

Molecular phylogeny of wild Hops, *Humulus lupulus* L.

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We have analysed wild hops collected widely from the Northern Hemisphere, assessing the genetic diversity and the geographical distribution of haplotypes, to investigate the evolution and phylogeny of hops, *Humulus lupulus*. The haplotypes were characterized by the nuclear ribosomal DNA spacer region (length and DNA sequence) and chloroplast DNA noncoding regions (DNA sequences). The results indicated that primary divergence into European (including Caucasus and Altai hops), and Asian-North American types, was 1.05 ± 0.28 to 1.27 ± 0.30 million years ago. Although an Eastern boundary for European nuclear haplotype distribution was unclear due to the ambiguous origin of Northern Chinese samples, the

European hop group showed a wide geographical distribution across Eurasia from the Altai region to Portugal. The low genetic variation in this group suggested rapid and recent expansion. The North American hop group showed high diversity, and is considered to include hops that have migrated from Asia. Japanese and Chinese hops were identified as genetically distinct. This study has shown that wild hops in each growing region are genetically differentiated with considerable genetic diversity. It gives insights into the evolution and domestication of hops that are discussed.

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Introduction

The hop plant, *Humulus lupulus*, is a dioecious perennial species, and only female cones are used for beer brewing. Hops are used to impart bitterness, flavour and preservation properties to modern beers, as established in Europe. However, similar beers first appeared in Ancient Mesopotamia and Egypt as early as the Neolithic (Behre, 1999).

The genus *Humulus* consists of three species, *H. lupulus*, *H. japonicus* and *H. yunnanensis* (Small, 1978; Neve, 1991). Wild hops of *H. lupulus* are widely distributed throughout the Northern Hemisphere, Europe, Asia and North America (Small, 1978; Neve, 1991). Small (1978) classified *H. lupulus* into a number of taxonomic varieties; var. *lupulus* for European wild hops and cultivars, var. *cordifolius* for Japanese wild hops, and var. *neomexicanus*, *pubescens* and *lupuloides*, for North American hops. The varieties differ in morphological characteristics such as number of lobes on the leaf and hairs on the bine. North American and Japanese wild hops resemble each other morphologically, suggesting a genetically close relationship, while they differ widely from European hops (Neve, 1991).

Commercial hop production occurs in many countries. Hop cultivars have been roughly classified into two groups, one group consisting of European landraces while the other has a genetic composition derived from North American wild hops (Seefeldler *et al*, 2000; Jakše *et al*, 2001). However, analysis of five microsatellite loci showed limited genetic variation among major hop cultivars throughout the world. It has been suggested that the use of a restricted number of hop varieties for breeding has caused this narrow range of genetic variation (Jakše *et al*, 2001).

Some important characteristics of modern cultivars, such as disease resistance and high levels of alpha-acids (which are converted to bitter compounds in beer), are known to originate from wild hops (Neve, 1991). Pillay and Kenny (1996) reported a difference between European and North American hops in the 18s-26s ribosomal DNA length (rDNA). Ribosomal DNA occurs in large tandem repeats, which are affected by gene conversion and unequal crossing over. The intergenic spacer (IGS) was found to exhibit sufficient variation to enable the study of closely related cultivars, populations and individuals. Murakami (2001) also found significant nucleotide variation among wild hops in the external transcribed spacer (ETS) of the IGS region of 18s-26s rDNA (IGS), although the number of wild hops studied was limited.

The history of hops and their usage is surrounded by uncertainty. It is not known when hops were first used

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for beer brewing in Europe (Wilson, 1975; Delyser and Kasper, 1994). How hops were domesticated in Europe is also unknown although pollen records suggest hop usage had already started in the early Roman era (Wilson, 1975). It has been assumed that the origin of European cultivars and wild hops was either from central Europe, brought by people migrating from the East, or simply from cultivation of native wild hops. However, it has been suggested that European wild hops themselves originated from past introductions and they do not represent an autochthonous European plant (Rybáček, 1991; Moir, 2000). Little is known about the phylogenetic relationships among wild hops and their genetic variability, nor about their origin and migration routes (Neve, 1991; Pillay and Kenny, 1996; Murakami, 2000, 2001). Phylogenetic analysis among wild hops from around the world could give insights into the evolution and domestication of hops.

In this paper, we analyze a large number of wild hops collected from around the world to assess their genetic diversification based on nuclear and chloroplast DNA differences. Nuclear DNA analyses made use of the IGS length of 18s-26s rDNA and the 537 bp sequence of ETS. A total of 12 noncoding regions of chloroplast DNA (cpDNA) were sequenced (8 kb in total nucleotides). Such regions are generally considered to be free from evolutionary constraint and where a relatively high rate of molecular evolution is expected (Demesure *et al*, 1995).

Materials and methods

Plant materials

The wild hops, *H. lupulus*, used in this study were collected widely from the Northern Hemisphere: European

countries (Austria, Czech Republic, Germany, Hungary, Italy, Poland, Portugal and Ukraine), the Caucasus region (located between the Black Sea and the Caspian Sea), the Altai region (close to western China, located on boundaries of Russia, Mongolia, Kazakhstan and China), China, Japan and North America (Canada and the USA) as shown in Figure 1 and Appendix 1. Unfortunately, records of the locality of collection of Chinese hops were ambiguous. Three samples from China were recorded as just 'Mongolia', which could be either Mongolia or inner Mongolia autonomous region. Similarly, two Chinese samples were described as 'Southern Chinese region', which could be either Sichuan or Yunnan province. In this study, the 'Mongolia' samples are described as Northern Chinese hops, and the Sichuan or Yunnan province samples are termed Southern Chinese hops.

All materials were maintained as rootstocks, at the authors' organizations. *H. japonicus* was collected in Yokohama city, Japan.

DNA analyses

DNA was extracted using a Plant DNA extraction mini kit (Quiagen), followed by purification according to Murakami (2000).

IGS length and ETS sequence were examined among 147 individuals as detailed in Table 1, and PCR was performed according to the method of Murakami (2000). PCR fragments were purified by a High Pure PCR Products Purification kit (Boehringer Mannheim), and sequencing of ETS nucleotides was performed by the method described by Murakami (2001).

For the cpDNA analyses, primers (Table 2) designed by Demesure *et al* (1995) for specific noncoding regions were used, and 125 individuals were examined (Table 1).

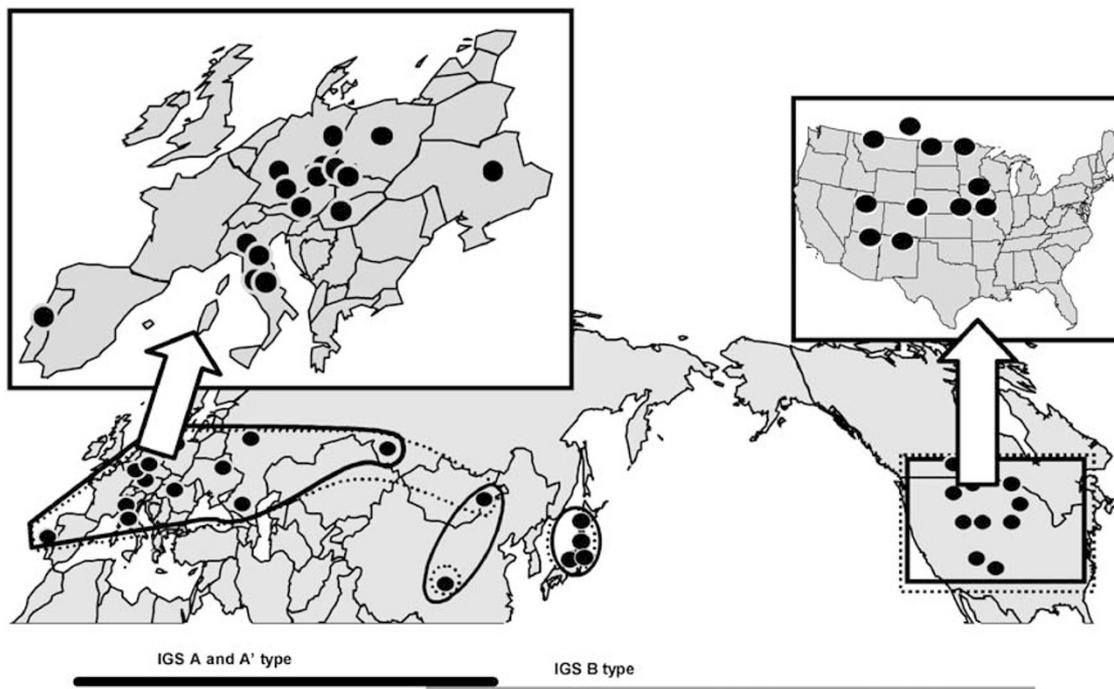


Figure 1 Geographical locality of samples and distribution of haplotypes. Black circle indicates the location of samples used in the analyses. Solid and dotted lines show distribution of cpDNA and ETS sequence defined haplotypes, respectively. Distribution of two IGS length haplotypes is also shown as horizontal bars.

Table 1 Summary of haplotypes defined by nuclear and chloroplast DNA

Area	Country	Haplotypes (number of samples)			
		Nuclear		Chloroplast	
		IGS	ETS	Sequence	Indel
Europe	Germany	A(12)	E(12)	cpE(12)	inE(12)
	Czech	A(36)	E(36)	cpE(18)	inE(18)
	Portugal	A(20)	E(20)	cpE(14)	inE(14)
	Other ^a	A(11)	E(11)	cpE(11)	inE(11)
Altai		A(2)	E(2)	cpE(2)	inE(2)
Caucasus		A(1)	E(1)	cpE(1)	inE(3)
		A'(2)	E'(2)	cpE'(2)	
Asia	Northern China ^b	A(3)	E(3)	cpNC(3)	inNC(3)
	Southern China ^c	B(2)	SC(2)	cpSC(2)	inSC(2)
	Japan	B(34)	J(25) J'(9)	cpJ(34)	inJ(34)
North America	Canada USA	B(24)	NA1(19)	cpNA1(3)	inNA1(2)
			NA2(1)		inNA1'(1) ^d
			NA3(2)	cpNA2(15)	inNA2(15)
			NA4(2)	cpNA3(8)	inNA3(8)

^aIncluding samples from Austria (number of individuals: $n = 1$), Hungary ($n = 2$), Italy ($n = 5$), Poland ($n = 1$) and Ukraine ($n = 2$).

^bCollected in either Mongolia or inner Mongolia autonomous region. It was not determined clearly due to poor information on collection.

^cCollected in either Sichuan or Yunnan province. It was not determined clearly due to poor information on collection.

^dThe inNA and inNA1' differed by one indel, but the remaining sequence was identical as cpNA1.

Table 2 cpDNA noncoding region analysis

Primer pair	Location of the primer on cpDNA	Annealing (°C)	Extention (min)	Fragment size (approx. kb)	Primer for PCR and sequencing, sequence (5'-3')	No. of nucleotide (bp)
<i>trnH</i>	tRNA-His(GUG)	65	1	1.6	ACGGGAATTGAACCCGCGCA	604
<i>trnK</i>	tRNA-Lys(UUU)exon1				CCGACTAGTTCGGGTTCGA	545
<i>trnC</i>	tRNA-Cys(GCA)	53	3	2.6	CCAGTTCAAATCTGGGTGTC	975
<i>trnD</i> (1)	tRNA-Asp(GUC)				GGGATTGTAGTTCAATTGGT	1160
<i>trnD</i> (2)	tRNA-Asp(GUC)	53	1	1.2	ACCAATTGAACTACAATCCC	541
<i>trnT</i>	tRNA-Thr(GGU)				CTACCACTGAGTAAAAGGG	595
<i>psbC</i>	psII 44kd protein	53	1	1.6	GGTCGTGACCAAGAAACCAC	545
<i>trnS</i> (1)	tRNA-Ser(UGA)				GGTTCGAATCCCTCTCTCTC	577
<i>trnM</i>	tRNA-Met(CAU)	55	3	3.0	TGCTTTCATACGGCGGGAGT	568
<i>rbcL</i>	RuBisCo large subunit				GCTTTAGTCTCTGTTTGTGG	686
<i>psaA</i>	PS I (P700 apoprotein A1)	65	3	4.0	ACTTCTGGTTCGGCGAACGAA	705
<i>trnS</i> (2)	tRNA-Ser(GGA)				AACCACTCGGCCATCTCTCCTA	712
					Total	8213

Figures in parentheses of primer pair indicate different locus or region if they have the same name.

The PCR product of a single band was partially sequenced from the primer used in PCR, and, in the cases of *trnC* and *trnD*(1) regions, additional primers, designated as *trnC*plus (TATCTTGAGTCTATATT TTA TAC) and *trnD*(1)plus (GCATTTATTGCTACTGCACT), respectively, were designed and used for further sequencing. Sequencing was performed by a Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) and the ABI PRISM™ 3100 Genetic Analyzer.

Sequences aligned using Clustal W (Thompson *et al*, 1994) were analysed using MEGA2 (Kumar *et al*, 2001) and the neighbor joining tree was evaluated by repeating

the analysis using 1000 bootstrap samples. For relative rate testing, *H. japonicus* was used as an out-group. Kimura's two-parameter method was used to determine the nucleotide substitution rate per site. Combined ETS and cpDNA noncoding sequence analysis was also carried out by the maximum parsimony method implemented in MEGA2, using the close neighbor interchange algorithm for branch swapping, random addition, 500 replicates and 100 bootstrap samples. Indels found in cpDNA noncoding regions can also provide evolutionary information (Giribet and Wheeler, 1999) and were used to build a reduced median network using the Network

software (Bandelt *et al*, 1995). The sequences used in this study have been registered in DDBJ under the accession numbers AB112490–AB112539, AB112596–AB112655 and AB112750–AB112769.

Results

Nuclear DNA

IGS length: The primers for IGS PCR resulted in multiple bands and in order to improve specificity, the '18s-IGS' primer was changed to '19' (Murakami, 2001). A length difference in the single amplified band was found among the wild hops, and it distinguished three haplotypes (Table 1). European, Altai and northern Chinese hops showed a single band of approximately 4.0 kb, designated as IGS A type. Caucasus hops were found to have either IGS A type or a 3.8 kb band, designated as IGS A' type. Samples from southern China, Japan and North America were characterized by a band of approximately 3.2 kb, designated the IGS B type.

ETS sequence difference: The 537 bp ETS sequence revealed two haplotypes, differing by two nucleotides, in European, Altai and Caucasus hops. They were designated as E and E', and were linked with the IGS A and A' types, respectively. Northern Chinese hops showed haplotype E, while southern Chinese hops had a different DNA sequence that was designated SC. Two haplotypes differing by a single nucleotide (haplotype J and J') were found in Japan. In North American hops, four haplotypes were found, designated as haplotype NA1 through NA4, and the average nucleotide variation among them was 2.27. The number of individuals having each haplotype defined by the ETS sequence is shown in Table 1.

Nucleotide substitution rates among hops grown in each area (group) varied from 0.0113 ± 0.0045 (Japan *versus* southern China) to 0.0170 ± 0.0057 (Europe *versus* Japan). No significant rate differences among the haplotypes were found by the relative rate test using *H. japonicus* as an out-group. Neighbor joining tree analysis of the haplotypes showed four clades as (i) Europe–Altai–Caucasus–northern China, (ii) North America, (iii) Japan and (iv) southern China, respectively (Figure 2a).

cpDNA

Sequence differences: In all, 12 cpDNA noncoding regions were combined, and were analysed by the relative rate test using *H. japonicus* as an out-group. A significantly faster nucleotide substitution rate ($P < 0.01$) was found in European haplotypes, and it seemed to be caused by two regions, *trnH* and *trnS(1)*. These two regions were therefore excluded, and the remaining 7032 bp of nucleotides were used for the analyses of DNA sequence. Eight haplotypes were distinguished by nucleotide differences found in the cpDNA noncoding regions. Among hops in Europe, Altai and Caucasus, two haplotypes differed by a single nucleotide, and they were designated as cpE and cpE', respectively. The cpE' haplotype was, however, found only in two of the three Caucasus samples. A two nucleotide difference could distinguish northern Chinese hops (haplotype cpNC) from southern ones (haplotype cpSC). In the North

American hops, three haplotypes were identified (haplotype cpNA1 through cpNA3), and the average nucleotide difference among them was 3.67. There was no variation among Japanese hops (haplotype cpJ). While the nucleotide differences among Chinese, Japanese and North American hops were relatively small, from 0.0009 ± 0.0003 to 0.0013 ± 0.0004 , the comparisons between the haplotypes in Europe, Altai and Caucasus, and the others showed higher values, from 0.0020 ± 0.0005 to 0.0024 ± 0.0006 (Table 3). Construction of a neighbor joining tree showed four clades: (i) Europe–Altai–Caucasus, (ii) Japan, (iii) Northern–Southern China and (iv) North America. (Figure 2b).

Estimation of divergence time: Evolutionary divergence time was estimated using the molecular clock hypothesis from the combined sequence of cpDNA noncoding regions. The heterogeneity of the nucleotide substitution rate within the genus *Humulus* was determined by the relative rate test using *Cannabis sativa*, another member of the Cannabinaceae, as an out-group. Its *rbcL* sequence is available on the DNA data Bank (DDBJ AJ390068). The test revealed nonsignificant rate variation between *H. lupulus* and *H. japonicus* at the third codon position. To estimate the time of divergence between *H. lupulus* and *H. japonicus*, we used a previously reported nucleotide difference of the *rbcL* (Murakami, 2000) and the rate of 1.23×10^{-9} per site per year, used for analyses of many species (Xiang *et al*, 2000). The time of divergence between *H. lupulus* and *H. japonicus* was calculated as 6.38 million years ago which would give an averaged nucleotide substitution rate per site per year of 0.93×10^{-9} , for combined cpDNA noncoding regions.

Similarly, the time of divergence among Chinese, Japanese and North American haplotypes was estimated as between 0.46 ± 0.17 and 0.69 ± 0.21 million years ago, while the European haplotypes were estimated to have diverged from the others between 1.05 ± 0.28 and 1.27 ± 0.30 million years ago (Table 3).

Maximum parsimony analysis of combined ETS and cpDNA noncoding region

Combined sequences of parsimony-informative sites in ETS and cpDNA noncoding regions gave eight haplotypes in North America (ETS and cpDNA haplotype combinations: NA1cpNA1, NA2cpNA1, NA2cpNA2, NA3cpNA1, NA3cpNA2, NA3cpNA3, NA4cpNA1 and NA4cpNA3), while two haplotypes were found in Europe. One haplotype was found both in Southern China and Japan. Northern Chinese hops were excluded from this analysis because of their ambiguous origin.

Parsimony analysis of the 47 informative sites generated three minimal trees with a tree length of 60 substitutions. The trees showed the same topology except for the branching pattern within the North American clade. The overall consistency index was 0.850 and the retention index 0.869. The bootstrap consensus tree is shown in Figure 2c where the primary division is into European and Asian–North American types. The bootstrap support was high for the European lineage (100%) and moderate (72%) for the Asian–North American lineage.

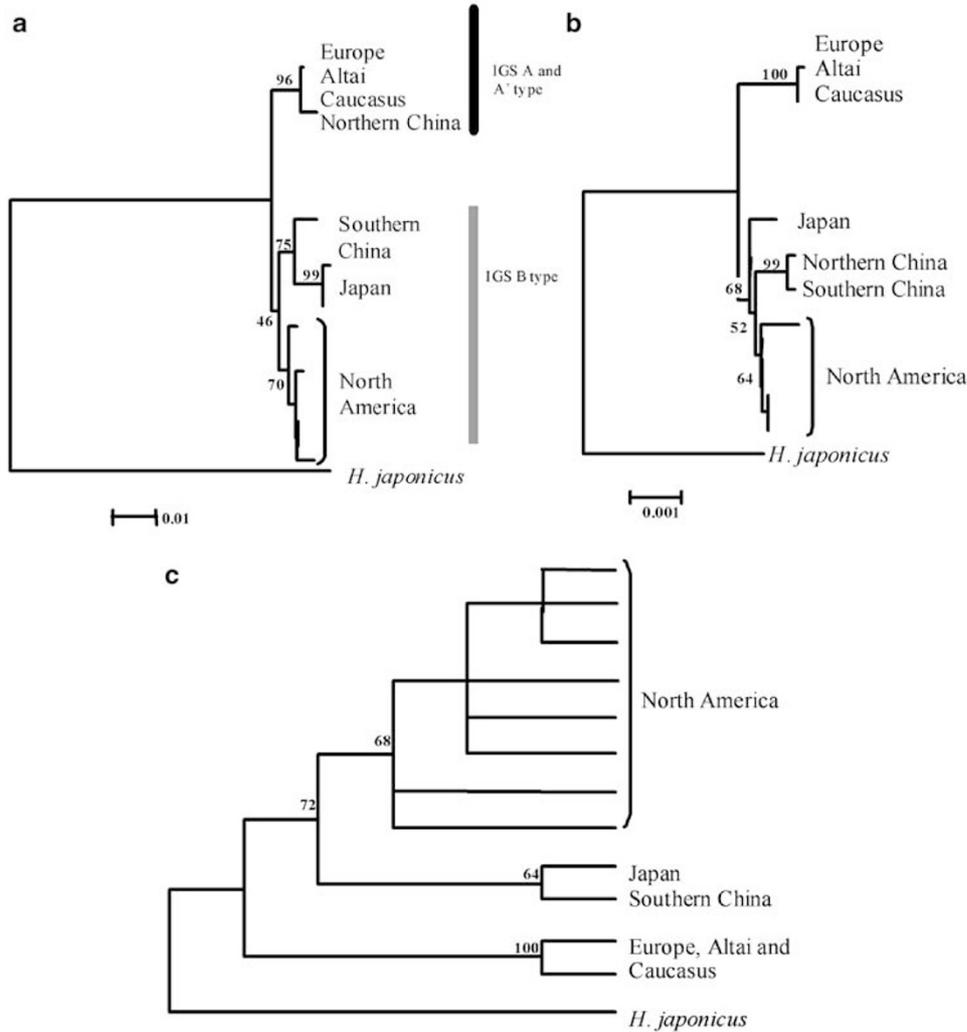


Figure 2 Phylogenetic trees of haplotypes. (a) Neighbor joining (NJ) tree of haplotypes identified by partial external transcribed spacer sequence (537 bp) and intergenic spacer (IGS) type of nuclear 26S-18S rDNA. For each clade, a 1000 bootstrap value is shown. Vertical bars show IGS types having IGS A and B type with approximately 4.0 and 3.2 kb lengths, respectively. IGS A' type with approximately 3.8 kb length is found only in two Caucasus hops. The scale bar indicates nucleotide substitution rate per site determined by Kimura's two-parameter method. (b) NJ tree of haplotypes identified by combined sequence (7032 bp) of cpDNA noncoding regions. For each clade, a 1000 bootstrap value is shown. The scale bar indicates the same as shown in Figure 2a. (c) Maximum parsimony analysis of combined ETS and cpDNA noncoding region. Northern Chinese hop samples were excluded from this analysis because of their ambiguous origin (see text). A total of 47 informative sites generated three minimal trees with tree length of 60 substitutions, an overall consistency index of 0.850 and retention index of 0.869.

Table 3 Nucleotide substitution rate per site and estimated divergence time based on the difference of combined cpDNA noncoding regions

	Europe	China	Japan	North America	<i>H. japonicus</i>
Europe		0.0024 ± 0.0006	0.0020 ± 0.0005	0.0020 ± 0.0005	0.0120 ± 0.0012
China	1.27 ± 0.30		0.0013 ± 0.0004	0.0013 ± 0.0003	0.0122 ± 0.0012
Japan	1.05 ± 0.28	0.69 ± 0.21		0.0009 ± 0.0003	0.0115 ± 0.0012
North America	1.05 ± 0.26	0.69 ± 0.18	0.46 ± 0.17		0.0117 ± 0.0012
<i>H. japonicus</i>	6.45 ± 0.66	6.55 ± 0.64	6.16 ± 0.65	6.28 ± 0.64	

The top diagonal indicates nucleotide substitution rate per site between the populations using Kimura's two-parameter method, and the bottom diagonal shows divergence time ($\times 10^6$ years) estimated by the averaged rate, 0.93×10^{-9} per site per year. All values are obtained using cpDNA noncoding regions, accounting for 7032 bp, excluding *trnH* and *trnS(1)*.

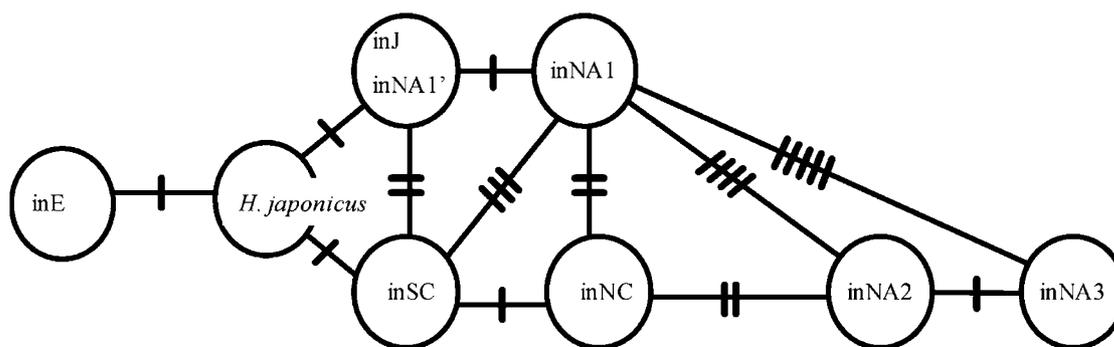
Structural differences among hops

Seven relatively large indels of at least 10 bp were found in the cpDNA sequenced, and they could distinguish seven haplotypes as detailed in Tables 1 and 4. A reduced median network between the haplotypes is

shown in Figure 3, which also contains the position of *H. japonicus*. The primary split of the network separating inE (Europe–Caucasus–Altai) haplotype from the others occurs at this position. From the latter, two lineages appear distinguishing Japanese haplotype, inJ, from

Table 4 Indels (>10 bp) found in cpDNA noncoding regions

Area (haplotype)	DNA region				trnD(2)	trnH	trnM
	trnC 1	2	3	4			
Europe,Caucasus and Altai (inE)	—	—	—	—	—	Δ13	Δ84
Southern China (inSC)	—	—	—	Δ10	—	Δ13	—
Northern China (inNC)	—	—	—	Δ10	—	—	—
Japan (inJ)	—	—	—	—	—	—	—
North America (inNA1')	—	—	—	—	—	—	—
(inNA1)	—	—	+32	—	—	—	—
(inNA2)	—	Δ180	—	Δ10	+22	—	—
(inNA3)	+30	Δ180	—	Δ10	+22	—	—
Out group <i>H. japonicus</i>	—	—	—	—	—	Δ13	—

**Figure 3** Reduced median network of haplotypes defined by indels found in all cpDNA noncoding regions. A vertical bar on the connected line between haplotypes indicates one indel.

southern Chinese haplotype, inSC. One of haplotypes found in North America, inNA1', shows the same indel as that of inJ, and it connects to inNA1 haplotype. The inNA1 and inNA1' haplotypes differed by one indel (Table 4), but the remaining cpDNA sequence was identical.

The network places Northern Chinese haplotype, inNC, following inSC haplotype. Other haplotypes, inNA2 and inNA3, are on the line from inNC haplotype, showing four and five indel differences from inNA1 haplotype, respectively.

Discussion

Molecular phylogenies by nuclear DNA, cpDNA and their combined sequence

This study has shown that the phylogenetic trees reconstructed from nuclear DNA (ETS) and cpDNA sequence data agree in terms of topology, except for the position of northern China (discussed later). The haplotypes, characterized by ETS, were deeply and primarily divided

into European, including Caucasus and Altai hops, and Asian-North American types, although only a poor bootstrap value (46%) supported the Asian-North American clade (Figure 2a). Analyses both of cpDNA noncoding regions (Figure 2b) and the combined sequence (Figure 2c) resulted in the same topology showing two clades of Europe and Asia-North America, with the bootstrap values on the trees (Figure 2b and c) moderately supporting the Asian-North American clade, 68 and 72%, respectively. Morphological, cytological and chemical studies have also indicated a close relationship between Asian and North American hops (Neve, 1991; Stevens *et al*, 2000). Thus, Chinese, Japanese and North American wild hops may be closely related to each other.

This study has shown the primary evolutionary divergence to be into European and Asian-North American clades and, although Chinese samples were limited in number, the geographic distribution suggests that this deep divergence occurred in China. Our study differentiated two clades, the European clade located in Western China and the Asian-North American clade in Eastern China, respectively (Figure 1). In addition to the

haplotype distribution shown here, support for the origin of the genus *Humulus* and hops, *H. lupulus*, being in China, as hypothesized by Neve (1991) is provided by the presence of related species, *H. japonicus* and *H. yunnanensis*. These are indigenous only to Eastern Asia (Small, 1978; Neve, 1991) and are not found in North America.

North American hops and migration

Significant differences in anatomical characteristics among North American hops have been found, and three taxonomic varieties have been identified (Small, 1978). This study showed four and three haplotypes defined by nuclear and chloroplast DNA nucleotides, respectively. At the molecular level, these results confirm a genetic diversity within North American hops. Estimated divergence time among haplotypes within the North American clade varied from 0.08 (cpNA1 versus cpNA2) to 0.54 (cpNA1 versus cpNA3) million years ago, suggesting that the North American population includes both recent and long-diverged haplotypes.

The network of indels places North American haplotypes on the lines extended from Chinese and Japanese haplotypes (Figure 3), suggesting that North American hops are derived from genetically distinct Asian populations, nowadays recognized as Chinese and Japanese hops. This suggests that a heterozygous hop population migrated into North America or, alternatively, that multiple migrations from distinct populations have occurred.

If the origin of hops is, indeed, China (Neve, 1991), migration must have occurred between the continents. In many plant species, an eastern Asia – eastern North America disjunction – has been found (Liu *et al*, 2002). It is considered to have been formed several million years ago in the late Tertiary period, as estimated from the divergence time between the species *Caulophyllum*, *Penthorum*, *Phryma*, *Podophyllum*, *Decumaria*, *Campsis* and *Cornus*, in each region. However, one exceptional species, *Menispermum*, diverged and migrated within the last 0.31 million years, that is, the Quaternary period (Xiang *et al*, 2000). Our study indicates that North American and Asian hops diverged from 0.46 ± 0.17 to 0.69 ± 0.21 million years ago in the Quaternary period, and hop migration into North America would appear to have proceeded as for *Menispermum* species.

Northern Chinese hops

Northern Chinese hops gave inconsistent results from the nuclear DNA and cpDNA analyses. Nuclear DNA (ETS sequence and IGS length) of Northern Chinese hops was identified unequivocally as of European type, while the sequence of their cpDNA noncoding was close to that of Southern Chinese hops, only differing by two nucleotides, but was rather different from that of European haplotypes, cpE and cpE', differing by 17 and 16 nucleotides, respectively. These results suggest the possibility that the Northern Chinese samples were a hybrid of Chinese and European hops.

Historical evidence of the origin of hop production in northern China indicates that it began with introduced female hop cultivars from Europe (Barth *et al*, 1994). Introgression between these introduced cultivars and a wild native population may have produced a haplotype

having a mixture of European nuclear and Chinese cytoplasmic DNA. It is clear that further studies are needed to clarify whether they are, indeed, genuine indigenous wild plants.

European hops

The entire geographical distribution of the European nuclear haplotypes remains unresolved due to the ambiguous origin of our Northern Chinese samples. However, it is evident that the European haplotypes, determined by nuclear DNA and cpDNA, show a wide distribution ranging between Portugal and the Altai region.

Fossil pollen data has shown that European tree species, for example, Oak (*Quercus*), Pine (*Pinus*) and Alder (*Alnus*), migrated and expanded throughout Europe after the last glacialiation, and a high migration rate was observed, representing from a few hundreds to thousands meters per year (Ferris *et al*, 1999). Scots pine (*Pinus sylvestris*) is distributed from Scotland to east Siberia, and the current population structure in Europe was formed 7000–900 years ago (Dvornyk *et al*, 2002). In North America, lodgepole pine (*Pinus contorta*) expanded into a range of 2200 km over 12 000 years (Marshall *et al*, 2002). The distribution of European hop haplotypes found in our study covers a range of 7000 km, calculated as the distance between Portugal and the Altai region. This wide distribution suggests that migration, or gene flow, occurred across Eurasia at a high rate, similar to those for European trees (Ferris *et al*, 1999).

It has also been suggested that European wild hops themselves originated from past introductions, brought by people migrating from the East, and that they do not represent an autochthonous European plant (Rybáček, 1991; Moir, 2000). If human behavior is involved in hop migration, it would be expected that population size would be reduced. One model of founder speciation involves severe but short-term founder events that reduce microsatellite allele variation by loss of rare alleles without substantially reducing heterozygosity, the test for heterozygosity excess could detect such kind of founder events (Cournuet and Luikart, 1996). A recent microsatellite DNA study of 11 loci (Murakami *et al*, 2006) analysed wild hops, in almost the same samples used in this study. It found highly polymorphic microsatellites, but no significant excess heterozygosity ($P > 0.05$) in European wild hops, thus providing no evidence of recent founder events of this type.

Nuclear and chloroplast DNA analyses in this study have indicated that genetic differentiation of European hops was found only in the Caucasus region. A phylogenetic tree based on microsatellite DNA also showed the Caucasus hops to be deeply divergent from a large cluster of European hops (Murakami *et al*, 2006). This differentiation could have arisen if the Caucasus region was genetically isolated from other European populations, perhaps as one of the refugia located in southern Europe area during glacial periods. A similar effect has been observed in some species of European tree (Ferris *et al*, 1999).

Thus, our study of European haplotype distribution has allowed important aspects of their history to be deduced. The current distribution suggests a similar history to European trees, which are thought to have

been affected by the last glacial periods. After the last glaciation, hops may have expanded from southern refugia and, since a founder event was not detected, this may have occurred naturally rather than through human association.

Microsatellite analyses indicate that cultivars share alleles with the European native population and would be classified within this population (Jakše et al, 2004; Murakami et al, 2006, unpublished data). This strongly suggests that traditional cultivars were simply selected from natural wild populations, presumably because of their superior qualities for beer production.

Breeding and genetic resources

Although all modern cultivars have much common ancestry (Seefelder et al, 2000; Jakše et al, 2001), some important characteristics have been derived from wild hops. It appears that high alpha-acids content and resistance to *Verticillium* wilt disease originated from North American wild hops (Neve, 1991). In this study, North American wild hops were confirmed to be distinct and a high degree of genetic variation still remains within them. Similarly, Japanese and Chinese hops are identified as genetically differentiated hops and thus wild germplasm from these regions may be a new genetic resource to expand genetic variation for breeding. Indeed, a new characteristic, a high level of resistance to aphids, was found in a wild male hop collected in Japan (Darby, 1999).

In addition to the agronomic and brewing characteristics of hops, their pharmacological properties have recently been studied with promising results (eg Stevens et al, 1998). Different compositions of medicinal components have been found in wild hops (Stevens et al, 2000). This strongly suggests that wild hop resources have potential in new applications as well as within the brewing industry.

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Appendix 1

Summary of sample.

<i>Region</i>	<i>Country</i>	<i>District or origin</i>	<i>Number of individual</i>
Europe	Germany	Berlin and Ostsee	4
		Frankfurt	1
	Czech Republic	Munich	7
		Žatec	16
		Litoměřice and Ústěk	8
		Central Moravia	5
		Eastern Bohemia	7
	Portugal		20
	Italy	Garda lake	1
		Nera valley	2
		Cascia valley	1
		Norcia valley	1
		Salzburg	1
		Platten lake	2
		Kazimierz	1
Ukraine		2	
Caucasus	Russia		3
Altai	Russia	Biysk town	2
Asia	China	Inner Mongolia or Mongolia	3
		Sichuan or Yunnan	2
	Japan	Hokkaido island	4
		Northern main island	14
	Central main island	16	
North America	Canada	Saskatchewan	1
		Montana	7
	US	North Dakota	2
		Minnesota	1
		Missouri	1
		Utah	1
		Corolado	5
		Iowa	1
		Nebraska	2
		Arizona	2
		New Mexico	3
Total		149	