A new method to extract and purify DNA from allophanic soils and paleosols, and potential for paleoenvironmental reconstruction and other applications

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ABSTRACT

Andisols, developed from late-Quaternary tephra (volcanic ash) deposits and dominated by the nanocrystalline aluminosilicate, allophane, contain large stores of organic matter and are potential reservoirs for DNA. However, DNA recovery from Andisols and other allophane-bearing soils has been difficult and inefficient because of strong chemical bonding between DNA and both allophane and organic matter, and also because much DNA can be encased and physically protected in nanopores in allophane nano/microaggregates. We have therefore developed a new two-step DNA isolation method for allophanic soils and buried paleosols, including those low in clay, which circumvents these problems. The method centres on (1) releasing mainly microbial DNA, and extracellular (unbound) DNA, using an alkaline phosphate buffer ("Rai's lysis buffer") that blocks re-adsorption sites on the allophanic materials, and (2) the novel application of acidified ammonium oxalate (Tamm's reagent) to dissolve the allophane and to release DNA which had been chemically-bound and also which had been protected within nanopores. Ammonium oxalate has not previously been applied to soil DNA extraction. DNA yields up to 44.5 μg g^{-1} soil (oven-dry basis) were obtained from three field-moist natural allophanic soil samples from northern New Zealand using this two-step method. Following extraction, we evaluated different DNA purification methods. Gel electrophoresis of the extracted DNA followed by gel purification of the DNA from the agarose gel, despite some DNA loss, was the only purification method that removed sufficient humic material for successful DNA amplification using the polymerase chain reaction (PCR) of multiple gene regions. Sequencing of PCR products obtained from a buried allophanic paleosol at 2.2-m depth on a sandy Holocene tephra yielded endemic and exotic plants that differed from the European grasses growing currently on the soil's surface. This difference suggests that the DNA extraction method is able to access (paleo)environmental DNA derived from previous vegetation cover. Our DNA extraction and purification method hence may be applied to Andisols and allophane-bearing paleosols, potentially offering a means to isolate paleoenvironmental DNA and thus facilitate reconstruction of past

environments in volcanic landscapes, datable using tephrochronology, and also aid biodiversity understanding of andic soils and paleosols.

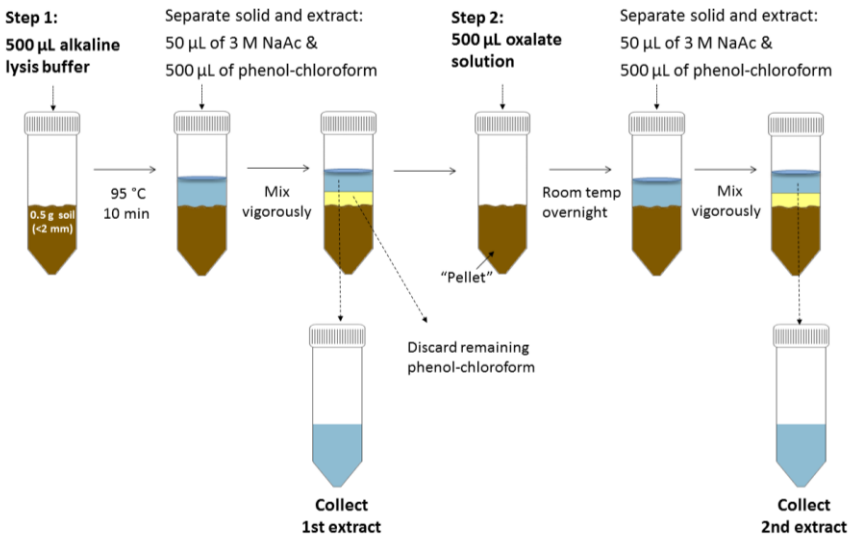
Keywords:

Andisols; allophane; DNA extraction; DNA purification; paleopedology; tephra

Highlights:

- We developed a new 2-step method to extract DNA from allophanic soils and paleosols
- It uses alkaline lysis buffer then acid oxalate to dissolve allophane and release DNA
- We purified the extracted DNA using DNA purification kits and gel electrophoresis
- DNA yields up to 44.5 µg g^{-1} were obtained from natural allophanic soil materials
- A buried andic paleosol yielded 1.9 µg g^{-1} amplifiable DNA of antecedent vegetation

Graphical abstract:



(≡ Fig. 3)

1. Introduction

Allophane-containing soils, which include most Andisols, strongly sequester organic matter (Dahlgren et al., 2004; McDaniel et al., 2012; Matus et al., 2014) and strongly adsorb deoxyribonucleic acid (DNA)[1] (Cai et al., 2006; Saeki et al., 2008, 2011; Huang et al., 2014), and hence enable carbon storage and possible preservation of DNA. Consequently, Andisols and allophanic soils on Holocene or late Pleistocene tephras potentially represent a valuable source of soil environmental DNA which could be used to reconstruct past environments or climates of the late Quaternary period through paleopedology. In addition, the extraction of environmental DNA from Andisols, which can comprise fungal, microbial, plant (including recombinant plant DNA), insect, and animal DNA (e.g. Fierer and Jackson, 2006; Levy-Booth et al., 2007; Staudacher et al., 2011; Pedersen et al., 2015), would offer enhanced insight into the biodiversity of these soils as well as possible application to forensic soil analysis (e.g. Horswell et al., 2002; Fitzpatrick, 2013; Young et al., 2014, 2015). However, the potential use of such a resource has been hampered mainly because of the difficulty in extracting adsorbed DNA from allophanic materials (see section 2). An effective method for DNA recovery from Andisols and buried allophanic paleosols (soils of past environments or landscapes) is therefore required to enable paleoenvironmental DNA (Rawlence et al., 2014) to be investigated, and to aid research involving soil biodiversity. The development of such a method,

[1]Abbreviations: asl, above sea level; bp, base pair; BP, before present (present = AD 1950); cal, calendar or calibrated; CTAB, cetyltrimethylammonium bromide; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; *g*, gravitational acceleration (9.81 m s^{-2}); humics, humic substances including humic acids; HTS, high-throughput sequencing; IPTG, isopropyl β-D-1 thiogalactopyranoside; kb, kilo base pair; LB, Luria-Bertani broth; ODS, oven-dry soil; pa, per annum; PalEnDNA, paleoenvironmental DNA; PEG, polyethylene glycol; PCR, polymerase chain reaction(s); PVP, polyvinylpyrrolidone; Rai's lysis buffer, buffer of Rai et al. (2010); rpm, revolutions per minute; SDS, sodium dodecyl sulphate; TAE, Tris-acetic-EDTA; TE, 10 mMTris-HCl, 1 mM EDTA; TEM, transmission electron microscope; UV, ultraviolet; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

106 and its potential applications including paleoenvironmental reconstruction, form the main focus of
107 this paper.

108 The key roles of allophane and organic matter in DNA adsorption and extractability are
109 summarised before we outline the basis of our new approach to extract and purify DNA from
110 allophanic soils or paleosols (section 2). Our extraction method was developed using initial
111 experiments involving associations of synthetic allophane and salmon-sperm DNA with/without
112 bead-beating and is based on firstly extracting microbial DNA and blocking adsorptive sites on
113 allophanic materials, and then releasing (rather than displacing) additional DNA bound to allophane
114 spherules and nanoaggregates by dissolving the allophane using acidified ammonium oxalate
115 (section 3). The results of the DNA extraction and purification (from both synthetic
116 allophane–salmon-sperm-DNA complexes and field-moist natural allophanic soils), including (for
117 the first time) the successful polymerase chain reaction (PCR) amplification and sequencing of plant
118 DNA extracted from a buried Holocene paleosol, are reported in section 4. The efficacy of the
119 method and some implications are then discussed (section 5) before the conclusions (section 6).

120

121 **2. Roles of allophane and organic matter in DNA adsorption and extractability**

122 Allophane provides Andisols, and other allophanic soils and buried paleosols, with many of
123 their unique chemical and physical properties (Qafoku et al., 2004; McDaniel et al., 2012; Yuan and
124 Wada, 2012; cf. Takahashi and Dahlgren, 2016). Such properties are defined as "andic soil
125 properties" in *Soil Taxonomy* and these help delimit the Andisol order (Ahrens and Arnold, 2012;
126 McDaniel et al., 2012; Soil Survey Staff, 2014). Allophane is a nanocrystalline aluminosilicate
127 comprising tiny spherules ~3.5 to 5.0 nm in diameter and with a chemical composition in the range
128 $(1–2)SiO_2 \cdot Al_2O_3 \cdot (2–3)H_2O$ (Abidin et al., 2007; Harsh, 2012). It has been shown previously that
129 extracting DNA from allophane-bearing volcanic soils is more difficult than from non-allophanic
130 (non-volcanic) soils using the same extraction method (Rai et al., 2010). The strong bonding between

131 the allophane and DNA (Hashizume and Theng, 2007; Huang et al., 2014) can be attributed to the
132 small particle size of such clay minerals, an extremely high specific surface area (up to ~1125 $m^2 g^{-1}$)
133 (Allbrook, 1985; Parfitt, 2009; Kaufhold et al., 2010; Yuan and Theng, 2012), and chemical
134 attractions with variable surface-charge characteristics that arise via $(OH)Al(OH_2)$ groups at wall
135 perforations of its outer gibbsitic octahedral sheet [$Al(OH)_3$] (Cai et al., 2006; Buurman et al., 2007;
136 Saeki et al., 2010; Calabi-Floody et al., 2011; Harsh, 2012; Matsuura et al., 2014). Huang et al. (2014,
137 2016) showed that the DNA phosphate group was bound to the outer gibbsitic octahedral sheet on
138 the surface of allophane spherules or to organic matter, or both.
139 In addition, allophane spherules tend to form very stable nanoaggregates (aggregated
140 clusters of allophane spherules up to about 100 nm in diameter) together with microaggregates
141 (aggregated clusters of allophane nanoaggregates up to several hundred micrometres in diameter)
142 (Calabi-Floody et al., 2011; Huang et al., 2016). Associated nanopores, which are tiny spaces
143 between spherules within nanoaggregates (typically ≤2 nm in diameter), and between
144 nanoaggregates within microaggregates (typically <100 nm in diameter), are too small and markedly
145 tortuous in structure to be accessible to microbes and enzymes (Chevallier et al., 2010; Filimonova et
146 al., 2011, 2016; Huang et al., 2016). These nanolabyrinthic networks of nanopores amidst the
147 allophane aggregates demonstrably provide "refuges" to any disseminated DNA in Andisols and
148 such a protection mechanism enables substantial amounts of DNA (potentially as much as ~80% of
149 the total) to be preserved (Huang et al., 2016).

150 Furthermore, the abundant organic matter in Andisols is also highly adsorptive and has a
151 strong affinity for DNA (Saeki and Sakai, 2009). Adsorptive humic substances including humic acids
152 ("humics" hereafter) in soils can be co-extracted with DNA and prevent DNA amplification by
153 inhibiting the enzyme activity in PCR (Lloyd-Jones and Hunter, 2001; Saeki et al., 2011; Young et
154 al., 2014). Most commercial soil DNA extraction kits involve bead-beating, which mechanically
155 disrupts both microbial cells and soil aggregates, and various extraction reagents (Young et al., 2014),
156 but they do not produce sufficient yields for samples with highly adsorptive minerals, such as

allophane or ferrihydrite, or efficiently purify DNA from samples with high amounts of humics (Lloyd-Jones and Hunter, 2001; Cai et al., 2006; Saeki and Sakai, 2009; Saeki et al., 2011). Also, methods that utilise commercial columns, flocculation, and gel electrophoresis for inhibitor removal lead to considerable loss of DNA (Miller et al., 1999) and so they are not ideal for purifying environmental DNA. Therefore, an effective DNA extraction and purification scheme for allophane must be able to efficiently extract DNA and remove PCR inhibitors whilst optimising DNA yield.

A limited number of studies have evaluated DNA extraction from "volcanic materials" including allophanic clays (Herrera and Cockell, 2007), but the common reagents involving ion/cation exchange or chelation could neither release bound DNA from allophane nor isolate DNA from humics (Herrera and Cockell, 2007; Miao et al., 2014). Consequently, DNA extraction methods applicable to allophanic soils have required ongoing development (e.g. Ikeda et al., 2004; Takada-Hoshino and Matsumoto, 2004; Dong et al., 2006). In combination with a commercial kit and bead-beating, one method able to extract environmental DNA from allophanic soil materials used skim milk, which functions as an adsorption competitor (Takada-Hoshino and Matsumoto, 2004, 2005); mainly clay- and organic-rich topsoils (soil 'A' horizons; typically 0 to 15 cm depth) comprising DNA from microbes, insects, and plants, were tested but with limited efficacy with respect to DNA extractability. In addition, Takada-Hoshino and Matsumoto (2004, p.18) showed that "skim milk did not increase the amount of DNA extracted from sandy soil", and so on this basis the potential application of the skim-milk method to sandy (weakly weathered or strongly microaggregated) allophanic soils or paleosols is liable to be unsuccessful. Moreover, proteins in skim milk have large molecular weights and therefore are likely to be too large to fit into nanopores in nanoaggregates, especially those with a high degree of tortuosity. Even if the skim-milk method were capable of extracting partial DNA from sandy soils or paleosols, a more universally effective method is required to retrieve preserved DNA bound both chemically on allophane and physically within nanopores in allophanic buried soil horizons (Huang et al., 2016).

182 *2.1. A new approach to extract and purify DNA from adsorptive allophanic soils*

183 As noted earlier, we posit that much more genetic information could be obtained by applying
184 a more efficient method of DNA extraction to most Andisols or to allophanic paleosols. We have
185 therefore developed a two-step DNA isolation method for allophanic materials that breaks down the
186 strong bonding between allophane and chemically-bound DNA, and DNA adsorbed physically in
187 nanopores, and liberates relatively high yields of amplifiable DNA (including from sandy soil
188 horizons or paleosols). Our method is based on the premise that DNA is likely to be more easily
189 extractable if the allophane spherules and nanoaggregates are physically disrupted and, notably, if
190 allophane is chemically dissolved to release the DNA (including that encapsulated within nanopores).

191 Our approach uses an initial alkaline lysis buffer extraction (based on Rai et al., 2010,
192 described below), with and without bead-beating, followed by acidified ammonium oxalate, also
193 known as Tamm's reagent (Tamm, 1922). The alkaline lysis buffer has been shown to extract DNA
194 quantities of up to 20−40 μg g^{-1} soil from Japanese allophanic soils (Rai et al., 2010), whereas
195 acidified ammonium oxalate has been widely used to extract Al and Si, and other metals such as Fe,
196 from nanocrystalline minerals including allophane and ferrihydrite by means of their dissolution (e.g.
197 Parfitt and Childs, 1988; Wada, 1989; Parfitt, 2009; Churchman and Lowe, 2012). To our knowledge,
198 this key ingredient, Tamm's reagent, has not previously been used to release effectively all adsorbed
199 DNA by complete dissolution of the allophane.

200 Furthermore, we tested the procedure using field-moist soil rather than soil that had been
201 dried in the laboratory. Fine pores are largely lost, the texture of the soil becomes coarser, and
202 dispersion is difficult when allophanic soils are oven-dried, and these effects are irreversible
203 (Allbrook, 1983; Churchman and Payne, 1983; Gray and Allbrook, 2002; Harsh et al., 2002). In
204 addition, drying of soils strengthens bonds between minerals and organic matter, which tend to
205 increase in covalent character (Kleber et al., 2015). Hence it is likely that prior laboratory drying of
206 allophanic soils alone could prevent the complete removal of adsorbed DNA. Results involving the

DNA extractions (yields) from the soil samples are expressed relative to the mass of oven-dry soil (ODS), however.

We also explored several DNA purification techniques prior to, and after, DNA extraction. The techniques trialled included (1) manufactured activated charcoal prior to DNA extraction to absorb soluble humics, (2) two commercial DNA clean-up kits after DNA extraction, and (3) gel electrophoresis followed by use of GenScript QuickClean II Gel Extraction Kit.

3. Materials and methods

3.1. Samples for DNA extraction

3.1.1. Synthetic allophane–salmon-sperm-DNA complexes

For proof of principle, synthetic allophane and salmon-sperm DNA were used to test whether our DNA extraction method was able to extract DNA strongly bound to synthetic allophane. Pure aluminosilicate allophane was synthesized following the method of Ohashi et al. (2002), and chemical analysis of the fine white product (Fig. 1a) with both oxalate and pyrophosphate extractions showed an allophane yield of 95% with an Al/Si ratio of 1.2. Ultra-high-resolution transmission electron microscope (TEM) images of the synthetic allophane (after Huang et al., 2016) showed that the allophane was consistently spherical in shape with an average diameter of 10−15 nm (Fig. 1b and 1c), approximately two- to three-fold larger than that reported for natural allophane spherules (typically ~3.5 to 5 nm) (e.g. McDaniel et al., 2012). This larger spherule size is presumably a consequence of the precipitation of synthetic allophane in the laboratory setting without growth constraints imposed in the milieu of natural soil microenvironments (Churchman and Lowe, 2012).

Because allophane spherules are heat-sensitive (Woignier et al., 2007), the synthetic product was frozen quickly with liquid nitrogen, followed by freeze-drying for subsequent analysis.

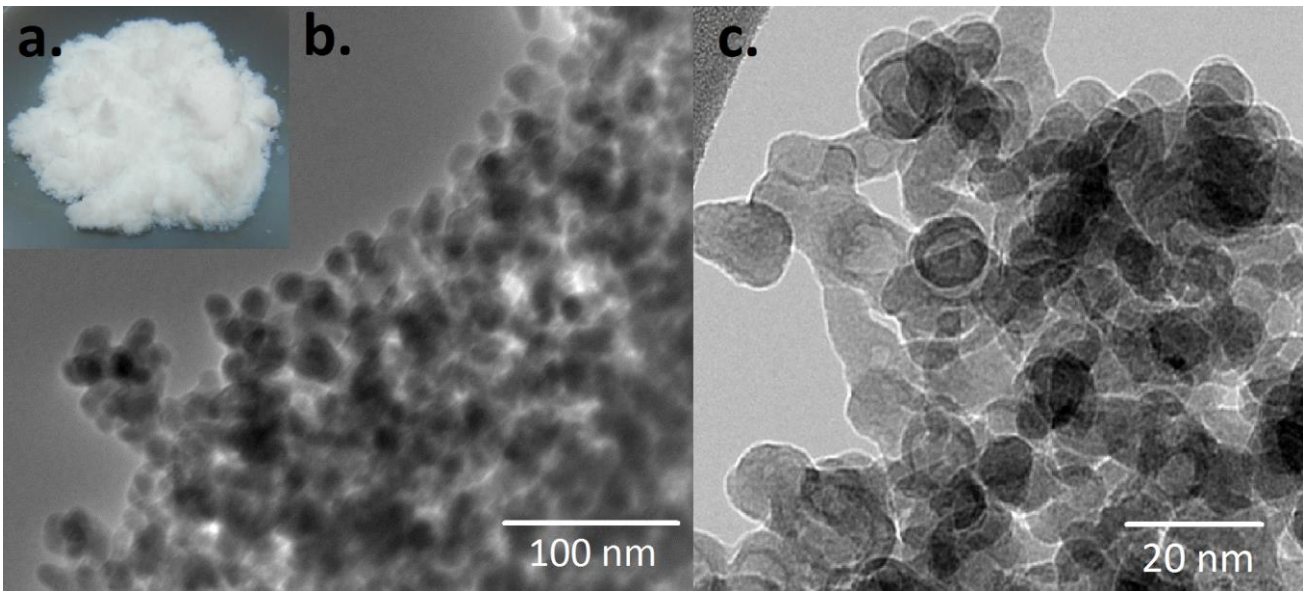


Fig. 1. Synthetic allophane (**a**) and its spherical morphology seen using transmission electron microscopy (TEM) (**b**) and (**c**). *Reproduced from* Huang et al. (2016, p. 43) *with permission from Elsevier*.

To carry out DNA adsorption, 90 µg of salmon-sperm DNA was added into a suspension containing 25 mg of synthetic allophane and mixed on a rotator for 3 h. Then the DNA-clay suspension was centrifuged at 10,000 × *g* and the supernatant was analysed spectrophotometrically to ascertain that all additive DNA had bonded to the synthetic allophane with no detectable DNA remaining in the supernatant. The complex of synthetic allophane and salmon-sperm DNA was stored at 4 °C in the dark for subsequent DNA extraction.

3.1.2. Natural allophanic soil samples (paleosols)

We selected a site near Rotorua in central North Island, New Zealand, where a well-drained Andisol had previously been characterised (Cole, 1970; Lowe et al., 2010, 2012). This multilayered soil profile of the Rotomahana soil series (Fig. 2) was excised laterally in two stages for close to 1 m to remove surface (roadside) contamination. Allophanic materials were sampled from buried soil

horizons developed on three weakly weathered Holocene rhyolitic (siliceous) tephras, namely Taupo, Whakatane, and Rotoma, details of which (including ages) are given in Table 1. The depths of samples below the modern land surface were ~0.8 m (3ABb horizon developed on Taupo tephra), ~1.4 m (4Bwb horizon developed on Whakatane tephra), and ~2.2 m (5Ahb soil horizon developed on Rotoma tephra) (Fig. 2).

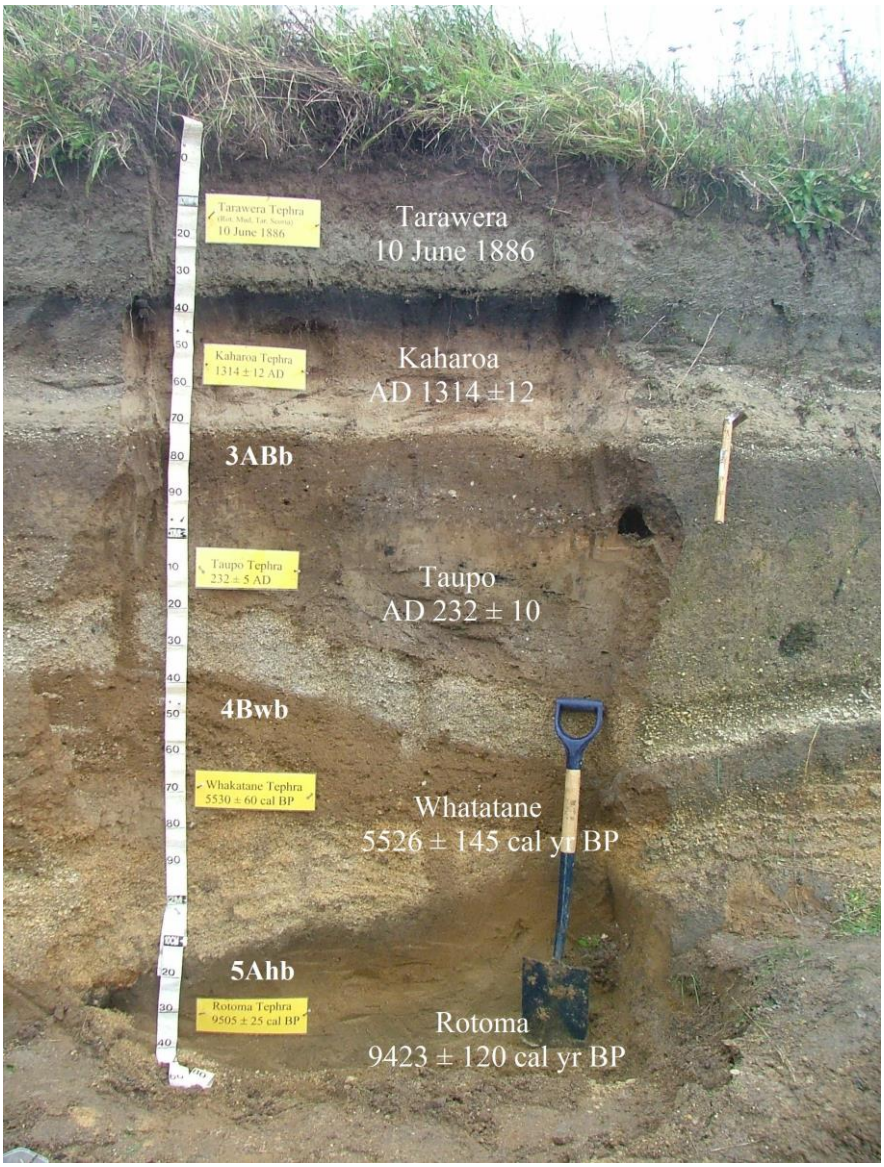


Fig. 2. Multilayered Andisol at Brett Rd near Mt Tarawera comprising five separate tephra deposits (names and ages/dates as marked; see also Table 1) and associated soil horizons. We sampled the buried allophanic soil horizons on Taupo (denoted 3ABb), Whakatane (4Bwb), and Rotoma (5Ahb) tephras (horizonation from McDaniel et al., 2012, after Soil Survey Staff, 2014). The suffix 'b' denotes an identifiable soil horizon with pedogenic features developed before its burial (Soil Survey Staff, 2014). Scale divisions on tape = 10 cm. Photo: D.J. Lowe.

Table 1. Selected properties of allophanic material from three buried soil horizons of an Andisol[a], northern New Zealand.

Buried soil (horizonation)	pH (H_2O)	Sand content[b] wt%	Clay content[c] wt%	Allophane content[d] %	Ferrihydrite content[d] %	Total carbon %
Taupo (3ABb)[e]	6.6	72.6	3.4	39	6.1	11.9
Whakatane (4Bwb)[f]	6.8	62.9	3.9	38	9.5	10.2
Rotoma (5Ahb)[g]	6.6	70.2	5.0	47	4.9	6.3

[a] Well-drained multilayered soil (of the Rotomahana soil series, Hewitt, 2010) located on Brett Road, Rerewhakaaitu (near Mt Tarawera), North Island (Fig. 2), at 38° 17' 53.53" S, 176° 28 52.83" E, elevation 452 m asl, rainfall ~1464 mm pa (Leathwick et al., 2003; Lowe et al., 2012; McDaniel et al., 2012, p. 33.38). It is a Fine-loamy/medial, mixed/glassy, active, mesic Typic Udivitrand (Soil Survey Staff, 2014). Note that the uppermost material, labelled 'Tarawera' in Fig. 2, comprises mainly pre-weathered materials of the so-called Rotomahana Mud Member of the Tarawera Tephra Formation (Froggatt and Lowe, 1990) erupted on 10 June 1886. With a clay content of ~20% (Parfitt et al., 1981), the clays in this deposit include interstratified montmorillonite/mica, montmorillonite, mica, kaolinite, and mordenite, as well as allophane (Kirkman, 1976).

[b] Sand fractions (0.063–2 mm) were determined by wet sieving, oven drying, and weighing (Green, 1987). Data are from equivalent buried soil horizons on the same tephras at nearby sites as reported by Lowe and Green (1992).

[c] <2 μm size fraction determined using the pipette method after prolonged shaking of samples with glass beads (method after Churchman and Tate, 1986).

[d] As percentage of clay fraction.

[e] Taupo tephra deposited AD 232 (± 10 years) (date is 95% probability range from Hogg et al., 2012); soil formed on it represents ~1082 years at land surface before its burial by Kaharoa tephra in AD 1314 (± 12 years) (date is 95% probability range from Hogg et al., 2003).The 3ABb horizon sampled is ~0.8 m below the modern land surface (Fig. 2).

[f] Whakatane tephra deposited 5526 ± 145 calendar (cal) years before present (BP) (age is 95% probability range from Lowe et al., 2013) (present = AD 1950); soil formed on it represents ~3808 years at land surface before its burial by Taupo tephra in AD 232 (± 10 years). The 4Bwb horizon sampled is ~1.4 m below the modern land surface (Fig. 2).

[g] Rotoma tephra deposited 9423 ± 125 cal years BP (age is 95% probability range from Lowe et al., 2013); soil formed on it represents ~3897 years at land surface before its burial by Whakatane tephra 5526 ± 145 cal years BP. The 5Ahb horizon sampled is ~2.2 m below the modern land surface (Fig. 2).

Modern root growth was observed through all horizons and tephra layers of the profile; the current land surface underlies roadside pasture. Therefore, samples were sieved through a 2-mm mesh to remove gravel and visible plant roots, and the <2-mm fractions were thoroughly homogenised and stored at field-moist conditions in plastic bags at 4 °C prior to analysis. Moisture contents (to enable analyses to be expressed on an ODS basis) and pH were measured using the methods described by Blakemore et al. (1987). Total carbon contents of the freeze-dried clay fractions were examined using a Leco TruSpec carbon/nitrogen determinator. Allophane and

297 ferrihydrite contents were estimated according to the oxalate/pyrophosphate-dissolution based
298 methods of Parfitt and Wilson (1985) and Parfitt and Childs (1988), as documented by Blakemore et
299 al. (1987).

300

301 *3.2. DNA extraction*

302 Three independent one-step extraction methods were initially applied to the previously
303 prepared synthetic allophane–salmon-sperm-DNA complexes, and to the natural allophanic soils on
304 tephras (Taupo, Whakatane and Rotoma): (1) alkaline lysis buffer, (2) alkaline lysis buffer with
305 bead-beating, and (3) ammonium oxalate extraction. Subsequently, we applied a two-step DNA
306 extraction method using the alkaline lysis buffer followed by ammonium oxalate extraction to both
307 the synthetic allophane and natural allophanic soils. For each extraction, 25 mg of the synthetic
308 allophane–salmon-sperm-DNA complex and 0.5 g of natural (field-moist) allophanic soil material
309 were processed. All DNA extractions were carried out in triplicate.

310

311 *3.2.1. Alkaline lysis buffer*

312 The alkaline DNA extraction buffer of Rai et al. (2010) ("Rai's lysis buffer" hereafter)
313 consisted of 1% sodium dodecyl sulphate (SDS), 100 mM Tris-HCl (pH Buffer), 200 mM
314 ethylenediaminetetraacetic acid (EDTA), and 500 mM Na_2HPO_4 (final pH at 8.6). The SDS (a
315 surfactant) is used to lyse (break down) the cell membranes, which are composed mainly of lipids, to
316 release DNA, the lysis being boosted by heating; the EDTA chelates metal ions including Al^{3+} and
317 hence prevents deoxyribonuclease (DNase) from degrading DNA; and the phosphate blocks the
318 adsorptive sites on the allophane to minimise re-adsorption of newly-released DNA. Sample material
319 was mixed with 500 µL of Rai's lysis buffer and incubated at 95 °C with shaking for 10 min on a
320 thermomixer. For the synthetic allophane, the supernatant was collected by centrifugation at 10,000

× *g* for 10 min. 50 µL of 3 M sodium acetate (1/10 of the volume of Rai's lysis buffer) and 500 µL of phenol-chloroform (an equal volume to that of Rai's lysis buffer) were specifically added only to the natural allophanic soil samples, followed by vigorous mixing, 10 min shaking, and centrifugation at 10,000 × *g* for 10 min, to generate two immiscible liquid layers and to separate the aqueous phase containing DNA (upper layer) from an organic phase (bottom layer), and the upper aqueous supernatant was collected (Fig. 3).

3.2.2. Alkaline lysis buffer (Rai's lysis buffer) with bead-beating

After adding 500 µL of Rai's lysis buffer to synthetic allophane and natural allophanic material, three 2-mm glass beads (provided by Biospec, USA) (Li et al., 2011) were added, followed by beating three times at maximum speed for 30 s with an interval of 30 s between each period of beating, and then samples were incubated at 95 °C with shaking on a thermomixer for 10 min. The extracts from synthetic allophane and natural allophanic soils were collected following the methods described in section 3.2.1.

3.2.3. Ammonium oxalate extraction

To chemically dissolve synthetic allophane and natural allophanic soil material to release bound DNA, oxalate extraction was carried out. Usually, for analysing the Al/Si ratio of allophanic samples, the oxalate extraction is undertaken with 0.2 M oxalate solution (pH 3) for 4 hours in the dark (Blakemore et al., 1987; Dahlgren and Saigusa, 1994). Here, we increased both the concentration of oxalate solution and the reaction time to enhance the efficiency of oxalate extraction for DNA. 1 M acidified ammonium oxalate salt was prepared by homogenising 18 g of ammonium oxalate and 10.8 g of oxalic acid powder in sterile deionised water, the final volume being made up to 200 mL at pH 3. 500 µL of this oxalate solution was added to each sample, followed by overnight

shaking for allophane dissolution. The extracts from synthetic allophane and natural allophanic soils were collected following the methods described in section 3.2.1.

3.2.4. Two-step DNA isolation: alkaline lysis buffer (Rai's lysis buffer) followed by ammonium oxalate extraction

We developed our two-step DNA extraction method consisting of (step 1) DNA extraction using Rai's lysis buffer, followed by (step 2) overnight DNA extraction using acidified ammonium oxalate extraction from the residual material (i.e. the synthetic or the natural allophane "pellet" remaining after step 1 of the extraction) (Fig. 3). After step 1 of the extraction, 50 μL of 3 M sodium acetate and 500 μL of phenol-chloroform were specifically added only to the natural allophanic soil samples, and the aqueous supernatant (upper layer) was collected and remaining phenol-chloroform (bottom layer) was removed by a fine transfer pipette; the remaining soil underwent step 2 of the extraction (ammonium oxalate extraction). In contrast, synthetic allophane was directly treated with acidified ammonium oxalate after extraction by step 1. The supernatant collected after extraction by step 1 is denoted as the '1st extract' in Fig. 3, and the supernatant obtained from step 2 is recorded as the '2nd extract'. The extracts from the first and second extractions were kept separate.

3.3. DNA quantification and sample purification

The fragment lengths of extracted DNA were assessed via gel electrophoresis and ethidium bromide staining, using 1 kb Plus DNA Ladder (Invitrogen) as a reference. For the extracts from synthetic allophane, DNA yield was evaluated via gel electrophoresis together with a 1 kb Plus DNA Ladder (Invitrogen). DNA extracts derived from the natural allophanic soils on tephras (Taupo, Whakatane, and Rotoma) were evaluated spectrophotometrically, and the reading (in ng μL^{-1}) was multiplied by the final volume of extract (μL) and then divided by the oven-dry mass of soil material

used for the DNA extraction. Note that the co-extracted humics substantially interfered with the spectrophotometric measurement at 260 nm, and therefore the quantification of DNA yields may be overestimated.

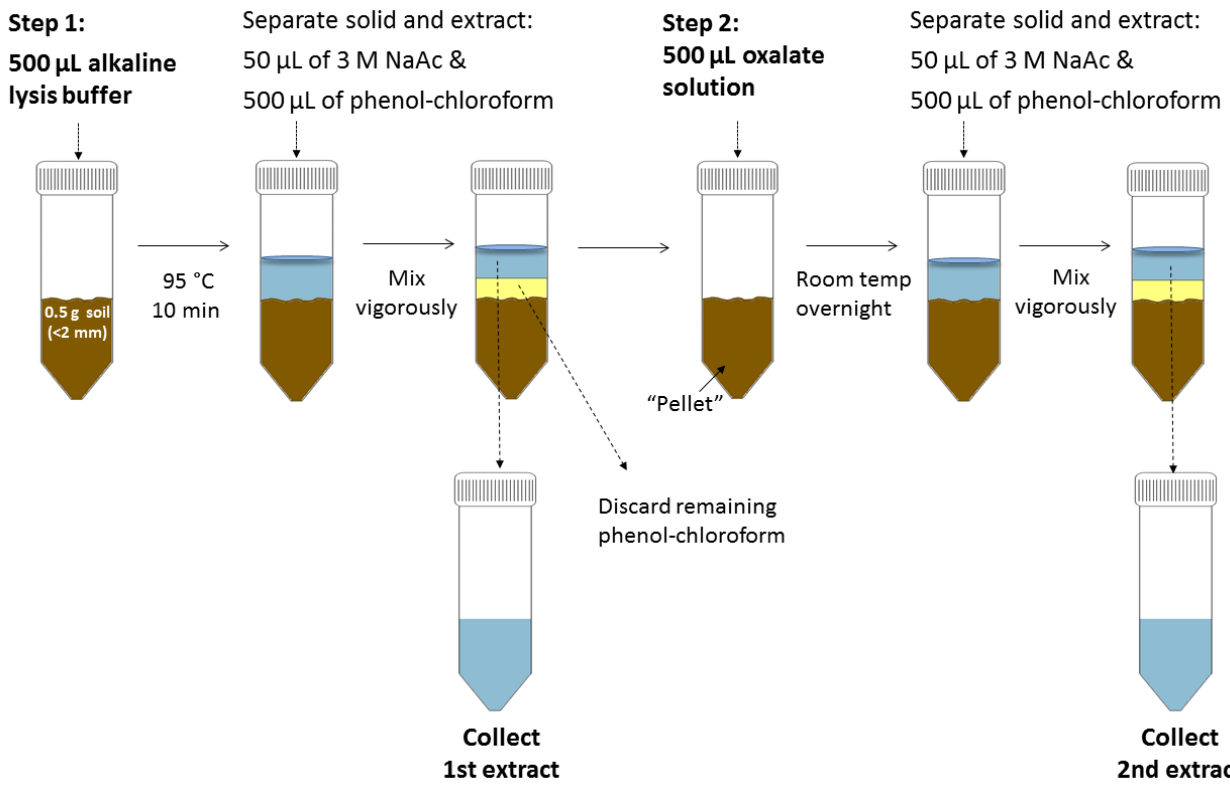


Fig. 3. Simplified schematic depicting the two-step DNA extraction method involving Rai's lysis buffer (step 1) followed by acidified ammonium oxalate treatment (step 2) for DNA extraction from natural allophanic soil samples.

To remove co-extracted humics and impurities from the natural allophanic soils, each extract (described in sections 3.2.1 to 3.2.4) was gently mixed with 5 M NaCl (1/3 of the volume of the collected supernatant/extract) and 10% cetyltrimethylammonium bromide (CTAB) (1/3 of the volume of the collected supernatant/extract), followed by 65 °C incubation and mixing for 10 min on a thermomixer. The mixture was then thoroughly shaken with 750 µL of chloroform for 10 min on a rotator at room temperature, and then centrifuged at 10,000 × *g* for 10 min. The upper layer was collected and gently mixed with an equal volume of 20% polyethylene glycol 8000 (PEG 8000),

incubated at room temperature for 20 min followed by centrifugation for 15 min at10,000 × *g*. The supernatants were removed, and the precipitates were washed with 1 mL of 100% ethanol and 1 mL of 70% ethanol followed by centrifugation at 10,000 × *g* for 2 min. After the ethanol had been decanted, the precipitates were air dried and then resuspended with 30 µL TE (10 mM Tris-HCl and 1 mM EDTA). DNA concentrations in 30 µL of TE were evaluated at 260 nm spectrophotometrically using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA), and DNA yields from the natural allophanic soils were then calculated by the volume of TE and sample concentration. PCR amplification of the DNA in TE was carried out with selected primers (see section 3.4). However, the A_{260}/A_{280} NanoDrop readings and the poor PCR amplification rate indicated that further purification to remove inhibitors was required.

3.3.1. Further DNA purification of DNA extracts from buried soil horizon on Rotoma tephra for polymerase chain reaction (PCR)

DNA extracts derived from the buried soil on Rotoma tephra using the two-step DNA extraction method were further purified for PCR and subsequent DNA sequencing. The soil horizon (5Ahb) developed on the Rotoma tephra was selected for testing DNA purification for two main reasons: (1) it represents nearly c. 4000 years of development and weathering whilst at the land surface (before being buried by the Whakatane and other tephras since c. 5500 cal years ago) and, of the paleosols examined, had the highest clay and allophane content (Table 1) (its clay content at 5% is still low); and (2) it is now at a depth of c. 2.2 m and so is beyond the range of most topdown pedogenic processes (Fig. 2) (e.g. Schaetzl and Thompson, 2015).

To further purify the DNA extracts from the buried soil horizon on Rotoma tephra, two commercial kits (Zymo Research Genomic DNA Clean & Concentrator Kit and GenScript QuickClean PCR Purification Kit) were used following the manufacturers' protocols. A third approach involved mixing the DNA extract with 5 µL of nucleic acid stain GelRed for gel

electrophoresis (1% agarose gel). The gel was illuminated with a blue-light illuminator (Safe Imager, Invitrogen) and the DNA band or smear was extracted for gel purification using the GenScript QuickClean II Gel Extraction Kit. In addition, the use of humic-acid removal solution and activated charcoal prior to DNA extraction was also included (described below in section 3.3.1.1). The efficiency of each of these five purification techniques (Table 2) was assessed with regard to final DNA yield, purity, and amplification success using four primer pairs (section 3.4).

Table 2. DNA yield, purity, and PCR amplification success observed from DNA extracts from the buried soil (5Ahb horizon) on Rotoma tephra subjected to five different purification methods following the two-step DNA isolation method.

Purification method	DNA yield[a] (μg g^{-1}dry soil)	DNA purity		PCR amplifications with selected primers[b]				
		A_{260}/A_{280}	A_{260}/A_{230}	16s rDNA	Lep	rbcL	trnL c−d	trnL g−h
1. Two-step DNA extraction followed by chloroform treatment	8.7 (2.3)	1.6	0.6	−	−	−	−	−
2. Two-step DNA extraction followed by Zymo Research Genomic DNA Clean & Concentrator Kit	3.2 (0.8)	1.2	0.2	+	−	−	−	−
3. Two-step DNA extraction followed by GenScript QuickClean PCR Purification Kit	8.6 (1.0)	1.3	0.2	+	−	−	−	−
4. Humics-removal prior to two-step DNA extraction followed by chloroform[c]	10.0 (2.6)	1.6	0.9	−	−	−	−	−
5. Two-step DNA extraction followed by gel electrophoresis and GenScript QuickClean II Gel Extraction Kit	1.9 (0.5)	1.6	1.7	+	−	+	+	+

[a]Final DNA concentration in 30 μL TE was evaluated spectrophotometrically (see text, section 3.3). Reported as mean values (± 1 std dev) of three assays.
[b]Presence (+) or absence (−) of amplicons on agarose gel after amplification with touchdown PCR.
[c]DNA precipitated from the humics removal step remained inhibiting to DNA amplification, presumably because of the presence of unknown inhibitors.

427 *3.3.1.1. Humics removal using activated charcoal prior to two-step DNA extraction*

428 The combination of 10 mmol L^{-1} $MgCl_2$ and 1% activated charcoal has been found to
429 effectively remove most humics and PCR inhibitors from soil samples (Sharma et al., 2014). $MgCl_2$
430 precipitates organic matter by chemical flocculation and the porous charcoal absorbs soluble humics
431 and other impurities. Activated charcoal (or biochar) is usually manufactured after pyrolysis at
432 400−500 °C for 5 to 10 hours (Kloss et al., 2012), and sometimes up to 800 °C (Gaskin et al., 2008),
433 and hence DNA from any original tissues is likely to be destroyed after treatment at such
434 temperatures. To test this assumption, we attempted to extract DNA from the residual activated
435 charcoal used in our study, but no quantifiable DNA was detected nor amplified.

436 A humics-removal solution (Li et al., 2011) was used in combination with a charcoal
437 suspension and $MgCl_2$ solution prior to cell lysis/DNA isolation (i.e. as the initial step of the DNA
438 extraction). The humics-removal solution comprised 0.1 M Tris, 0.1 M $Na_4P_2O_7$, 0.1 M Na_2EDTA,
439 1% polyvinylpyrrolidone (PVP) (w/v), 0.1 M NaCl, and 0.05% Triton X-100 (v/v), pH 10. 400 µL of
440 the humics-removal solution, 50 µL of 10% charcoal suspension, and 50 µL of 100 mM $MgCl_2$ were
441 added to 0.5 g of moist soil, followed by vortexing or shaking for 5 min. The humics-rich
442 supernatant was removed after centrifugation at 10,000 × *g* for 2 min. The remaining soil in the
443 reaction tube was then treated with the two-step DNA extraction method, and extracted DNA was
444 purified with chloroform and ethanol (see section 3.3) for DNA quantification and PCR
445 amplification.

446

447 *3.4. Polymerase chain reaction (PCR) and DNA sequencing of extract from the buried soil horizon*
448 *on Rotoma tephra*

449 Four universal primers (Table 3) were used to assess PCR amplification success resulting
450 from the five different purification methods (Table 2). PCR amplification was carried out in a total

volume of 50 µL, containing 43.75 µL of master mix (1.5 mM of Mg^{2+}, 200 µM of each dNTP, 1× Hot Fire Buffer), 1 µL of primers (20 pmol/µL), 0.25 µL of Taq DNA polymerase (Solis BioDyne), and 5 µL (~20 ng) of purified DNA template. To increase specificity and sensitivity, PCR amplifications were performed using a touchdown protocol (Korbie and Mattick, 2008) as follows: (1) heating at 95 °C for 15 min to activate the polymerase; then (2) 10 cycles consisting of 95 °C for 20 s, 60 °C for 20 s , and 72 °C for 1 min, with the annealing temperature being decreased by 1 °C after each cycle; then (3) 30 cycles consisting of 95 °C for 20 s, 50 °C for 20 s, and 72 °C for 1 min; and (4) a final extension step at 68 °C for 10 min. PCR products were visualised by electrophoresis on a 1% (w/v) agarose gel in 1× TAE buffer. The rbcL and *trn*L PCR products derived from the two-step extraction followed by gel purification were prepared for DNA sequencing. For each primer pair, PCR products from the first and second extraction were combined before being ligated into pBluescript SK+ vectors according to the manufacturer's instructions, followed by cloning into *Escherichia coli* DH5 α and Sanger sequencing with T3 and T7 primers.

Table 3. Universal primers used for PCR amplification from the buried soil horizon (5Ahb) on Rotoma tephra. The primer pairs of rbcL and *trn*L include rbcLb F−rbcLa R, *trn*L c−h, and *trn*L g−h.

Name	Sequence	Target taxa[a]	Reference
16s F	GGTTAAGTCCCGCAACGAGCGC	Bacteria	Greisen et al. (1994)
16s R	AGGAGGTGATCCAACCGCA	Bacteria	Greisen et al. (1994)
Lep F1	ATTCAACCAATCATAAAGATATTGG	Insects	Hebert et al. (2013)
Lep R1	TAAACTTCTGGATGTCCAAAAAATCA	Insects	Hebert et al. (2013)
rbcLb F	AGACCTWTTTGAAGAAGGTTCWGT	Plants	Dong et al. (2014)
rbcLa R	GTAAAATCAAGTCCACCRCG	Plants	Micklos et al. (2013)
*trn*L c F	CGAAATCGGTAGACGCTACG	Plants	Taberlet et al. (2007)
*trn*L h R	CCATTGAGTCTCTGCACCTATC	Plants	Taberlet et al. (2007)
*trn*L g F	GGGCAATCCTGAGCCAA	Plants	Taberlet et al. (2007)

[a]Length of target fragment with 16s primers: 450 bp; with Lep primers: 658 bp; with rbcLb F−rbcLa R primers: 350 bp; with *trn*L c−h primers: ~200 bp; with *trn*L g−h: 40 bp.

Table 4. Summary of the new method for DNA extraction and its purification from Andisols and allophanic soils or buried paleosols.

Procedure	Description
	0.5 g field-moist soil (<2-mm fraction)[a] in a 2-mL centrifuge tube
Humics removal	Add 400 µL of humics-removal solution, 50 µL of 10% charcoal suspension, and 50 µL of 100 mM $MgCl_2$, and then vortex or shake for 5 min. Centrifuge at 10,000 × *g* for 2 min. Decant the supernatant.
1st step of DNA extraction	Add 500 µL DNA extraction buffer to the soil pellet, vortex, and then incubate the tube at 95 °C for 10 min with shaking in a thermomixer. Add 50 µL of 3 M sodium acetate and 500 µL of phenol-chloroform into the tube. Shake the sample vigorously and mix on a rotator for 10 min and decant the supernatant after another centrifugation at 10,000 × *g*. Collect the top aqueous layer into a new 2-mL tube for subsequent DNA purification.
2nd step of DNA extraction	In the original tube, decant the remaining phenol-chloroform and then add 500 µL of acidified ammonium oxalate to the residual soil pellet (i.e. soil remaining from 1st extraction). Put sample on a rotator at room temperature for overnight mixing. Add 50 µL of 3 M sodium acetate and 500 µL of phenol-chloroform into the tube. Shake the sample vigorously and mix on rotator for 10 min. Decant the supernatant after centrifugation. Collect the top aqueous layer into a new 2-mL tube for subsequent DNA purification.
DNA gel purification	Mix the collected extract with 5 M NaCl (the volume ratio of NaCl to extract is 1/3) and 10% CTAB (the volume ratio of CTAB to extract is 1/3), followed by 65 °C incubation and mixing for 10 min on a thermomixer. The mixture is then shaken with 750 µL of chloroform for 10 min on a rotator at room temperature and centrifuged at 10,000 × *g* for 10 min. Collect the upper layer and mix it with an equal volume of 20% PEG 8000, and leave sample at room temperature for at least 10 min. After centrifugation for 15 min at 10,000 × *g*, discard supernatant carefully and keep the precipitate. Rinse the precipitate with 1 mL of 100% ethanol, and re-suspend the precipitate with 30 µL of TE[b]. Load the DNA solution on a 1% agarose gel and electrophoresis for 30 min, and then cut out the DNA band within the gel using a sterile blade. Place the cut DNA band (in the agarose gel) into a 1.5-mL tube and add 3 volumes of DNA binding buffer to 1 volume of gel slice from the GenScript QuickClean II Gel Extraction Kit; solubilise the gel at 55 °C for 10 min, and purify the DNA using the kit instructions.

Footnotes next page

[a]Field-moist soil is used to help prevent irreversible changes to the allophane-organic complexes and to reduce possible bond strengthening between these on drying (see text). Yields are expressed relative to the mass of oven-dry soil, however, and hence moisture contents (oven-dry) need to be measured.

[b]TE buffer contains 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

NCBI BLAST and an unpublished database of New Zealand endemic and exotic plants (held at Landcare Research, Lincoln, New Zealand, a central government-owned research centre) were used to analyse the sequences.

3.5. Summary of new method

The two-step DNA extraction and gel purification method is summarised in Table 4 (see also Fig. 3). The development of this protocol forms a key part of our paper, and its efficacy and performance are discussed further in section 4.

4. Results

4.1. DNA extraction from synthetic allophane–salmon-sperm-DNA complexes

Using the synthetic allophane–salmon-sperm-DNA complex, only acidified ammonium oxalate was able to successfully extract DNA from synthetic allophane (Fig. 4), indicating that acidified ammonium oxalate treatment indeed provides a means to dissolve allophane spherules and nanoaggregates to release DNA bound to the allophanic matrix. In contrast, Rai's lysis buffer, both alone and with bead-beating, was ineffectual, yielding no DNA (Fig. 4).

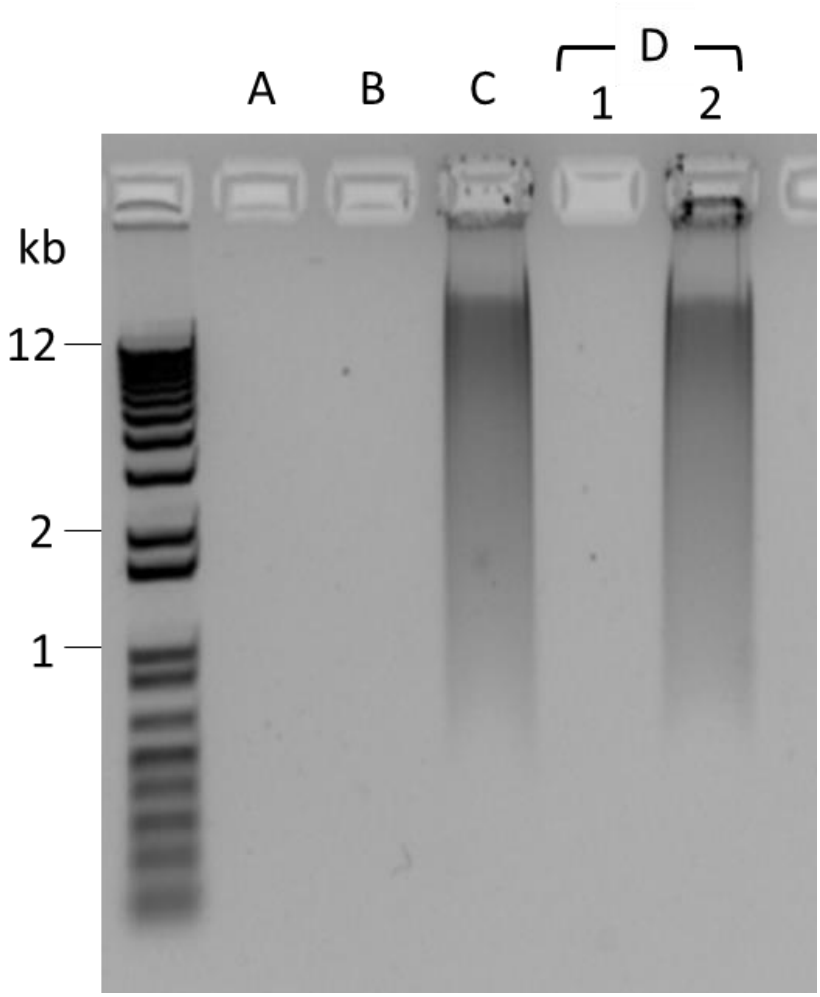
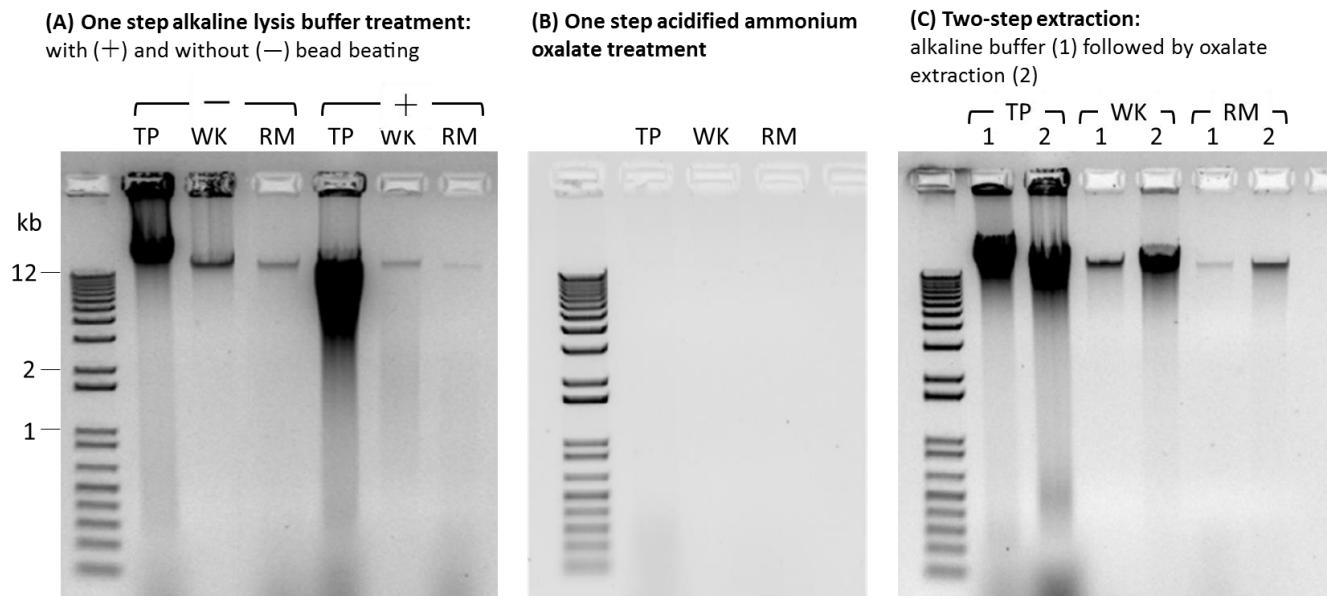


Fig. 4. Gel photo of salmon-sperm DNA extracted from synthetic allophane using four different methods. A: Rai's lysis buffer; B: Rai's lysis buffer with bead-beating; C: ammonium oxalate extraction; D-1: first step of two-step DNA extraction; D-2: second step of two-step DNA extraction (Fig. 3).

4.2. DNA extraction from the natural allophanic soils on Taupo, Whakatane, and Rotoma tephras

4.2.1. Effects of Rai's lysis buffer and bead-beating on DNA yield and fragment length

DNA extraction from three soil samples using Rai's lysis buffer allowed 26.8, 4.7, and 7.1 μg g^{-1} soil (ODS basis) of DNA to be extracted from the soil horizons on Taupo, Whakatane, and Rotoma tephras, respectively, and bead-beating combined with Rai's lysis buffer seemed not to increase DNA yield from the three soil samples (Fig. 5A). Instead, the mechanical lysis step involving bead-beating appeared to fragment (shear) the DNA from >12 kb to 2−12 kb (Fig. 5A), particularly evident in the Taupo extracts.



510

511 **Fig. 5.** Gel photos of extracted DNA from samples of natural allophanic soil horizons on Taupo (TP),
512 Whakatane (WK), and Rotoma (RM) tephras using different methods, showing the relative concentrations and
513 lengths of extracted DNA.

514

515 *4.2.2. Effects of oxalate extraction on DNA yield*

516 The effect of ammonium oxalate extraction differed between synthetic allophane and natural
517 allophanic soils. Using the synthetic allophane–salmon-sperm-DNA complex, acidified ammonium
518 oxalate yielded a higher DNA yield than treatment with alkaline lysis buffer (Fig. 4). In contrast,
519 when a one-step ammonium oxalate treatment was applied to natural allophanic soils, no DNA yield
520 was observed (Fig. 5B). This finding indicates that the natural allophanic system differs somewhat
521 from the synthetic model. However, when ammonium oxalate was used in the two-step DNA
522 isolation method, DNA was obtained upon ammonium oxalate treatment at step 2 for all three soil
523 horizons on the tephra layers (Figure 5C), leading to a DNA yield of up to 44.5 μg g^{-1} (measured
524 spectrophotometrically) from the buried soil horizon (3ABb) on Taupo tephra. This result shows that
525 the use of Rai's lysis buffer prior to ammonium oxalate treatment is essential for effectively releasing
526 mainly microbial DNA, and any unbound (extracellular) DNA, from natural allophanic soils. We
527 deduce that this finding is likely to be a consequence of residual alkaline buffer from step 1

528 increasing the pH of the solution in step 2 (soil pH at this step was measured as 6). The additional
529 DNA obtained from each soil upon step 2 with ammonium oxalate treatment is likely to be the
530 chemically-bound DNA, and physically-adsorbed DNA within nanopores in the allophane
531 nano/microaggregates, as demonstrated for the synthetic allophane–salmon-sperm-DNA complex
532 previously.

533

534 *4.3. DNA purification, PCR amplification, and DNA sequencing of the buried allophanic soil on*
535 *Rotoma tephra*

536 Purification of the DNA extract from the soil horizon (5Ahb) on Rotoma tephra using NaCl,
537 CTAB, and chloroform (see section 3.3) resulted in 8.7 μg g^{-1} soil (ODS basis) of DNA, but the
538 purified DNA was not amplifiable with selected primers (Table 2). The poor A_{260}/A_{230} reading of the
539 purified DNA using NaCl, CTAB, and chloroform showed a significant presence of impurities at 230
540 nm which inhibited PCR. Attempts to remove humics using the humics-removal solution and
541 charcoal prior to the two-step DNA extraction resulted in no significant improvement in DNA purity
542 nor amplification success (Table 2). Nevertheless, the use of the humics-removal process prior to
543 two-step DNA extraction increased the overall DNA yield by ~1 μg g^{-1}.

544 Following the use of Zymo Research and GenScript DNA clean-up kits on extracted DNA
545 from the soil horizon on Rotoma tephra, 3.2 and 8.6 μg g^{-1} soil (ODS basis) of purified DNA were
546 obtained, respectively. However, in both cases PCR amplification was only successful for one of the
547 five primer pairs, 16S rRNA (Table 2 and Fig. 6). This result is expected because bacterial DNA is
548 ubiquitous and generally present in a much higher concentration than insect or plant DNA (e.g.
549 Pietramellara et al., 2009), and so is more easily amplified than targets of lower abundance. In
550 contrast, the gel purification approach led to successful PCR amplification of four primer pairs
551 (except Lep primers) from the soil horizon on Rotoma tephra (1.6 of A_{260}/A_{280} and 1.7 of A_{260}/A_{230}),
552 despite lower total DNA yields (1.9 μg g^{-1} soil, ODS basis).

553 Successful PCR amplification of the plant chloroplast *trn*L and rbcL genes allowed us to
554 identify five plant families, including native New Zealand forest and scrub genera/species (Table 5)
555 and an exotic species (*Eucalyptus*).

556

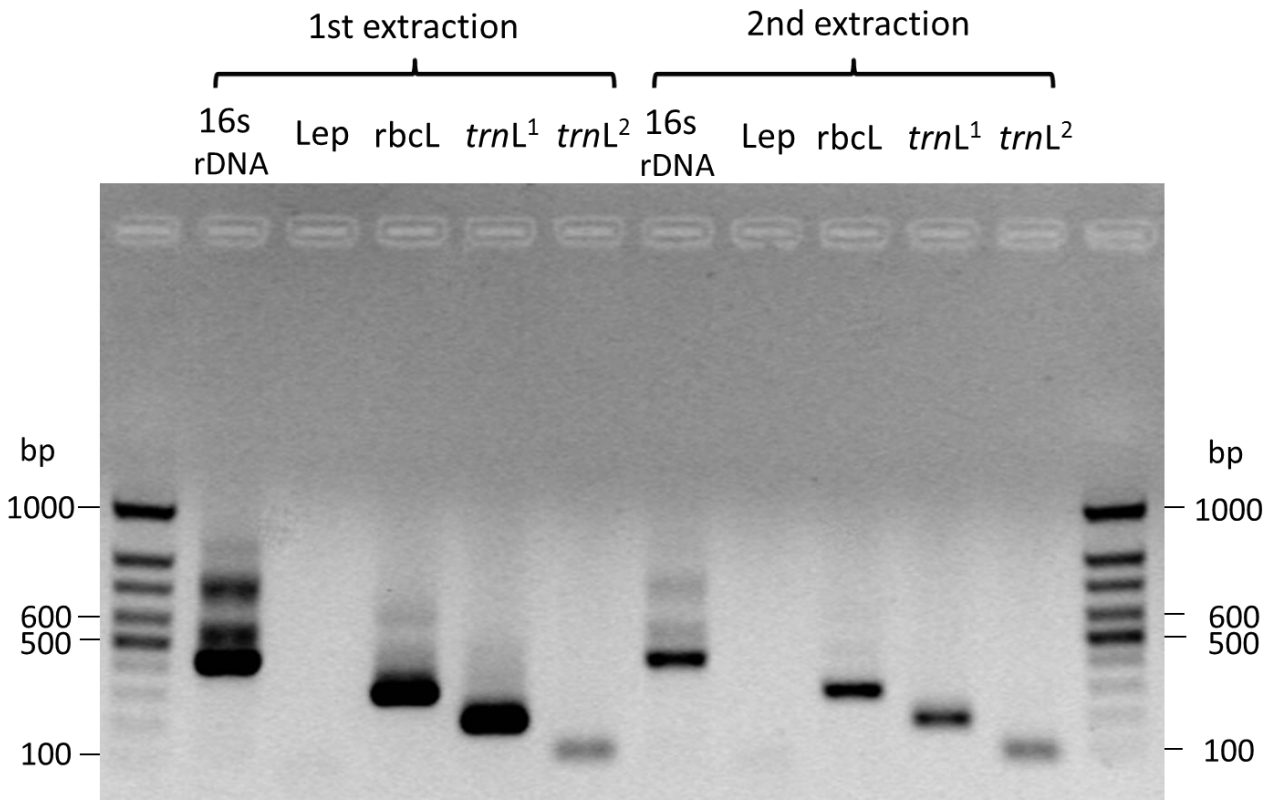
557 **Table 5.** New Zealand endemic plants detected in the buried soil horizon (5Ahb) on Rotoma
558 tephra subjected to the two-step DNA extraction protocol, gel purification method, and PCR with
559 selected primers.

Family	Plant taxa	Primer pair and % similarity to reference
Myrtaceae	*Leptospermum scoparium*[a]	*trn*L g−h (98−99%) and *rbcL* (99%)
Araliaceae	*Hydrocotyle*	rbcL (97%)
	Other genera (multiple)	*trn*L c−h (99%) and *trn*L g−h (100%)
Griseliniaceae	*Griselinia lucida*[a]	rbcL (98%)
Podocarpaceae	*Podocarpus*	*trn*L c−h (95−98%)

560 [a]NCBI accessions of *trn*L and rbcL genes of *Leptospermum scoparium* are KF591267 and
561 HM850121; NCBI accession of rbcL gene of *Griselinia lucida* is L11225.

562

563



564

565 **Fig. 6.** Image of gel electrophoresis of PCR products with 16S rDNA (bacterial), Lep (insect), rbcL (plant),
566 *trn*L c−h (plant)[1], and *trn*L g−h (plant)[2] primers. The DNA templates were extracted from the buried
567 allophanic soil horizon (5Ahb) on Rotoma tephra (Fig. 2) using the two-step extraction. Each extract was
568 purified using gel electrophoresis followed by GenScript QuickClean II Gel Extraction Kit and amplified
569 separately with different primers. PCR was undertaken using DNA extracted from multiple subsamples from
570 the same soil horizon on Rotoma tephra, and the PCR amplicons were very similar to the results shown here.
571

572

573 **5. Discussion**

574 *5.1 DNA extraction*

575 For proof of principle, salmon-sperm DNA extraction from its association with synthetic
576 allophane using various methods showed that only ammonium oxalate extraction was effective at
577 releasing salmon-sperm DNA, whereas Rai's lysis buffer − either with or without mechanical bead-
578 beating − showed no significant DNA yield. Previous studies have shown that the use of bead-
579 beating (in conjunction with chemical extraction) can increase DNA yield from some soils (e.g.

Ikeda et al., 2004; Li et al., 2011) but the efficacy depends on soil characteristics (Burgmann et al., 2001). In allophanic soils, the nano- and microaggregates are not able to be broken down easily (Matus et al., 2014; Huang et al., 2106), and this stability helps explain the ineffectiveness of the bead-beating. Additionally, the high concentrations of EDTA and phosphate in Rai's lysis buffer, respectively, prevent DNase from degrading the released microbial (and unbound) DNA and block this DNA from being adsorbed onto allophane, or humics, immediately following cell lysis. However, in our study, the use of a synthetic allophane–salmon-sperm-DNA complex highlights that EDTA and phosphate (i.e. Rai's lysis buffer) together with bead-beating are not sufficient for the isolation of extracellular DNA that has already become bound to allophane because of the highly adsorptive nature of allophane. Conversely, the increase in DNA yield from synthetic allophane using oxalate extraction to solubilize the allophane suggests that ammonium oxalate dissolution firstly releases DNA bound to the allophane by disrupting chemical bonds between DNA phosphate groups and Al-hydroxyl groups (Parfitt, 1980, 1990; Wada, 1989), and secondly it also releases DNA encased and physically protected within nanopores. This is an important finding because it demonstrates that the use of ammonium oxalate not only enables substantial environmental DNA to be extracted from allophanic soils but also offers a potential approach for differentiating between (1) ancient extracellular DNA both chemically bound to the matrices and physically adsorbed, and (2) modern or near-modern DNA.

The application of ammonium oxalate extraction on natural allophane soils was only successful following the initial extraction with Rai's lysis buffer. The DNA yield obtained upon one-step ammonium oxalate extraction was much lower than that derived in the two-step ammonium oxalate extraction, or negligible. This outcome indicates that Rai's lysis buffer is required prior to oxalate treatment to enable effective dissolution of natural allophane and hence the release of DNA from allophanic soils. The first extraction using Rai's alkaline lysis buffer appeared to buffer the pH of the soil solution so that the ammonium oxalate extraction (at pH 3) would not severely damage the extracted DNA because the extract obtained after the second step of DNA extraction remained at pH

606 6. In fact, the DNA yield obtained using Rai's buffer was significantly higher for the natural
607 allophane soils compared with that from the synthetic allophane where DNA yield with Rai's buffer
608 was negligible. This difference may be partially due to the increase in microbes and unbound DNA
609 present in natural allophanic soil materials compared with the synthetic allophane, which contained
610 chemically-bound DNA and DNA secured within nanopores. This result further suggests that Rai's
611 lysis buffer targets mainly soil bacterial/fungal DNA together with unbound DNA in soils, whereas
612 oxalate targets a second source of DNA, the chemically-bound DNA and the DNA adsorbed
613 physically within the networks of nanopores. Analyses to test this hypothesis are currently in
614 progress.

615 For reasons noted earlier in section 2.1, we used field-moist rather than laboratory-dried soil
616 in applying our extraction method to natural allophanic soils because the process of drying an
617 allophanic soil sample could, in itself, prevent the complete removal of adsorbed DNA from it.

618

619 *5.2 DNA purification*

620 Following ammonium oxalate treatment (either as a single treatment or as step 2 of the two-
621 step extraction), the abundance of co-extracted humics inhibits the PCR reaction and therefore the
622 removal of such PCR inhibitors is imperative. In comparing various DNA purification methods, we
623 found that gel electrophoresis followed by DNA recovery from the gel was the most efficient method
624 for successful PCR amplification across four gene regions (one bacterial region and three plant
625 regions), even though the final DNA yield was reduced. This result accords with the findings of
626 Harnpicharnchai et al. (2007) and Miao et al. (2014). After gel electrophoresis, we observed that the
627 brownish humics were separated from DNA by migrating faster than the extracted DNA to the
628 positively-charged electrode.

Despite the high DNA loss, gel electrophoresis followed by gel purification enabled the DNA extracted from the sand-rich buried andic soil horizon (5Ahb) on Rotoma tephra at the Brett Rd site to be successfully amplified, cloned, and sequenced via Sanger sequencing. From this, DNA of several New Zealand indigenous plants, and an exotic plant, were identified in the 5Ahb horizon (Table 5). However, the plants currently growing on the land surface at the Brett Rd site today are largely European grasses (composed mainly of *Poaceae, Fabaceae, Asteraceae, Ranunculaceae*, *Oxalidaceae*, and *Plantaginaceae*). Therefore, the plant DNA extracted from the buried soil horizon on Rotoma tephra may have originated from previous vegetation growing at the site.

Other DNA purification methods used in this study were less efficient at removing humics and, as a result, PCR amplification was largely unsuccessful. Chloroform, used in the purification of the extracts from the soil horizons on Taupo, Whakatane and Rotoma tephras, forces a separation of the organic and aqueous phases. DNA is soluble in the aqueous phase because the negatively-charged DNA backbone makes the molecules chemically polar. Conversely, proteins and the hydrophobic humics remain in the organic phase (chloroform) of two immiscible liquids. The chloroform treatment, however, was not able to eliminate hydrophilic humics in solution or humics bound to DNA, and hence PCR of the treated DNA remained inhibited and PCR amplification was thus unsuccessful. The humics-removal undertaken before the two-step DNA extraction increased the DNA yield from the soil sample on Rotoma tephra; however, PCR amplification of all five gene regions failed. This result suggests that the removal of co-extracted humics prior to DNA extraction may improve DNA recovery from allophanic soils, but further purification post-extraction is still required. The use of two commercial DNA purification kits brought about substantial loss of DNA, and the poor A_{260}/A_{230} readings following purification indicate the presence of impurities which resulted in unsuccessful PCR amplification in three of the five gene regions. The poor purification observed using these commercial kits could be due to simultaneous binding of both humics and DNA to the silica gel column and co-elution of both into the buffer (Miao et al., 2014).

654 *5.3 Potential applications of the DNA extraction method*

655 DNA sequencing results from the buried soil horizon (5Ahb) on Holocene-aged Rotoma
656 tephra showed that vegetation dating to a time before that of the present can be accessed using the
657 DNA extraction protocols developed in this study (Table 4, Fig. 3). Therefore, our method should be
658 useable to access paleoenvironmental DNA (PalEnDNA) that has been protected within such soils,
659 enabling the novel study of past terrestrial environments or paleoclimates using such DNA extracted
660 from successions of allophanic paleosols on tephra deposits of known age. Even though New
661 Zealand's late Quaternary vegetation and past climates have been generally well reconstructed from
662 pollen and plant macrofossils preserved in peats and lake sediments, and from various other climate-
663 environment proxies such as phytoliths and speleothems (e.g. Kondo et al., 1994; Newnham et al.,
664 1995, 1999, 2013; Williams et al., 2010; Barrell et al., 2013), (paleo)environmental DNA preserved
665 in subfossils and sediments is able to provide considerable insightful information about past
666 environmental change not always evident using the classical methods (Jørgensen et al., 2012;
667 Pedersen et al., 2013; Rawlence et al., 2014; Birks and Birks, 2015).

668 Our new extraction and purification method thus potentially adds an important new
669 PalEnDNA-based tool for studying climatic and environmental change through paleopedology using
670 andic soils and buried paleosols in extensive volcanic terrains. The effectiveness of such studies is
671 enhanced because tephra layers provide a powerful means – tephrochronology – both for dating the
672 soils and paleosols and for connecting them chronostratigraphically to other paleoenvironmental sites
673 (e.g. Sanborne et al., 2006; Lowe, 2011; Barrell et al., 2013).

674

675

676

677

678 **6. Conclusions**

679 (1) We have developed a new two-step DNA isolation method for allophanic soils (including
680 Andisols) and buried paleosols that successfully bypasses previous difficulties in extracting DNA
681 from such materials relating to the strong chemical bonding between DNA and allophane and
682 organic matter, and to the physical adsorption (protection) of DNA encapsulated in nanolabyrinthic
683 nanopores in allophane nano- and microaggregates. The method centres around (1) using an alkaline
684 phosphate buffer ("Rai's lysis buffer") to release mainly bound microbial DNA (and any unbound
685 DNA) by SDS, EDTA, and heat, and by blocking the released DNA from adsorptive sites on
686 allophanic materials using phosphate; and (2) the novel application of acidified ammonium oxalate
687 (Tamm's reagent) to dissolve the allophane and to release both chemically-bound DNA and DNA
688 within nanopores. DNA yields up to 44.5 μg g^{-1} soil (ODS basis) were obtained from three (field-
689 moist) natural allophanic soil samples (i.e. samples were not dried prior to extraction) in northern
690 New Zealand using this two-step method.

691 (2) Following the DNA extraction, we evaluated different DNA purification methods. Gel
692 electrophoresis followed by gel purification of the DNA, despite some DNA loss, was the only
693 purification method that sufficiently removed inhibitors (humics), allowing successful PCR of
694 multiple gene targets.

695 (3) Sequencing of PCR products obtained from a buried allophanic paleosol at 2.2-m depth
696 on a Holocene tephra (Rotoma tephra), even though weakly weathered and low in clay, in northern
697 New Zealand yielded 1.9 μg g^{-1} soil (ODS basis) of amplifiable DNA comprising endemic and exotic
698 plants that differed from the European grasses growing currently at the land surface.

699 (4) The DNA extraction and purification protocols we have developed offer a means to
700 extract DNA and successfully amplify multiple gene regions from allophane-bearing soils and
701 paleosols to study past environments or climates in volcanic terrains where such soils/paleosols are
702 widespread and potentially datable using tephrochronology and other geochronological methods.

703 Further, the method potentially provides a way of studying biodiversity in such soils/paleosols as
704 well as having possible application in forensic soil analysis.

705 (5) The use of acidified ammonium oxalate, in conjunction with Rai's lysis buffer, could be
706 the key for releasing adsorbed DNA chemically bound to allophane and physically encapsulated
707 within allophane nano/microaggregates, enabling detailed pictures of past environments and
708 biodiversity changes through time to be obtained. However, to further support the hypothesis that
709 microbial and extracellular DNA, and the DNA adsorbed (bound) on the allophane spherules and in
710 nanopores within aggregates, are isolated using Rai's alkaline lysis buffer and ammonium oxalate,
711 respectively, additional investigations using high-throughput sequencing (HTS) are required to
712 determine the DNA quality and taxonomic profiles recovered in the different DNA isolation steps.

713

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