

# Selection for High Oridonin Yield in the Chinese Medicinal Plant *Isodon* (Lamiaceae) Using a Combined Phylogenetics and Population Genetics Approach

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## Abstract

Oridonin is a diterpenoid with anti-cancer activity that occurs in the Chinese medicinal plant *Isodon rubescens* and some related species. While the bioactivity of oridonin has been well studied, the extent of natural variation in the production of this compound is poorly known. This study characterizes natural variation in oridonin production in order to guide selection of populations of *Isodon* with highest oridonin yield. Different populations of *I. rubescens* and related species were collected in China, and their offspring were grown in a greenhouse. Samples were examined for oridonin content, genotyped using 11 microsatellites, and representatives were sequenced for three phylogenetic markers (ITS, *rps16*, *trnL-trnF*). Oridonin production was mapped on a molecular phylogeny of the genus *Isodon* using samples from each population as well as previously published Genbank sequences. Oridonin has been reported in 12 out of 74 species of *Isodon* examined for diterpenoids, and the phylogeny indicates that oridonin production has arisen at least three times in the genus. Oridonin production was surprisingly consistent between wild-collected parents and greenhouse-grown offspring, despite evidence of gene flow between oridonin-producing and non-producing populations of *Isodon*. Additionally, microsatellite genetic distance between individuals was significantly correlated with chemical distance in both parents and offspring. Neither heritability nor correlation with genetic distance were significant when the comparison was restricted to only populations of *I. rubescens*, but this result should be corroborated using additional samples. Based on these results, future screening of *Isodon* populations for oridonin yield should initially prioritize a broad survey of all species known to produce oridonin, rather than focusing on multiple populations of one species, such as *I. rubescens*. Of the samples examined here, *I. rubescens* or *I. japonicus* from Henan province would provide the best source of oridonin.

**Citation:** Harris ESJ, Cao S, Schoville SD, Dong C, Wang W, et al. (2012) Selection for High Oridonin Yield in the Chinese Medicinal Plant *Isodon* (Lamiaceae) Using a Combined Phylogenetics and Population Genetics Approach. PLoS ONE 7(11): e50753. doi:10.1371/journal.pone.0050753

**Editor:** Ting Wang, Wuhan Botanical Garden, Chinese Academy of Sciences, China

**Received:** June 12, 2012; **Accepted:** October 25, 2012; **Published:** November 27, 2012

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**Funding:** This work was supported by grants from the National Institutes of Health National Cancer Institute (U19 CA128534) and the Bernard Osher Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Oridonin is a bioactive diterpenoid produced by plant species in the genus *Isodon* (Schrader, ex Benth.) Spach (Fig. 1). Several studies have shown that oridonin has potent *in vitro* and *in vivo* activity against human cancer cells [1–3], including ovarian [4], breast [5], colorectal [6], laryngeal [7], leukemia [8], liver [9], and prostate [10] cancers. Oridonin was first isolated from the plant *Isodon japonicus* (Burm. f.) H. Hara whose common name in Japanese is *orido* [11], hence the chemical name, and shortly after was also detected in other species of *Isodon*, most notably the Chinese medicinal plant *Isodon rubescens* (Hemsl.) H. Hara [12,13]. In fact, the majority of recent studies that evaluate the bioactivity of oridonin have focused on *I. rubescens* as the source of the compound [10,14–16].

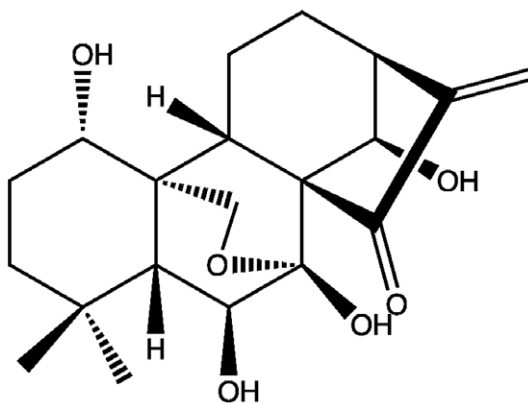
The aerial portions of *I. rubescens* have traditionally been used as a folk medicine in Henan province, central China for the treatment of sore throat, inflammation and gastrointestinal problems [17] where it is usually called *donglingcao* (???). The medicinal uses of *I. rubescens* were first documented in the 1977 edition of the Pharmacopoeia of the People's Republic of China [18]. As such, *I. rubescens* is a relatively recent introduction to the Traditional Chinese Medical literature. Although *I. rubescens* was not included in editions of the Chinese Pharmacopoeia after 1977, it was reintroduced in the most recent edition [19].

While much work has been done to characterize the chemical structure and diversity of diterpenoids in *I. rubescens* and other species of *Isodon* [3,17,20–22], relatively little is known about the evolutionary or ecological context of these abundant compounds

[but see 23], or their variation in nature. The main goal of this paper is to summarize the results of a preliminary study of the natural variation of oridonin production using a unique combination of phylogenetic and population genetic approaches. This work was done to guide researchers in selecting populations of *I. rubescens* and related species that produce the most oridonin, since to-date there is no reported *de novo* synthesis of oridonin. This project was carried out in the context of a larger endeavor to evaluate more than 200 species of commonly used Chinese medicinal plants for their bioactivity [24]. The study of oridonin production in *Isodon* was to be used as an example that could be applied to any potential screening discoveries of promising bioactive chemicals that would require additional plant material for follow-up pharmacological studies and clinical trials.

While oridonin has been extensively studied for therapeutic potential, it should be noted that it is not the only bioactive diterpenoid in *Isodon* [10]. Furthermore, it is possible that synergy or additivity of multiple diterpenoids may contribute to higher bioactivity of the whole extract of *I. rubescens* as compared to oridonin alone [16]. Therefore, the results of this study may not necessarily be extended to selection of other diterpenoids in *Isodon* or for the total bioactivity of *Isodon* extracts.

The genus *Isodon* is comprised of about 100 species and occurs primarily in Asia, with a few species in Africa [25]. The center of diversity of the genus is in China, where 77 species of the genus are located [26]. Although the classification of *Isodon* has been well studied, recent molecular phylogenetic work has placed the traditional subgeneric classification in question [27]. In addition, a study of *Isodon* in Japan using chloroplast sequence markers found low phylogenetic resolution and many species were polyphyletic. The phylogenetic patterns were attributed to some combination of a recent radiation, hybridization, or incomplete lineage sorting of ancestral polymorphisms in the genus *Isodon* [28]. Given the low phylogenetic resolution between *Isodon* species, published studies of the high diversity of diterpenoids in both *Isodon* [17] and *I. rubescens* [21], as well as suggestions of variability in diterpenoid production according to growing environment [29,30], the expectation for this study was that oridonin production would be highly variable, both throughout *Isodon* and within *I. rubescens*. This variability would be indicated by multiple gains and losses of oridonin production in the evolutionary history of *Isodon*, and also by variation of oridonin production between individual plants, both within the same population as well as different populations of *I. rubescens* and also between parents and offspring. To evaluate these expectations, oridonin production was



**Figure 1. Chemical structure of oridonin.**  
doi:10.1371/journal.pone.0050753.g001

**Table 1. Information for *Isodon* populations collected in this study.**

Population	Location	Province	Latitude	Longitude	Parents, Microsat. Genotyped (N)	Parents, Tested for Oridonin (N)	Offspring, Microsat. Genotyped (N)	Offspring, Tested for Oridonin (N)
<i>enanderianus</i> QD	Qiongdongnan Prefecture	Guizhou	27° 7.027'N	108° 7.069'E	11	6	13	6
<i>enanderianus</i> TR	Tongren Prefecture	Guizhou	27° 44.159'N	108° 48.888'E	12	5	4	4
<i>henryi</i> XN	Xinxian city	Henan	31° 37.374'N	114° 49.112'E	12	7	10	7
<i>japonicus</i> XX	Xinxiang city	Henan	35° 38.725'N	113° 33.118'E	1	1	3	3
<i>lophanthoides</i> var. <i>micranthus</i> TR	Tongren Prefecture	Guizhou	27° 44.159'N	108° 48.888'E	1	1	0	0
<i>rubescens</i> HB	Hebi city	Henan	35° 38.533'N	114° 6.414'E	11	11	15	9
<i>rubescens</i> JY	Jiyuan city	Henan	35° 11.081'N	112° 30.183'E	14	14	9	9
<i>rubescens</i> QY	Qiyang city	Henan	35° 12.848'N	112° 43.756'E	10	9	12	9
<i>rubescens</i> XX	Xinxiang city	Henan	35° 38.68'N	113° 33.059'E	10	10	12	9
<i>rubescens</i> YC	Yichang city	Hubei	30° 11.527'N	110° 39.656'E	11	11	12	8
			TOTAL		93	75	90	64

Details include population name (species name with location code), location information, as well as the number of wild-collected parents and greenhouse-grown offspring from each population that were microsatellite genotyped and tested for oridonin content. Location codes are used throughout the text.  
doi:10.1371/journal.pone.0050753.t001

mapped onto a phylogeny of *Isodon* to determine the extent of evolutionary lability and to identify species or clades that might be the best sources of the compound. In addition, oridonin production was compared in microsatellite-genotyped individuals of wild-collected parents and greenhouse-grown offspring to determine heritability and populations with the highest chemical production.

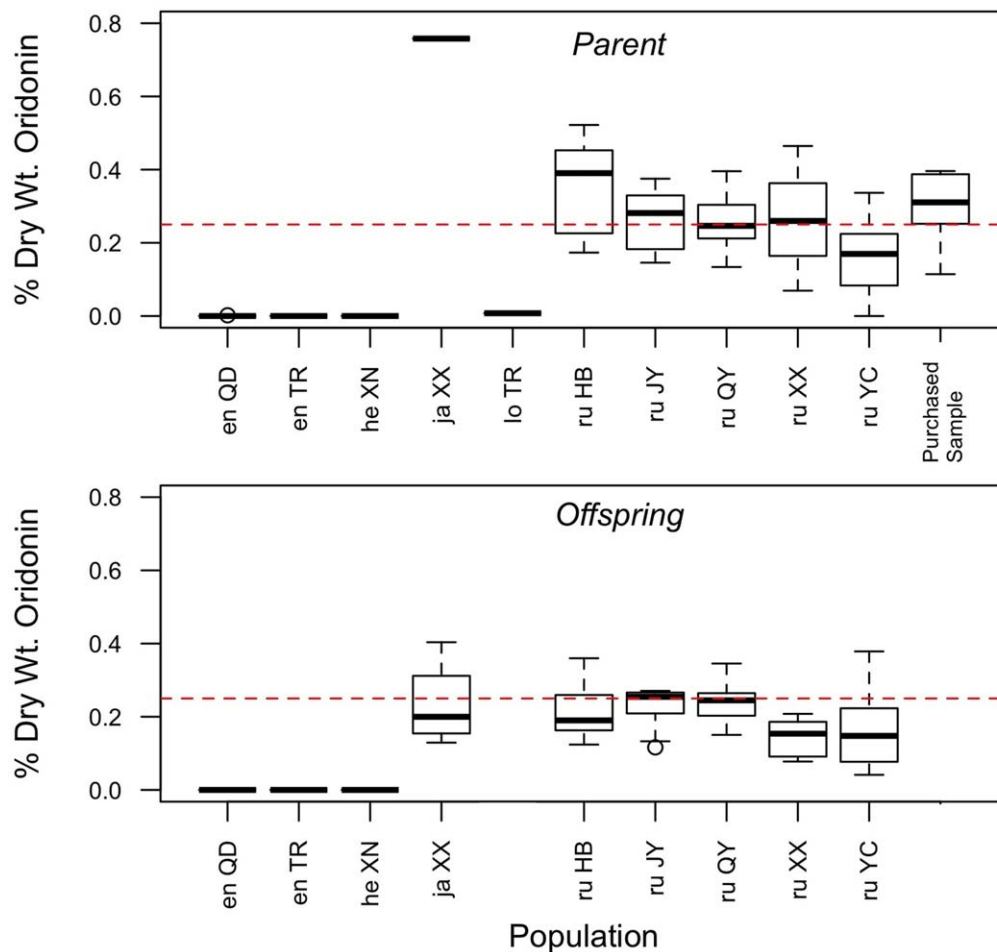
**Materials and Methods**

**Sample Collection**

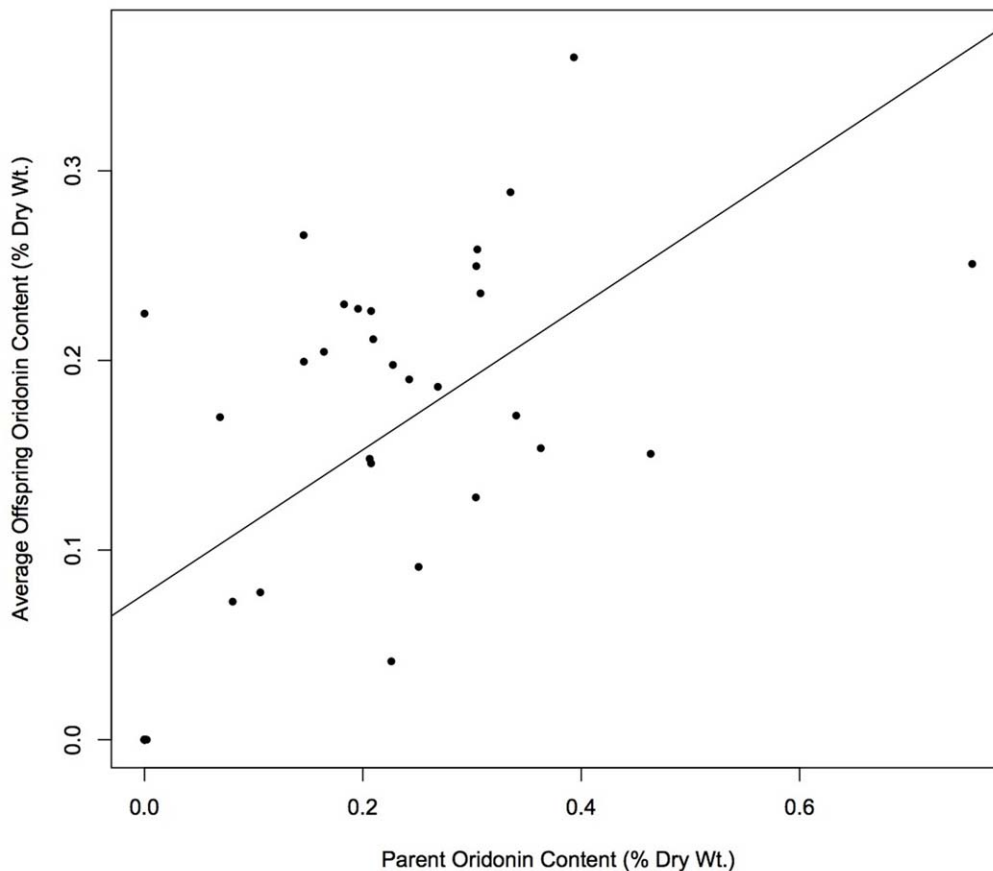
Samples of *I. rubescens* and related species were collected from the People’s Republic of China in October and November of 2009 in Henan, Hubei, and Guizhou provinces. Samples were collected from a total of eight sites, with about 10 to 15 individuals collected at each site. Five populations consisted of the species *I. rubescens*, four of which were collected in the area of traditional use of *donglingcao* in Northern Henan province. One population of *I. rubescens* from Henan province represented a *donglingcao* cultivation site (JY). Two populations from Guizhou were determined to be the species *Isodon enanderianus* (Hand.-Mazz.) H.W. Li and one population from southern Henan province was identified as *Isodon henryi* (Hemsl.) Kudô. In addition, individuals of *I. japonicus* and *I.*

*lophanthoides* var. *micranthus* (C.Y. Wu) H.W. Li were collected growing at the same collection sites of *I. rubescens* and *I. enanderianus*, respectively. Location details for each population are shown in Table 1.

The collection of each individual consisted of a pressed voucher specimen, leaves in silica desiccant for DNA and chemical analyses, and seeds, if available. Representative voucher specimens are deposited at the Gray Herbarium of Harvard University (GH) and are noted in Table S1. In addition to wild-grown samples collected for the project, five samples of the herbal medicine *donglingcao* were purchased in Zhengzhou city, Henan province. Collection activities for this study were carried out under the auspices of a larger collaborative project between Harvard Medical School and the Beijing University of Chinese Medicine. Details about the collaboration agreements and the relevant Chinese government authorities that reviewed them are provided in a publication about the larger project [24]. No specific permits or permissions beyond those in the collaboration agreements were required for the collections in this study. Furthermore, collections were not conducted on privately owned or protected areas and no species of *Isodon* are endangered or protected.



**Figure 2. Boxplot diagram of oridonin yield in parents and offspring.** Populations are indicated by a two-letter species code (en = *enanderianus*; he = *henryi*; ja = *japonicus*; lo = *lophanthoides*; ru = *rubescens*) and a two-letter location code as provided in Table 1. The dashed line indicates the minimum required quantity of oridonin in the herb *donglingcao* (0.25% dry wt.), as specified in the 2010 edition of the Pharmacopoeia of the People’s Republic of China [19]. doi:10.1371/journal.pone.0050753.g002



**Figure 3. Heritability of oridonin production.**

doi:10.1371/journal.pone.0050753.g003

### Greenhouse

Seeds from parents representing each population were planted on April 7, 2010 in seedling trays. Seeds were grown in 3B Mix potting soil, which includes peat moss, pine bark, perlite, starter nutrients, wetting agent, and limestone (Fafard; Agawam, Massachusetts). A total of 1824 seeds were planted, averaging 27 seeds from each individual that was producing seeds at the time of collection. Seeds were collected from between four and 11 individuals per population. Of the total, 439 seeds germinated, and 308 plants survived to harvest. Seedlings were transplanted after two weeks to individual pots. Plants were arranged randomly with respect to parent population source, and re-arranged periodically during the growing period. Offspring individuals were harvested on August 10, 2010, which represents the typical harvest time of *I. rubescens* [19]. Leaves were harvested and immediately placed in silica desiccant.

### Extraction and Chromatography of Oridonin

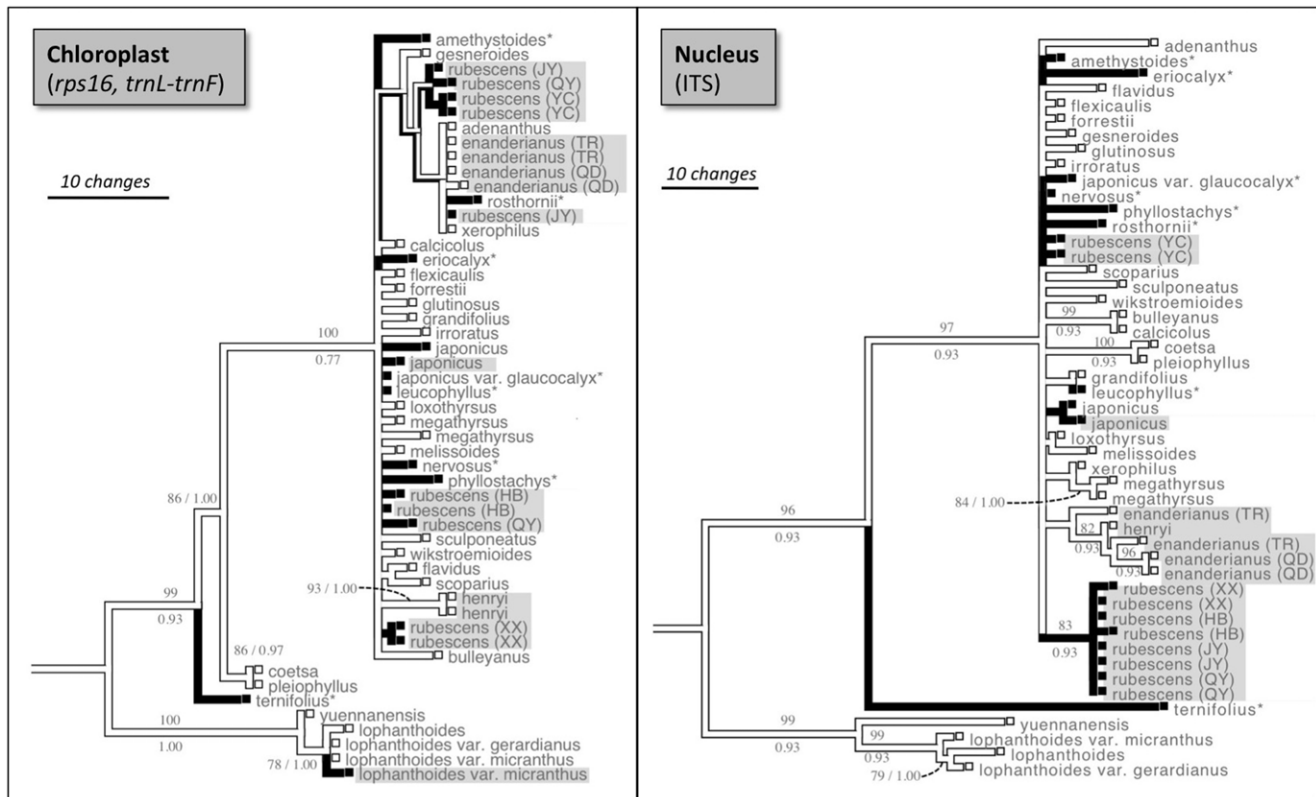
Between 200–500 mg of dried leaf samples were ground using a mortar and pestle to prepare for chemical analyses. The ground samples were put in a 40 mL vial (National Scientific Co.) and 10 mL of 1:1 dichloromethane : ethanol were added. The solution was sonicated for 30 min, centrifuged for 5 min, then filtered into a new 40 mL vial using Whatman Grade 1 Filter Paper (Whatman; Piscataway, NJ) without disturbing the pellet. The filtrate was placed in a cold room at 4°C and the filtrant was resuspended in 10 mL of 1:1 dichloromethane:ethanol and left at room temperature overnight. The mixture was then filtered, and

resulting filtrate was combined with the filtrate of the first step. The combined filtrate was dried in a Savant SPD2010 Speedvac (Thermo Scientific; Waltham, MA) for 5–6 hrs. After the extract was completely dry, 20 mL of LC/MS grade methanol was added and the resulting mixture sonicated for 30–60 min. Once fully dissolved, 150 µL of the resulting solution was added to a 1 mL syringe (Luer Lock Tuberculin Syringe; BD; Franklin Lakes, NJ) with filter (Acrodisc LC 13 mm Syringe Filter with 0.2 µm PVDF membrane; Pall Corporation; Port Washington, NY), and filtered into a 2 mL screw top vial (Agilent Technologies; Santa Clara, CA) using a vial insert.

Ten µL of extracted and filtered samples were run on an Agilent 1200 Series HPLC/6130 Series MS (Agilent Technologies; Santa Clara, CA), with a Phenomenex Luna C18 column (100 mm×4.60 mm 5 micron; Phenomenex; Torrance, CA) using a gradient elution of 15% to 100% CH<sub>3</sub>CN in water with 0.1% formic acid over 35 min at a flow rate of 0.7 mL/min. To quantify oridonin content, standard oridonin was run at seven concentrations from 1.5 ng/µL to 100 ng/µL. Oridonin content was quantified by comparison with a standard curve constructed from integrated peak areas at both UV 254 nm and MS+347. Oridonin content was expressed as percent dry weight (% dry wt.) of the plant.

### Heritability and Population Comparison of Oridonin Content

The heritability of oridonin yield was assessed by plotting the average offspring oridonin amount against parent oridonin



**Figure 4. Presence of oridonin when mapped onto a molecular phylogeny of *Isodon*.** Phylograms represent strict consensus of most parsimonious (MP) trees, using sequence data from the chloroplast (*rps16*, *trnL-trnF*; strict consensus of 140 MP trees) and nucleus (ITS; strict consensus of 390 MP trees). Presence of oridonin is indicated by dark colored branches. Taxa reported polymorphic for oridonin production (some populations produce oridonin, others do not) are shown with an asterisk. Samples newly sequenced for this study are shown with gray shading. Parsimony bootstrap (BS) values are shown above branches, Bayesian posterior probabilities (PP) are shown below branches. Support values are shown only for branches with BS>75. Outgroup taxon (*C. xanthanthus*) used for ancestral state construction not shown. doi:10.1371/journal.pone.0050753.g004

amount. The regression coefficient ( $b_{op}$ ) of offspring oridonin yield against parental yield was estimated and tested for significance using a t-test. The one parent-offspring heritability formula was used to calculate the narrow-sense heritability ( $h^2$ ) [31].

### DNA Extraction and Sequencing

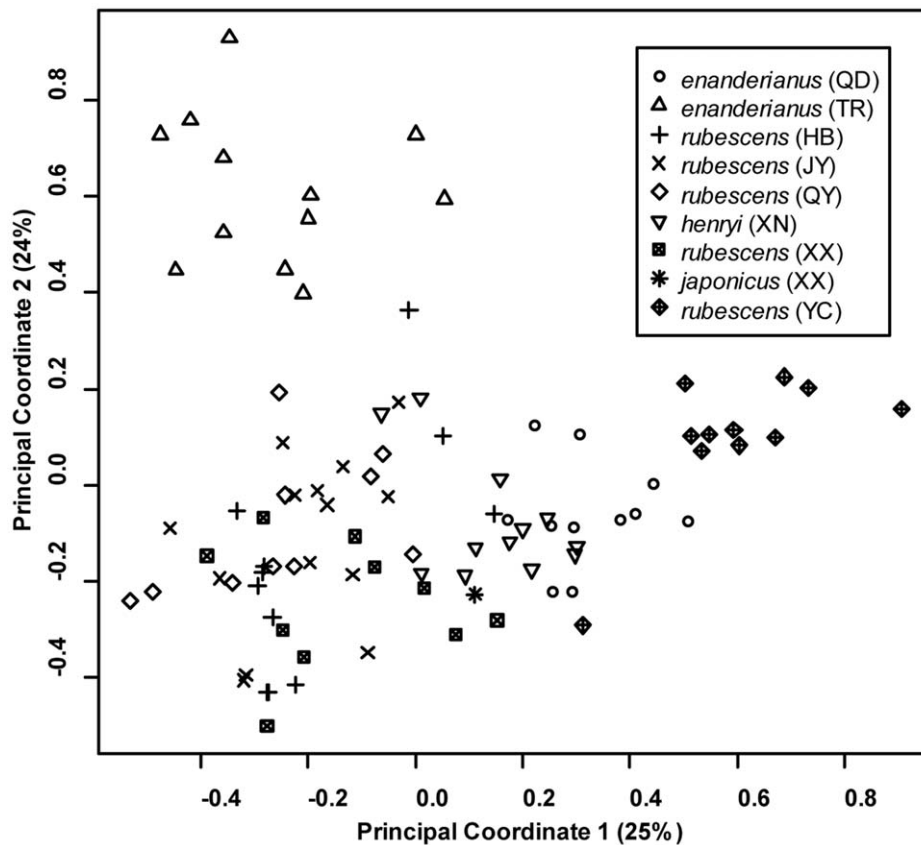
To prepare samples for DNA extraction, a small amount of leaf tissue, typically 1 cm<sup>2</sup>, was ground for 20 s in a 2 mL screw cap microcentrifuge tube (Sarstedt; Nümbrecht, Germany) using a 1/4" ceramic sphere (MP Biomedicals; Solon, Ohio) in a Thermo-

**Table 2. Genetic variability of *Isodon* averaged across 11 microsatellite loci.**

Population	N (parent)	N (offspring)	N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	F	F <sub>mat</sub>	T <sub>m</sub> -T <sub>s</sub>
enanderianus QD	11	13	9.091 (1.004)	0.685 (0.042)	0.806 (0.028)	0.147 (0.047)	0.199 (0.090)	-0.159 (0.378)
enanderianus TR	12	4	8.455 (1.012)	0.718 (0.041)	0.764 (0.038)	0.053 (0.042)	-0.200 (0.008)	-0.070 (0.035)
henryi XN	12	10	5.909 (0.814)	0.636 (0.072)	0.675 (0.048)	0.049 (0.090)	0.010 (0.075)	-0.395 (0.125)
japonicus XX	1	3	1.000 (0.234)	0.273 (0.141)	0.136 (0.070)	n/a	n/a	n/a
rubescens HB	11	15	7.727 (0.702)	0.684 (0.048)	0.779 (0.028)	0.128 (0.046)	-0.200 (0.036)	0.250 (0.483)
rubescens JY	14	9	9.273 (0.954)	0.645 (0.047)	0.756 (0.043)	0.143 (0.045)	-0.011 (0.156)	0.387 (0.212)
rubescens QY	10	12	6.000 (0.714)	0.643 (0.072)	0.676 (0.064)	0.043 (0.057)	0.518 (0.201)	0.544 (0.404)
rubescens XX	10	12	4.455 (0.623)	0.459 (0.072)	0.568 (0.061)	0.203 (0.104)	0.065 (0.037)	0.421 (0.521)
rubescens YC	11	12	5.727 (0.634)	0.525 (0.072)	0.650 (0.053)	0.191 (0.087)	0.049 (0.113)	0.296 (0.419)

For each population, the mean value and, in parentheses, standard error for sample size (N), number of alleles (N<sub>A</sub>), observed heterozygosity (H<sub>O</sub>), expected heterozygosity (H<sub>E</sub>), and fixation index (F) is shown for parental microsatellite data. The maternal inbreeding coefficient (F<sub>mat</sub>) and multi-locus population out-crossing rate (T<sub>m</sub>) are shown based on the combined parental and offspring microsatellite data in the program MLTR.

doi:10.1371/journal.pone.0050753.t002



**Figure 5. Plot of principal coordinate analysis of microsatellite variability in *Isodon* populations.**  
doi:10.1371/journal.pone.0050753.g005

Savant Bio 101 FastPrep FP120 (Qiogene; Carlsbad, California). Total genomic DNA was extracted using a Qiagen DNeasy Plant Mini Kit (Qiagen; Valencia, California), following the instructions of the manufacturer. Two individuals for each population were sequenced for three phylogenetic markers: *rps16*, *trnL-trnF*, and ITS. One individual each of *I. japonicus* and *I. lophanthoides* was also sequenced. PCR mixes included 2  $\mu$ L of genomic DNA, 1  $\mu$ L of forward and reverse primer, and 15  $\mu$ L of water in a Bioneer Premix tube (Bioneer; Oakland, California). The *rps16* region was amplified with the primers *rpsF* and *rpsR2* [32], using a touchdown PCR protocol as follows: 95°C for 2:30; 20 cycles of 95°C for 20 seconds; 60°C for 20 s reduced by 0.5°C per cycle, 72°C for 30 s; then 15 cycles of 95°C for 20 s, 50°C for 20 s and 72°C for 30s, followed by a 72°C extension step of 10 min. The *trnL-trnF* region was amplified with the primers *TabC* and *TabF* [33] using the following PCR protocol: 95°C for 3 min; 30 cycles of 95°C for 1 min, 50°C for 30 s, 72°C for 1 min, followed by an extension step of 72°C for 7 min. The ITS region was amplified using the ITS5 and ITS4 primers [34] using the following PCR protocol: 96°C for 1 min; 40 cycles of 96°C for 10 s, 48°C for 30 s, 72°C for 20 s +4 s per cycle; and 72°C for 7 min. PCR reactions were visualized using a 1X TAE agarose gel, cleaned using the Qiagen Qiaquick PCR cleanup kit, and sequenced using an ABI Hitachi 3730XL DNA Analyzer (Applied Biosystems; Carlsbad, California).

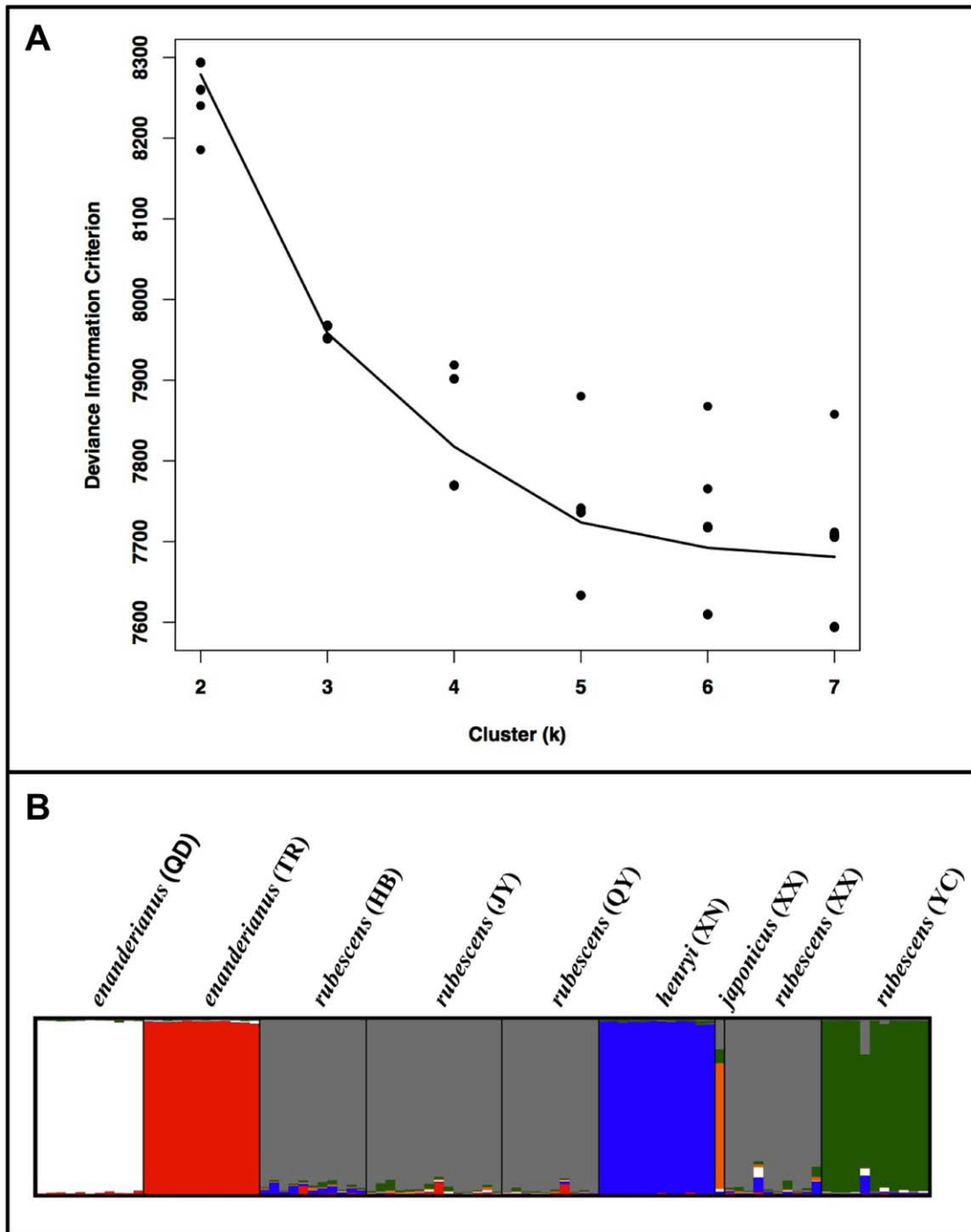
### Phylogenetic Analyses

Sequences were assembled in Geneious Pro 5.4.3 (Biomatters, Auckland, New Zealand). In addition to 18 samples newly

sequenced for this paper, 40 samples were included from a previous study [27] to provide phylogenetic context and to help with the analysis of patterns of oridonin evolution. *Coleus xanthanthus* C. Y. Wu & Y. C. Huang was used as the outgroup, using Genbank sequences from another study [27]. A list of all sequences and associated Genbank identification numbers is provided in Table S1. Sequences were first aligned using MUSCLE [35] as implemented in Geneious, then manually edited in Jalview [36]. Ambiguously aligned regions, such as polynucleotide repeats and regions at the end of sequences were identified by eye, and excluded in Mesquite Version 2.74 [37].

Incongruence between the different gene regions was tested using the incongruence length difference (ILD) test [38] as implemented in PAUP\* 4.0b10 [39]. The ILD test was conducted using the heuristic search method with 1000 homogeneity replicates of 1000 random addition sequence replicates each, saving one tree per addition sequence replicate.

Maximum parsimony (MP) analyses were done in PAUP using parsimony ratchet commands created in PAUPRat [40]. Each PAUPRat analysis was done with 1000 ratchet iterations, with 15% of the characters perturbed on each iteration. The PAUPRat commands were implemented five separate times and the most parsimonious trees from each run were concatenated in PAUP to make a strict consensus tree. Support for the MP trees was assessed using parsimony bootstrap. Each bootstrap analysis consisted of 1000 bootstrap replicates each consisting of 100 heuristic search random addition sequence replicates, saving one tree per replicate. The bootstrap analysis was done five times. The bootstrap trees from each analysis were concatenated in PAUP without excluding



**Figure 6. Results of the Bayesian clustering analysis of microsatellite variation in the *Isodon* samples.** A) Plot of the deviance information criterion versus the number of clusters (k). B) Posterior probability of cluster membership (designated by colors) for each individual represented as barplots.

doi:10.1371/journal.pone.0050753.g006

duplicate trees, and a majority rule consensus tree was constructed using tree weights to provide bootstrap support values.

The best model of evolution for maximum likelihood (ML) and Bayesian analyses was determined using jModeltest 0.1.1 [41,42]. Based on the Akaike information criterion, the TrN+ $\Gamma$  model was selected for the chloroplast data and the GTR+ $\Gamma$  model was selected for the ITS data. The ML analyses were conducted in PAUP and in the online version of RAxML [43] as implemented

through the Cyberinfrastructure for Phylogenetic Research (CIPRES) web portal [44] using parameters specified by jModeltest. For ML analyses conducted in PAUP, the heuristic search method was used with 100 random addition sequence replicates. Bayesian analyses were conducted using MrBayes 3.1 [45,46]. Each Bayesian analysis consisted of four Markov chains running for 1,000,000 generations, and sampling every 100 generations. Since the exact jModeltest specifications could not

be implemented in MrBayes, a general model (i.e., GTR+ $\Gamma$ ) using estimated parameters was used for both the chloroplast and ITS datasets. The trees and parameters corresponding to the first 30% of samples were discarded as the burnin in order to calculate the best tree and posterior probability support values.

### Evolutionary Gains and Losses of Oridonin Production

Oridonin is known to occur in at least 12 out of 74 species (16%) of *Isodon* examined for diterpenoid diversity [20]. In a subset of species known to produce oridonin, production may be polymorphic, as indicated by the fact that some studies have reported oridonin in those species and others have not. For the purposes of testing evolutionary lability of oridonin production, all species with at least one report of oridonin production were coded as “present”, whereas species without any report of oridonin production were coded as “absent.” To evaluate the number of evolutionary gains and losses in oridonin production, the presence or absence of oridonin production was mapped onto the strict consensus tree of the MP analysis using the program Mesquite Version 2.74 [37]. Oridonin presence/absence was empirically determined for the samples newly sequenced in this study, and derived from the literature for other species of *Isodon* that were included in the phylogeny. Table S2 provides a list of species of *Isodon*, whether they produce oridonin, and associated references. The amount of evolutionary lability in oridonin production was estimated using the retention index (RI), and by comparing the parsimony tree length of the oridonin character (i.e., # evolutionary steps) with a null distribution created by randomly reshuffling the oridonin presence/absence character among the samples included in the study. The random distribution was created in Mesquite Version 2.74 by reshuffling the character 1000 times. The tree length comparison was conducted when resolving polytomies as “soft,” since this is the more conservative option for detecting evolutionary gains and losses of a character.

### Amplification and Scoring of Microsatellites

Eleven microsatellites were amplified in both parent and offspring samples in multiplex mixtures using fluorescently labeled primers. The resulting amplicons were mixed with a GeneScan 500 LIZ Size Standard (Applied Biosystems) and separated on an Applied Biosystems 3730xl sequencer (Applied Biosystems). Alleles were scored using GeneMapper v3.0 (Applied Biosystems). Additional details about the microsatellite regions and the methods used to amplify them are provided in [47].

### Population Genetic Analysis

Statistical tests of population genetic variation were conducted within GENALEX v6.41 [48]. First, the effect of pooling parents and offspring was tested to see if it increased Wright’s inbreeding coefficient ( $F_{IS}$ ) for each population. Due to a significant increase in  $F_{IS}$  in several populations, analyses of population genetic variation involve only parents. Each microsatellite locus was tested for deviation from Hardy-Weinberg equilibrium (HWE) using chi-square tests. Due to the large number of tests ( $n = 99$ ), the nominal level of statistical significance ( $\alpha = 0.05$ ) was adjusted by Dunn-Šidák correction ( $1 - (1 - \alpha)^{1/n}$ ) to 0.00052. The number of alleles ( $N_A$ ), observed ( $H_O$ ) and expected heterozygosity ( $H_E$ ), and fixation index ( $F = 1 - (H_O/H_E)$ ) were averaged across loci for each population. Using the combined microsatellite data from the maternal parent and offspring, the Multi-locus Mating System Program (MLTR; [49]) was employed to calculate the maternal parent inbreeding coefficient ( $F_{mat}$ ) and the biparental inbreeding rate ( $T_m - T_s$ ). The biparental inbreeding rate is estimated here as

the difference between the multi-locus population out-crossing rate ( $T_m$ ) and the single locus out-crossing rate ( $T_s$ ).

The spatial pattern of microsatellite variation was examined in order to identify distinct population clusters and the relationship between genetic distance and geographic distance. A principal coordinate analysis (PCoA) was first employed to summarize microsatellite genetic variation among all individuals. The eigenvectors of the PCoA were calculated from a covariance matrix with data standardization using the program GENALEX. The clustering of individuals from each population was examined based on the first two principal coordinates. A Bayesian clustering algorithm implemented in the program TESS v2.3.1 [50] was also employed, using the admixture model with correlated allele frequencies [51] to account for any migrants in the dataset, following recommendations of Francois and Durand [52]. TESS was run by setting the cluster (“k”) value incrementally from two to seven with 20 independent runs at each k value. A burn-in period of 75,000 sweeps was followed by MCMC sampling for 500,000 sweeps. The optimal k value was determined by examining the deviance information criterion (DIC) and the 20 independent runs at this value of k were summarized using the program CLUMPP [53] with the Greedy algorithm. The program DISTRUCT [54] was used to graphically display the output.

Microsatellite genetic data were also examined for genetic isolation by distance (IBD) and compared to chemical distance. A matrix of genetic distance [55] was compared to Euclidean and log-transformed Euclidean geographic distance. Genetic distance was also compared to a matrix of untransformed and log-transformed absolute difference of oridonin amounts (% dry wt.) in both parents and offspring. A mantel test of matrix correspondence was conducted using GENALEX, with statistical significance assessed after 999 permutations.

## Results

### Repeatability of Oridonin Concentration Determination

A total of 144 unique samples were tested for oridonin concentration, including 75 from parent populations, 64 from greenhouse grown offspring, and five of purchased herbal samples. Representative LC/MS chromatograms are provided in Table S4. In order to test the reliability of the analytical method used in this study, a subset of 10 samples were run on multiple days and the resulting oridonin concentration values for those samples were compared. On average, the relative uncertainty in the detected value of oridonin was  $\pm 12\%$  of the detected value. Oridonin yield in parents and offspring of each population is shown in Fig. 2.

### Heritability of Oridonin Yield

The dry weight of oridonin for each offspring was averaged among siblings and regressed on the value of the maternal parent (Fig. 3) yielding a regression coefficient ( $b_{po}$ ) of 0.381 (standard error  $\pm 0.083$ ). This coefficient was highly statistically significant based on a t-test ( $p = 0.00007$ ). Narrow-sense heritability ( $h^2$ ) was estimated as 0.761. When a similar regression was performed using only populations of *I. rubescens* (HB, JY, QY, XX, YC), the correlation between parent and offspring oridonin content was no longer statistically significant, yielding a regression coefficient of 0.157 (standard error  $\pm 0.131$ ;  $p = 0.244$ ).

### Phylogenetic Analyses

The aligned chloroplast data included a total of 1579 nucleotide characters, of which 1478 were constant, 51 were parsimony informative, and 50 were parsimony uninformative. The ITS dataset included 572 nucleotide characters, of which 404 were



constant, 80 were parsimony informative, and 88 were parsimony uninformative. The ILD test indicated that the ITS partition was significantly different from the chloroplast partition (*rps16*, *trnL-trnF*) ( $P=0.001$ ), whereas the two chloroplast markers were not significantly incongruent from one another ( $P=0.698$ ). Therefore, chloroplast and ITS data were examined separately in all subsequent phylogenetic analyses.

The phylogenetic topologies resulting from the MP, ML, and Bayesian analyses were similar, mainly differing in the amount of resolution of shallower nodes. Parsimony ratchet analyses of the chloroplast data yielded 140 unique equally parsimonious trees (length,  $L=130$  steps; consistency index,  $CI=0.86$ ; retention index,  $RI=0.95$ ). The ITS parsimony analysis yielded 960 unique equally parsimonious trees ( $L=285$ ;  $CI=0.78$ ;  $RI=0.84$ ).

### Evolutionary Gains and Losses of Oridonin Production

In the context of the chloroplast MP strict consensus phylogeny, oridonin production has five evolutionary steps ( $CI=0.06$ ;  $RI=0.2$ ), which is within the distribution of randomly reshuffled characters (mean = 7.116; standard deviation = 1.221). Oridonin production has three evolutionary steps ( $CI=0.33$ ;  $RI=0.89$ ) in the context of the ITS MP strict consensus phylogeny, however is lower than the values for randomly reshuffled characters (mean = 7.9958; standard deviation = 1.479). The pattern of oridonin presence/absence as mapped on the chloroplast and ITS strict consensus phylogenies is shown in Fig. 4.

### Population Genetic Diversity and Spatial Genetic Structure of *Isodon*

Given the lack of phylogenetic resolution between populations collected for this study, the microsatellite data for all populations, regardless of species designation, were analyzed together. The microsatellite loci were first tested for deviations from Hardy-Weinberg equilibrium using chi-square tests (Table S3). After controlling the significance level for multiple testing, only loci 38 and 107 in the *rubescens* (XX) population had significant tests. Based on the low mean number of alleles across loci and the high fixation index in this population (Table 2), it is likely that these loci deviate from Hardy-Weinberg equilibrium for biological reasons rather than the presence of null alleles. Therefore, these two loci were included in all downstream analyses. The mean number of alleles across loci varied across populations, with *enanderianus* (QD) and *rubescens* (JY) having the highest polymorphism, and *rubescens* (XX) having the least. Since *rubescens* (JY) is a cultivated population from wild collected parent material, the observation of higher polymorphism might be expected. All populations show a deficit of average observed heterozygosity relative to average expected heterozygosity. The populations *rubescens* (XX) and *rubescens* (YC) have the largest fixation indices, but  $F$  does not exceed 0.2 in any population. Only *rubescens* (QY) has a large maternal fixation index ( $F_{mat}$ ), but the large standard error makes this estimate unreliable. Similarly, the estimated biparental inbreeding rates ( $T_m - T_s$ ) have large standard errors, and only two populations, *rubescens* (QY) and *rubescens* (JY), have estimated rate greater than zero.

The first two coordinates from the principal coordinate analysis account for 25% and 24% of the genetic variation, respectively. A plot of the first two components (Fig. 5) suggests that three populations form distinct clusters, and the remaining populations exhibit a large degree of overlap. Individuals from *rubescens* (YC) and *enanderianus* (QD) can be distinguished along the first coordinate axis, while *enanderianus* (TR) is distinguished along the second coordinate axis. The DIC values in the Bayesian clustering analysis approach an asymptote at six clusters (Fig. 6A and 6B), with only a slight increase at seven clusters. No single individual is

assigned to the seventh cluster when  $k=7$ . Focusing on  $k=6$ , unique clusters include *enanderianus* (QD), *enanderianus* (TR), *henryi* (XN), *japonicus* (XX), *rubescens* (YC), and a cluster where *rubescens* (JY), *rubescens* (QY), *rubescens* (HB), and *rubescens* (XX) are combined.

A pattern of isolation by distance is evident in the Mantel test of microsatellite variation and geographic distance. There is a significant correlation with Euclidean distance ( $N=92$ ,  $r_{xy}=0.327$ ,  $p=0.001$ ) and the correlation is stronger for log-transformed Euclidean distance ( $N=92$ ,  $r_{xy}=0.401$ ,  $p=0.001$ ). Similarly, when all populations in this study were included, genetic distance was significantly correlated with chemical distance in both parents ( $N=73$ ,  $r_{xy}=0.200$ ,  $p=0.002$ ) and offspring ( $N=64$ ,  $r_{xy}=0.113$ ,  $p=0.005$ ). However, when only populations of *I. rubescens* were compared (HB, JY, QY, XX, YC) there was no statistical correlation between chemical and genetic distance in either parents ( $N=55$ ,  $r_{xy}=0.038$ ,  $p=0.263$ ) or offspring ( $N=44$ ,  $r_{xy}=0.031$ ,  $p=0.331$ ).

## Discussion

### Oridonin Yield

Average yields per population show that oridonin is at higher concentrations in the area of traditional production of *donglingcao* in Henan province (Fig. 2). Four of these high yield populations comprise a single genetic cluster and all were identified as *I. rubescens*. The sample of *I. japonicus* (XX) collected growing sympatrically with *I. rubescens* had the highest oridonin yield of all individuals. The southern population of *I. rubescens* from Hubei province (YC) had intermediate values of oridonin and was genetically distinct from *I. rubescens* in Henan province. Of individuals that produced oridonin, fewer than half were above the 0.25% oridonin dry weight limit specified by the Chinese Pharmacopoeia for samples of *donglingcao* [19]. Oridonin yield was generally higher in parents than offspring, with 37% of parent samples above the Chinese Pharmacopoeia limit, as compared to 21% of greenhouse grown offspring. The reason for the higher concentration of oridonin in parent samples is unclear, but may be due to lower environmental stress faced by greenhouse-grown offspring (e.g., no insect damage, regular watering) and/or the differences in timing of the plant collection. Interestingly, four out of five purchased herbal samples of *donglingcao* were above the Chinese Pharmacopoeia limit, despite evidence of adulteration (e.g., multiple banding of PCR amplicons of phylogenetic markers such as ITS; visible leaf material from other species).

### Heritability of Oridonin Production

Estimates of oridonin yield show that there is a strong heritable component to the underlying variation in production of the chemical ( $h^2=0.761$ ). This pattern is corroborated by the test of correlation between chemical and genetic distance. However, the contribution of additive genetic variance to the overall genetic variation is not clear, because our estimate of heritability assumes that there is no genotype-environment interaction and we are not able to test this assumption directly. The heritability of oridonin production is less clear when examining only populations of *I. rubescens*, either in the context of comparing average offspring values with parents to calculate heritability, or by examining correlations between genetic and chemical distance. The pattern of results in these two cases (i.e., all populations vs. only populations of *I. rubescens*) is suggestive that when seeking populations that produce the most oridonin, the selection of the species with highest yield should be prioritized before selecting populations within species. In the case of *Isodon*, it would be important to first select a wide taxonomic breadth to test for

oridonin content, as opposed to focusing on variation of oridonin production within only one species (e.g., *I. rubescens*). However, the lack of heritability or correlation with genetic distance in oridonin content of only *I. rubescens* samples may be due to the smaller sample size. Additional samples of *I. rubescens* would be helpful to clearly determine whether heritability within *I. rubescens* is in fact low. This, in turn, would help to determine whether there is a genetic component to variation in oridonin production between populations of a single species, such as *I. rubescens*, and thus, whether additional sampling for high oridonin yield *within* species is necessary.

### Phylogenetic Origins of Oridonin

The distribution of oridonin production on the chloroplast phylogeny cannot be distinguished from a random mapping of the character. By contrast, the distribution of oridonin production has less homoplasy than expected by chance in the context of the ITS phylogeny. The difference in these results is most likely because *I. rubescens* is not monophyletic in the chloroplast phylogeny, but is better resolved in the ITS phylogeny (Fig. 4). A phylogenetic study of *Isodon* species from Japan based on chloroplast markers did not resolve any species as monophyletic [28]. The authors attributed this pattern to incomplete lineage sorting of ancestral polymorphisms or hybridization, and suggested that nuclear gene markers such as ITS might be better at resolving phylogenetic relationships [28]. Because the ITS phylogeny is potentially more reliable, a tentative conclusion is that oridonin arose at least three times in *Isodon*, or less than would be expected if it were random (i.e., shows phylogenetic inertia). However, given the low resolution of the phylogeny, this number could be an underestimate. Further work with additional nuclear phylogenetic markers would be helpful for clarifying the patterns of oridonin evolution.

Oridonin is present in some populations and absent from others in at least seven of the 12 species of *Isodon* that have been found to produce the chemical (*I. amethystoides*, *I. eriocalyx*, *I. japonicus*, *I. nervosus*, *I. phyllostachys*, *I. rosthornii*, *I. ternifolius*; see Table S2). The inter-specific variability of oridonin production was also corroborated by the low quantity of oridonin found in this study in the sample of *I. lophanthoides* var. *micranthus* and one parent sample of *I. enanderianus*, two species that have previously not been reported to produce oridonin. Furthermore, the offspring of the *I. enanderianus* sample that had a small oridonin yield did not produce oridonin. Therefore it is reasonable to conclude, as others have [29,30], that oridonin production can occur in some populations and not others of the same species.

### Population Genetic Variation and Spatial Genetic Structure

Patterns of microsatellite genetic variability are fairly uniform across the *Isodon* populations, with the exception of samples from the population *rubescens* (XX) that have a reduced level of variation. Based on the average number of alleles in this population and heterozygosity patterns across loci, it is unlikely that null alleles are causing this deficit in variation. Instead, it is more likely that the reduction in variation in the *rubescens* (XX) population is due to biological factors, such as a recent population bottleneck (or founder event), or as a consequence of selfing. We used estimates of the maternal fixation rate and the biparental inbreeding rate [49] to test whether selfing is evident in any of the sampled populations. However, the estimates of inbreeding that we obtained have a large sampling error and cannot be distinguished from zero. While selfing is clearly not a dominant component of the mating strategy of *Isodon*, we are unable to clearly estimate inbreeding rates in these populations.

A principal coordinate analysis indicates that there is extensive overlap in genetic variation in most populations of *Isodon*, with clear separation of the three southern populations from Hubei and Guizhou provinces, *rubescens* (YC), *enanderianus* (QD), and *enanderianus* (TR). Notably, these three populations can be distinguished from one another based on the first two principal coordinates. Bayesian analysis of genetic structure suggests that the *Isodon* samples collected for this study are composed of six population clusters. In addition to the three southern populations, *henryi* (XN) and *japonicus* (XX) are also identified as distinct clusters. The clusters identified in the Bayesian analysis are geographically separated, with the exception that the sympatric sample of *I. japonicus* is distinct from *I. rubescens* at the Xinxiang (XX) site. One complicating factor of this analysis is that the populations also exhibit isolation by distance. Previous studies have shown that clustering methods can over-predict the number of unique clusters if there are large sampling gaps with respect to geographical distance [56]. However, it is noteworthy that different species of *Isodon* appear to form distinct clusters, though this may simply due to the fact that with the exception of the sympatric species at XX, the different species are separated by large distances.

### Conclusions

1. Oridonin has been reported in 12 out of 74 *Isodon* species examined for diterpenoids. Oridonin has arisen at least three times in *Isodon*, but further phylogenetic work is needed to clarify patterns of oridonin evolution.
2. *Isodon* populations are characterized by high levels of gene flow, even between different species. Gene flow, however, is restricted geographically and genetic differentiation is observed among distant populations as evident in both the isolation-by-distance and structure analysis.
3. Microsatellite genetic distance is correlated with quantitative difference in oridonin production when all individual samples of this study are considered.
4. Oridonin production is strongly heritable when comparing different species of *Isodon*. The strong heritability is indicative that *Isodon* would respond well to selection and cultivation of species with high oridonin yield.
5. Oridonin yield does not appear to be heritable or correlated with genetic distance when only samples of *I. rubescens* are considered. However, additional sampling of multiple populations of *I. rubescens* is needed to confirm this.
6. Collectively, these results suggest that initial screening of *Isodon* populations for oridonin production should prioritize a broad survey of all species known to produce oridonin, rather than focusing on multiple populations of one species.
7. Of the samples in this study, populations of *I. rubescens* or *I. japonicus* from Henan province would be the best source of oridonin.

### Supporting Information

**Table S1 Sample collection information and Genbank accession numbers.** Samples in bold collected by study authors.

(XLS)

**Table S2 Summary of literature reports of oridonin as detected in species of *Isodon*.**

(DOC)

**Table S3 Chi-square test for deviation from Hardy-Weinberg equilibrium.** Significant tests indicated (Dunn-Šidák corrected  $\alpha = 0.00052$ ) in bold with asterisk. (XLS)

**Table S4 Representative chromatograms of *Isodon rubescens* and *Isodon japonicus*.** (DOC)

## Acknowledgments

The authors would like to acknowledge Janet Sherwood for greenhouse assistance; Sally Andrews, Jackie Craigue, Jane Craycroft, Kim Ravenscroft, Bruce Littlefield, Weidong Lu, Susan Rodliff, Robert Scholten, and

Patricia Wilkinson for help at the Osher Research Center; Kathy Lubuglio, Donald Pfister, and Michaela Schmuell for help at the Harvard University Herbaria; Baoyu Ji, Hanwei Li, Mengqi Liu, and Peng Xu for help at Henan College of Traditional Chinese Medicine; Qjuling Wang and Fulai Yu at the Beijing University of Chinese Medicine; and, Xiwen Li at the Kunming Institute of Botany for help with specimen identification.

## Author Contributions

Conceived and designed the experiments: ESJH SC WW ZZ DME JC. Performed the experiments: ESJH SC CD ZJ. Analyzed the data: ESJH SDS. Contributed reagents/materials/analysis tools: SC SDS CD WW DME JC. Wrote the paper: ESJH SDS.

## References

- Ikezoe T, Chen SS, Tong XJ, Heber D, Taguchi H, et al. (2003) Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. *Int J Oncol* 23: 1187–1193.
- Leung CH, Grill SP, Lam W, Han QB, Sun HD, et al. (2005) Novel mechanism of inhibition of nuclear factor-kappa B DNA-binding activity by diterpenoids isolated from *Isodon rubescens*. *Mol Pharm* 68: 286–297.
- Bai NS, He K, Zhou Z, Tsai ML, Zhang L, et al. (2010) Ent-Kaurane diterpenoids from *Rabdosia rubescens* and their cytotoxic effects on human cancer cell lines. *Planta Med* 76: 140–145.
- Chen S, Cooper M, Jones M, Madhuri TK, Wade J, et al. (2011) Combined activity of oridonin and wogonin in advanced-stage ovarian cancer cells. *Cell Biol Toxicol* 27: 133–147.
- Cui Q, Tashiro SI, Onodera S, Minami M, Ikejima T (2007) Autophagy preceded apoptosis in oridonin-treated human breast cancer MCF-7 cells. *Biol Pharm Bull* 30: 859–864.
- Gao FH, Hu XH, Li W, Liu H, Zhang YJ, et al. (2010) Oridonin induces apoptosis and senescence in colorectal cancer cells by increasing histone hyperacetylation and regulation of p16, p21, p27 and c-myc. *BMC Cancer* 10: 610. Available: <http://www.biomedcentral.com/1471-2407/10/610>. Accessed 12 December 2011.
- Kang N, Zhang JH, Qiu F, Chen S, Tashiro SI, et al. (2010) Induction of G2/M phase arrest and apoptosis by oridonin in human laryngeal carcinoma cells. *J Nat Prod* 73: 1058–1063.
- Zhou GB, Chen SJ, Wang ZY, Chen Z (2007) Back to the future of oridonin: again, compound from medicinal herb shows potent antileukemia efficacies in vitro and in vivo. *Cell Res* 17: 274–276.
- Wang H, Ye Y, Chu JH, Zhu GY, Li YW, et al. (2010) Oridonin induces G2/M cell cycle arrest and apoptosis through MAPK and p53 signaling pathways in HepG2 cells. *Oncol Rep* 24: 647–651.
- Meade-Tollin LC, Wijeratne EMK, Cooper D, Guild M, Jon E, et al. (2004) Ponicidin and oridonin are responsible for the antiangiogenic activity of *Rabdosia rubescens*, a constituent of the herbal supplement PCSPEs. *J Nat Prod* 67: 2–4.
- Fujita E, Fujita T, Katayama H, Shibuya M (1967) Oridonin, a new diterpenoid from *Isodon* species. *Chem Commun* 6: 252.
- Henan Institute of Medical Sciences (河南省医学科学研究所药理药化组等) (1978) Oridonin: a new antitumor subject. *Chin Sci Bull (科学通报)* 23: 53–56.
- Sun H, Chao J, Lin Z, Marunaka T, Minami Y, et al. (1982) The structure of rubescensin-c - A new minor diterpenoid isolated from *Rabdosia rubescens*. *Chem Pharm Bull* 30: 341–343.
- Chen S, Gao J, Halicka HD, Huang X, Traganos F, et al. (2005) The cytostatic and cytotoxic effects of oridonin (Rubescensin), a diterpenoid from *Rabdosia rubescens*, on tumor cells of different lineage. *Int J Oncol* 26: 579–588.
- Ikezoe T, Yang Y, Bandobashi K, Saito T, Takemoto S, et al. (2005) Oridonin, a diterpenoid purified from *Rabdosia rubescens*, inhibits the proliferation of cells from lymphoid malignancies in association with blockade of the NF-kappa B signal pathways. *Mol Cancer Ther* 4: 578–586.
- Wong AM, Zhang YJ, Kesler K, Deng M, Burhenn L, et al. (2010) Genomic and in vivo evidence of synergy of a herbal extract compared to its most active ingredient: *Rabdosia rubescens* vs. oridonin. *Exp Ther Med* 1: 1013–1017.
- Sun HD, Huang SX, Han QB (2006) Diterpenoids from *Isodon* species and their biological activities. *Nat Prod Rep* 23: 673–698.
- Chinese Pharmacopoeia Commission (1979) Pharmacopoeia of the Peoples Republic of China: 1977. Beijing: People's Medical Publishing House.
- Chinese Pharmacopoeia Commission (2010) Pharmacopoeia of the People's Republic of China: 2010 (Chinese Version). Beijing: China Medicine and Technology Publishing House. 3382 p.
- Sun HD (2001) Diterpenoids from *Isodon* species (香茶菜属植物二萜化合物). Beijing: Science Publishing House. 387 p.
- Han J, Ye M, Chen HB, Dai SJ, Guo D (2005) Determination of diterpenoids and flavonoids in *Isodon rubescens* by LC-ESI-MS-MS. *Chromatographia* 62: 203–207.
- Li CY, Wang EQ, Cheng Y, Bao JK (2011) Oridonin: An active diterpenoid targeting cell cycle arrest, apoptotic and autophagic pathways for cancer therapeutics. *Int J Biochem Cell Biol* 43: 701–704.
- Li H, Pu JX, Li J (2012) Diterpenoids Chemodiversity of the Genus *Isodon* Spach from Lamiaceae (唇形科香茶菜属二萜类化合物分布规律). *Plant Div Res (植物分类与资源学)* 34 In press.
- Eisenberg DM, Harris ESJ, Littlefield BA, Cao SG, Craycroft JA, et al. (2011) Developing a library of authenticated Traditional Chinese Medicinal (TCM) plants for systematic biological evaluation – Rationale, methods and preliminary results from a Sino-American collaboration. *Fitoterapia* 82: 17–33.
- Li HW (1988) Taxonomic review of *Isodon* (Labiatae). *J Arnold Arb* 69: 289–400.
- Li XW, Hedges IC (1994) *Isodon*. In: Wu, ZY and Raven, P, editors. *Flora of China*, Vol. 17 (Verbenaceae through Solanaceae). Beijing: Science Press, and St. Louis: Missouri Botanical Garden Press. pp. 269–291.
- Zhong JS, Li J, Li L, Conran JG, Li HW (2010) Phylogeny of *Isodon* (Schrad. ex Benth.) Spach (Lamiaceae) and related genera inferred from nuclear ribosomal ITS, *trnL-trnF* region, and *rps16* intron sequences and morphology. *Syst Bot* 35: 207–219.
- Maki M, Yamashiro T, Dohzono I, Suzuki K (2010) Molecular phylogeny of *Isodon* (Lamiaceae) in Japan using chloroplast DNA sequences: Recent rapid radiations or ancient introgressive hybridization? *Plant Species Biol* 25: 240–248.
- Han QB, Li ML, Li SH, Mou YK, Lin ZW, et al. (2003) Ent-kaurane diterpenoids from *Isodon rubescens* var. *lushanensis*. *Chem Pharm Bull* 51: 790–793.
- Han QB, Xiao WL, Shen YH, Sun HD (2004) Ent-kaurane diterpenoids from *Isodon rubescens* var. *rubescens*. *Chem Pharm Bull* 52: 767–769.
- Falconer D, Mackay TFC (1996) Introduction to quantitative genetics, 4<sup>th</sup> edition. Essex: Longman. 438 p.
- Oxelman B, Liden M, Berglund D (1997) Chloroplast *rps16* intron phylogeny of the tribe Sileneae (Caryophyllaceae). *Plant Syst Evol* 206: 393–410.
- Taberlet P, Gelly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17: 1105–1109.
- White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. *PCR protocols: A guide to methods and applications*. New York: Academic Press. pp. 315–322.
- Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32: 1792–1797.
- Clamp M, Cuff J, Searle SM, Barton GJ (2004) The Jalview java alignment editor. *Bioinformatics* 20: 426–427.
- Maddison WP, Maddison DR (2011) Mesquite: a modular system for evolutionary analysis. Version 2.74. Available: <http://mesquiteproject.org>. Accessed 16 January 2012.
- Farris JS, Källersjö M, Kluge AG, Bult C (1995) Constructing a significance test for incongruence. *Syst Biol* 44: 570–572.
- Swofford DL (2002) PAUP\*: Phylogenetic analysis using parsimony (\*and other methods). Sunderland: Sinauer. 142 p.
- Sikes DS, Lewis PO (2001) PAUPRat: PAUP\* implementation of the parsimony ratchet. Distributed by the authors. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs.
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52: 696–704.
- Posada D (2008) jModelTest: Phylogenetic model averaging. *Mol Biol Evol* 25: 1253–1256.
- Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.
- Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE)*. New Orleans. pp. 1–8.
- Huelsbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17: 754–755.
- Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

47. Harris ESJ, Klooster MR (2011) Development of microsatellite markers for the medicinal plant *Isodon rubescens* (Lamiaceae) and related species. *Am J Bot* 98: e293–e295.
48. Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6: 288–295.
49. Ritland K (2002) Extensions of models for the estimation of mating systems using  $n$  independent loci. *Heredity* 88: 221–228.
50. Chen CB, Durand E, Forbes F, François O (2007) Bayesian clustering algorithms ascertaining spatial population structure: a new computer program and a comparison study. *Mol Ecol Notes* 7: 747–756.
51. Durand E, Jay F, Gaggiotti OE, François O (2009) Spatial inference of admixture proportions and secondary contact zones. *Mol Biol Evol* 26: 1963–1973.
52. François O, Durand E (2010) Spatially explicit Bayesian clustering models in population genetics. *Mol Ecol Resour* 10: 773–784.
53. Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23: 1801–1806.
54. Rosenberg NA (2004) DISTRUCT: A program for the graphical display of population structure. *Mol Ecol Notes* 4: 137–138.
55. Smouse PE, Peakall R (1999) Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. *Heredity* 82: 561–573.
56. Frantz AC, Cellina S, Krier A, Schley L, Burke T (2009) Using spatial Bayesian methods to determine the genetic structure of a continuously distributed population: clusters or isolation by distance? *J Appl Ecol* 46: 493–505.