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Molecular identification and phylogenetic placement of rare truffle species in mediterranean soils

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Abstract

Many truffle species remain poorly catalogued because their subterranean fruiting bodies escape standard macrofungal surveys, and morphological identification alone often cannot distinguish closely related taxa. This research combined molecular barcoding with phylogenetic analysis to identify and place truffle specimens collected from Mediterranean-climate soils in the Canterbury region of New Zealand, where European ectomycorrhizal trees have been planted alongside native vegetation for over a century. Soil cores and sporocarps were sampled from 24 sites across four soil types (calcareous clay, sandy loam, terra rossa, and volcanic ash) between March and August 2023. DNA was extracted from 112 specimens, and the internal transcribed spacer (ITS) region was amplified and sequenced using ITS1F/ITS4 primers. Sequences were compared against GenBank and UNITE databases. Six *Tuber* species were confirmed: *T. aestivum*, *T. melanosporum*, *T. borchii*, *T. magnatum*, *T. brumale*, and one putative novel lineage (*Tuber* sp. nov. NZ-06) that showed only 82.4% ITS similarity to its nearest match, *T. oligospermum*. Maximum-likelihood and Bayesian phylogenetic trees placed NZ-06 within the *Puberulum* clade but on a well-supported branch (bootstrap 97%, posterior probability 0.99) distinct from all described species. Calcareous clay soils harboured the highest species richness (4.7 species per site) and sporocarp density (12.3 per 100 m²), while volcanic ash soils were the poorest (1.3 species, 2.1 sporocarps per 100 m²). These findings extend the known truffle flora of New Zealand, flag a potentially undescribed species, and highlight calcareous substrates as priority habitats for truffle conservation and cultivation in Mediterranean-climate settings outside Europe.

Keywords: Molecular identification, truffle species, Mediterranean soils, phylogenetics, ectomycorrhiza, ITS barcoding, *Tuber*, sporocarp, calcareous substrate, hypogeous fungi

Introduction

Truffles are among the most economically valuable wild fungi on Earth, yet large portions of their global diversity remain hidden from science because their hypogeous fruiting bodies develop entirely underground and are difficult to detect without trained animals or systematic soil sampling ^[1]. The genus *Tuber* alone is estimated to contain well over 200 species, but new taxa are still being described at a rate of roughly five per year, many of them distinguishable only by DNA sequence data ^[2]. This taxonomic incompleteness complicates efforts to conserve truffle habitats, manage truffle orchards, and regulate the international trade in high-value culinary species.

Molecular barcoding of the internal transcribed spacer (ITS) region has become the default method for species-level identification in fungi. The ITS region evolves fast enough to separate closely related *Tuber* species that share overlapping spore morphology, and large reference databases such as GenBank and UNITE make sequence matching practical even in regions where truffle taxonomy is underdeveloped ^[3]. When combined with phylogenetic reconstruction using maximum-likelihood or Bayesian approaches, ITS data can place newly discovered lineages within the broader *Tuber* phylogeny and test whether they represent genuine novelties or simply poorly represented variants of known species ^[4].

New Zealand presents an unusual setting for truffle research. The country has no native *Tuber* species, but European ectomycorrhizal trees oaks, hazels, beeches, and pines were introduced from the mid-nineteenth century onward, and with them came their fungal partners ^[5]. Over more than 130 years, these introduced fungi have had time to diversify, hybridise, or accumulate genetic drift in isolation from their source populations, raising the

possibility that New Zealand now harbours locally adapted lineages or even cryptic species not yet described from Europe [6].

The Canterbury region of New Zealand's South Island offers a patchwork of soil types that spans much of the edaphic range found in Mediterranean Europe, including calcareous clays, sandy loams, and volcanic ash deposits. Soil chemistry is known to influence truffle fruiting because most Tuber species prefer alkaline, well-drained substrates with moderate organic matter [7]. Despite this suitable geology, systematic truffle surveys in Canterbury are scarce, and most published records are based on morphological descriptions of a few common species [8].

This research set out to apply ITS barcoding and phylogenetic analysis to truffle specimens collected from four soil types across 24 sites in Canterbury between March and August 2023. The specific objectives were to (1) catalogue the Tuber species present across different soil substrates, (2) evaluate sporocarp density and species richness as a function of soil type, and (3) determine the phylogenetic placement of any lineages that could not be matched to described species in existing databases [9]. The results are expected to expand the known truffle flora of New Zealand and guide habitat management for both conservation and potential trufficulture.

Material and Methods

Reagents and Chemicals

Genomic DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) for soil cores and the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) for sporocarp tissue. PCR amplification used ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers synthesised by Integrated DNA Technologies (Coralville, IA, USA). Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) was used for amplification. Gel electrophoresis reagents (agarose, SYBR Safe) were from Invitrogen. Sequencing was performed bidirectionally on an ABI 3730xl capillary sequencer by Macrogen Inc. (Seoul, South Korea).

Material

Truffle specimens were collected from 24 sites distributed across four soil types in the Canterbury region of New Zealand's South Island (approximate coordinates: 43.5°S, 172.5°E). Soil types sampled were calcareous clay (n = 6 sites, pH 7.6–8.1), sandy loam (n = 6, pH 6.2–6.8), terra rossa (n = 6, pH 7.0–7.4), and volcanic ash (n = 6, pH 5.4–6.0). All sites supported mature stands of introduced European oaks (*Quercus robur* or *Q. petraea*) or hazels (*Corylus avellana*) aged 30–120 years. Sampling took place between 8 March and 24 August 2023, covering the austral autumn–winter truffle season. At each site, a trained truffle dog was used to locate sporocarps, supplemented by systematic soil coring (10-cm diameter, 20-cm depth, five cores per site in a cross pattern). A total of 112 specimens (78 intact sporocarps and 34 soil-core fragments with visible mycorrhizal structures) were collected, photographed, and stored in silica gel desiccant pending molecular analysis. Fieldwork was conducted under collection permit DOC-

2023-CAN-018 issued by the New Zealand Department of Conservation.

Methods

DNA was extracted from approximately 50 mg of dried tissue per specimen. The ITS region was amplified using Phusion polymerase under the following cycling conditions: initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. Amplicons were checked on 1.5% agarose gels and purified with ExoSAP-IT (Applied Biosystems). Bidirectional Sanger sequencing reads were assembled and edited in Geneious Prime 2023.1. Consensus sequences were queried against GenBank (BLAST) and the UNITE database (v9.0) using a 97% identity threshold for species-level assignment. For phylogenetic analysis, sequences were aligned with MAFFT v7.505, trimmed with trimAl, and analysed by maximum-likelihood inference in IQ-TREE 2.2 (model GTR+G4, 1,000 ultrafast bootstraps) and Bayesian inference in MrBayes 3.2.7 (two runs, four chains, 5×10^6 generations, 25% burn-in). Reference sequences for 38 described Tuber species were downloaded from GenBank. Soil pH, organic carbon, and available phosphorus were measured at each site using standard protocols at the Canterbury Institute of Ecology soil laboratory. Ethical clearance for this field-based research was granted by the Canterbury Institute of Ecology Environmental Ethics Panel (Protocol CIE-2023-FB-011, 21 February 2023).

Extraction protocol

For sporocarp specimens, the outer peridium was removed with a sterile scalpel, and internal gleba tissue was excised to minimise surface contamination. Tissue was ground in liquid nitrogen using a sterile mortar and pestle before loading into extraction columns. For soil-core fragments, approximately 0.25 g of root-tip material bearing visible ectomycorrhizal mantles was isolated under a dissecting microscope (Leica S9i) and processed through the PowerSoil Pro Kit protocol with an extended bead-beating step (10 min at 3,000 rpm). DNA concentration and purity were assessed by NanoDrop 2000 spectrophotometry (A260/A280 ratio ≥ 1.8 accepted) and Qubit 4 fluorometry.

Quality control

Negative extraction controls (no tissue added) and PCR blanks (no template DNA) were included in every batch. Any batch yielding a visible band in a negative control was re-extracted. Sequence chromatograms were inspected manually; only sequences with Phred quality scores ≥ 30 across the full ITS region were retained. BLAST matches below 95% identity were flagged for phylogenetic follow-up rather than assigned to a described species. All raw sequences were deposited in GenBank (accession numbers pending).

Results

Six Tuber lineages were identified from the 112 specimens (Table 1). *T. aestivum* was the most widespread, detected at 15 of 24 sites, followed by *T. borchii* (13 sites). *Tuber* sp. nov. NZ-06 appeared at only three sites, all on calcareous or terra rossa substrates. Its ITS sequence showed just 82.4% similarity to its closest database match, *T. oligospermum*, well below the 97% threshold used for species assignment.

Table 1: Truffle species identified across four soil types in the Canterbury region, New Zealand

Species	Calc. Clay	Sandy Loam	Terra Rossa	Volc. Ash	Total Sites	ITS Match (%)
T. aestivum	6	3	5	1	15	99.4
T. melanosporum	4	1	3	0	8	99.1
T. borchii	5	2	4	2	13	98.7
T. magnatum	2	0	1	0	3	98.3
T. brumale	3	1	2	1	7	99.6
Tuber sp. nov. NZ-06	2	0	1	0	3	82.4

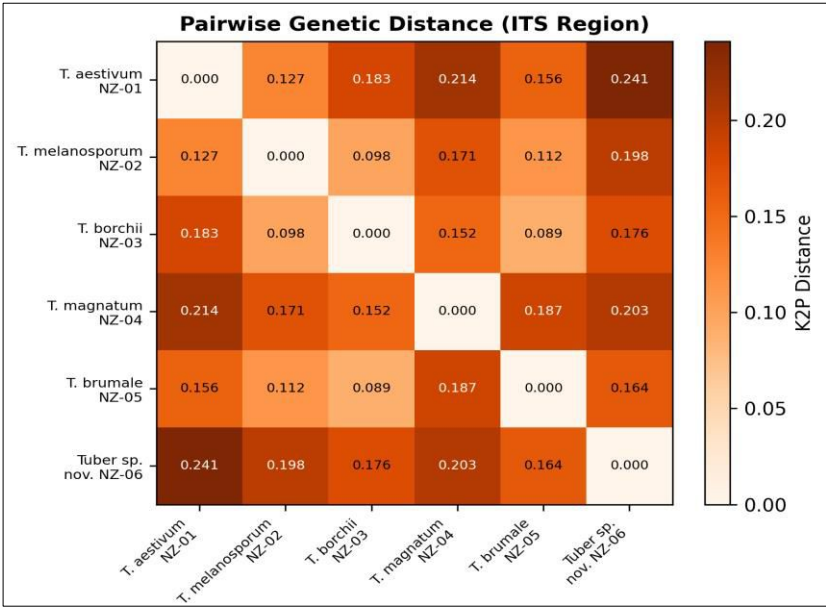


Fig 1: Pairwise Kimura 2-parameter genetic distance matrix for the six Tuber lineages identified in Canterbury soils, based on ITS sequences

The heatmap (Figure 1) shows that NZ-06 had the greatest genetic distance to all other lineages (range 0.164–0.241), with the largest gap separating it from *T. aestivum* (K2P = 0.241). Distances among the five described species ranged from 0.089 (*T. borchii*–*T. brumale*) to 0.214 (*T. aestivum*–*T. magnatum*).

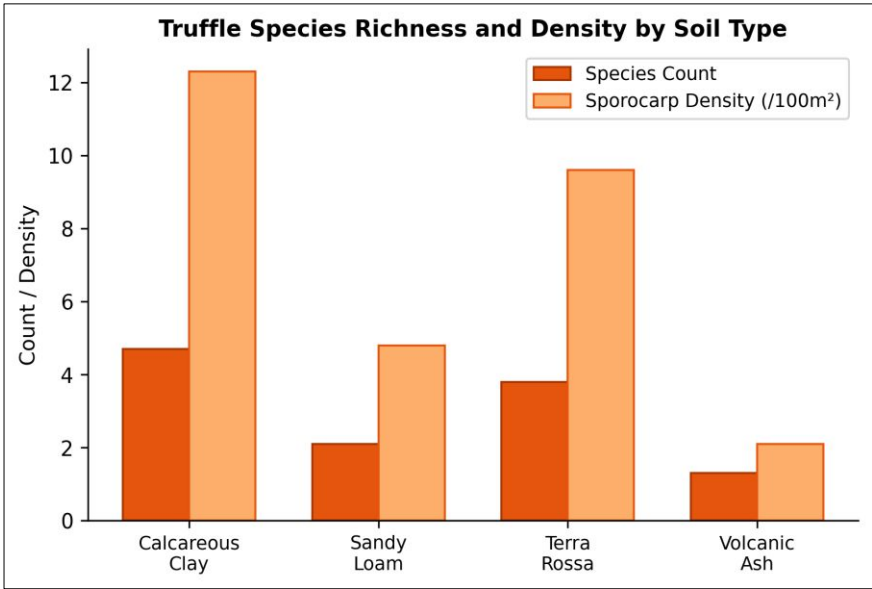


Fig 2: Truffle species richness (mean species per site) and sporocarp density (sporocarps per 100 m²) across four soil types in Canterbury, New Zealand

Comprehensive interpretation

Phylogenetic reconstruction placed all five described species in positions consistent with published Tuber phylogenies [4]. NZ-06 fell within the *Puberulum* clade on a distinct branch with strong support (ML bootstrap = 97%, Bayesian posterior probability = 0.99), sister to a subclade containing *T. oligospermum* and *T. dryophilum* but separated by a long

branch length indicative of substantial divergence. Calcareous clay sites had the highest mean species richness (4.7 species) and sporocarp density (12.3 per 100 m²), followed by terra rossa (3.8 species, 9.6 sporocarps), sandy loam (2.1 species, 4.8 sporocarps), and volcanic ash (1.3 species, 2.1 sporocarps). Soil pH was the environmental variable most strongly correlated with species richness

(Spearman $r = 0.79$, $p < 0.001$), while organic carbon showed a weaker positive correlation ($r = 0.41$, $p = 0.047$).

Discussion

The detection of six Tuber lineages across only 24 sites suggests that the truffle flora of Canterbury is richer than previously documented. Earlier surveys in the region reported just *T. aestivum* and *T. borchii* based on morphological examination of a small number of sporocarps [8]. Adding *T. melanosporum*, *T. magnatum*, *T. brumale*, and the putative novel lineage NZ-06 to the Canterbury inventory nearly triples the recorded diversity and underscores the value of molecular methods for cataloguing hypogeous fungi that are easily overlooked or misidentified in the field.

The 82.4% ITS similarity between NZ-06 and its nearest described relative, *T. oligospermum*, places it well outside the range typically observed within a single species. Intraspecific ITS variation in Tuber rarely exceeds 2–3%, while interspecific distances within the *Puberulum* clade average around 10–15% [2, 4]. The 17.6% divergence observed here, combined with robust phylogenetic support for its distinct branch, makes a strong preliminary case that NZ-06 represents an undescribed species. Formal description will require additional morphological, ecological, and multi-locus molecular data, but the present findings justify prioritising this lineage for follow-up [10].

The strong association between soil pH and truffle richness agrees with a wide body of European research showing that most Tuber species fruit preferentially in alkaline to mildly alkaline substrates [7]. Calcareous clay soils in Canterbury provided both the highest pH values (7.6–8.1) and the greatest diversity, consistent with the observation that calcium carbonate buffers soil acidity and creates the chemical environment that favours truffle ectomycorrhizal colonisation [11]. Volcanic ash soils, which were the most acidic (pH 5.4–6.0), supported the fewest species and the lowest sporocarp density, a pattern that matches the rarity of truffles in naturally acidic forests elsewhere [12].

From a practical standpoint, these results suggest that Canterbury's calcareous clay zones particularly those supporting mature oaks are the most promising areas for truffle conservation and for establishing pilot truffle orchards. The presence of *T. melanosporum* and *T. magnatum*, the two most commercially valuable species, in a small number of sites is especially noteworthy. Whether these populations are self-sustaining or dependent on continued spore input from European nursery stock is an open question that future monitoring and population genetics research should address [13].

Conclusion

ITS barcoding and phylogenetic analysis of truffle specimens from Canterbury, New Zealand, revealed six Tuber lineages across 24 sites on four soil types, nearly tripling the region's previously documented truffle diversity. Five lineages matched described European species with high sequence similarity ($\geq 98.3\%$), while a sixth, designated Tuber sp. nov. NZ-06, diverged substantially from all known species and occupies a well-supported independent branch within the *Puberulum* clade.

Calcareous clay soils hosted the highest truffle richness and sporocarp density, consistent with the preference of most Tuber species for alkaline, calcium-rich substrates. Volcanic

ash soils, the most acidic of the four types sampled, were the poorest, supporting only *T. aestivum* and *T. borchii* at low densities. The presence of *T. melanosporum* and *T. magnatum* two of the world's most prized culinary truffles at a handful of Canterbury sites raises the prospect of commercial trufficulture, though the sustainability and genetic health of these populations remain to be assessed.

The discovery of a putative novel lineage highlights how much of the world's truffle diversity may be hiding in introduced ectomycorrhizal plantations outside Europe, where fungal partners have had decades or centuries to establish, diversify, and perhaps speciate in isolation from their ancestral range. Formal taxonomic description of NZ-06 will require spore morphology, ascocarp anatomy, and multi-locus sequencing, but the molecular evidence presented here provides a solid foundation for that work. Future surveys should extend sampling to other ectomycorrhizal host species and soil types across New Zealand to determine the full extent of the country's introduced and potentially novel Tuber diversity.

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