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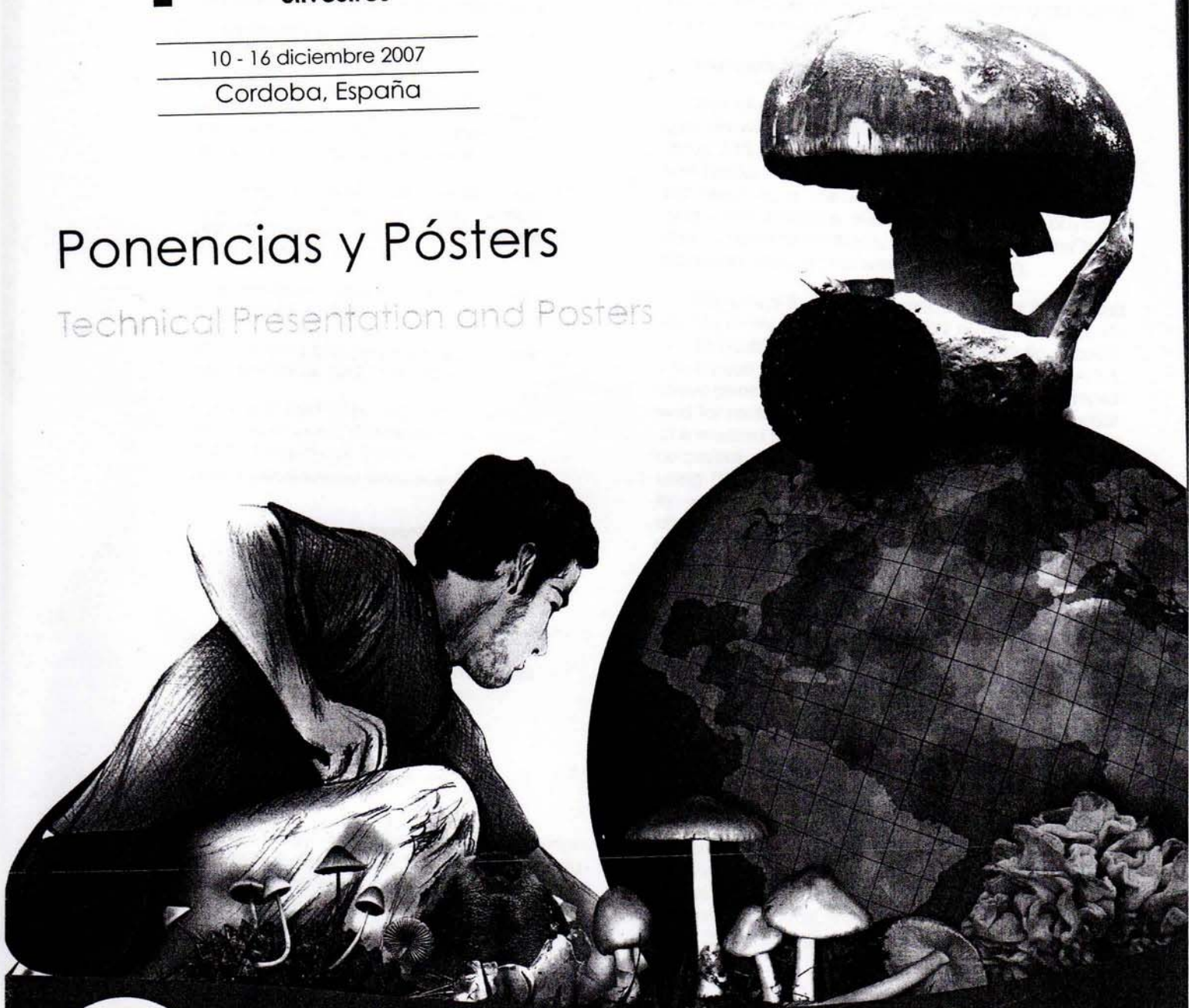
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Molecular phylogeny, historical biogeography and evolution of truffles

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Objectives

The name "truffle" is a broad term that com-
prises species of the genus *Tuber* belonging to the As-
comycota, Pezizales order and Tuberales family.
Index Fungorum recognizes 227 species, subspecies
and varieties of *Tuber*, however the truffle classifi-

cation based on the morphological features of as-
comata and spores has led to many controversies.
Currently, only 70-75 species have been validated in
the world, 32 out of which in Europe. The genus *Tuber*
is only found in the northern hemisphere, between
25 ° and 60 ° latitude, spread over diverse climates:
from tropical to Mediterranean, temperate, cold-
temperate and cold-continental climates.

A combination of different data sets and analysis
methods was used as part of a long-standing pro-
ject aimed at understanding the molecular phylo-
geny of *Tuber*: i.e. current distribution patterns of the
genus *Tuber*, phylogenetic reconstruction, dispersal
and vicariance analysis and molecular clocks (Wang
et al. 2006ab, Wang et al. 2007 and Jeandroz et al.
in press). In addition, we have investigated the tru-
ffle evolution and identified genomic regions which
could be involved in the speciation events.

Materials and Methods

The phylogenetic relationships among the *Tuber*
species were reconstructed using a dataset of 18S
rRNA, 5.8S rRNA, 5.8S-ITS2 rRNA et β -tubuline, Elonga-
tion Factor 1 Alpha and Protein Kinase C sequences
obtained from species harvested in Asia, Europe
and North America. We applied different phyloge-
netic inference methods: Maximum Parsimony (MP),
Bayesian analysis and Neighbour Joining.

We tested the global molecular clock hypothesis
for 18S rRNA, 5.8S rRNA, 5.8S-ITS2 rRNA, β -tubulin, EF-
1 α , 18S+5.8S rRNA and 18S+5.8S+ β -tubulin sequen-
ces by use of Tajima's relative rate test in MEGA 3.1.
These genes, combined or on their own, were employ-
ed for molecular clock estimates after construction
of linearized trees using MEGA 3.1. We reconstructed
ancestral areas with dispersal-vicariance analysis
using DIVA v. 1.1 in the Northern Hemisphere. Final-
ly, we used genomic suppression subtractive hybrid-
ization (gSSH) to isolate specific sequences of the
black truffle *T. melanosporum* which are absent in
the white truffle *T. borchii*. The obtained clones were
sequenced and tested for their specificity through a
dot blot experiment.

Results

The molecular phylogeny divided the genus *Tu-
ber* into five distinct clades (see Figures 1 and 2 in
Jeandroz et al., *In press*). The basal clade I (*Aestivum-
group sensu lato*) contained only European species.
It consists of three subclades corresponding to *T.
macrosporum*, *T. magnatum* and *Aestivum-group
sensu stricto*. The *Excavatum-group* (Clade II) was re-
presented by the European species, *T. excavatum*.
The *Rufum-group* (clade III) was divided in four sub-
clades. Subclade III-1 corresponded to misidentified
Chinese individuals, reclassified as *Tuber* sp. 1 (Wang
et al., 2007). Subclades III-2, III-3 and III-4 correspon-

ded, respectively, to the following Chinese species: *T. liaotongense*, *T. huidongense* and *T. taiyuanense*. The grouping of the remaining taxa depended on the phylogenetic methods that were used. In the Bayesian tree, *T. rufum* and *T. ferrugineum* were grouped into the same subclade. A different topology was observed in the MP tree, but was supported by a low bootstrap value of 50%. The two American species grouped together in the MP tree, while they corresponded to separate lineages in the BI tree.

Clade IV, identified as the *Melanosporum*-group, consisted of two European species (*T. melanosporum* and *T. brumale*) and two Asiatic species (*T. indicum* and *T. pseudoexcavatum*).

Clade V contained the species belonging to the *Puberulum*-group and was divided into four subclades. Subclade V-1 was formed by one European species, *T. scruposum* and by one American species, *T. whetstonense*. Subclade V-2 was formed by three European species, *T. foetidum*, *T. maculatum* and *T. rapaeodorum*. These two latter species appeared to be paraphyletic. Subclade V-3 was only composed of Chinese species, two misidentified species reclassified as *Tuber* sp. 3 and *Tuber* sp. 4 (Wang et al., 2007). Subclade V-4 was comprised of four European species, *T. borchii*, *T. dryophilum*, *T. oligospermum* and *T. puberulum*, two Chinese species, *T. liui*, and one misidentified species reclassified as *Tuber* sp. 2 (Wang et al., 2007).

Tajima's relative rate test showed that *Tuber* 18S rRNA, 5.8S rRNA, 5.8S-ITS2 rRNA and β -tubulin sequences evolved in a clock-like manner. According to the global molecular clock analysis, the radiation of the genus *Tuber* could have started between 271 and 140 Mya. Clade I (*Aestivum*-group *sensu lato*), clade II (*Excavatum*-group), clade III (*Rufum*-group), clade IV (*Melanosporum*-group) and clade V (*Puberulum*-group) would have diverged, respectively, between 160 Mya and 140 Mya, 111 Mya ago, 70 Mya, between 85 Mya and 25 Mya and between 65 Mya and 53 Mya.

The DIVA analysis inferred the ancestral distribution from the different nodes and the reconstruction required 9 dispersals. Equally optimal distributions were obtained for several nodes suggesting different possible biogeographical histories. Considering each group separately, the DIVA analysis showed a European ancestral distribution of the ancestors of the *Magnatum*-, *Macrosporum*- and *Aestivum*-groups. Ambiguous ancestral distributions were instead observed for the ancestors of the four other groups: E or EA for the *Excavatum*-, *Puberulum*- and *Rufum*-groups and E or A for the *Melanosporum*-group.

Using the gSSH we identified specific genomic fragments from *T. melanosporum*. Sixty three % of the sequences did not show homology in databases,

16% showed homology with hypothetical protein sequences, 16% showed homology with retrotransposon sequences and 5% showed homology with bacterial sequences.

Conclusion

Molecular phylogeny allowed us to identify several discrepancies with the classical taxonomy of the genus *Tuber* and to propose a phylogenetically based classification. According to the MP and Bayesian molecular phylogenies (Jeandroz et al., In Press), the genus *Tuber* appeared to be organized in five distinct clades. Although giving slightly different results, the two analyses clearly showed that *T. panniferum*, previously considered a part of the *Rufum*-group, was phylogenetically far removed from the *Rufum*-group, and instead belongs to the *Aestivum*-group *sensu lato*. Both phylogenetic analyses also showed that the European species, *T. magnatum*, until now included in the *Puberulum*-group, was phylogenetically well-differentiated from the other species of this group and belonged to the *Aestivum*-group *sensu lato*.

We also showed that the *Macrosporum*-group (*T. macrosporum*, *T. foetidum*, *T. malençonii*, *T. pseudoexcavatum* and *T. regianum*), as defined by Rioussat et al., (2001), appeared polyphyletic. At least two species were reclassified into other groups, *T. pseudoexcavatum* in the *Melanosporum*-group and *T. foetidum* in the *Puberulum*-group.

According to the different sequences used for molecular clocks, the radiation of the genus *Tuber* could have started between 271 and 140 Mya. The supercontinent of Pangea would have begun to separate into two masses, the Gondwana and the Laurasia, late in the Triassic Period (245 to 208 Mya) or early in the Jurassic period (195 Mya). Consequently, we cannot exclude a Pangean origin of the common *Tuber* ancestor or its presence in only the northern part of the Pangea. During the Cretaceous, the South Atlantic Ocean opened, but North America was still connected to Europe through the North Atlantic Land Bridge. Forty-five million years ago, North America and Greenland split away from Europe and the North Atlantic Bridge was broken. To explain the presence of the genus *Tuber* both in America and Eurasia, the existence of a migration through the North Atlantic Bridge has to be taken in consideration for the species, which would have diverged before 45 Mya. According to the molecular clock analysis, we estimated the divergence time between the North American species *T. quercicola*, *T. candidum* and *T. whetstonense* and the European species belonging to the same group, respectively, as 42, 40 and 18 Mya. Consequently, we can roughly estimate that the radiation of these American species occurred after breakdown of the North Atlantic

Bridge. Nevertheless, given the usually large confidence intervals, dispersal across the North Atlantic Bridge cannot be completely excluded. Another route of terrestrial migration between North America and Eurasia occurred during the Eocene via the Bering Land Connection between Siberia and Alaska. We hypothesize that three groups, the *Puberulum*-group, the *Rufum*-group, and perhaps the *Melanosporum*-group, could have migrated towards North America with their host from the middle of the Miocene via the Bering Land Bridge.

The DIVA ancestral area reconstructions suggested that the common *Tuber* ancestor originated from the Laurasian region, from what later became Europe or from a larger area, which later became Eurasia. In conjunction with divergence time estimates and probable intra- and inter-continental land connections, we were able to propose two scenarios of intra- and inter-continental diversification of the genus according to the geographic distribution of the more recent common ancestor, Europe or Eurasia. Reconstruction of ancestral distribution areas requires 9 dispersals and the two proposed biogeographical scenarios mainly implied intra-continental dispersal events between Europe (E) and Asia (A) (6/9). Only one dispersal appeared characteristic of each of the two scenarios, while the other dispersals were common to both scenarios. Whatever the hypothesis may be, a first diversification event led to the *Aestivum*-*Magnatum*-*Macrosporium*- and *Panniferum*-groups only being found in Europe. These four groups represent the oldest lineage. However, it is currently impossible to choose between the two hypotheses of the origin of the genus *Tuber*: Despite the limitations of our current phylogenetic hypothesis (non exhaustive taxa sampling), we were able to show the relative role of dispersal-vicariance events in shaping distribution patterns in the genus *Tuber*. Dispersal through ancient land connections appeared to have been more important for the biogeography of *Tuber* rather than vicariance, although the real biogeography was no doubt more complicated.

Finally, we demonstrate that gSSH can be used to identify specific genomic regions between mycorrhizal fungal genomes. In addition, the results suggest that retrotransposons might have played a role in truffle evolution. This hypothesis could soon be verified since the genome of the black truffle *Tuber melanosporum* will become available in the next months.

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