337] and [2514–118,057], respectively). The estimated mutation rate per generation was $\mu = 0.001$ [1e⁻³–3e⁻³]. We used 1000 pseudo-observed data sets to check the coverage property of the marginal posterior distributions estimated with our approach. As seen from the histograms of the posterior quantiles, the marginal posterior distributions were slightly overestimated on average (Fig. S5, Supporting information). These estimations should therefore be regarded with caution, particularly the divergence time estimations that presented biased posterior distributions. However, the observed values fell within the simulated data ($P = 0.35$), suggesting that the assumed model is capable of reproducing the observed summary statistics (the 8 PLS components, in our case).

We checked that the power of the analysis was sufficient to discriminate between the competing models. For model $b$ against the other two models, the type I error rate was 0.22 and the mean type II error rate was 0.20. Overall, ABC analyses provided strong support for the independent domestication of CTPs and FTPs from *P. jishanensis* and *P. rockii* ssp. atava, respectively.

### Discussion

Independent domestcations of the common and flare tree peonies: a model of parallel and convergent evolution

In combination, the STRUCTURE and ABC analyses presented here provide strong support for the hypothesis of independent domestications of cultivated common and flare tree peony varieties in China from different wild peony species, *Paeonia jishanensis* and *P. rockii* ssp. atava, respectively. These findings are consistent with the morphological features of the cultivated groups (CTPs and FTPs) (Rehder 1920; Li 2005; Cheng 2007). The CTPs are dwarf shrubs less than one metre in height; they are typically characterized by the presence of nine (often less than 15) leaflets per compound leaf and red carpels and filaments, characteristics that are very similar to those of wild *P. jishanensis* (Li 2005, 2011). The cultivated FTPs are taller than one metre, usually have more than 15 leaflets per compound leaf and display the yellowish or white carpels and...
filaments typically found in *P. rockii* ssp. *atava* (Cheng *et al.* 2005; Li 2005; Cheng 2007). The allopatric distributions of the two wild contributors, *P. jishanensis* and *P. rockii* ssp. *atava*, across China also point to independent geographical domestications of cultivated tree peonies. Together with the cultivated olive (Besnard & Berville 2000; Breton *et al.* 2006; Besnard *et al.* 2013), this study is one of the rare existing examples of documented independent geographical domestications in perennial tree crops. Our results do not clearly define the contribution of *P. decomposita* to flare tree peony domestication because of the low numbers of samples of this species in our survey. To answer this question, further sampling that includes a subspecies studied by Zhou (2007), that is, *P. decomposita* ssp. *rotundiloba* from Maoxian County in Sichuan province, is needed.

The STRUCTURE results obtained in this study support a more recent domestication of FTPs than CTPs by indicating little differentiation between FTPs and its wild progenitor, *P. rockii* ssp. *atava*, in contrast to the clear differentiation that was found between CTPs and *P. jishanensis*. Although the ABC estimations of divergence time *sensus stricto* of CTPs and FTPs from their respective progenitors should be regarded with caution, the estimated divergence time between *P. rockii* ssp. *atava* and FTPs was half the time of CTPs from *P. jishanensis*. Together, the genetic findings are in accordance with historical clues suggesting that domestication of FTPs may be more recent than domestication of CTPs. Traditional CTP cultivars were referred to as ‘the king of flowers’ in the Tang Dynasty (approximately 627–850) (Li 2005, 2011), while the FTPs were developed later during the Ming to the Qing Dynasties (around 1398–1797) (Cheng *et al.* 2005; Li 2011). Despite their independent domestications, FTP and CTP cultivars share a common domestication syndrome with respect to the number and size of flowers (multiple vs. single in wild species) and the number and colours of the petals (colourful, e.g., patchy white, pink, purple and red vs. mostly white in wild species or subspecies). Cultivated tree peonies thus represent an interesting case of parallel and convergent evolution, with similar phenotypes in the two groups despite their independent domestication from different locations and different species. The relatively recent domestication of tree peonies and the fact that they display a strong syndrome with respect to a few traits provides a good model for studying the genes and processes involved in adaptation (Hohenlohe *et al.* 2010). In addition, this study suggests that future studies targeting the genes involved in the marked domestication syndrome are warranted.

In contrast to previous suggestions (Zhou 2007), we show here that hybridizations have not been a major force in shaping the genomics of cultivated tree peonies. The low level of gene flow between CTPs and FTPs and between cultivated and wild tree peonies contrasts with the pattern found in other cultivated fruit tree crops, which experience frequent wild-to-crop gene flow and vice versa (Duputie *et al.* 2007; Miller & Gross 2011; Cornille *et al.* 2012, 2013; Delplancke *et al.* 2012). Although tree peonies are outcrossing species, they can be vegetatively propagated, more easily than fruit trees that require grafting. Early propagation by clonality may have prevented introgression between wild and cultivated peonies. The lack of widespread clonal structure found is due to our sampling scheme, which was designed to avoid the use of multiple samples from a given variety.

**Moderate but higher genetic diversity in cultivated peonies than in wild peony species: implications for the conservation of genetic resources and future breeding programmes**

As a human-mediated evolutionary process, domestication impacts contemporary patterns of genetic variation in cultivated populations. The moderate level of genetic diversity estimated in cultivated and wild tree peony species in this study is consistent with findings from previous studies (Pei *et al.* 1995; Zou *et al.* 1999; Meng & Zheng 2004; Yuan *et al.* 2010, 2011, 2012; Zhang *et al.* 2011). No clear footprints of recent genetic bottlenecks were detected in cultivated peonies in this study. In particular, the genetic diversity of the cultivated CTPs and FTPs appeared higher than the diversity of their wild progenitors, *P. jishanensis* and *P. rockii* ssp. *atava*. This unexpected result is not due to introgression in cultivated tree peonies as introgression events were found to be rare, nor is it due to undersampling, because we have well covered the present-day distribution of these wild peony species. Genetic bottlenecks are expected to occur during domestication, and the allelic diversity in domesticated species is expected to be a subset of that found in their wild progenitors (Gross & Olsen 2010). Footprints of bottlenecks have been reported in several annual seed-propagated crops, such as maize, wheat, soybean and sunflower (Tenaillon *et al.* 2004; Hyten *et al.* 2006; Liu & Burke 2006; Haudry *et al.* 2007). Long generation times and overlapping generations in trees can reduce the occurrence of domestication bottlenecks (Austerlitz *et al.* 2000). The particular breeding system of cultivated trees may also limit the loss of genetic diversity due to selection; as with apples (Cornille *et al.* 2012), many cultivated tree peonies have been selected from seedlings derived from open pollination in gardens.

In contrast to the lack of recent genetic bottlenecks in cultivated tree peonies, a bottleneck footprint has
occurred in a few populations of wild tree peonies. The lower diversity of wild tree peony species or subspecies, such as *P. rockii* ssp. *atava* and *P. jishanensis*, compared to cultivated peonies is likely due to their distribution decreasing in extent during recent decades because of habitat change caused by natural factors and human overexploitation (Li 2005; Hong 2010; Yuan et al. 2011, 2012). Several wild peony species have been listed as rare and endangered taxa in the Red Book of Chinese Plant Species (Fu 1992). In contrast, the diversity in domestic cultivars is maintained by vegetative propagation that occurs as part of agricultural activity. Continuous habitat destruction and fragmentation in wild tree peonies is expected to result in smaller and more isolated populations, raising the inbreeding coefficient at the species level and further increasing the risk of population extinction, as reported for *P. rockii* (Cheng 2007; Yuan et al. 2011, 2012). Given the low genetic diversity and fragmentation of wild populations, more efficient and stringent conservation policies should be established for wild tree peonies to maintain their genetic variation as a resource for future breeding programmes.

### Conclusion

This study provides the first example of the independent domestications of perennial tree crops and illustrates the complexity of the process of domestication of such crops, which can involve contributions to the genetic makeup of cultivated tree genomes from multiple species, populations and locations (Myles et al. 2010; Cornille et al. 2012; Besnard et al. 2013). The results of this study also provide crucial insights into the domestication history of some of the most famous ornamental trees, the peonies, that are valuable for improving current tree peony breeding strategies. Future research requires extending the investigation of the origin of the worldwide peony gene pool far from its centre of origin in China.

The parallel and convergent evolution of cultivated tree peonies represents an interesting case study for understanding the mechanisms of domestication, diversification and adaptation in plants. Future studies focusing on adaptive genetic variation in tree peonies will likely yield important insights into the processes of evolution. It would be interesting for instance to determine whether the same genes, or even the same mutations, are involved in the traits shared by the FTPs and CTPs.

### Acknowledgements

We thank Myriam Heuertz and two referees for comments on a previous version of this manuscript. We also deeply thank Mr. Jiajue Li, Mr. Xingyun Cheng, Mrs. Yialing Tian, Dr. Jia Wang, Mr. Jianhong Wang and others for their help with the sample collections. We thank Dr. Shi-Liang Zhou for kindly providing the experimental platform. This study was funded by the National Natural Science Foundation of China (No. 31171984 and 31070617) and Technologies R&D Program of Shanghai (10391901200).

### References


© 2013 John Wiley & Sons Ltd


© 2013 John Wiley & Sons Ltd


Yuan JH, Cheng FY, Zhou SL (2012) Genetic structure of the tree Peony (Paeonia rockii) and the Qinling Mountains as a geographic barrier driving the fragmentation of a large population. PLoS ONE, 7, e39455.


J.Y. and F.C. conceived and designed the experiments and provided samples; J.Y., F.C. and Y.H. obtained funding; J.Y. performed the experiments; J.Y. and A.C. analysed the data and contributed analysis tools; J.Y., A.C., T.G. and Y.F. wrote the article.

Data accessibility

Sampling locations and microsatellite genotypes are deposited in the DRYAD repository, doi:10.5061/dryad.vk43j.
Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The proportions of ancestry of genotypes of cultivated common tree peonies, flare tree peonies and the four wild tree peony species, *Paeonia jishanensis*, *Paeonia rockii* ssp. *atawa*, *Paeonia rockii* ssp. *rockii* and *Paeonia decomposita* (*N* = 553) from *K* = 2 to *K* = 8 ancestral gene pools (‘clusters’) inferred with the STRUCTURE program using the full data set. Each individual is represented by a vertical bar partitioned into *K* segments representing the amount of ancestry of its genome in *K* clusters. For cases in which several clustering solutions (‘modes’) were represented within replicate runs, the proportion of simulations represented by each mode is given.

Fig. S2 The proportions of ancestry of genotypes of cultivated common tree peonies, flare tree peonies and the four wild tree peony species, *Paeonia jishanensis*, *Paeonia rockii* ssp. *atawa*, *Paeonia rockii* ssp. *rockii* and *Paeonia decomposita* (*N* = 323) from *K* = 2 to *K* = 8 ancestral gene pools (‘clusters’) inferred with the STRUCTURE program using the pruned data set (i.e. excluding clonal and related individuals). Each individual is represented by a vertical bar partitioned into *K* segments representing the amount of ancestry of its genome in *K* clusters. Where several clustering solutions (‘modes’) were represented within replicate runs, the proportion of simulations represented by each mode is given.

Fig. S3 Bayesian inference of the number of ancestral gene pools (‘clusters’, *AK*) from cultivated common tree peonies, flare tree peonies and the four wild tree peony species or subspecies, *Paeonia jishanensis*, *Paeonia rockii* ssp. *atawa*, *Paeonia rockii* ssp. *rockii* and *Paeonia decomposita* (*N* = 553).

Fig. S4 The proportions of ancestry in two ancestral gene pools inferred with the STRUCTURE program and based on the full data sets for (a) the common tree peony (red, *N* = 72, left) and (b) the flare tree peony (purple, *N* = 103, left) and each of four wild tree peony species (right) and (c) for the common tree peony (red, left) and the flare tree peony (purple, right). Details are provided in Tables S6 and S7.

Fig. S5 Distribution of the posterior quantiles based on 1000 pseudo-observed data sets. *P*-values were computed with a Kolmogorov–Smirnov test of uniformity of the posterior quantile distribution.

Table S1 Description of the cultivated common tree peony, flare tree peony and wild tree peony accessions analysed with their geographic origins, providers, latitudes (N), longitudes (E), altitudes and sampling sizes.

Table S2 Repeat motifs, annealing temperatures (Ta), primer sequences and range of alleles detected for each of the 14 microsatellite loci.

Table S3 The genotypes of the 553 accessions at 14 microsatellite loci.

Table S4 Genetic polymorphism within the cultivated common and flare peonies and the four wild peony species or subspecies at the population level.

Table S5 Mean membership coefficient and the α value at *K* = 2 between (a) cultivated common tree peonies and (b) flare tree peonies (Genepool 1) and each of the four wild tree peony species or subspecies and (c) between the cultivated common tree peonies and flare tree peonies determined using the STRUCTURE program.

Table S6 The mean proportions of assignment to each of the two ancestral gene pools in pairwise comparisons (*K* = 2) including common tree peonies as Genepool 1 and each of the four wild tree peony species or subspecies or flare tree peonies as Genepool 2.

Table S7 The mean proportions of assignment to each of the two ancestral gene pools in pairwise comparisons (*K* = 2) including flare tree peonies as Genepool 1 and each of the four wild tree peony species or subspecies or common tree peonies as Genepool 2.
Dear Author,

During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper’s edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

<table>
<thead>
<tr>
<th>Query reference</th>
<th>Query</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AUTHOR: Please identify and encircle the forename and surname of all authors.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AUTHOR: Please provide full address details for affiliation “¶”.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AUTHOR: Please provide fax number for all corresponding authors.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AUTHOR: Gross &amp; Olsen 2011 has not been included in the Reference List, please supply full publication details.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AUTHOR: Iwata et al. 2000 has not been included in the Reference List, please supply full publication details.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AUTHOR: Li 2006 has not been included in the Reference List, please supply full publication details.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AUTHOR: Zhang et al. 2009 has been changed to Zhang et al. 2011 so that this citation matches the Reference List. Please confirm that this is correct.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AUTHOR: Arnaud-Haond &amp; Belkhir 2006 has been changed to Arnaud-Haond and Belkhir 2007 so that this citation matches the Reference List. Please confirm that this is correct.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AUTHOR: Garza &amp; Williamson 2001 has not been included in the Reference List, please supply full publication details.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AUTHOR: Wegmann (2009 and 2010) has been changed to Wegmann et al. (2009, 2010) so that this citation matches the Reference List. Please confirm that this is correct.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>AUTHOR: Blackman et al. (2011) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>AUTHOR: Please check author name “I Joly H” for reference Delplancke et al. (2013).</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>AUTHOR: Flowers et al. (2012) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: He et al. (2011) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Hufford et al. (2012) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Kiani et al. (2010) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Li et al. (2010) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Matsuoka et al. (2002) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Please provide the page range for reference Mevik and Wehrens (2007).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Please check page range for reference Molina et al. (2011).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Molina et al. (2011) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Morrell et al. (2011) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Tellier et al. (2011) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Tian et al. (2009) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Wills and Burke (2006) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Yang et al. (2012) has not been cited in the text. Please indicate where it should be cited; or delete from the Reference List.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: If you would like the figures in your article to appear as colour in print, please promptly post or courier the completed hard copy of the Colour Work Agreement Form (including payment information) to this mailing address: Customer Services (OPI) John Wiley &amp; Sons Ltd European Distribution Centre New Era Estate, Oldlands Way Bognor Regis West Sussex PO22 9NQ The form and charge information can be found online at: <a href="http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-294X/homepage/ForAuthors.htm">http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-294X/homepage/ForAuthors.htm</a>.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AUTHOR: Please provide the publisher Location for reference Zhou (2007).</td>
<td></td>
</tr>
</tbody>
</table>
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)

The latest version of Acrobat Reader can be downloaded for free at: [http://get.adobe.com/reader/](http://get.adobe.com/reader/)

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We’ve picked out some of these tools below:

1. **Replace (Ins) Tool** – for replacing text.

   ![Replace (Ins) Tool](image)

   How to use it:
   - Highlight a word or sentence.
   - Click on the Replace (Ins) icon in the Annotations section.
   - Type the replacement text into the blue box that appears.

2. **Strikethrough (Del) Tool** – for deleting text.

   ![Strikethrough (Del) Tool](image)

   How to use it:
   - Highlight a word or sentence.
   - Click on the Strikethrough (Del) icon in the Annotations section.

3. **Add note to text Tool** – for highlighting a section to be changed to bold or italic.

   ![Add note to text Tool](image)

   How to use it:
   - Highlight the relevant section of text.
   - Click on the Add note to text icon in the Annotations section.
   - Type instruction on what should be changed regarding the text into the yellow box that appears.

4. **Add sticky note Tool** – for making notes at specific points in the text.

   ![Add sticky note Tool](image)

   How to use it:
   - Click on the Add sticky note icon in the Annotations section.
   - Click at the point in the proof where the comment should be inserted.
   - Type the comment into the yellow box that appears.
5. **Attach File Tool** — for inserting large amounts of text or replacement figures.

How to use it
- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp Tool** — for approving a proof if no corrections are required.

How to use it
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups Tools** — for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

How to use it
- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: