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Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation

**Running title: A fig tree with multiple pollinator fig wasps**

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### **Competing interests**

The authors declared that they have no competing interests.

## **Abstract**

The ways that plant-feeding insects have diversified is central to our understanding of terrestrial ecosystems. Obligate nursery pollination mutualisms provide highly relevant model systems of how plants and their insect associates have diversified and the over 800 species of fig trees (*Ficus*) allow comparative studies. Fig trees can have one or more pollinating fig wasp species (*Agaonidae*) that breed within their figs, but factors influencing their number remain to be established. In some widely distributed fig trees, the plants form populations isolated by large swathes of sea, and the different populations are pollinated by different wasp species. Other *Ficus* species with continuous distributions may present genetic signatures of isolation by distance, suggesting more limited pollinator dispersal, which may also facilitate pollinator speciation. We tested the hypothesis that *Ficus hirta*, a species for which preliminary data showed genetic isolation by distance, would support numerous pollinator species across its range. Our results show that across its range *Ficus hirta* displays clinal genetic variation and is pollinated by nine parapatric species of *Valisia*. This is the highest number of pollinators reported to date for any *Ficus* species and it is the first demonstration of the occurrence of parapatric pollinator species on a fig host displaying continuous genetic structure. Future comparative studies across *Ficus* species should be able to establish the plant traits that have driven the evolution of pollinator dispersal behaviour, pollinator speciation and host plant spatial genetic structure.

**Keywords:** coevolution, dispersal, gene flow, mutualism, plant growth form, speciation.

## **Introduction**

Insects feeding on plants represent one of the most common forms of trophic interaction seen in terrestrial ecosystems, and the relationship between plants and insects has generated the most species-rich animal taxa ever seen on the planet (Price 1980, 2002). These insects are typically associated with one or a few related host plants, so different plants host different insects (Strong, Lawton, & Southwood, 1984). Processes such as local adaptation, the geography of speciation, and the consequences of secondary sympatry are key issues in understanding the dynamics of community diversification (Mittelbach, & Schemske 2015). However, almost no studies have explored how the insect community on a plant changes across its geographic range (Leather 1986, Lawton, Lewinsohn & Compton 1993, Lewinsohn, & Roslin 2008). A study across Papuan rainforests found little variation in herbivorous insect communities over distances of up to 1000 km (Novotny et al., 2007, Craft et al., 2010) while a study of non-pollinating fig wasps on *Ficus rubiginosa* Desf. ex Ventenat in Australia detected, within a set of seven widespread morphospecies, four pairs of parapatric cryptic species, along a 2000 km transect (Darwell, & Cook, 2017).

In nursery pollination mutualisms, pollinating insects breed in floral structures of the plants they pollinate (Dufay, & Anstett, 2005). Insects involved in nursery pollination mutualisms constitute particularly favorable biological models for investigating the geographic variation of diversity on a host plant as the insect's prevalence on a host is high and their presence is easy to detect. Furthermore, some of these mutualisms are species rich allowing comparative studies. Nursery pollination systems such as those involving *Yucca* and *Yucca* moths (Pellmyr, 2003), *Glochidion* and *Epicephala* moths (Kawakita, 2010) and fig trees (*Ficus*) and fig-wasps (*Agaonidae*) (Cook, & Rasplus, 2003), were initially envisioned as systems in which a single species of pollinating insect breeds in and pollinates a single host plant

species. This simple pattern is now largely rejected because of accumulating examples where one insect species pollinates several host species (Pellmyr, 1999; Hembry et al., 2018; Cornille et al., 2012; Wachi, Kusumi, Tzeng, & Su, 2016; Wang, Cannon, & Chen, 2016), or, cases where several insect species pollinate a single host (Darwell, et al., 2014; Li, Wang, & Hu, 2015, Yang et al., 2015). A general model of plant-insect diversification in these nursery pollination mutualisms must therefore take into account the factors influencing their relative rates of speciation and explain why some of the plants have a single pollinator when others have two or more.

Fig trees (*Ficus*) represent the most species-rich group of plants offering nursery pollination rewards and the most diversified lineage within the Moraceae (Brunn-Lund, Verstraete, Kjellberg, & Rønsted, 2018). Fig trees are pollinated by female fig wasps that enter *Ficus* inflorescences to lay their eggs. Because fig wasps carry pollen from their natal plants, they only transfer pollen from those plants in which their larvae can develop. This is a more direct link between suitability for offspring development and subsequent pollen transfer than in any other brood pollination mutualism and may favour co-adaptation (Anstett, Hossaert-McKey, & Kjellberg, 1997). However, a more rapid rate of speciation among fig wasps, compared with their hosts, is to be expected given their much shorter generation times (Petit & Hampe, 2006; Thomas, Welch, Lanfear, & Bromham, 2010). From this perspective, the long-held assumption that each fig tree was pollinated by a single species of pollinator was problematic.

More extensive sampling, and molecular techniques that have facilitated separation of morphologically close ('cryptic') species have revealed an increasing number of *Ficus*

species with several pollinators (Darwell, al-Beidh, & Cook, 2014). However, we still know little about how genetic diversity is structured within *Ficus* species and how this is related to the distributions and genetic diversity among the fig wasp pollinators that it supports (Bain et al., 2016; Wachi, Kusumi, Tzeng, & Su, 2016; Rodriguez et al., 2017). Where several pollinator species share a single *Ficus* host, they are generally sister species (Yang et al., 2015). The recorded exceptions, where non-sister pollinator species occupy the same host species, have almost all been within two *Ficus* clades that have diversified recently, and this appears to have favoured host shifts (Cruaud et al., 2012; Jousselin et al. 2008; Machado, Robbins, Gilbert, & Herre, 2005; Yang et al., 2015). The main modes of fig wasp speciation therefore appear to vary across lineages.

Widely-distributed plants grow in areas with a wide range of environmental conditions, which will vary in suitability for their associated insects, including their pollinators. Reflecting this, plant-eating insects usually only occupy a sub-set of the ranges of their hosts (Strong, Lawton, & Southwood, 1984), but fig trees require an effective and specific pollinator to be present wherever they grow. Widely distributed *Ficus* species would therefore be predicted to support additional species of pollinator compared to species with narrow, more climatically homogeneous, ranges. Only a few widely-distributed *Ficus* species have been sampled at multiple sites across their geographic ranges. *Ficus racemosa* L. is pollinated by a single fig wasp across India and by another in China and SE Asia, with additional sister species of pollinators in the island of Borneo and Australia (Bain et al., 2016; Kobmoo, Hossaert-McKey, Rasplus, & Kjellberg, 2010). More localised differentiation is evident among island populations of *F. septica* Burm. f., in the Philippines (Conchou, Cabioch, Rodriguez, & Kjellberg, 2014; Lin, Yeung, Fong, Tzeng, & Li, 2011;

Rodriguez et al., 2017), and along elevation gradients (Segar et al., 2017, Souto-Vilarós et al., 2018), where several different pollinators are present.

The absence of divergence among the pollinators of *F. racemosa* across large swathes of its continental range is likely to reflect their ability to disperse over large distances. Spatial genetic structuring across the range of a plant species provides an indication of the geographical extent of their gene flow, and in the case of *Ficus* it has revealed a widely-varying extent of dispersal among their pollinators. Paternity analyses have shown that some fig wasps transport pollen between large fig trees growing over 100 km apart (Ahmed, Compton, Butlin, & Gilmartin, 2009), whereas population structuring shows that other, smaller, species have much more localised gene flow (Chen, Zhu, Compton, Liu, & Chen, 2011; Liu, Compton, Peng, Zhang, & Chen, 2015). These differences in dispersal behaviour among the pollinators of different trees appear highly likely to influence differentiation and speciation of both their own populations as well as those of the host plants they pollinate.

Previous data has shown that *Ficus hirta* Vahl presents a pattern of spatial genetic structure suggesting genetic isolation by distance across continental South-East Asia (Yu, & Nason, 2013). Here, based on extensive sampling, we describe the population genetic structure of *F. hirta* and its pollinating fig wasps across most of their continental range. We addressed the following questions (1) To what extent is the population structure of the plant co-incident with that of its pollinator(s) – have they diversified at the same spatial scales? (2) Even in the absence of major geographic barriers, is this widespread host tree pollinated by a diverse assemblage of fig wasps within its range? And (3) if so, what are the ecological consequences – do individual plants have the chance to receive pollination services from more than one fig

wasp species? This is the first study comparing spatial genetic structuring in a widespread fig species and its pollinating wasps that encompasses most of their ranges. It is also the first broad scale study of joint genetic structuring between pollinator fig wasps and a fig tree species displaying genetic isolation by distance. We discuss the results in terms of the factors that may have resulted in the patterns of diversification we observed and what they suggest about modes of speciation.

## **Materials and Methods**

### **(a) Study species**

Like all *Ficus* species, *F. hirta* (family Moraceae, subgenus *Ficus*, section *Eriosycea*, Berg, 2003) has unique protogynous inflorescences called figs. These are hollow and lined internally with numerous tiny male and female florets. Pollination can only be achieved when pollen is carried by female fig wasps from their natal fig into receptive figs through a narrow aperture called the ostiole. Like about half of all *Ficus* species, *F. hirta* is functionally dioecious, with figs that differ in floral structure on ‘male’ and ‘female’ trees. Figs of the former support the development of the fig wasp offspring that when mature can transport pollen to receptive figs. In contrast, the figs on female trees develop seeds, and no pollinator offspring. *Ficus hirta* is a species of secondary, disturbed habitats, and typically grows as a shrub or treelet. It can reach a maximum height of about 5 m, but most mature individuals are much shorter (Berg, & Corner, 2005). Its figs are small and produced in the leaf axils. Female figs ripen to a bright red and are mainly eaten by birds (Corlett, 2006). As many as 50 figs can be present on a tree, but their development is asynchronous, which reduces the peak numbers of figs available for pollination or dispersal at any one time and can also allow pollinator cycling between figs on the same male tree (Jia, Dan, Zhang, & Chen, 2007).



The distribution of *F. hirta* extends northwards from the island of Java (Indonesia) in the south to China in the north and westwards into north-east India, Nepal and Sikkim (Berg, & Corner, 2005). This extensive geographical range (covering over 30 degrees of latitude and 30 degrees of longitude) encompasses a range of tropical and sub-tropical biomes. Berg (2007) restricted *F. hirta* to what was previously known as *F. h.* subsp. *hirta*. In the following, we will use the name *F. hirta* for *F. hirta* sensu Berg (2007). Following that definition, *F. hirta* has a disjunct distribution. South of Thailand it is absent from Peninsular Malaysia and it is only present in South Sumatra and North Java (Berg, & Corner, 2005). Population genetic studies, mainly from China, detected some spatial genetic structuring across *F. hirta* populations and concluded that low nuclear differentiation, combined with high inter-population differentiation and geographic structuring of chloroplast variation, indicated that gene flow via seed dispersal was more limited than via dispersal of pollen (Yu, & Nason, 2013). Genetic differentiation in nuclear genes between populations of *F. hirta* on the Chinese mainland and Hainan Island has also been detected, and corresponding differences were also present between the populations of the tree's pollinators (Tian et al., 2015). Two morphologically-distinguishable pollinator fig wasps have been described from the figs of *F. hirta*. *Valisia javana javana* Mayr was reared from *F. hirta* figs collected in Java, and a second sub-species, *Valisia j. hilli* Wiebes was described from figs collected in Hong-Kong (Wiebes, 1993).

## **(b) Sampling**

Between 2006 and 2014, we sampled the leaves of *F. hirta* (31 locations) and its pollinating fig wasps (32 locations) across mainland South-East Asia and south to the island of Java

(Table S1, Supporting information). The samples were up to 4100 km apart (North-South), and reached the northern, eastern and southern limits of the range of *F. hirta*.

Within locations, *F. hirta* individuals were typically sampled at intervals of 3–5 m, with no individuals located more than 200 m apart. Their leaves were collected and dried in plastic bags containing silica gel. At each location, 10-30 figs containing mature fig wasp offspring were also removed from the plants and placed individually in fine-mesh bags, where the fig wasps were allowed to emerge. The pollinators were then separated from other species of fig wasps and preserved in 95% ethanol that was stored at -20°C until DNA extraction. A single female fig wasp per fig was used for genetic analyses.

### **(c) Pollinating wasps**

#### **(i) Pollinating wasp DNA extraction, amplification and sequencing**

The mitochondrial genetic marker mtCOI was sequenced from an average of 9.9 individuals per location (range 1–28, total 330), and microsatellite loci were genotyped from an average of 18.9 other individuals from the same locations (range 2-32, total 568). To complement the data, the nuclear ITS2 nuclear gene was amplified for 201 individuals.

Genomic DNA was extracted from the whole body of each fig wasp using the EasyPure Genomic DNA Extraction Kit (TransGen, Beijing, China). A 681bp fragment of the mtCOI gene was then sequenced following the protocol used in previous studies (Tian et al., 2015). A 689 bp fragment of the ITS2 gene was amplified in 201 individuals using the universal primer pair (ITSR : 5'- CGCCTGATCTGAGGTCGTGA-3' , ITSF : 5'- ATTCCCGCACCACGCCTGGCTGA-3' (Lopez-Vaamonde, Rasplus, Weiblen, & Cook,

2001) and the same PCR amplification reaction volume as for the COI gene. The reaction was optimized and programmed on a MJ Thermal Cycler (PTC 200) as one cycle of denaturation at 94°C for 5min, 35 cycles of 30 s denaturation at 94°C, 30 s at a 55°C annealing temperature, and 30 s extension at 72°C, followed by 8 min extension at 72°C. All amplified PCR products were purified using QIAquick spin columns (Qiagen) and were sequenced in an ABI 3730xl capillary sequencer using BigDye Terminator V 3.1 chemistry (Applied Biosystems).

Previously published protocols were also used to genotype individuals at nine unlinked microsatellite loci (1-78, 1-141, A34, A80, A99, B30, C25, F17, H33) that had been previously developed for *Valisia j. hilli* (Tian, Yu, Zhang, & Nason, 2011). The alleles used in the present study were the same as those used previously to analyze South-East China and Hainan populations (Tian et al., 2015).

#### **(ii) Pollinating fig wasp sequence analysis**

We did not detect any indications of pseudo-genes, such as multiple peaks in chromatograms, stop codons or frame shift mutations (Song, Buhay, Whiting, & Crandall, 2008). Sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) with manual corrections.

Previous work has shown that the fig wasp genus *Valisia*, including samples collected from *Ficus hirta*, is monophyletic (Cruaud et al., 2010). We downloaded from GenBank the COI sequences of the eight *Valisia* species available including one sequence (FJ619191) of *V.*

javana. Published phylogenies have shown that the genera *Ceratosolen* and *Kradibia* constitute an outgroup relative to all other pollinating fig wasps (Cruaud et al., 2010; Cruaud et al., 2012). We included representatives of these two genera (2 species of *Ceratosolen* and 11 species of *Kradibia*) in the phylogenetic analysis.

Dated phylogenetic trees that included the downloaded sequences and all our sequenced haplotypes were estimated using Bayesian methods. The best-fit model, GTR+I+G, was selected by hierarchical likelihood ratio tests in the program Modeltest 3.7 (Posada, & Crandall, 1998), where GTR+I+G was favoured. We ran BEAST 2.3.1 (Bouckaert et al., 2014) to explore the best combinations of substitution, clock and population models, with 10 million generations. Twice the difference in Ln harmonic mean of the likelihood of each model combination (LnBF) (Nicholls et al., 2010) was calculated with TRACER 1.6 to assess the preferred models, based on the LnBF table (Kass, & Raftery, 1995). Using the GTR+I+G model, we ran BEAST under a strict clock, an uncorrelated exponential relaxed clock or lognormal relaxed clock with each population model (constant size, exponential growth, yule process, birth-death process). Bayes Factors indicated that the combination of an uncorrelated exponential clock and the exponential population growth model was the best (with LnBFs from 0.47 to 198.19 and most > 15).

Two independent runs of 30 million iterations were performed, with genealogies and model parameters sampled every 1000 iterations. The chain convergence was checked based on ESSs (effective sample sizes) viewed in TRACER1.6. All ESSs for each parameter had to be larger than 200. The phylogenetic tree was summarized by TREEANNOTATOR 1.8.1, and then viewed by FIGTREE 1.3.1 (Rambaut, 2006). The dates of the most recent common

ancestor were scaled by a mutation rate of 1 because no suitable fossil record was available to calibrate node ages. We used the published 34.8 (46.5-24.2) Ma age for the crown group of the genus *Valisia* that had been estimated previously for a very similar set of *Valisia* species that used numerous genetic markers and a comprehensive set of species within Agaonidae (Cruaud et al., 2012). We used the phylogenetic tree to detect candidate species characterized by low within-clade genetic distances and much larger between-clade genetic distances.

We calculated Kimura-2-parameter (K2P) distances within and between clades for COI haplotypes using MEGA 6.06. Tajima's D, Fu and Li's D and Fu and Li's F (Fu, & Li, 1993) were used to detect signatures of population expansion or selection using DnaSP 5.0 (Librado & Rozas, 2009). We then explored the relationships of COI haplotypes within each clade using phylogenetic networks built separately for each clade with TCS 1.21 (Clement, Posada, & Crandall, 2000) using the 95% statistical parsimony criterion as a connection limit, with loops in the network resolved following Crandall & Templeton's methods (1993). Variation in COI sequences among populations within clades and within populations was partitioned using hierarchical analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.0 (Excoffier et al., 2005). Significance tests were based on 10,000 permutations.

We used jMOTU as a complementary method to detect molecular operational taxonomic units (MOTU) using a range of threshold differences. All the COI sequences used in the phylogenetic analysis were analysed using jMOTU at cut-offs from 0 to 21 bases (Jones,

Ghoorah, & Blaxter, 2011). The aggregation parameter was 97%, and the input minimum sequence percentage was set to 95%.

As ITS2 evolves more slowly than COI, we applied a simplified analysis of the data. We calculated Kimura-2-parameter (K2P) distances within and between clades as for COI haplotypes using MEGA 6.06. and we used a maximum likelihood tree to reconstruct the phylogenetic relationships based on all ITS haplotypes. The ML tree was reconstructed using MEGA 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), and node supports were assessed based on 2000 bootstrap replicates.

### **(iii) Pollinating fig wasp microsatellite data analysis**

Classical indices of genetic diversity were estimated using GenALEX 6.1 (Peakall, & Smouse, 2006). In order to represent the global data, we performed a factorial correspondence analysis following Nenadić, & Greenacre (2007) as implemented in GENETIX (Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme, 1996-2004). Because multiple locus population genetics data should not be analysed using phylogenetic trees, we constructed an unweighted neighbour joining tree of multilocus microsatellite genotypes using DARwin v6 (Perrier & Jacquemoud-Collet, 2006), thus grouping genotypes according to the numbers of shared alleles, without taking into account gene evolution. Bootstrap number was set at 2000. Finally, we used Bayesian clustering to assign multilocus microsatellite genotypes to clusters using STRUCTURE 2.2 (Pritchard, Stephens, & Donnelly, 2000). The admixture ancestry and correlated allele frequencies model was used with five independent runs each of 500,000 MCMC iterations and 500,000 burn-in steps. We ran STRUCTURE with varying K values (the number of clusters) from 2 to 30 (the

maximum number of populations available for microsatellite analysis) to explore how different values of K resulted in the assignment of species and populations to different clusters. The resulting assignment patterns were explored to detect co-occurrence of different clades within a sampling location. Results obtained from the factorial correspondence analysis, neighbour joining tree and Bayesian clustering were compared.

The presence of a relationship between (log) geographic distance and genetic differentiation  $F_{ST}/(1-F_{ST})$  values (isolation by distance) was evaluated with GenALEx 6.1 within the two clades detected in the above analysis and for which data from more than three locations were available, using a Mantel test with 10,000 permutations. When structuring was detected within clades, the distribution of pairwise  $F_{ST}$  values was explored to detect potential structuring into sub-clades.

#### **(d) *Ficus hirta***

##### **(i) *Ficus hirta* DNA extraction and amplification**

We used previously-published methodological details for cpDNA and nuclear microsatellite sequencing and genotyping (Yu, & Nason, 2013), and re-used all the previously published cytoplasmic data. The set of analyzed microsatellite loci was reduced to avoid some amplification/reading problems, leaving seven microsatellite loci available: FS4-11, Frub38, Frub398 and Frub436 (Yu, & Nason, 2013), and FH3, FH10 and FH47 (Zheng, Nason, Liang, Ge, & Yu 2015). Using these, we analyzed plant microsatellite data from two locations in South-East China and two locations in Hainan, and cytoplasmic data from 14 locations in South-East China and Hainan. This was in addition to the microsatellite data and

cytoplasmic data from 17 locations elsewhere within the range of the plant, so we had a total of 21 locations with microsatellite data and 31 locations with cytoplasmic data.

### **(ii) *Ficus hirta* cytoplasmic DNA analysis**

The sequences of the two cpDNA regions were concatenated (1367 bp) and then aligned using Muscle in Mega 6.06 with manual corrections. A matrix of combined sequences for trnL-trnF and trnS-trnG was constructed, and haplotypes were distinguished on the basis of nucleotide and insertion/deletion differences. The same procedures as for wasp COI data were used to determine polymorphism indices, detect signatures of population expansion and establish haplotype networks.

We used AMOVA to test the significance of cpDNA differentiation among populations (999 permutations) and tested for genetic isolation by distance by conducting a Mantel test of the correlation between  $F_{ST}/(1-F_{ST})$  and log transformed geographical distance for all population pairs (999 permutations).

### **(iii) *Ficus hirta* microsatellite analyses**

Parameters of genetic diversity were calculated as for pollinator microsatellite data. As with the pollinating wasp microsatellite data, we performed a factorial correspondence analysis, we build a neighbour joining tree, and we used Bayesian clustering to assign multilocus microsatellite genotypes to clusters. The number of clusters was set to vary from 1 to 21.



The relationship between  $F_{ST}$  and geographic distance was plotted to visualize genetic differentiation. The significance of the correlation between  $F_{ST}/(1-F_{ST})$  and the log geographic distance was evaluated as with the insects.

## **Results**

### **(a) Pollinating fig wasps**

#### **(i) Pollinating fig wasp gene sequences**

After alignment and exclusion of sites with gaps, 502 bp long COI sequences were obtained for phylogenetic analysis. A total of 162 haplotypes were obtained and 267 COI polymorphic sites were identified from the 330 sequenced fig wasps (Table S2, Supporting information). Haplotype sequences have been deposited in GenBank under accession Nos. KR873011 - 47 and MF472722 - 846. A 689 bp fragment of the ITS2 gene was amplified in 201 individuals. A total of 18 haplotypes were obtained. The sequences have been deposited in GenBank under accession Nos. MF467418-467426 and MF467428-467436.

The Bayesian COI tree separated the *Ficus hirta* pollinating fig wasps into nine clades (Fig. 1). Gene sequence differences were weak within clades (Kimura-2-parameter = 0.001-0.014) and high between clades (0.064-0.272) (Fig. 1, Fig. S1, Table S3, Supporting information). The between-clade distances are similar to those between currently recognized Agaonidae species (Chen, Compton, Liu, & Chen, 2012; Yang et al., 2015).

The ITS2 maximum likelihood tree separated the *Ficus hirta* pollinating wasps into seven clades (Fig. S2, Table S4, Supporting information). ITS2 data was obtained for several

individuals from each of 30 of the 31 locations for which COI data was obtained (Table S1, Supporting information). The ITS2 sequences grouped the genotypes according to sampling location (Table S5) in the same way as the COI sequences (Table S2, Supporting information), except that the ITS2 sequences provided less resolution and pooled the locations that gave COI clade 2 and COI clade 9 and pooled the locations that gave COI clade 6 and COI clade 7. Hence, cytoplasmic and nuclear sequence data provided consistent location-level assignment to clades, although the ITS2 sequences provided less resolution.

The program jMOTU groups sequences into clades (MOlecular Taxonomic Units) so that the minimum difference, expressed in numbers of bases, between two different MOTUs is larger than the chosen cutoff value. With increasing cutoff values, the number of inferred MOTUs drops rapidly and then stabilizes when within-species variation is included within a single MOTU, before dropping again when closely related species are pooled. The number of clades inferred from the COI sequences dropped to 33 MOTUs for a cutoff value of 9 and remained constant up to a cutoff value of 11 (Fig. S3, Supporting information). For these cutoff values, all the clades recognized in the COI tree were separated into single MOTUs except for clade 7, which was separated into 3 MOTUs and clade 6 that was also separated into 3 MOTUs. Starting at the cutoff value of 17 bases (which is the 2.5% cutoff threshold value proposed as a general rule-of-thumb for species discrimination with COI; Jones, Ghoorah, & Blaxter, 2011) and up to a cutoff value of 21 bases, jMOTU gave 29 MOTUs. Each of the 9 clades recognized from the phylogenetic tree (Fig. 1) was recognized as a single MOTU and the two sequences of fig wasps (AY842415 and HM802690) collected from *Ficus langkokensis* grouped into a single MOTU. Hence, the jMOTU analysis supports

the proposition that each clade recognized in the COI tree is a separate operational taxonomic unit, i.e. a species.

Overall, the different analyses of sequence data support the conclusion that clades 1-9 can be considered as distinct species, and this conclusion is applied from here on (as sp1-sp9).

The pollinators collected in Singapore (sp5) formed part of a distinct clade that had the largest gene sequence differences relative to the other species pollinating *F. hirta* (for cytoplasmic COI, Fig. 1, Table S3, Supporting information, for nuclear ITS2, Fig. S2, Table S4, Supporting information). This clade included a COI GenBank sequence (AY642456) of a fig wasp collected from *F. androchaete* Corner, a *Ficus* species endemic to Borneo, where *F. hirta* is absent (Berg, & Corner, 2005). The other major clade comprised sp1-4 and sp6-9 reared from *F. hirta*, plus the GenBank sequence for *Valisia esquiroliana* Chen & Chou. The dated tree obtained with BEAST (Fig. S1, Supporting information) suggests that separation of sp5 from the remaining eight species associated with *F. hirta* began about 16 (11-22) Ma and the two tightly knit subgroups of species (sp2+sp9) and (sp4+sp6+sp7) diverged from each other about 8 Ma (Fig. S1, Supporting information). These two species groups were also recovered in the ITS2 phylogeny (Fig. S2, Supporting information). The estimated dates of divergence among the taxa within these subgroups that we are recognizing as distinct species varied between 4.6 (sp2+sp9 and sp4+sp6+sp7) and 6.0 (sp1+sp3) Ma.

A striking feature was the limited overlap in the distribution of species. We only observed examples of a pair of species occurring together in two locations, location CS (with sp2 and a low frequency of sp3) in North Thailand and Wu in North East Thailand (with sp4 together

with small numbers of sp7) (Fig. 2a). In addition, GenBank accession FJ619191 belonged to sp3 and was recorded from XI in SW China, an area where we only collected sp2. Where species were collected from several sites their distributions were generally geographically coherent, but sp7 has a disjoint distribution. It is present in East Thailand and in Java, but absent from peninsular Malaysia where its host plant is also absent and is apparently replaced in peninsular Thailand by sp8 (Fig. 2a).

AMOVA revealed considerable haplotype variation within species, as well as within and among locations (Table S6, Supporting information). The exception was sp5, where haplotype diversity was strikingly low, with only two almost identical haplotypes represented in the 17 individuals (Fig. S4, Supporting information). In the two more extensively sampled species we observed negative deviations from neutral assumptions in both sp1 (Tajima's D value = -1.51,  $p=0.055$ ; Fu and Li's D = -2.75,  $p<0.05$  and Fu and Li's F = -2.69,  $p<0.05$ ) and sp2 (Tajima's D = -1.88,  $p<0.05$ ; Fu and Li's D = -3.49,  $p<0.01$  and Fu and Li's F = -3.40,  $p<0.01$ ). Deviations were not significant in the other species. The COI haplotype networks (Fig. S4, Supporting information) revealed a subdivision in sp1 into a South-East China group of haplotypes, a Hainan Island group of haplotypes and a Vietnamese group of haplotypes, with some haplotype exchange between South-East China and Hainan Island.

#### **(ii) Pollinating fig wasps microsatellite data**

Diversity indices for microsatellite markers are given in Table S7, Supporting information. Pairwise genetic differentiation between location values ( $F_{ST}$ ) are given in Table S8, Supporting information.

The first 8 components of the discriminant analysis separated the 9 species (Fig. S5, Supporting information). The analysis also shows differentiation within sp1 according to geographic origin (China, Hainan, and North Vietnam), as suggested by the COI analysis (Fig. S4, supporting information). While ITS2 sequences did not allow separation of sp2 from sp9 and sp6 from sp7, the discriminant analysis of microsatellite data separates them without ambiguity on component 2. Hence, the discriminant analysis confirms the presence of 9 nuclear genetic clusters corresponding to the species detected using the cytoplasmic COI sequences.

In the neighbour joining tree (Fig. 3a), the different species are again separated, except for sp5, which seems to be close to sp1 from Vietnam, a result that is not compatible with the nuclear and cytoplasmic sequence data. The subdivision of sp1 into three geographic entities is supported. The close proximity between sp4 and sp6 is also supported, as is the slight separation within sp7 depending on geographic origin.

The bar plots of assignments of individuals to clusters using STRUCTURE with variable numbers of clusters are presented in Fig. S6a, Supporting information. Irrespective of the different values of K, assignments to clusters are generally strong, except for the south China locations for sp1 for which mixed assignments suggest the presence of some variation among locations. From K=6 to 22, the populations of sp1 from Hainan island and Vietnam are each assigned to their own cluster. For K=11, all the species are separated into different clusters except for sp4 and sp6 (Fig. 4a). For K=20 and above, sp4 and sp6 are separated into distinct clusters. For K=22 and above the individuals of sp7 collected in Thailand were perfectly

separated from those collected in Java. Consistently, the separation of sp3 to sp9 is somewhat unstable across the different values of K. As with the COI data, and despite different individuals being used for COI and microsatellite analyses, the microsatellite data suggested assignment of one individual to sp3 in location CS and another individual to sp7 in location Wu.

Genetic isolation by distance could only be explored for sp1 and sp2 as the other species were only sampled in 1-3 locations. There was significant isolation by distance, as shown by the relationship of  $F_{ST}/(1-F_{ST})$  with distance (Mantel test) in sp1 ( $R^2 = 0.13$ ,  $P = 0.008$ ), but not in sp2 ( $R^2 = 0.023$ ,  $P = 0.459$ ). However, the signature of isolation by distance in sp1 was generated by the differentiation of sp1 into the three genetically differentiated geographic entities already detected in the COI, in the discriminant and in the STRUCTURE analyses (located in South-East China, Hainan and Vietnam, Fig. 5), despite obtaining a single ITS2 haplotype for sp1 (with 33, 11 and 9 individuals genotyped respectively). There was no genetic isolation by distance within any of these three geographic entities.

## **(b) *Ficus hirta***

### **(i) *Ficus hirta* cpDNA analysis**

We detected 24 haplotypes (H1–H24). Sequences of the trnL-trnF and trnS-trnG regions have been deposited in GenBank under accession Nos. GQ452019 - 32 and MF467405 - 16. We did not detect any signature of selection or of population expansion on cpDNA sequences. The cpDNA network and the distribution of these haplotypes among populations are presented in Fig. 2b and Table S9, Supplementary information. The haplotype network is centered on haplotype H8 and no haplotype differed from H8 by more than 6 mutations

(Fig. 2b). Haplotype H8 was observed throughout the range of *F. hirta*. Haplotype diversity was high in the north-central part of our sampling zone, where sampling density was highest. AMOVA confirmed significant differentiation among populations ( $F_{ST} = 0.799$ ;  $P < 0.001$ ). The colors correspond to different branches of the network and their geographic distribution suggests spatial structuring. The regression of population pairwise  $F_{ST}$  on the natural logarithm of geographical distance was also significant ( $R^2 = 0.0472$ ,  $P = 0.003$ , Mantel test), confirming that there was spatial genetic structure among populations of the plant.

#### **(ii) *Ficus hirta* microsatellite analysis**

Genetic diversity parameters are given in Table S10, Supplementary information.

The first four components of the discriminant analysis organized the data along a North-South axis, except for the genotypes from Singapore, which were placed close to the northern genotypes (Fig. S7, Supporting information). The discriminant analysis suggests much more continuous genetic variation in *F. hirta* than in its pollinators (compare Fig. S5 and S7, Supporting information).

As with the discriminant analysis, the neighbour joining tree separates the data along a north-south axis (Fig. 3b). The Singapore samples and the Trang samples each form highly homogeneous clusters. As in the discriminants analysis, the Trang samples are placed with southern locations while the Singapore samples are placed with northern locations.

The bar plots of assignments of individuals to clusters of varying total number using STRUCTURE are presented in Fig. S6b, Supporting information. For  $K=2$ , genotypes from all northern locations plus Singapore were mainly assigned to cluster one and genotypes from southern locations were mainly assigned to cluster two (Fig S6b, Supporting information). The geographic distribution of assignments (Fig. 2a) showed: 1) progressive geographic genetic variation of *F. hirta* along a north-south axis, 2) some geographic east-west geographic genetic structure and 3) a placement of the Singapore samples that does not correspond to its geographic location. For  $K=4$ , genotypes from the northern locations had mixed assignments to two clusters, supporting an east-west spatial genetic structure, genotypes from Singapore still clustered with genotypes from the north, and genotypes from Trang formed a distinct group (Fig. 4b).

We also explored spatial genetic structure by plotting genetic differentiation between locations against geographic distance (Fig. 6). Comparisons between locations showed a general pattern of genetic isolation by distance ( $R^2 = 0.204$ , permutation test,  $P = <10^{-3}$ ). Singapore was an exception, as it was most similar to the geographically distant northern locations. Pairwise genetic differentiation between location values ( $F_{ST}$ ) are given in Table S11, Supporting information.

Taken together, the factorial analysis, the STRUCTURE assignments to clusters and the neighbour-joining tree all suggest that the genetic diversity of *F. hirta* is organized according to a pattern of genetic isolation by distance. Despite their southern location, the Singapore genotypes belong with the northern genotypes. Furthermore, the local pollinator (sp5) is only distantly related to the other pollinator species.



## **Discussion**

### **Species status of the pollinators**

Although different pollinating wasp individuals were genotyped using COI, ITS2 and microsatellites, the parapatric distribution of the wasp clades allows direct comparison of results obtained with the different markers. Cytoplasmic and nuclear markers subdivide the pollinating wasps into the same nine clades. The COI divergence between clades (ranging from 5.4% to 28%, with most values above 10%) is larger than the divergence previously reported between sister-species of fig pollinating wasps that share the same host (2.4%-7.4%; Yang et al., 2015). Furthermore, the nine clades can be separated morphologically (Wiebes 1993; Rasplus JY, com. pers.) and we therefore conclude that *F. hirta* is pollinated by nine species of fig wasps in our study zone, which covered most but not all of the range of the plant.

Sp5 is not closely related to the other pollinators of *F. hirta*. It was recorded from Singapore, where our data confirms an earlier proposition that *F. hirta* is not native (Berg & Corner, 2005). We suggest that sp5 is the regular pollinator of another *Ficus* species that has colonized *Ficus hirta* in Singapore. Fig trees planted outside their native range are known to similarly be capable of supporting successful development by fig wasps that usually breed in the figs of another *Ficus* species (Cook, & Segar, 2010; Wang, Cannon, & Chen, 2016). Sequences from GenBank suggest that *Valisia esquiroliana* Chen & Chou is nested within the group of *Valisia* species associated with *F. hirta*. This fig wasp was described from *Ficus triloba* Buch.-Ham. ex Voigt, a species closely related to *F. hirta* (Berg, 2007; Berg, &

Corner, 2005). Genetic analyses incorporating these two *Ficus* species will be required to clarify their evolutionary relationships.

Genetic differentiation was detected within the distribution of pollinator sp1. Its populations are divided into three genetic groups located in continental China, Hainan Island and Vietnam. Such differentiation could be a first step towards speciation. Experimental data is needed to determine whether these genetic groups are inter-fertile. Reproductive isolation could arise rapidly in fig pollinating wasps as they display a high prevalence of *Wolbachia* that could cause cytoplasmic incompatibilities (Haine, & Cook, 2005) and they display systematic assortative mating due to mating in their natal figs before dispersal (Anstett, Hossaert-McKey, & Kjellberg, 1997).

### **The biogeography of *Ficus* and their pollinators**

*Ficus hirta* displays a signature of genetic isolation by distance with both nuclear microsatellites and the cytoplasmic haplotypes, with no marked subdivision into distinct gene pools. It has been proposed that speciation in nursery pollinators and their host plants may be decoupled (Hembry, & Althoff, 2016) and our results provide an example of this phenomenon.

The disjunct distribution of sp7, with a 2000km gap and with three species of pollinating wasps (sp5, sp8, sp9) located in between, suggests that pollinators can successfully colonise distant host plants and establish populations there. A similar example is known from Australia, where one pollinator of *F. rubiginosa* is present in the cool south and at a higher

altitude in the warmer north, resulting in a 1500 km distribution gap (Sutton, Riegler, & Cook, 2016). This suggests that pollinating fig wasp distributions are plastic, and do not necessarily reflect a classical biogeographic history. Indeed, the biogeographic history of *F. racemosa* populations differs from that of its pollinator species, despite their identical separation into large gene pools (Bain et al., 2016). Similarly, in *F. septica*, an ecologically divergent pollinating fig wasp has colonised a 2000 km range previously only occupied by three parapatric pollinating wasp species (Rodriguez et al., 2017). Thus, the distributions of fig pollinating wasp species seem to be dynamic and reveal cases of successful establishment of long distance migrants. The long-range dispersal abilities of at least some fig wasps should facilitate this (Ahmed et al., 2009).

The mismatch distributions of COI sequences in the two best-sampled species (sp1 and sp2) provide signatures of either selective sweeps or population expansion. This is similar to what has been found in the pollinators of *F. pumila* (Chen, Compton, Liu, & Chen 2012) and in a pollinator of *F. septica* (Lin, Yeung, & Li, 2008). It has been proposed that these signatures are due to recurrent regional extinctions of pollinating wasps during extreme climatic events, but not of their host plants, followed by population expansions (Tian et al., 2015). Contemporary examples of this phenomenon have been documented in Florida (hurricane), Southern France (frost) and the north of Borneo Island (El Nino-associated draught) (Tian et al., 2015). Climatic episodes such as these make fig wasp species ranges intrinsically dynamic.

The establishment of new disjunct populations by long distance migrants depends on a combination of suitable environmental and biological conditions in their new locations. Founder populations will also usually be small, which can lead to Allee effects. These result

from 1) genetic inbreeding and loss of heterozygosity, 2) demographic stochasticity (including sex-ratio fluctuations) and 3) a reduction in cooperative interactions when individuals are at low density (Courchamp, Clutton-Brock, & Grenfell, 1999). As a fig is often colonised by a single fig wasp and given that their offspring mate in their natal fig before dispersal, inbreeding among fig wasps is routine (Molbo, Machado, Herre, & Keller, 2004), and consequently we expect founder populations of fig wasps to be little affected by inbreeding. Furthermore, although offspring sex ratios are heavily female-biased, foundress females typically begin by laying male eggs in a clutch, so female offspring rarely have difficulty finding mates (Raja, Suleman, Compton, & Moore, 2008). Finally, foundress females compete within figs for oviposition sites, so no cooperation between them is expected (Herre, 1989). Consequently, pollinating fig wasps are probably not sensitive to Allee effects and they should be much less dependent on progressive geographic range expansion than many other organisms. Within this context, the parapatric distributions of many pollinating fig wasps suggest an important role for interspecific competition in shaping their distributions, as has been proposed for non-pollinating fig wasps (Darwell, & Cook, 2017).

### **Diversification in plant-insect mutualisms**

The total of nine pollinator species found on *F. hirta* represents the highest ever-reported number of pollinator species for a *Ficus* species. Almost as high pollinator diversity has been documented for some other *Ficus* species (five for *F. rubiginosa*, Darwell, al-Beidh, & Cook, 2014; five for *F. racemosa*, Bain et al., 2016; four for *Ficus septica*, Rodriguez et al., 2017). This is strikingly different from the comprehensively-known *Yucca* moth-*Yucca* association, where from a total of 24 pollinating moth species (Pellmyr et al., 2008) there

are only five instances of two co-pollinators sharing on a host (Smith et al., 2009). Available data on the less comprehensively investigated *Epicephala*-*Phyllanthaceae* association suggests an intermediate situation with up to four pollinator species associated with one host (Li, Wang, & Hu, 2015). The comparatively low pollinator diversity observed in *Yucca* moths could be related to several factors. First, *Yuccas* have much more restricted distribution ranges than *Ficus* species, with only the distribution ranges of the two most northern *Yucca* species reaching 2000 km (Althoff et al., 2012). Second, *Yucca* moths can exhibit prolonged diapause, a factor that will slow down the dynamics of population divergence (Powel, 1992). Third, assortative mating is not systematic, unlike in fig wasps (Powel, 1992).

In addition to their contrasting rates of differentiation, the population genetics of *F. hirta* and its pollinators are strikingly different. Whereas *F. hirta* exhibits clinal genetic variation with a signature of genetic isolation by distance, its pollinator species sp1 and sp2 lack any such signature. The same pattern has been observed for *F. pumila* L. and one of its pollinating wasps in South-East China (Liu, Compton, Peng, Zhang, & Chen, 2015). Hence, although pollinating fig wasps diversify faster than their host figs, they also display signatures of stronger gene flow. This apparent contradiction suggests that factors which facilitate speciation, such as a short generation time, infection by *Wolbachia* and assortative mating, can compensate for extensive gene flow and be decisive factors facilitating pollinating fig wasp speciation. Some non-pollinating fig wasps (NPFW, belonging to several families of Chalcidoidea) may disperse as widely as pollinator fig wasps (Sutton, Riegler, & Cook, 2016; Kjellberg, & Proffitt, 2016). NPFW have a largely similar biology to the pollinators, but exhibit varying intensities of local mate competition due to varying patterns of oviposition and mating sites. Obligatory assortative mating among NPFW ranges

from minimal (among species that mate outside natal figs), through intermediate (NPFW with offspring dispersed across numerous figs that mate in the cavity of their natal figs) to being highly similar to that of pollinating wasps (with offspring aggregated in a single or small number of figs and mating inside these natal figs) (Cook, & Rasplus, 2003). If assortative mating indeed facilitates speciation, then we would expect a correlation between NPFW reproductive behavior and speciation rates in different NPFW clades.

The mismatch between genetic structuring of host plant and fig wasps shows that, as in *Yucca* moths (Godsoe, Yoder, Smith, Drummond & Pellmyr, 2010) and *Epicephala* moths (Hembry et al., 2018), fine population-level phenotypic matching between mutualists is not required to allow population persistence. Nevertheless, on Hainan Island, the populations of *F. hirta* and its pollinator present the same signature of marked genetic differentiation from continental populations. Hence, the mismatch in population genetic structure is not consistent over the whole range of the association. Such differences in genetic co-structuring are also encountered among associations in other species-specific plant-insect interactions. For instance, in a specialized association between two ants and an ant-plant, with populations arranged as stepping stones, the ants and the plant presented very similar genetic signatures of population geographic expansion (Léotard et al., 2009). In a similar association showing a history of restriction to refugia followed by expansion leading to more continuous populations, the plants and insects had the same pattern of genetic geographic structuring, but different histories (Blatrix et al., 2017). The latter study also showed that the trees had evolved stronger reproductive isolation than their ant symbionts. Hence, available case studies suggest that patterns of diversification in plant-insect mutualistic interactions are not uniform among representatives of the same type of interaction. As more case studies become

available, exploring how variation is explained by the biology of individual species is the next challenge.

### **Relationships between tree characteristics and diversity of pollinators**

Pollinator diversity has now been explored throughout large parts of the distribution ranges of four widely-distributed Asian and Australasian *Ficus* species (*F. hirta*, *F. septica*, *F. racemosa* and *F. rubiginosa*) (Bain et al., 2016; Darwell, al-Beidh, & Cook, 2014; Rodriguez et al., 2017). Their pollinator fig wasps each form old monophyletic species complexes (dating back >10 Ma) associated with a single host (Bain et al., 2016; Cruaud et al., 2012), but the species groups display varying geographical scales of pollinator species diversification. The host figs also display varying scales of genetic differentiation. Further studies are required to investigate whether plant traits such as crop size, crop synchrony and growth form may combine to select for different pollinator dispersal behavior, and may result in pollinator and fig genetic diversity being expressed at different geographical scales.

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### **Data Accessibility**

Microsatellite genotype data, final assembly mtDNA sequences for all individuals of *Valasia javana* and ITS2 nrDNA sequences for each species and each haplotypes of *V. javana*, and microsatellite genotype data and final assembly cpDNA sequences for all individuals of *Ficus hirta* will be archived in the Dryad Digital Repository at <http://datadryad.org>, doi: 10.5061/dryad.k7t33tg.

### **Author Contributions**

YH designed research, collected samples, analyzed data and co-wrote the manuscript, SGC co-wrote the manuscript. FK contributed to data analyses and co-wrote the manuscript. EWT and ZLN collected samples, performed laboratory work, and analyzed data. DXX and YFC performed laboratory work. DYZ designed research. CLF and WW analyzed data. WT collected samples in Thailand. All authors contributed substantially to revisions.

## Figure legends

### Fig. 1

COI Bayesian phylogenetic tree of the *Valisia* fig wasp pollinators associated with *Ficus hirta*, including all GenBank sequences of pollinating wasps reared from *Ficus* subg. *Ficus* sect. *Eriosycea* Miq., and some sequences of *Ceratosolen* and *Kradibia* used as outgroups. Posterior probabilities of the nodes are indicated as percentages.

### Fig. 2

(a) The distributions of the pollinator fig wasp species associated with *Ficus hirta*. (b) The distributions of cpDNA haplotypes of *Ficus hirta*. Note the disjoint distribution of Sp7, the allopatric distribution of the closely related (according to COI data) Sp2 and Sp9 and the parapatric distribution of the closely related (according to COI data) Sp4, Sp6 and Sp7. For cpDNA, colours are used to show the localised distribution of haplotype H1 and of the different branches of the haplotype network.

### Fig. 3

Neighbour joining microsatellite genotype networks for a) the pollinating wasps and b) *F. hirta*. The colours indicate the most common pollinator species recorded at the location where a plant genotype was collected.

### Fig. 4

Bar plots of membership probabilities of individuals to the different clusters (as vertical bars) from the STRUCTURE analysis. a) Genetic structure of the pollinating wasps obtained at  $K=11$ ; b) Genetic structure of the plant host, *Ficus hirta*, obtained at  $K=4$ . For the pollinator, sp1 genotypes from continental China present mixed assignments to two clusters suggesting genetic isolation by distance, while genotypes from Hainan and genotypes from Vietnam are each assigned to a particular cluster. For *F. hirta*, there is no clear global pattern of clustering, suggesting clinal variation in gene frequencies. The Singapore genotypes (SNP) cluster with Hainan Island (South China) genotypes Ding and Wan.

### Fig. 5

Spatial genetic structuring of the pollinator fig wasp Sp1 based on nuclear microsatellite data. Genetic differentiation between locations is plotted according to geographic distance. The colours indicate the geographic origin of the locations being compared. Dark blue: comparison between two locations from South-East China; red: comparison between a location in South-East China and one in Hainan Island; green: comparison between a location from South-East China and one from Vietnam; violet: comparison between a location from Hainan Island and one from Vietnam; light blue, comparison between two locations from Vietnam; orange: comparison between the two Hainan Island locations. The species is structured into 3 genetic groups: South-East China, Hainan and Vietnam, the same structure as revealed by the cytoplasmic data (supplementary material, fig. S2).

### Fig 6

Pairwise genetic differentiation between locations in *Ficus hirta*, as a function of distance, based on microsatellite data. Red points: comparisons involving the Singapore location.



## Supporting information

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

**Table S1.** Sampling locations and sample sizes.

**Table S2.** Pollinating wasps, COI, haplotype data.

**Figure S1.** Dated Bayesian tree for pollinating wasp COI gene sequences.

**Table S3.** Pollinating wasp COI cytoplasmic gene sequence differences (Kimura-2-parameter) within (diagonal) and between species (below diagonal).

**Figure S2.** Pollinating wasps, ITS2 maximum likelihood phylogenetic tree.

**Table S4.** Pollinating wasp ITS2 nuclear gene sequence differences (Kimura-2-parameter) within (diagonal) and between species (below diagonal)

**Table S5.** Pollinating wasps, ITS2, haplotype data.

**Figure S3.** Pollinating wasps, COI data, number of MOTUs obtained with jMOTU depending on cut-off value.

**Table S6.** Pollinating wasps, COI, AMOVA analysis comparing within and among population sequence variation.

**Figure S4.** Pollinating wasps, within species COI haplotype networks.

**Table S7.** Pollinating wasps, microsatellite data.

**Table S8.** Pollinating wasps, microsatellite data, pairwise genetic differentiation between locations ( $F_{ST}$ )

**Figure S5.** Pollinating wasps, Factorial Component Analysis of individual multilocus microsatellite genotypes

**Figure S6.** Assignments of a) pollinating wasps and b) *Ficus hirta* microsatellite genotypes to cluster for various values of K using STRUCTURE

**Table S9.** *Ficus hirta*, trnLF and trnSG haplotype data.

**Table S10.** *Ficus hirta*, microsatellite data.

**Figure S7.** *Ficus hirta*, Factorial Component Analysis on multilocus microsatellite genotypes

**Table S11.** *Ficus hirta*, microsatellite data, pairwise genetic differentiation between locations ( $F_{ST}$ )

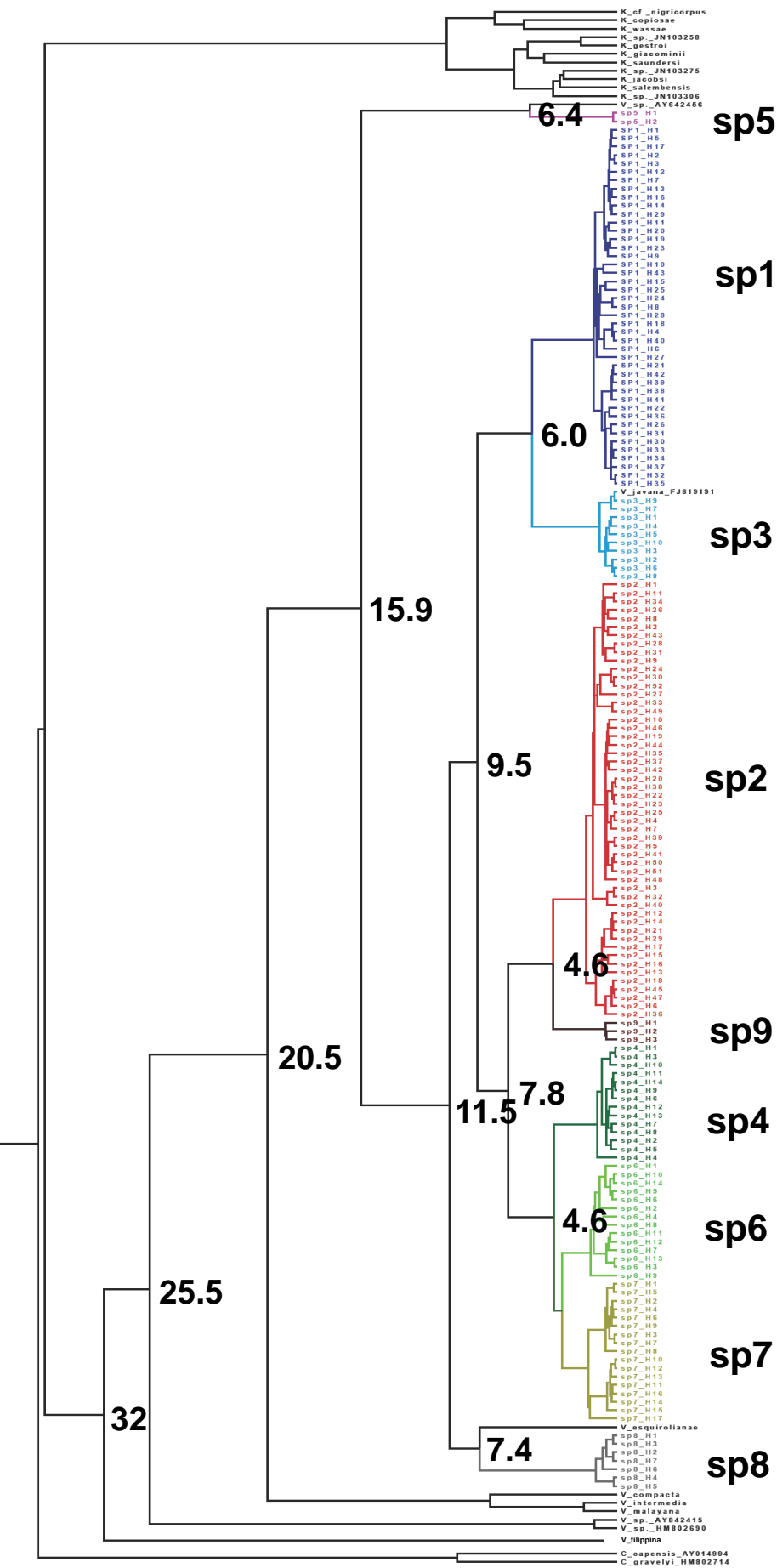
**Table S1.** Sampling locations and sample sizes. \* collected close to DAL at YL (lat 12.415, long 108.176). In bold, abbreviation used throughout the text.

Location	Name	Latitude	longitude	Wasp mtCOI	Wasp nSSR	Wasp ITS2	Fig cpDNA	Fig nSSR
S.E. China	<b>Guilin</b>	25.077	110.306				11	
	<b>Aomen</b>	22.199	113.640				10	
	<b>Dapu</b>	24.258	116.806				10	8
	<b>Xiamen</b>	24.742	118.072				10	
	<b>Ningde</b>	26.664	119.549	10	20	3	4	19
	<b>Shaxian</b>	26.419	117.818	7	27		9	
	<b>Suichuan</b>	26.476	114.239	11	20	3	9	
	<b>Xianggang</b>	22.424	114.306	8	16	5	6	
	<b>Huolu Mountain</b>	23.170	113.373	20	15	4	9	
	<b>Dinghu mountain</b>	23.166	112.543	14	24	9	10	
	<b>Hunan</b>	25.571	111.946	7	20	3		
	<b>Sandu</b>	25.984	107.874	6	18	2	10	
	<b>Nanning</b>	22.787	108.389	9	19	4	9	
Hainan	<b>Hai</b>	18.642	109.701				2	
	<b>Dingan</b>	19.697	110.328	8	19	6		24
	<b>Wan</b>	18.795	110.391	10	10	5	12	25
Yunnan	<b>Xishuangbanna</b>	21.913	101.264	3	0	3	9	
	<b>MengHai</b>	21.979	100.450	14	20	6	4	24
	<b>MengLun</b>	21.447	101.568	22	24	13	3	15
Thailand	Temple Hill <b>Tai</b>	18.894	98.858	15	24	12	18	17
	<b>Chao Son</b>	18.84	99.47	17	24	9	10	23
	Other side of TempleHill <b>QMS</b>	18.809	98.914	16	24	8		
	Taksinmaharat <b>TNP</b>	16.782	98.928	13	24	8	14	26
	<b>Wuwen</b>	14.443	105.273	7	24	6	7	24
	<b>CHantaburi</b>	12.774	102.096	12	18	10	9	24
	Trang <b>ST</b>	7.467	99.639	11	24	11	22	24
Vietnam	Vinh Yen <b>VH</b>	21.467	105.581	8	12	7	10	22
	Cuc <b>Phuong</b>	20.253	105.712	2	8	1	12	22
	Mengxi <b>NP</b>	17.579	106.308	2	2	2	12	19
	Hon Ba <b>HB</b>	12.248	108.796	28	32	14	10	24
	Dalat/Lac Thien ( <b>DAL/YL</b> )	12.157	108.137	4	12	4	12*	23*
	Kon Tum ( <b>KT</b> )	14.516	107.639	3	4	3	9	15
Singapore	<b>SiNgapore</b>	-1.312	103.816	17	24	18	12	23
Indonesia	<b>CInangneng</b>	-6.566	106.706	7	24	8		24
	<b>JAkarta</b>	-6.368	106.830	11	24	7	12	24
Cambodia	Pnom-penh <b>JP</b>	11.353	104.153	7	12	9		
	North <b>JF</b>	12.499	107.318	1				

**Table S2.** Pollinating wasps, COI, haplotype data. Sample size (N), number of haplotypes (K), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ), and COI haplotypes with number of individuals.

	Pop.	N	K	Hd	$\pi$	COI haplotype (number of individuals)
sp1	Ning	10	7	0.911	0.00346	2 (2), 3 (1), 5 (1), 6 (3), 12 (1), 13 (1), 14 (1)
	Sha	7	5	0.905	0.00406	1 (1), 2 (2), 3 (1), 4 (2), 5 (1)
	Sui	11	8	0.945	0.00358	1 (2), 4 (1), 5 (2), 6 (1), 10 (1), 11 (2), 13 (1), 15 (1)
	Xiang	8	7	0.964	0.00393	5 (1), 6 (2), 7 (1), 8 (1), 9 (1), 10 (1), 11 (1)
	Huo	20	9	0.789	0.00738	1 (1), 5 (9), 6 (2), 12 (1), 14 (1), 23 (3), 27 (1), 28 (1), 29 (1)
	DHS	14	9	0.912	0.00332	4 (2), 5 (4), 6 (2), 14 (1), 22 (1), 23 (1), 24 (1), 25 (1), 26 (1)
	Hu	7	2	0.571	0.00168	5 (4), 6 (3)
	Sand	6	4	0.800	0.00274	5 (1), 6 (3), 12 (1), 16 (1)
	Nan	9	7	0.917	0.00653	5 (1), 6 (1), 17 (3), 18 (1), 19 (1), 20 (1), 21 (1)
	Ding	8	5	0.786	0.00184	30, 31, 32, 33, 34 (4)
	Wan	10	6	0.867	0.00723	30 (3), 31, 34, 35 (3), 36, 37
	VH	8	5	0.786	0.00603	29(4), 38, 39, 40, 41
	CP	2	2	1	0.00294	42, 29
	NP	2	1	0	0	43 (2)
	<b>TOTAL</b>	<b>122</b>	<b>43</b>	<b>0.933</b>	<b>0.00018</b>	
sp2	XI	3	3	1.000	0.00685	1, 2,3
	MH	14	6	0.824	0.00494	1, 2(5), 4, 5(3), 6(3), 7
	ML	22	16	0.961	0.00835	5(3), 8, 9(3), 10,11,12,13(3),14,15,16,17,18,19,20, 21, 22
	Tai	15	14	0.990	0.00951	13,23,24,25,26,27,28,29(2),30, 31, 32, 33, 34, 35
	CS	16	11	0.908	0.00664	13(5), 28, 36,37(2), 38, 39, 40, 41, 42, 43, 44
	QMS	16	14	0.983	0.00901	13(2),26,28,31,37,39,45,46, 47, 48, 49(2), 50, 51, 52
		<b>TOTAL</b>	<b>86</b>	<b>52</b>	<b>0.972</b>	<b>0.00008</b>
sp3	TNP	13	9	0.910	0.00401	1, 2 , 3(4), 4, 5(2), 6, 7, 8, 9
	CS-1.12	1	1			10
		<b>TOTAL</b>	<b>14</b>	<b>10</b>	<b>0.923</b>	<b>0.00365</b>
sp4	HB	28	14	<b>0.881</b>	<b>0.00422</b>	1 (2),2 ,3,4,5,6,7 (8),8 ,9 ,10,11(6), 12,13(2),14
sp5	SNP	17	2	<b>0.309</b>	<b>0.00045</b>	1 (3),2(14)
sp6	DAL	4	4	1	0.1371	1,2,3,4
	KT	3	3	1	0.01077	5,6,7
	Wu	6	6	1	0.00861	8,9,10,11,12,13
	JF	1	1			14
		<b>TOTAL</b>	<b>14</b>	<b>14</b>	<b>1.000</b>	<b>0.00073</b>
sp7	CI	7	5	0.857	0.0028	1,2(3),3,4,5
	JA	11	5	0.855	0.00315	2(3),6,7(2),8(3),9(2)
	CH	12	7	0.909	0.00258	10(2),11 (3),12(2),13,14,15(2),16
	Wu-11	1	1	-	-	17
		<b>TOTAL</b>	<b>31</b>	<b>17</b>	<b>0.944</b>	<b>0.00057</b>
sp8	ST	11	7	<b>0.909</b>	<b>0.00833</b>	1,2(3),3,4(2),5,6,7
sp9	JP	7	3	<b>0.714</b>	<b>0.00420</b>	1(3),2, 3(3)

**Figure S1.** Dated Bayesian tree for wasp COI gene sequences. Dates in million years. Age of the different nodes with 95% lower and upper highest posterior distribution inferred by BEAST reported between parentheses: sp 1-3 crown 6.0 (2.0-10.7); sp2-9 crown 4.6 (1.5, 8.3); sp 4-6-7 crown 4.6 (1.5, 8.1); clade (2-9)-(4-6-7) crown 7.8 (3.0, 13.5); clade (sp1-2-3-4-6-7-9) crown 9.5 (3.8, 15.9); clade (sp1-2-3-4-6-7-8-9) crown 11.5 (4.3, 19.2); clade (1-2-3-4-5-6-7-8-9) crown 15.9 (6.7, 26.3)



**Table S3.** Pollinating wasp COI cytoplasmic gene sequence differences (Kimura-2-parameter) within (diagonal) and between species (below diagonal). Within species differences are low (highlighted in green), confirming separation into different species. Note the limited difference between sp2 and sp9 (highlighted in blue) and the limited difference between sp4, sp6 and sp7 (highlighted in yellow). Sp5 stands out as highly divergent suggesting that it does not belong to the species group of *V. javana* (differences highlighted in red).

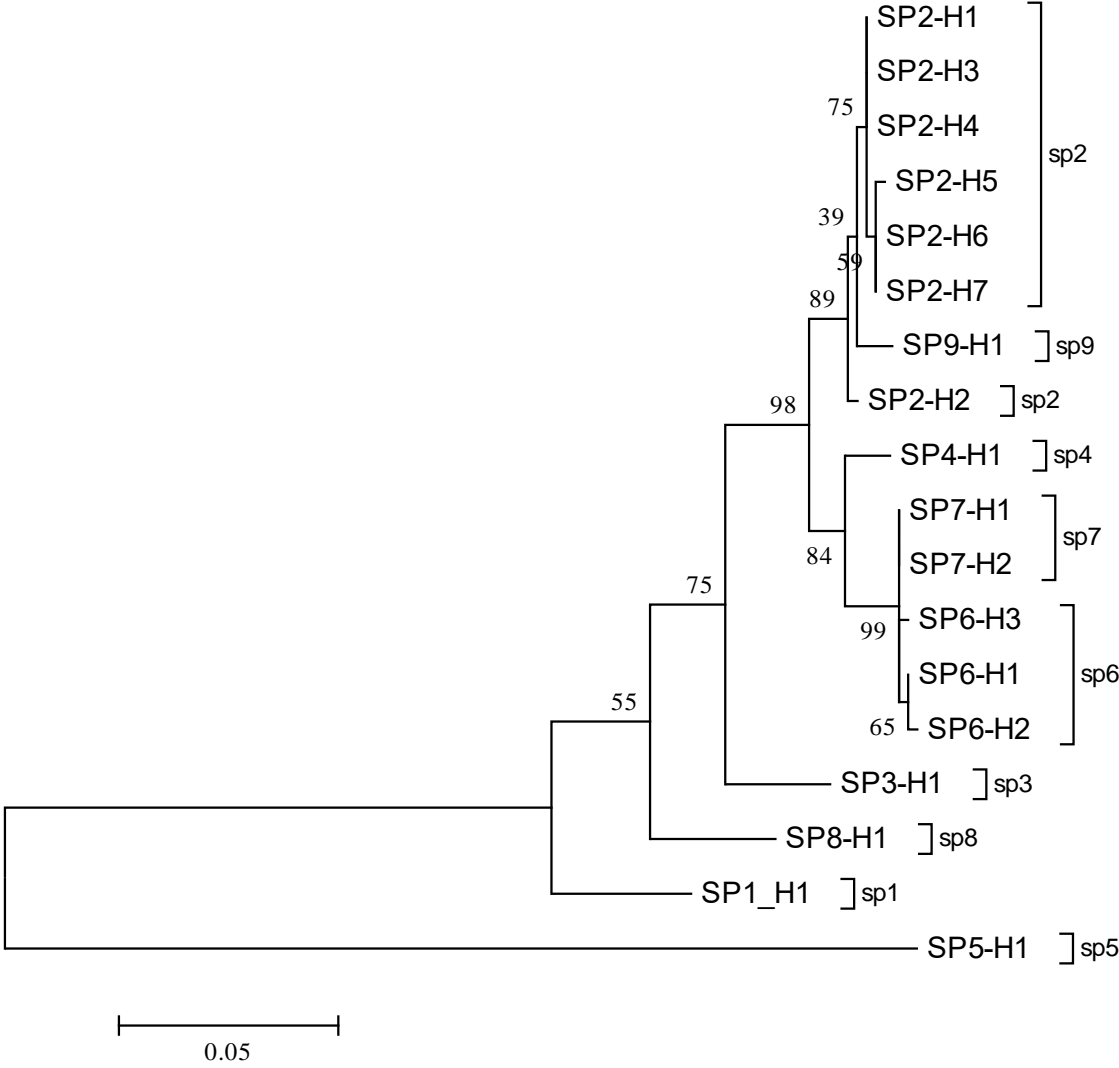
	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9
sp1	0.008								
sp2	0.135	0.014							
sp3	0.113	0.113	0.005						
sp4	0.130	0.113	0.118	0.006					
sp5	0.266	0.266	0.243	0.265	0.001				
sp6	0.124	0.110	0.126	0.068	0.281	0.012			
sp7	0.138	0.117	0.123	0.058	0.266	0.054	0.014		
sp8	0.122	0.121	0.123	0.137	0.243	0.133	0.123	0.009	
sp9	0.134	0.064	0.109	0.114	0.272	0.119	0.111	0.104	0.006

**Table S4.** Pollinating wasp ITS2 nuclear gene sequence differences (Kimura-2-parameter) within (diagonal) and between species (below diagonal). Within species differences are equal to zero. Note, as for COI sequences, the limited difference between sp2 and sp9 (highlighted in blue) and the limited difference between sp4, sp6 and sp7 (highlighted in yellow). Sp5 stands out as highly divergent suggesting that it does not belong to the species group of *V. javana* (highlighted in red).

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9
sp1	0								
sp2	0.1	0							
sp3	0.09	0.053	0						
sp4	0.104	0.029	0.057	0					
sp5	0.37	0.368	0.369	0.368	0				
sp6	0.105	0.036	0.06	0.025	0.383	0			
sp7	0.104	0.033	0.059	0.023	0.381	0.003	0		
sp8	0.077	0.075	0.068	0.079	0.399	0.08	0.079	0	
sp9	0.093	0.012	0.052	0.031	0.381	0.038	0.035	0.072	0

**Figure S2.** Pollinating wasps, ITS2 maximum likelihood phylogenetic tree. Number of individuals genotyped: 53, 51, 9, 14, 18, 13, 25, 11, 9 for sp1, sp2, sp3, sp4, sp5, sp6, sp7, sp8, sp9 respectively.

Note the clade comprising sp2, sp4, sp 6, sp7, sp9, the clade comprising sp2 and sp9 and the clade comprising sp4, sp6 and sp7 and the strong divergence of sp5. All these properties were also obtained with the COI data.

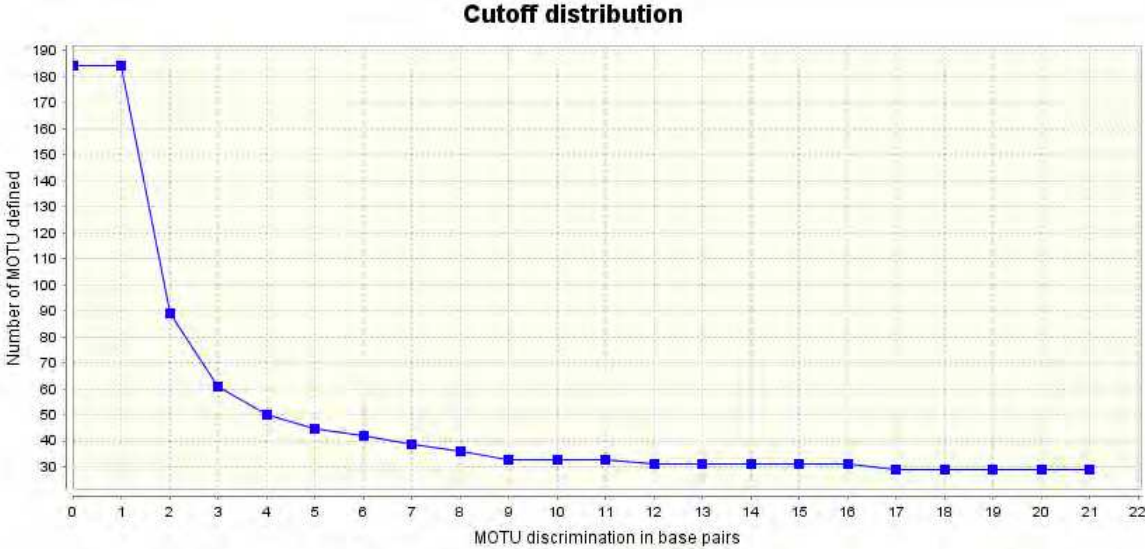


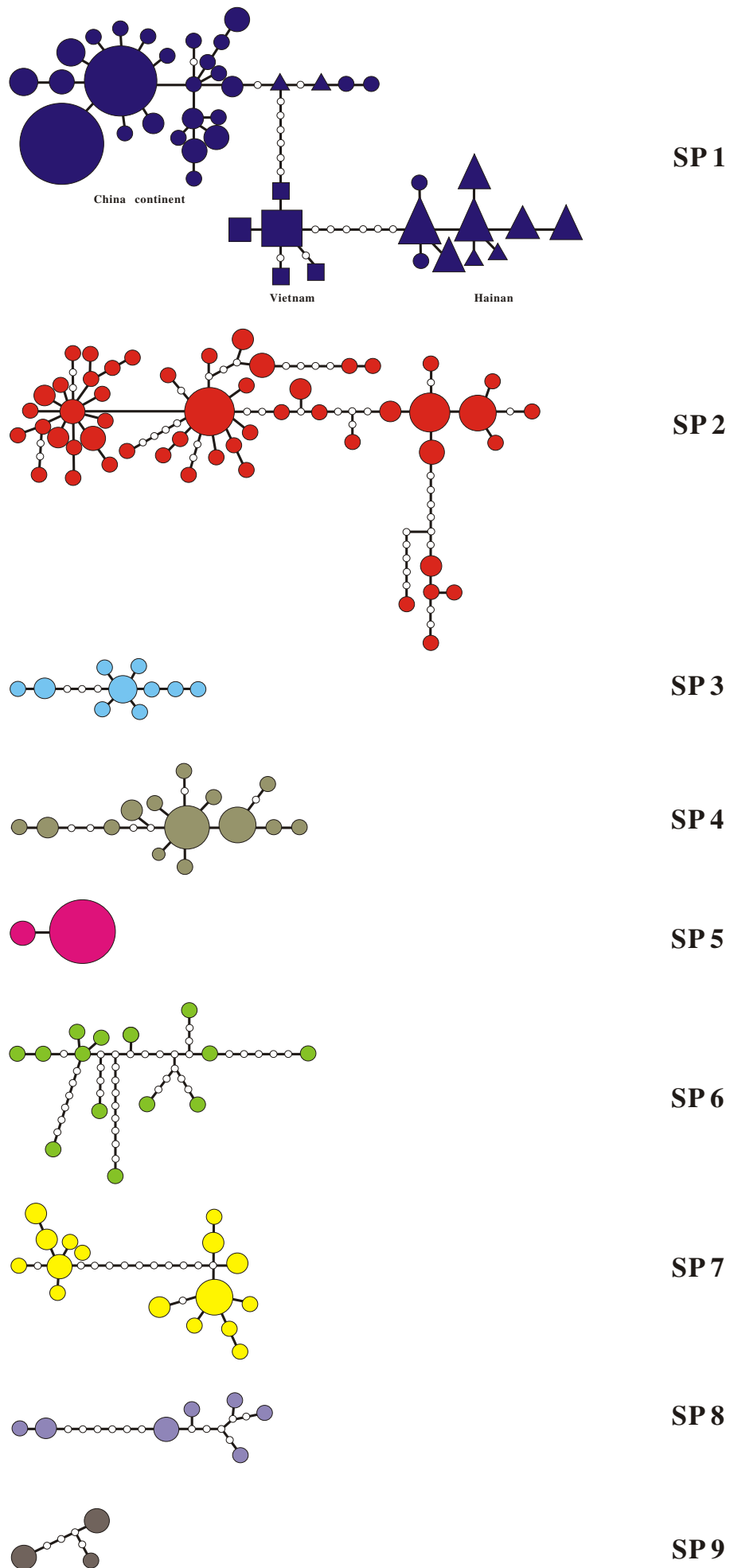
**Table S5.** Pollinating wasps, ITS2, haplotype data. Sample size (N), number of haplotypes (K), and ITS2 haplotypes with number of individuals.

	Pop.	N	K	ITS2 haplotype (number of individuals)
Sp1	Ning	3	1	Sp1-H1(3)
	Sui	3	1	Sp1-H1(3)
	Xiang	5	1	Sp1-H1(5)
	Huo	4	1	Sp1-H1(4)
	DHS	9	1	Sp1-H1(9)
	Hu	3	1	Sp1-H1(3)
	Sand	2	1	Sp1-H1(2)
	Nan	4	1	Sp1-H1(4)
	Ding	6	1	Sp1-H1(6)
	Wan	5	1	Sp1-H1(5)
	VH	7	1	Sp1-H1(7)
	CP	1	1	Sp1-H1(1)
	NP	2	1	Sp1-H1(1)
	Sp2	XI	3	1
MH		6	2	H3(1), H4(5)
ML		13	6	H1(1), H2(1), H3(5), H4(4), H5(1), H6(1)
Tai		12	6	H1(3), H2(1), H3(1), H4(5), H5(1), H7(1)
CS		9	5	H1(2), H2(1), H3(3), H4(2), H5(1)
QMS		8	4	H1(1), H3(2), H4(4), H5(1)
Sp3	TNP	8	1	Sp3-H1(8)
	CS1.12	1	1	Sp3-H2(1)
Sp4	HB	14	1	Sp4-H1(14)
Sp5	SNP	18	1	Sp5-H1(18)
Sp6	DAL	4	1	Sp6-H1(4)
	KT	3	1	Sp6-H2(3)
	Wu	6	1	Sp6-H3(6)
Sp7	CI	8	1	Sp7-H1(8)
	JA	7	1	Sp7-H1(7)
	CH	10	1	Sp7-H2(10)
Sp8	ST	11	1	Sp8-H1(11)
Sp9	JP	9	1	Sp9-H1(9)



**Figure S3.** Pollinating wasps, COI data, number of MOTUs obtained with jMOTU depending on cutoff value.





**Figure S4.** Pollinating wasps, within species COI haplotype networks. For sp1 the network is structured into three entities, South-East China (rounds), Vietnam (squares) and Hainan (triangles), the same structuring as for nuclear microsatellites. Generally, the species are highly diversified even within population, with sp6 an extreme with no repeated haplotype. Sp5 exhibits very limited diversity.

**Table S6.** Pollinating wasps, COI, AMOVA analysis comparing within and among population sequence variation. There is strong significant structuring among populations within pollinator species.

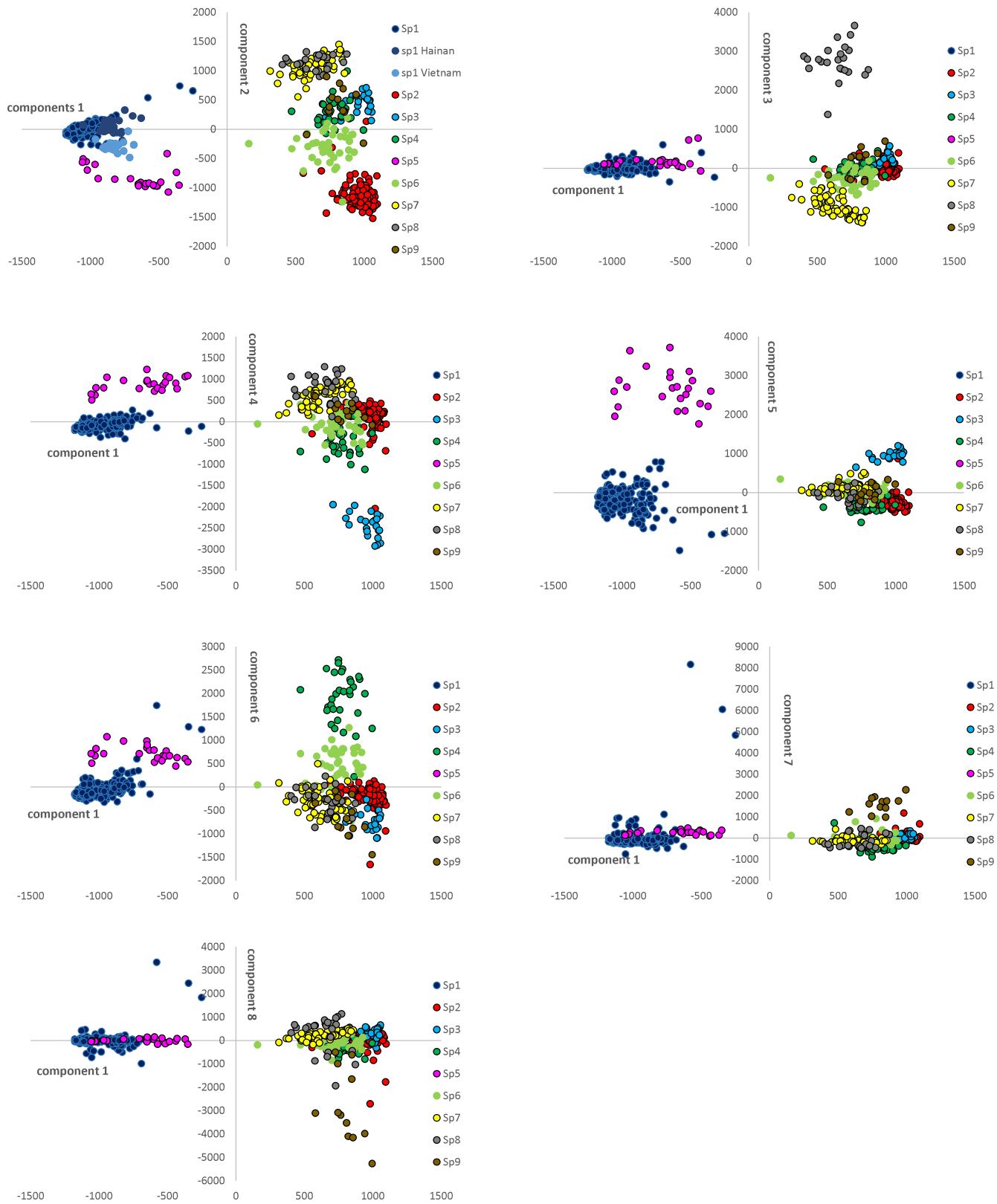
Species	number of samples	source of variation among populations	significance
sp1	122	44.61	<0.001
sp2	86	17.24	<0.001
sp3	--	--	--
sp4	--	--	--
sp5	--	--	--
sp6	14	12.64	0.00391
sp7	30	82.42	<0.001
sp8	--	--	--
sp9	--	--	--

**Table S7.** Pollinating wasps, microsatellite data. Number of alleles (Na), allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficients (Fis).

Species	Pop	Na	Ar	PAr	Ho	He	Fis
sp1	Ning	5.333	1.53	0.11	0.483	0.528	0.102
	Sha	5.556	1.56	0.1	0.393	0.562	0.323
	Sui	5.667	1.55	0.09	0.340	0.551	0.356
	Xiang	5.111	1.49	0.1	0.229	0.494	0.529
	Huo	6.444	1.64	0.15	0.367	0.632	0.441
	DHS	6.889	1.58	0.21	0.447	0.589	0.235
	Hu	6.778	1.61	0.22	0.472	0.601	0.222
	Sand	7.111	1.69	0.20	0.412	0.683	0.418
	Nan	6.667	1.55	0.16	0.538	0.557	0.021
	Ding	8.222	1.67	0.43	0.430	0.651	0.391
	Wan	5.222	1.59	0.41	0.344	0.539	0.321
	VH	4.667	1.49	0.14	0.457	0.495	0.073
	CP	4.000	1.45	0.18	0.408	0.446	0.049
NP	2.556	1.67	0.19	0.667	0.486	-0.367	
sp2	MH	4.556	1.46	0.14	0.273	0.408	0.390
	ML	4.667	1.38	0.10	0.207	0.345	0.420
	Tai	5.556	1.54	0.13	0.415	0.529	0.259
	QMS	5.222	1.45	0.11	0.387	0.442	0.103
	CS	6.556	1.51	0.16	0.431	0.481	0.190
sp3	TNP	5.000	1.50	0.36	0.421	0.512	0.169
sp4	HB	6.667	1.48	0.27	0.381	0.517	0.338
sp5	SNP	3.889	1.43	0.33	0.349	0.402	0.091
sp6	DAL	3.778	1.46	0.14	0.284	0.460	0.480
	KT	1.889	1.26	0.08	0.083	0.257	0.504
	Wu	5.333	1.11	0.13	0.365	0.469	0.166
sp7	CI	3.667	1.41	0.15	0.327	0.369	0.045
	JA	4.778	1.48	0.12	0.348	0.443	0.209
	CH	5.222	1.51	0.15	0.402	0.516	0.189
sp8	ST	7.111	1.59	0.59	0.521	0.607	0.093
sp9	JP	3.333	1.40	0.33	0.317	0.399	0.161

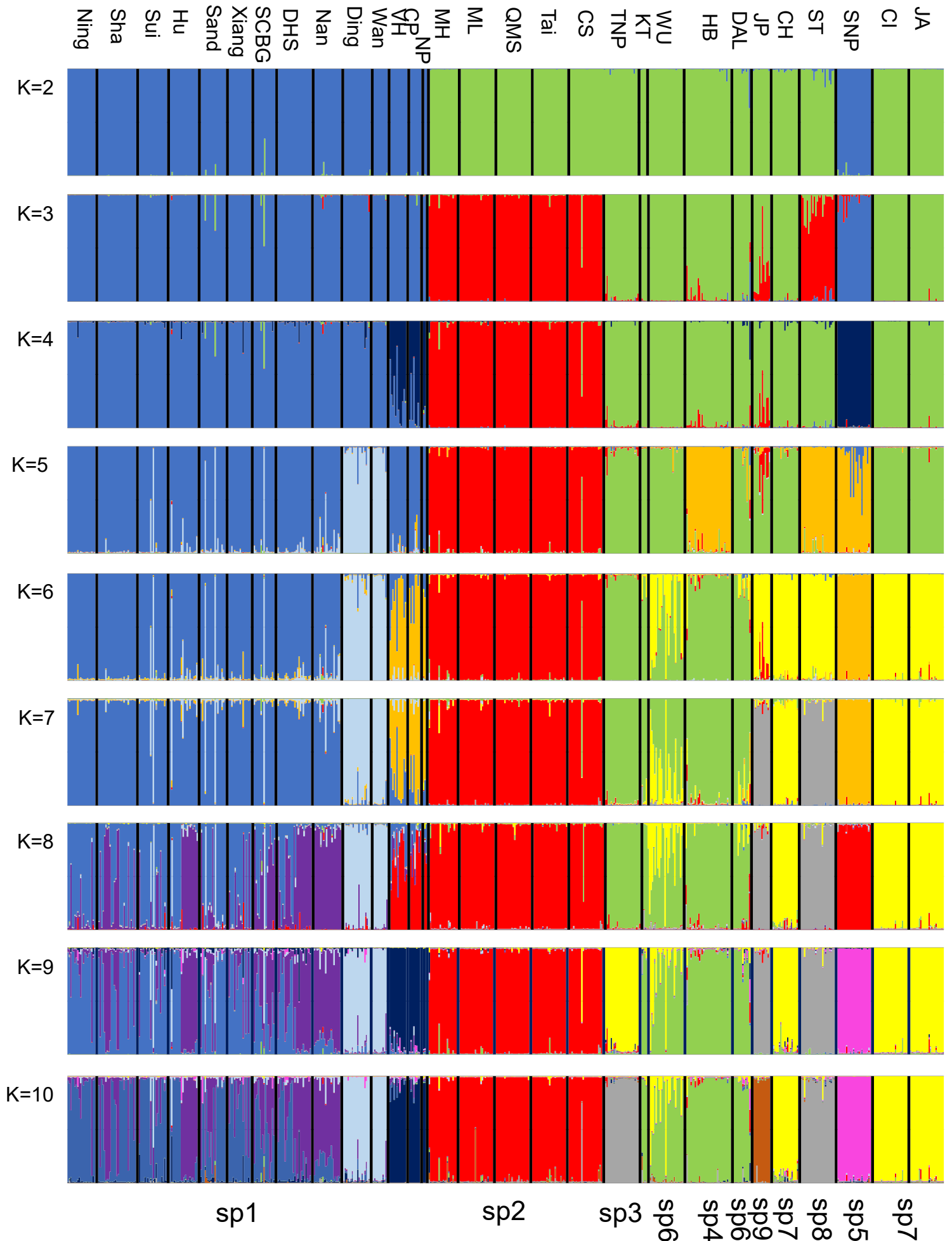
**Table S8.** Pollinating wasps, microsatellite data, pairwise genetic differentiation between locations ( $F_{ST}$ ). The border colours represent the different species. In green,  $F_{ST}<0.1$ , in blue  $0.1<F_{ST}<0.2$ , in yellow  $0.2<F_{ST}<0.3$ . Sp1 (dark blue) and sp2 (red) are highly homogeneous. Sp7 (yellow) is also highly homogeneous despite its disjoint distribution. Sp4 (dark green) and sp6 (light green) form a slightly less homogeneous group. Within sp1, continental China locations for a very homogeneous entity (locations Ning to Nan), the Hainan Island locations (Ding and Wan) form a slightly different homogenous entity, and Vietnamese locations (VH, CP, NP) form a third homogenous entity.

	Ning	sha	Sui	Hu	Sand	Xiang	SCBG	DHS	Nan	Ding	Wan	VH	CP	NP	MH	ML	QMS	Tai	CS	TNP	KT	Wu	HB	DAL	JP	CH	ST	SNP	CI	JA	
Ning	0.000																														
Sha	0.049	0.000																													
Sui	0.037	0.057	0.000																												
Hu	0.049	0.045	0.056	0.000																											
Sand	0.053	0.064	0.042	0.062	0.000																										
Xiang	0.026	0.070	0.042	0.053	0.052	0.000																									
SCBG	0.054	0.046	0.036	0.047	0.045	0.052	0.000																								
DHS	0.055	0.039	0.061	0.040	0.067	0.055	0.051	0.000																							
Nan	0.088	0.081	0.082	0.074	0.092	0.080	0.034	0.066	0.000																						
Ding	0.141	0.128	0.120	0.121	0.110	0.151	0.110	0.112	0.124	0.000																					
Wan	0.175	0.156	0.166	0.161	0.141	0.187	0.127	0.157	0.138	0.059	0.000																				
VH	0.204	0.197	0.166	0.201	0.139	0.195	0.187	0.176	0.254	0.226	0.308	0.000																			
CP	0.241	0.239	0.201	0.249	0.173	0.232	0.224	0.224	0.305	0.251	0.329	0.047	0.000																		
NP	0.211	0.194	0.162	0.191	0.147	0.197	0.181	0.176	0.258	0.237	0.310	0.080	0.112	0.000																	
MH	0.384	0.373	0.369	0.350	0.302	0.388	0.330	0.349	0.373	0.331	0.392	0.289	0.343	0.333	0.000																
ML	0.436	0.416	0.415	0.390	0.341	0.442	0.368	0.387	0.405	0.364	0.429	0.326	0.387	0.370	0.043	0.000															
QMS	0.372	0.358	0.355	0.334	0.290	0.379	0.316	0.334	0.351	0.313	0.366	0.297	0.320	0.324	0.038	0.037	0.000														
Tai	0.325	0.309	0.310	0.289	0.252	0.334	0.274	0.289	0.306	0.267	0.322	0.300	0.326	0.315	0.071	0.086	0.050	0.000													
CS	0.356	0.343	0.341	0.320	0.276	0.362	0.301	0.319	0.337	0.299	0.352	0.271	0.293	0.304	0.028	0.029	0.019	0.046	0.000												
TNP	0.347	0.332	0.326	0.315	0.260	0.351	0.291	0.314	0.333	0.294	0.340	0.348	0.376	0.344	0.347	0.365	0.309	0.250	0.301	0.000											
KT	0.496	0.472	0.465	0.449	0.387	0.500	0.421	0.448	0.465	0.429	0.490	0.484	0.516	0.485	0.416	0.463	0.431	0.315	0.330	0.324	0.000										
Wu	0.344	0.330	0.327	0.315	0.266	0.355	0.289	0.310	0.327	0.302	0.344	0.356	0.392	0.348	0.275	0.312	0.246	0.184	0.213	0.217	0.168	0.000									
HB	0.346	0.333	0.328	0.311	0.259	0.347	0.291	0.312	0.332	0.276	0.326	0.351	0.368	0.344	0.261	0.290	0.223	0.181	0.199	0.285	0.305	0.180	0.000								
DAL	0.353	0.340	0.331	0.318	0.268	0.357	0.297	0.325	0.342	0.279	0.341	0.354	0.367	0.338	0.297	0.340	0.279	0.204	0.237	0.238	0.212	0.082	0.188	0.000							
JP	0.383	0.363	0.363	0.344	0.297	0.395	0.323	0.351	0.361	0.317	0.378	0.398	0.426	0.398	0.352	0.395	0.341	0.298	0.318	0.321	0.385	0.293	0.313	0.328	0.000						
CH	0.295	0.286	0.285	0.272	0.228	0.305	0.252	0.266	0.294	0.277	0.320	0.308	0.347	0.302	0.340	0.369	0.315	0.254	0.302	0.212	0.297	0.165	0.287	0.212	0.250	0.000					
ST	0.289	0.277	0.268	0.260	0.215	0.295	0.230	0.260	0.257	0.238	0.280	0.304	0.331	0.301	0.270	0.296	0.247	0.215	0.231	0.221	0.302	0.205	0.210	0.228	0.228	0.222	0.000				
SNP	0.290	0.298	0.281	0.265	0.245	0.295	0.268	0.278	0.334	0.326	0.401	0.194	0.230	0.229	0.403	0.452	0.364	0.372	0.333	0.424	0.581	0.452	0.433	0.440	0.465	0.406	0.348	0.000			
CI	0.394	0.375	0.368	0.366	0.308	0.405	0.328	0.363	0.377	0.331	0.380	0.411	0.455	0.397	0.403	0.426	0.363	0.301	0.354	0.254	0.339	0.190	0.331	0.243	0.338	0.108	0.283	0.485	0.000		
JA	0.354	0.336	0.330	0.325	0.270	0.360	0.296	0.324	0.339	0.291	0.346	0.368	0.410	0.361	0.388	0.416	0.351	0.290	0.339	0.245	0.330	0.201	0.318	0.227	0.282	0.084	0.262	0.456	0.034	0.000	

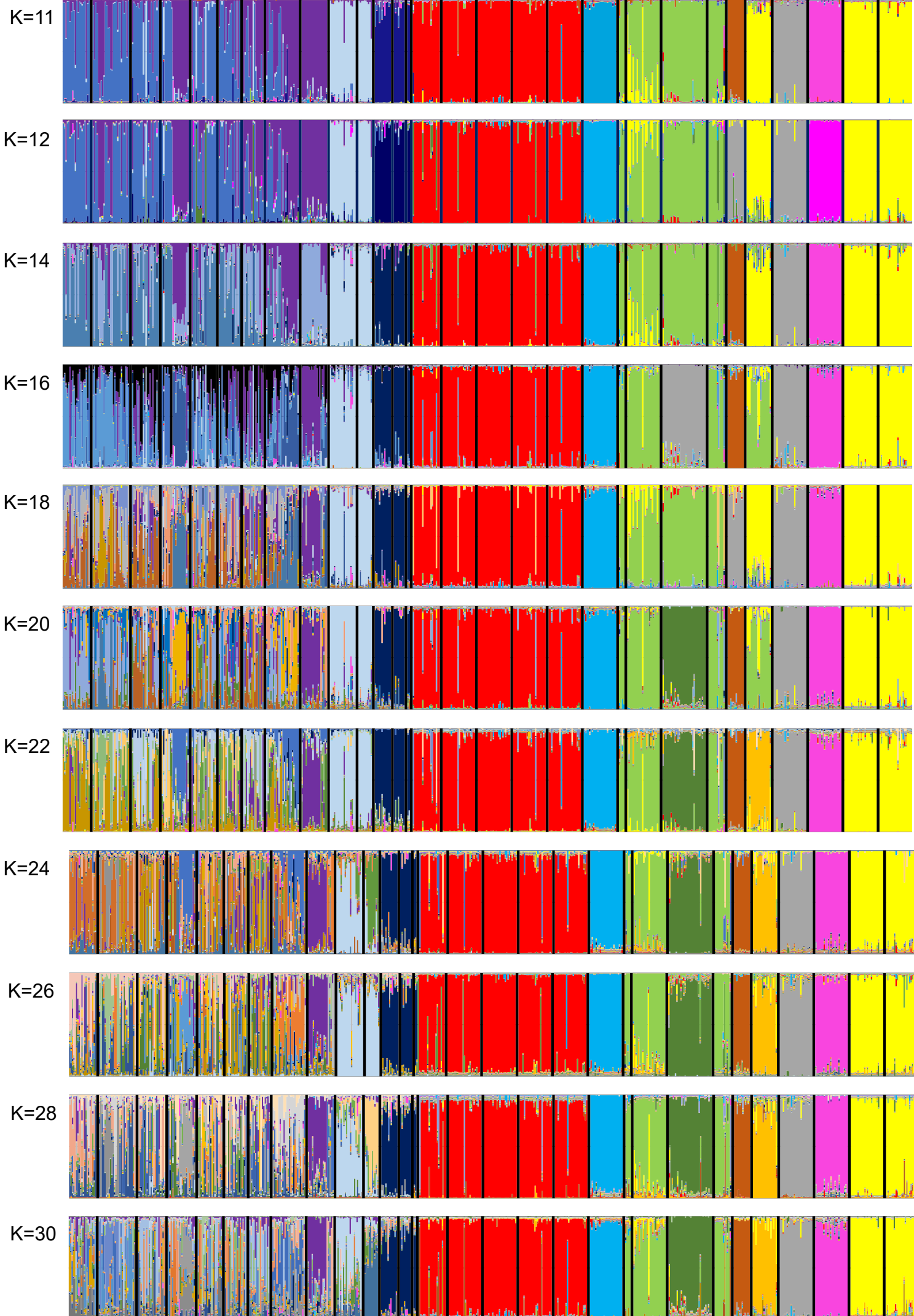


**Figure S5.** Pollinating wasps, Factorial Component Analysis of individual mut locus microsatellite genotypes. The first 6 components are informative for species separation. Every species is individualised in the analysis, e.g. sp2 on axis 2, sp7 and sp8 on axis 3, sp3 on axis 4, sp5 on axis 5, and sp4 is distinguished from sp6 on axis 6.

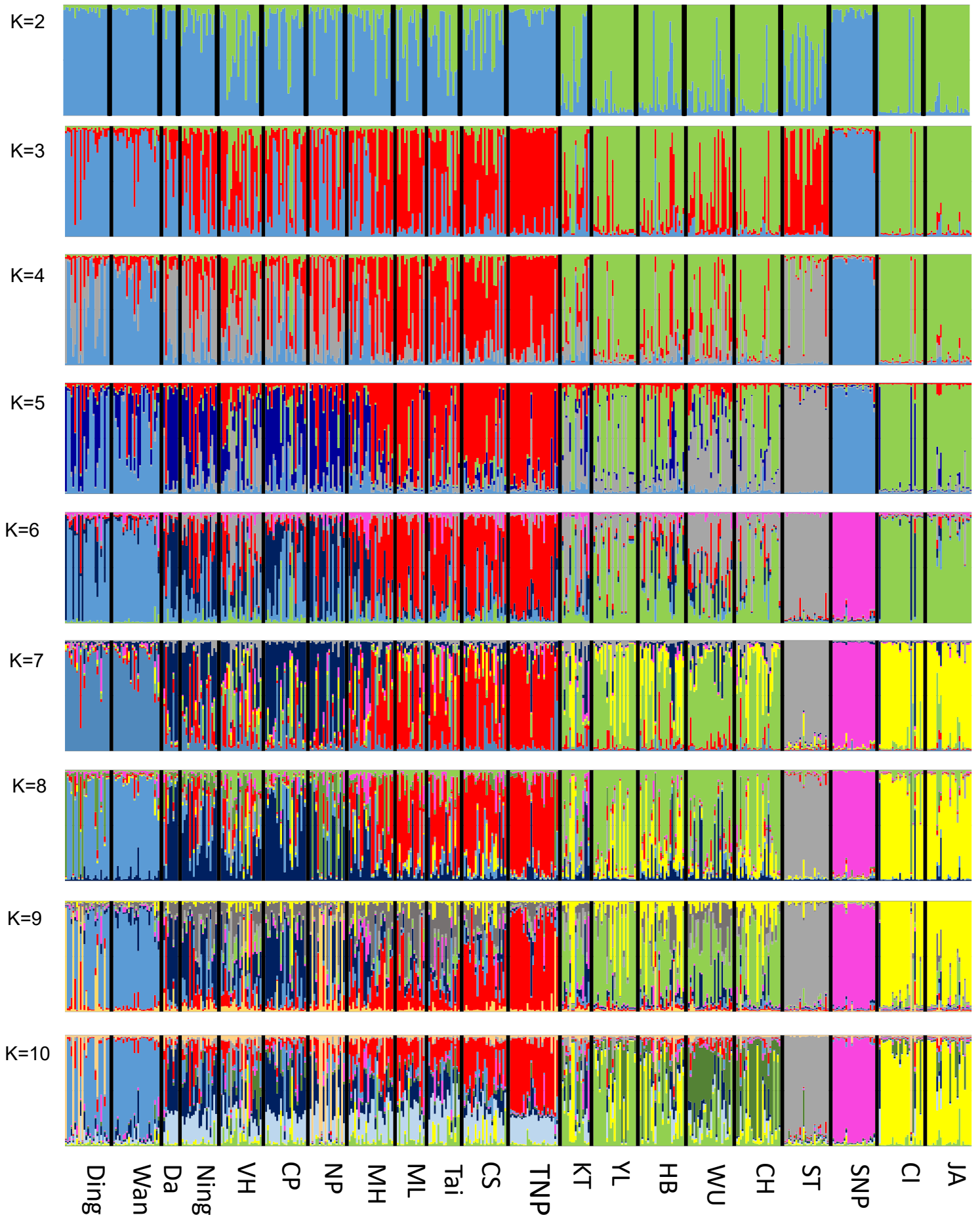
**Figure S6a.** Assignments of pollinating wasp individuals to clusters using STRUCTURE for K = 2 to K =30  
 Sp4 is separated from sp6 only for for K values of 20 or above. Sp1, is separated into 3 entities, Continental China locations (Ning to Nan), Hainan locations (Ding and Wan), and Vietnam locations (VH, CP, NP). Sp2 appears as a homogeneous entity (MH, ML, QMS, Tai, CS). Sp3 (TNP) is individualized for K>10.



**Figure S6a continued.** Assignments of pollinating wasp individuals to clusters using STRUCTURE for  $K = 2$  to  $K = 30$

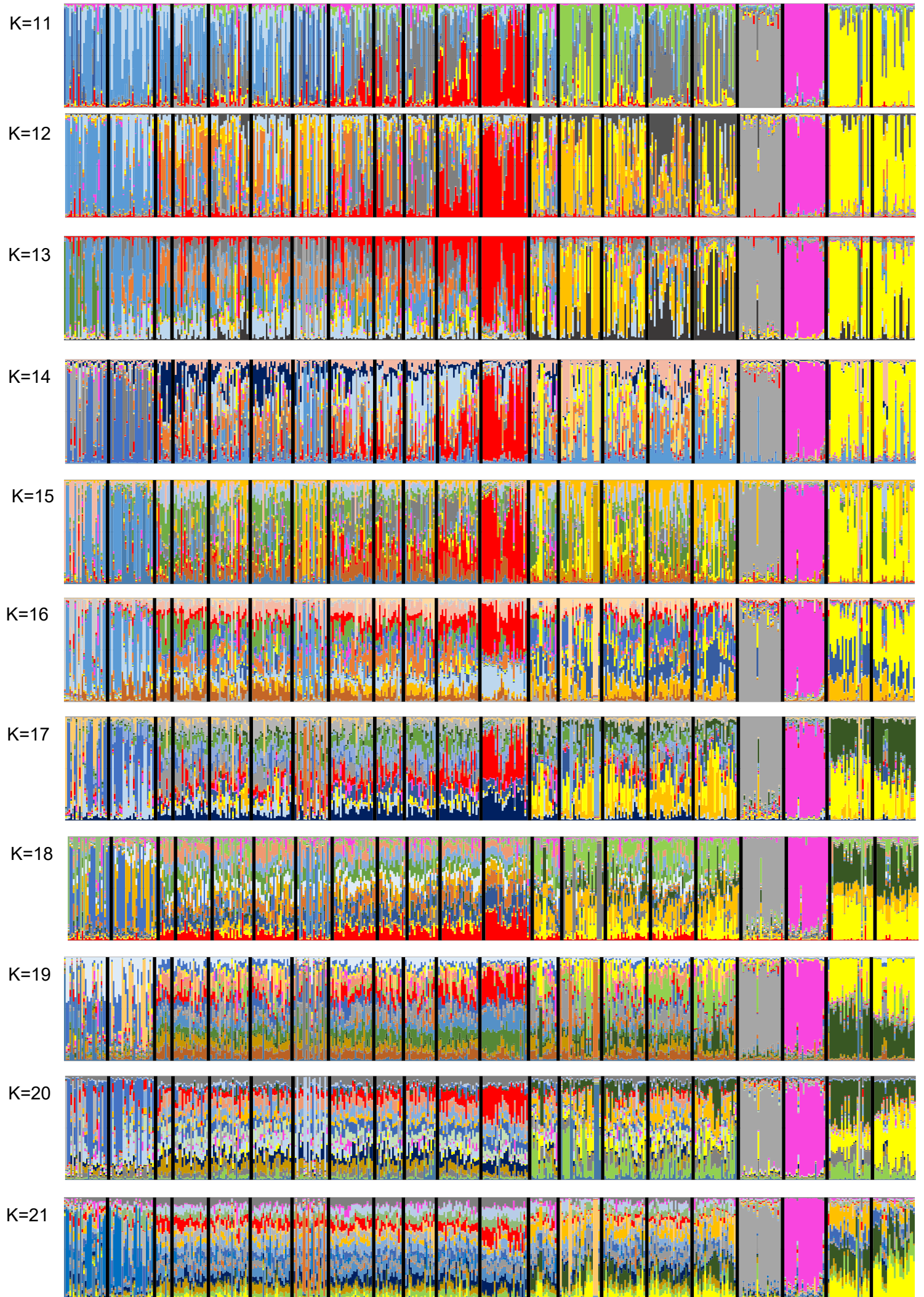


**Figure S6b.** Assignments of *Ficus hirta* individuals to clusters using STRUCTURE for  $K = 2$  to  $K = 21$ . For  $K=2$ , a north south gradient is apparent. Singapore belongs with the northern locations. At higher values of  $K$ , the peninsular location (grey) and the Singapore location (rosa) are separated from the other locations. The two Java locations (yellow) are also distinctive, but close to the central locations. In the north, the Hainan locations (Ding, Wan) are somewhat separated from the other locations.





**Figure S6b, continued.** Assignment of *Ficus hirta* individuals to cluster using STRUCTURE for K=12-21.

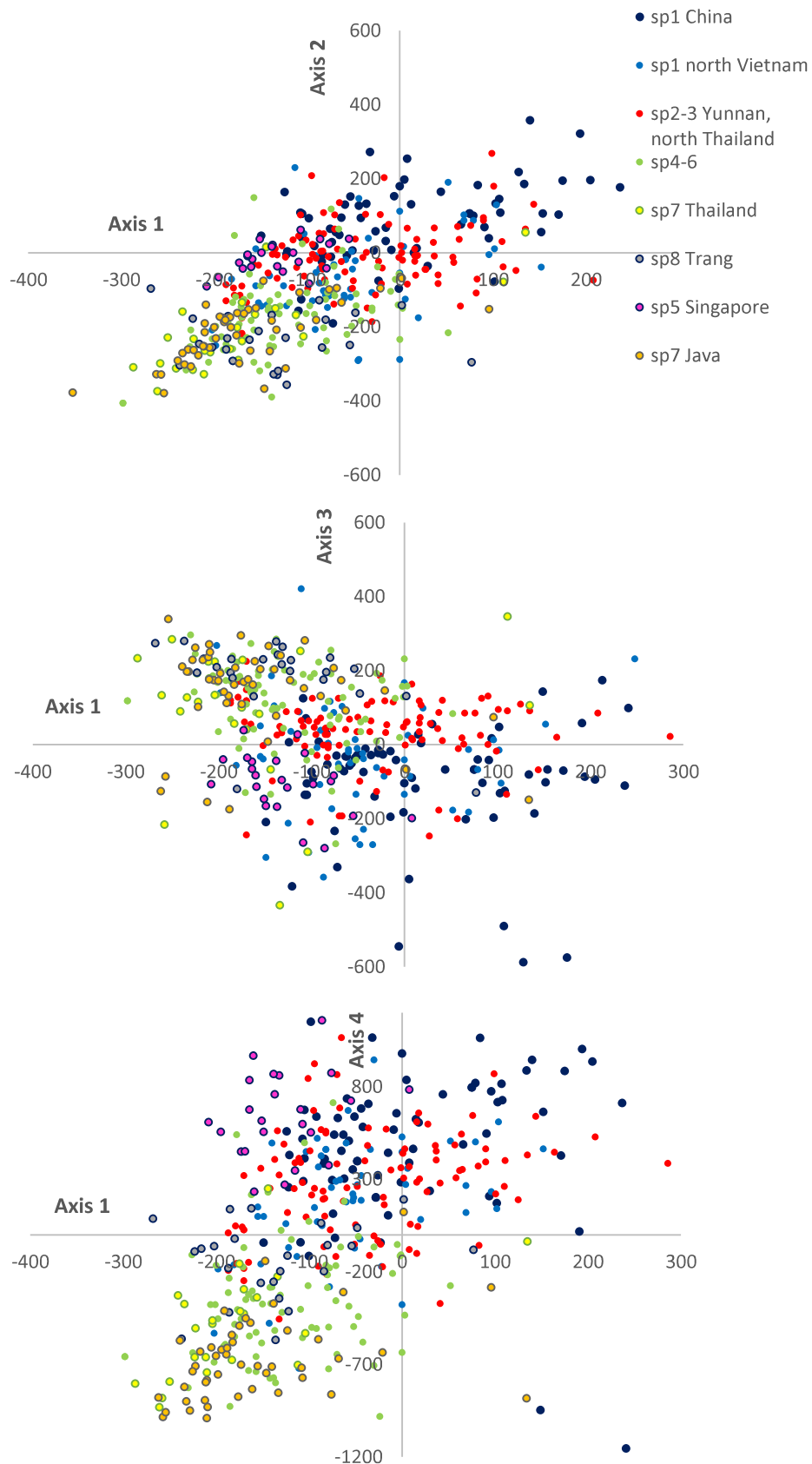


**Table S9.** *Ficus hirta*, trnLF and trnSG data. Sampled populations with their abbreviations. Sample size (N), number of haplotypes (K), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ), and cpDNA haplotypes with number of individuals.

Pop.	N	K	Hd	$\pi$	cpDNA haplotype (number of individuals)
Da	10	1	0	0	H1(10)
Xia	10	1	0	0	H1(10)
Sha	9	2	0.556	0.00082	H1(4),H3(5)
Ning	4	1	0	0	H1(4)
Xiang	6	1	0	0	H1(6)
Ao	10	3	0.511	0.00049	H1(1),H2(2),H3(7)
Sui	9	1	0	0	H3(9)
Sand	10	1	0	0	H10(10)
Gui	11	2	0.182	0.00013	H8(10),H11(1)
Huo	9	2	0.389	0.00028	H3(2),H8(7)
DHS	10	2	0.2000	0.00015	H3(9),H8(1)
Nan	9	1	0	0	H3(9)
Hai	2	1	0	0	H12(2)
Wan	12	1	0	0	H8(12)
XI	9	3	0.6667	0.00286	H4(4),H8(1),H12(4)
MH	4	1	0	0	H5(4)
ML	3	2	0.6667	0.00294	H18(1),H19(2)
Tai	18	5	0.4052	0.00051	H4(14),H5(1),H6(1),H7(1),H8(1)
Note: same population of Tai (9samples) in Yu et al. 2010					
CS	10	1	0	0	H20(10)
TNP	14	2	0.3626	0.00027	H7(11),H21(3)
Wu	7	1	0	0	H8(7)
CH	9	1	0	0	H8(9)
ST	22	2	0.4848	0.00036	H8(14),H9(8)
Note: same population of ST (10 samples) in Yu et al. 2010					
VH	10	2	0.3556	0.00079	H13(8),H14(2)
CP	12	2	0.545	0.00121	H15(6),H16(6)
NP	12	2	0.5303	0.00236	H13(7),H17(5)
HB	10	2	0.5556	0.00041	H22(5),H13(5)
KT	12	1	0	0	H13(9)
YL	12	2	0.4848	0.00036	H13(8),H23(4)
Note: the population is near to DAL					
SNP	12	1	0	0	H24(12)
JA	12	1	0	0	H8(12)
TOTAL	309	24	0.716	0.00134	

**Table S10.** *Ficus hirta*, microsatellite data. Number of alleles (Na), allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficients (Fis).

Pop	Na	Ar	PAr	Ho	He	Fis
Da	5.3	2.9	0.2	0.804	0.712	-0.307
Ning	8.0	3	0.21	0.781	0.766	-0.053
Ding	8.4	2.89	0.4	0.747	0.747	-0.200
Wan	7.1	2.75	0.25	0.704	0.713	-0.167
MH	7.7	2.96	0.2	0.771	0.763	-0.175
ML	6.6	2.93	0.1	0.738	0.749	-0.158
Tai	7.7	2.92	0.15	0.756	0.752	-0.011
CS	8.1	2.91	0.14	0.769	0.744	-0.233
TNP	7.1	2.84	0.15	0.761	0.745	-0.159
Wu	6.9	2.73	0.05	0.789	0.711	-0.023
CH	6.3	2.6	0.07	0.642	0.659	0.146
ST	7.1	2.44	0.23	0.606	0.582	-0.028
VH	7.6	2.98	0.18	0.629	0.759	-0.371
CP	8.1	2.92	0.15	0.747	0.760	-0.165
NP	9.1	3.05	0.58	0.614	0.778	-0.093
HB	5.9	2.44	0.09	0.616	0.590	-0.081
KT	6.4	2.83	0.08	0.800	0.728	-0.295
DAL	6.9	2.68	0.21	0.696	0.690	-0.064
SNP	5.4	2.32	0.21	0.553	0.571	-0.030
CI	6.7	2.39	0.14	0.576	0.599	0.237
JA	5.4	2.38	0.11	0.551	0.597	0.514
Mean	7.0			0.698	0.701	-0.066

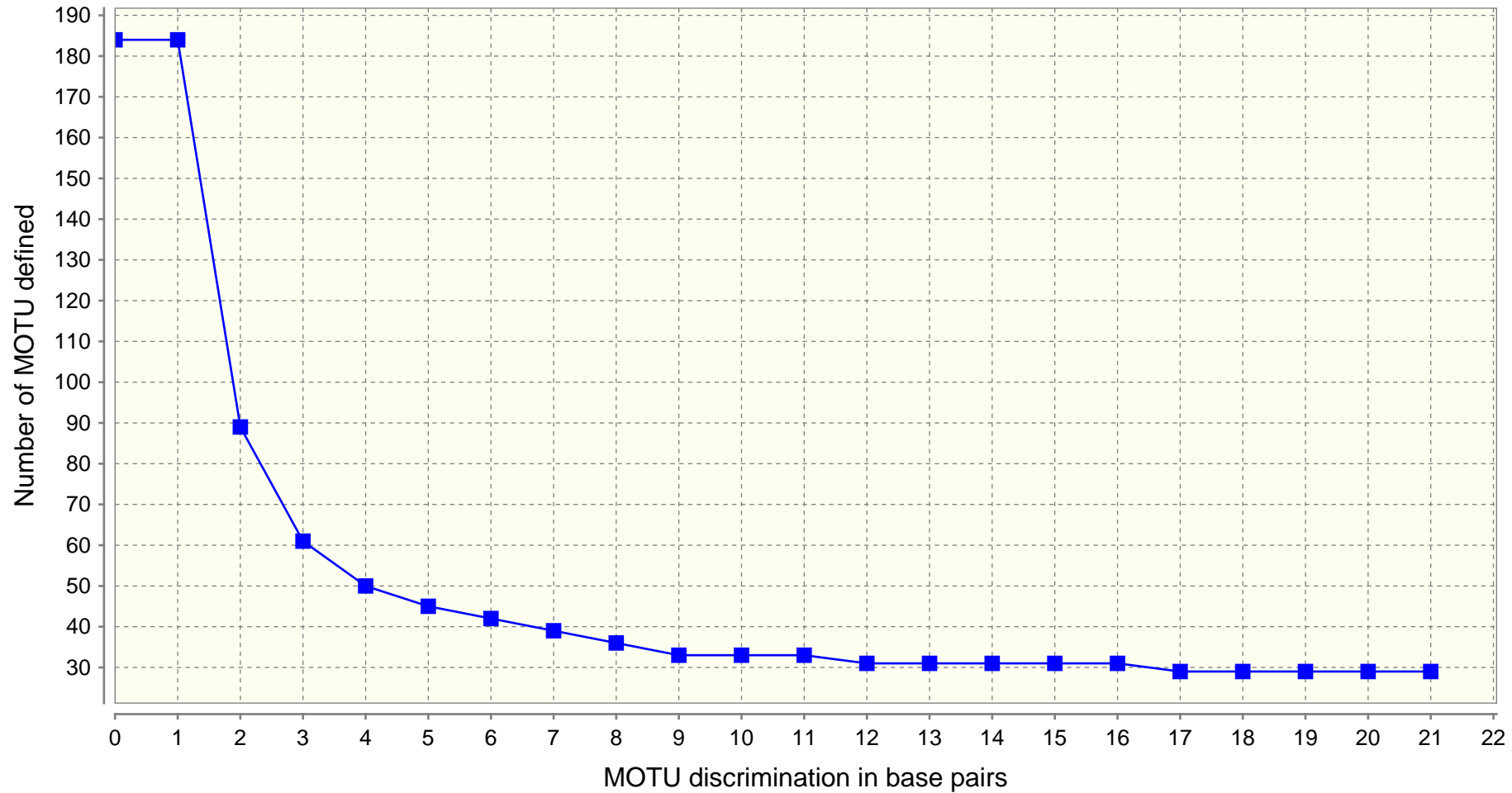


**Figure S7.** *Ficus hirta*, Factorial Component Analysis of multilocus microsatellite genotypes. Colours indicate main pollinating wasp species of the collection location. The order of the species legend follows a north-south direction. On all the axes north-south variation is present, with genotypes from Singapore presenting variable positioning.

**Table S11** Pairwise  $F_{ST}$  values, *Ficus hirta*. The locations are ordered from north to south. Values below 0.08 are highlighted in yellow.

	Ding	Wan	Da	Ning	VH	CP	NP	MH	ML	Tai	CS	TNP	KT	YL	HB	Wu	CH	ST	SNP	CI	JA
Ding	0.000																				
Wan	0.033	0.000																			
Da	0.062	0.064	0.000																		
Ning	0.055	0.062	0.065	0.000																	
VH	0.052	0.047	0.062	0.038	0.000																
CP	0.057	0.045	0.060	0.033	0.027	0.000															
NP	0.052	0.053	0.063	0.053	0.044	0.043	0.000														
MH	0.054	0.055	0.050	0.043	0.038	0.044	0.045	0.000													
ML	0.047	0.044	0.050	0.042	0.034	0.040	0.038	0.031	0.000												
Tai	0.071	0.064	0.077	0.051	0.048	0.048	0.047	0.042	0.032	0.000											
CS	0.041	0.038	0.066	0.049	0.034	0.047	0.034	0.039	0.021	0.040	0.000										
TNP	0.065	0.070	0.075	0.055	0.057	0.060	0.048	0.044	0.040	0.042	0.036	0.000									
KT	0.075	0.075	0.066	0.070	0.048	0.058	0.062	0.047	0.043	0.045	0.058	0.058	0.000								
YL	0.107	0.111	0.115	0.083	0.064	0.072	0.090	0.067	0.065	0.055	0.079	0.083	0.035	0.000							
HB	0.117	0.114	0.134	0.097	0.064	0.079	0.100	0.082	0.080	0.089	0.078	0.102	0.057	0.047	0.000						
Wu	0.091	0.095	0.089	0.052	0.056	0.047	0.066	0.045	0.052	0.037	0.068	0.071	0.035	0.031	0.057	0.000					
CH	0.114	0.115	0.126	0.094	0.090	0.077	0.109	0.094	0.088	0.077	0.102	0.107	0.049	0.038	0.078	0.041	0.000				
ST	0.162	0.178	0.153	0.123	0.144	0.149	0.133	0.142	0.134	0.115	0.141	0.130	0.114	0.149	0.202	0.117	0.158	0.000			
SNP	0.112	0.105	0.158	0.150	0.139	0.128	0.120	0.128	0.124	0.130	0.125	0.132	0.127	0.172	0.198	0.156	0.161	0.179	0.000		
CI	0.110	0.117	0.136	0.111	0.076	0.085	0.098	0.090	0.090	0.096	0.077	0.117	0.067	0.051	0.039	0.074	0.071	0.205	0.181	0.000	
JA	0.105	0.111	0.137	0.112	0.082	0.093	0.089	0.101	0.087	0.095	0.071	0.105	0.072	0.071	0.067	0.087	0.084	0.187	0.158	0.031	0.000

# Cutoff distribution



Metadata associated with Yu et al. Molecular Ecology DRYAD.xls

#### Sheet 1: Genotypic data of *Valisia javana*

Nuclear genotypes (9 loci) for 568 individuals of *Valisia javana*. Population refers to collection sites, with GPS coordinates provided in the main text. The sampling spanned a distance of over 4100 km. For each nuclear locus, alleles names are arbitrary (i.e., do not indicate size or sequence characteristics), and “0” indicates missing data.

#### Sheet 2: Genotypic data of *Ficus hirta*

Nuclear genotypes (7 loci) for 449 individuals of *Ficus hirta*. Population refers to collection sites, with GPS coordinates provided in the main text. The sampling spanned a distance of over 4100 km. For each nuclear locus, alleles names are arbitrary (i.e., do not indicate size or sequence characteristics), and “0” indicates missing data.

#### Notebook 1: Mitochondrial DNA sequence (COI) of *Valisia javana*

The aligned partial mtDNA COI sequences of *Valisia javana* with 330 individuals and the GPS coordinates for the sampling locations were included. Local population designations follow Sheet 1 and the main text.

#### Notebook 2: nuclear DNA sequence (ITS2) of *Valisia javana*

The aligned partial nrDNA ITS2 sequences of *Valisia javana* with species and haplotypes. Species and haplotype designations follow Table S5 in supplementary data and the main text.

#### Notebook 3: Chloroplast DNA sequence (trnLF+trnSG) of *Ficus hirta*

The aligned partial cpDNA (trnLF+trnSG) sequences of *Ficus hirta* with 309 individuals and the GPS coordinates for the sampling locations were included. Local population designations follow Sheet 2 and the main text.