論　文

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バイオマス炭化物による環境浄化の可能性[1]

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Possibility of environmental purifications by biomass charcoals

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Abstract

On the chemical analysis methods for pesticide residue in vegetables, some activated charcoals are often used as to adsorb pesticides. Those most are made from polymer materials, but a part is made from other things. For example ,sometimes activated charcoals are made from agricultural wastes, like nut shell, rice chaff, palm shell, bagasse, corn wastes etc. for environmental clean materials. In particular, the corncob charcoals (Dalian in China) can absorb a larger amount of metal ions from contaminated water compering with other wood charcoals. In this paper, the relationship between the carbonization conditions and the physicochemical properties and adsorbability of some environmental pollutants.

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コーンコブ炭化物による農作物へのカドミウムイオンの吸収抑制

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概要：中国産の廃棄コーンコブ（トウモロコシの実の芯）を原料とし，窒素気流中，電気炉で500～1000℃に加熱して炭化物を調製した。得られた炭化物をカドミウム水溶液に加え，バッチ方式により吸着実験を行った結果，コーンコブ炭化物は比較の木炭よりも高い吸着能を発揮し，炭化温度によって生じる細孔構造や表面電荷の違いが吸着能に影響を与えることがわかった。さらに，コマツ菜とキャベツの栽培実験において，土壌中にコーンコブ炭化物を混入したのち，カドミウム水溶液を滴下したところ，これら農作物の葉と根の両方の部位においてカドミウムイオンの含有量がコーンコブ炭化物を混入しない場合に比べて低くなることがわかり，コーンコブ炭化物には農作物への土壌カドミウムイオンの吸収抑制効果があることがわかった。

Absorption restraint of cadmium ion into crops by addition of corncob charcoal to soil

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Abstract: Charcoals were prepared by carbonization of waste corncob (core of corn berry)form China under a nitrogen gas flow at 500-1000℃ in an electric furnace. At the adsorption experiments of cadmium ion by the obtained corncob charcoals in aqueous solutions, the charcoals showed higher adsorbability compared to usual charcoals, depending on the difference in pore structure and surface charge caused by carbonization temperature. Moreover, at the cultivation experiments of Japanese mustard spinach and cabbage, the contents of cadmium ion in leaves and roots of these crops cultured in the soil with the charcoal were lower than that without the charcoal. This indicates that the corncob charcoal has the effect of absorption restraint of cadmium ion into the crops by its high adsorbability.

ユリの新発芽法「種子カット法」の開発

The “Seed-cutting method”: A novel aseptic method for inducing germination in *Lilium* species

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要　旨

本研究では岩手県に自生しているヤマユリを材料に，新しい発芽法である「種子カット法」の開発を行った．従来までの様々な発芽法と種子カット法の発芽試験を行った．主にユリ生産者が行っている「温度処理法」の発芽は75日を要したのに対し，「種子カット法」では6日で発芽し，発芽率は最も高かった．発芽時の生育形態は，地上発芽の形態を示し，本葉の出芽が確認された．世界に分布する野生ユリ18種に「種子カット法」を試した結果，供試全種について発芽および同じ生育形態が確認された．我々が開発した「種子カット法」によって，ユリ科植物の発芽期間を大幅に早めることが可能となった．

キーワード：ヤマユリ，種子カット法，温度処理法，地上発芽，本葉

Abstract

The present study describes a novel method for aseptically germinating *Lilium* species that grow wild in Iwate Prefecture, Japan. We compared seed germination characteristics in *Lilium auratum* using conventional horticulture germination techniques and a novel seed-cutting method. A total of 75 days was required to germinate the seeds using the conventional warm-and-cold storage technique that is most widely used by lily producers. However, using the seed-cutting method, germination only took six days and the rate of germination was higher than that observed with other methods. The growth form produced after germination by the seed-cutting method was typical of epigeal germination, and was characterized by the emergence of a true leaf. Germination and common growth forms were observed in all seeds from 18 global species of wild lily species

examined when the seed-cutting method was applied. Using this seed-cutting method will markedly decrease the period required to germinate members of the genus *Lilium*.
Key Words: epigeal, hot and cool method, *Lilium* auratum, seed-cutting method, true leaf

ヤマユリ（*Lilium auratum*[1]）図1は，地下に鱗茎状の球根（以下，球根）を有し，これをほぐして水洗後，天日乾燥したものを百合と称し，生薬として用いる．薬効は，清肺，養脾，止咳，清心安神の効果があるとして消炎，鎮咳，利尿，鎮静薬にする．日本に自生するユリでは，ヤマユリ，ササユリ，オニユリが用いられる[2,3,4,5]．

ユリの増殖には，リン片，分球，木子，珠芽を材料にした方法が行われており，組織培養では，リン片培養が一般的である．しかし，これらの増殖は親株の一部を使用するため，親株の持つ病気を全て受け継ぐというリスクを伴う．特にヤマユリはウイルス病などの病気に弱い種であることから，増殖や栽培が難しいとされている[2]．

ヤマユリの種子を利用した発芽法としては，ユリ生産者に普及している「温度処理法」と植物組織培養技術である「胚培養法」がある．前者は簡便な方法として取り入れられているが，実際には3段階の温度設定が必要であり,温度設定や種子の殺菌に失敗すると発芽しないという課題がある．後者は「温度処理法」と比較して発芽期間を短縮することができ，ユリ交雑種の未熟胚を発芽させる技術としても用いられているが，ヤマユリの場合は胚の摘出が難しいこと，また胚の殺菌処理の加減が難しいことなどの問題が発生することが確認されている[6]．小山田はこの対策としてスカシユリ（*L.maculatum*）を材料に「胚培養」を改良した「胚乳カット法」を考案しており，この手法によって生産したヤマユリの培養苗が紹介されている[7]．この手法は，殺菌した種子を材料にして胚周囲部位を残すようにメスまたはハサミを用いて胚乳部位を切り除き，胚周囲部位を培地に置床し発芽させるものである．この手法によって高い発芽率が維持できたが，胚乳を切り取る作業に時間を要すること，さらに発芽が安定しないこと等の改善の余地がみられる．

図1　開花したヤマユリ

そこで本研究では，新たな発芽法である「種子カット法」を開発することとした．これは種子の胚外側片面をメスで切り除き，胚を含んだ種子を培地において発芽させる簡便な方法である．これを従来までの発芽法と比較して有効性と実用性を検討した．また世界に分布する野生ユリ18種の種子を用いて発芽試験を行い，その効果を確認した．

1　材料および方法

試験開始前年までに岩手県内の山野においてヤマユリの自生地（遠野市，雫石町）を確認した．その翌年7月に同地においてヤマユリの有無と開花状況を確認し，10月末から11月に種子を含んださく果33個を回収して種子形成を調査した．この調査に使用した種子を発芽試験に用いた．

1) ヤマユリさく果の形態と種子形成の関係（試験1）

さく果を自生地から回収後，さく果の長さと径を

ノギスで計測し，電子天秤で総重量を計測した．その後，さく果から種子を全て取り出して種子数を調査し，種子に胚が含まれているものを有胚種子，胚が含まれていないものを無胚種子と位置付け，それぞれの数を調査した．

2) 種子カット法の開発（試験2）

試験1で得られた有胚種子（以下，種子）を材料に発芽試験を行った．まず種子はネットに入れて中性洗剤で洗い，水道水で洗浄した．そして蒸留水に24時間浸水した後，各試験に用いた．

発芽試験は，「実生発芽法[2)]」，「温度処理法[7)]」，「無菌播種法[8)]」，「胚培養法[6)]」，「胚乳カット法[7)]」，「種子カット法」の6つの方法による発芽試験区を設定し，それぞれ以下の条件で行った．

「実生発芽法」：まずバーミキュライトと人工培養土（メトロミックス350，ハイポネックス社）を同等混合した発芽用土（以下，用土）を作成し，これを発泡スチロール製トロ箱（縦44cm，横34cm，深さ18cm）に深さ15cmまで充填した．そしてその上に種子を200粒播き，種子の上に用土を1cm被覆した．播種後は，岩手県環境保健研究センター薬草園内（野外）にトロ箱を静置した（n=200）．

「温度処理法」：保存ビン（容量：100ml，SCHOTT社）にバーミキュライト80mlと種子50粒を混ぜ込んで充填して蓋をした．これを4ビン準備してインキュベータに移し，30℃で56日間，18℃で21日間，5℃で42日間になるよう設定した[7)]（n=200）．

無菌培養法の「無菌播種法」，「胚培養法」，「胚乳カット法」，「種子カット法」に使用する培地は，事前に3種の培地（MS[8)]，H[8)]，H改変培地；表1）を試験して選定した．1培養容器にリン片10個を移植し，各試験区に5容器準備した（n=50）．そして60日後の出芽率を目視により調査した．その結果，Hyponex改変培地（以下，培地）のみ70%以上の出芽率であったことから，この培地を選定し，以後の実験に使用した（表2）．その後の各実験の培養容器には，広口ガラス製の容器（容量：450ml，石塚硝子社）またはメリクロンフラスコ（容量：300ml，岩城硝子社）を用い，培地50mlを充填して使用した．1培養容器に20個ずつ播種し，各試験区に10容器ずつ準備した（n=200）．培養の環境条件は，照度2,000lx，16時間日長，設定温度20℃とした．

「無菌播種法」：種子から翼を除去した後（図2），これを0.3%次亜塩素酸ナトリウム溶液に15分間浸水させて，滅菌水で3回洗浄して培地に置床した（n=200）．

「胚培養法」：ヤマユリに限定した胚の摘出法を解説した方法はないため，胚のみをメスで切り出す摘出方法を実施した．この方法で摘出した胚を滅菌水で3回洗浄して培地に置床した（n=200）．

表1　発芽用培地の組成（Hyponex 改変培地）

成 分	添 加 量	MS5液の組成	添 加 量
Hyonex（6.5－6.0－19.0）	2.0 g/L	ミオイノシトール	100 mg/L
スクロース	30.0 g/L	ニコチン酸	0.5 mg/L
MS5液	2.0 mL/L	塩酸ピリドキシン	0.5 mg/L
ゲランガム	3.0 g/L	塩酸チアミン	0.1 mg/L
pH	5.7	グリシン	2.0 mg/L

表2　ヤマユリの発芽試験に用いる培地の選定

培 養 系	供試数	培地	出芽率の程度[z]
リン片培養	50	MS培地	＋＋
リン片培養	50	H培地	＋＋
リン片培養	50	H改変培地	＋＋＋

[z] ＋：30%以上，＋＋：50%以上，＋＋＋：70%以上

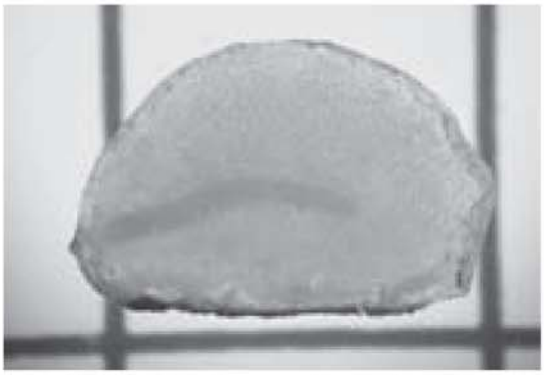

図２　翼を除去したヤマユリ種子　（マス目：５㎜）

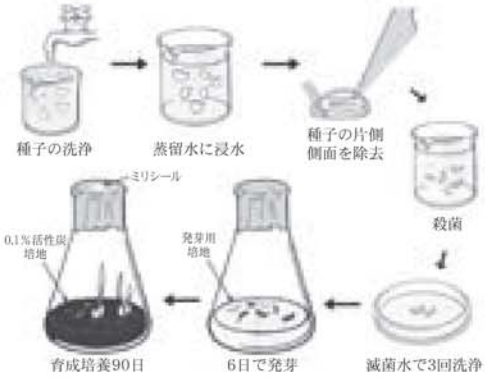

図３　種子カット法の工程

この処置とは別に，コンタミネーション（雑菌による培地の汚染）の発生を考慮して，摘出した胚を0.3%次亜塩素酸ナトリウム溶液に10秒間浸水させて滅菌水で3回洗浄して培地に置床する方法も同時に行った（n=200）．

「胚乳カット法」：種子から翼を除去した後，クリーンベンチ内において胚の周囲をメスで除去した．これを0.3%次亜塩素酸ナトリウム溶液に15分間浸水させ，滅菌水で3回洗浄して培地に置床した（n=200）．

「種子カット法」：種子から翼を除去した後，クリーンベンチ内において胚を残して種子の胚片側側面をメスで除去した．これを0.3%次亜塩素酸ナトリウム溶液に15分間浸水させ，滅菌水で3回洗浄して培地に置床した(n=200)．図3に「種子カット法」の工程を図示した．

以上の6つの方法による発芽試験区について，発芽の有無，発芽はじめ日数と発芽揃い日数，発芽数，発芽形態，コンタミネーションの有無を肉眼観察による分類法で調査した．

3) 育成培養の検討（試験3）

「種子カット法」で発芽させたヤマユリ幼植物体の生育促進を進める方法について検討するため，試験2で使用した培地への活性炭0.1％添加の有無と，培養容器への通気を行うミリシール設置の有無の各試験区を設定した．活性炭は培養植物の生長促進作用の効果があり[6,8]，またミリシールについても同様の効果が期待できる[9]．

「種子カット法」で発芽試験を開始して30日経過した幼植物体を1培養容器に10個体移植し，各処理区に2容器を準備した（n=20）．90日間培養した後に培養容器から取り出して葉数，根数を肉眼観察によって分類する方法で調査した．草丈と最大根長をノギスで，苗全体の生重量と球根重量を電子天秤で測定した．

4) 種子カット法由来培養苗の野外栽培試験（試験4）

2011年12月19日に「種子カット法」を実施し発芽させた幼植物体を0.1％活性炭添加培地に移し，ミリシールを設置して90日間培養したものを試験4に供試した．発泡スチロール製トロ箱に深さ15cmまで用土を充填して，その3cm深に球根を植えつけて岩手県環境保健研究センター薬草園内の栽培試験地にて野外栽培した．「種子カット法」を実施から17ヵ月後の2013年5月22日に「種子カット法」の苗から14個体のみ選抜して掘り出した．掘り出すことによる侵襲によって枯死する可能性もあることから，用いるサンプル数は14個体のみとした．2011年12月16日に「実生発芽法」を実施し，発芽させた実生苗をランダムに20個体を選抜して掘り出した．発芽法の違いによる苗の成長を比較するため，いずれも発芽試験開始から17ヵ月経過した2013年5月22日に，「種子カット法」と「実生発芽法」の苗の葉数，根数を肉眼観察によ

る分類する方法で調査して，草丈と最大根長を定規で，球根径と球根長をノギスで測定した．

5)「種子カット法」による 野生ユリ18種の発芽試験（試験5）

世界に分布する野生ユリ18種の種子を入手し，「種子カット法」を用いて発芽試験を行った．種子は東京山草会を中心に行っている種子交換会から2012年に入手できた日本産のユリ7種と，外国産のユリ5種，そして2013年に入手できた日本産のユリ1種と，外国産のユリ5種を供試した．発芽の有無を肉眼観察で確認し，発芽率を50％未満，50%以上，80%以上の3区分に分けて評価した．発芽した苗は，培養30日後に（試験3）で確認した育成培養に移行させて苗の生育を進めた．

6) 統計処理

すべての統計解析はRver.2.14.0（R Developmemt Core Team,2012）を使用した．

2　結果および考察

1) ヤマユリさく果の形態と種子形成の関係（試験1）

さく果の形態調査を行った結果，長さは59.0±2.1mm，径は26.8±0.7mm，重量は6.5±0.7gであった（平均±標準誤差）．種子数は1さく果あたり490.0±25.1個となり，有胚種子の形成率は69.0%であった．さく果の形態と種子数およびさく果の形態と有胚種子の形成率の関係を調査した結果，長さ，径および重量と種子数に正の相関が認められた．また，長さ，径および重量と有胚種子の形成率においても正の相関が認められた（図4，5）．このことから，より長く，より太いさく果を選択することによって苗生産を行う種子を計画的に確保できるようになると考えられる．

2) 種子カット法の開発（試験2）

表3に本研究で実施した発芽試験の結果を示した．発芽率は，「種子カット法」が他のどの方法よりも有意に高かった．「胚培養法」は，摘出した胚

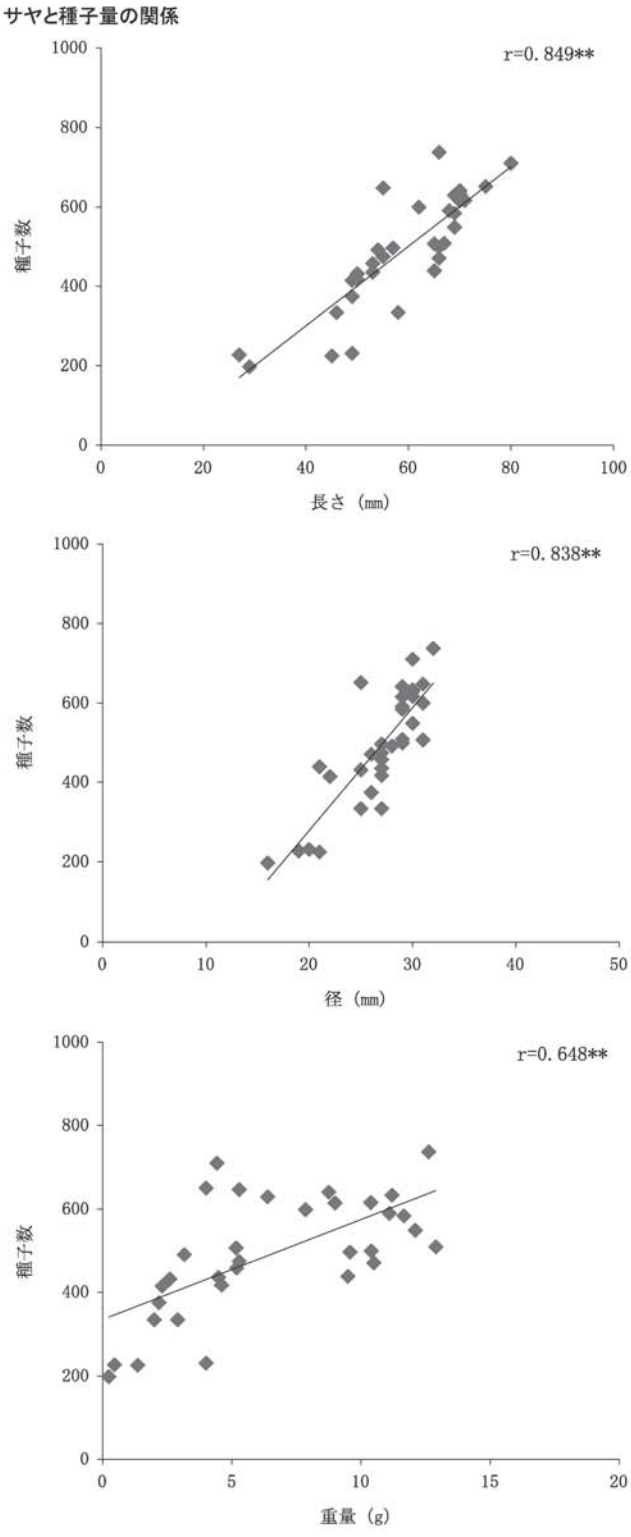

図4　さく果の長さ，径，重量と種子量の関係
** はピアソンの相関係数検定によって1％水準で有意差があることを示す．

を殺菌剤に浸けない試験では全てに培地汚染が発生し，殺菌剤に浸けた試験では，発芽が見られなかったことから，ヤマユリの苗生産には適さないと推察された．発芽はじめ日数は，「胚乳カット法」と「種子カット法」が他の方法より早く発芽が確認された．発芽揃い日数は，「種子カット法」の発芽揃い日数が最も早かった．「温度処理法」は容器の中で地下発芽するため，正確な日数を目視確認できなかった．

発芽時の生育形態を調査した結果，「温度処理法」と「無菌播種法」が地下発芽，「実生発芽法」と「胚乳カット法」，「種子カット法」が地上発芽を示した（図6）．「温度処理法」と「無菌播種法」の地下発芽はいずれも子葉が出芽した．「実生発芽法」，「胚乳カット法」，「種子カット法」の地上発芽はいずれも本葉が出芽した．ただし，「実生発芽法」は地上発芽が観察される前に地下発芽が起きているため，「胚乳カット法」と「種子カット法」の発芽とは異なる[2,7]．手法の違いによって発芽形態に違いが出た原因は，「温度処理法」と「無菌播種法」は胚が種子内部に一定期間とどまってから発芽する手法に対し，「胚乳カット法」と「種子カット法」は短期間のうちに種子のカット部から胚が培地に露出して培地上に発芽するためであると考えられる．ヤマユリはもともと地下発芽する植物でありその発芽期間は遅発芽の発芽習性であることが知られており[7]，これは胚乳内にある有機酸が発芽抑制作用をするためだといわれている[2]．「胚

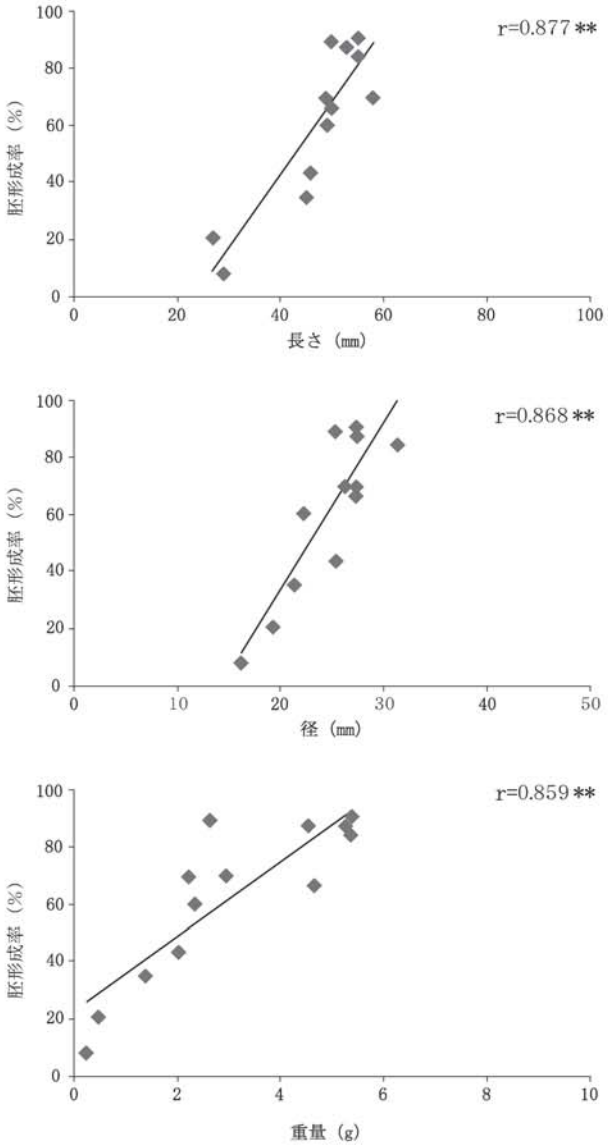

図5　さく果の長さ，径，重量と胚形成率の関係
**はピアソンの相関係数検定によって1%水準で有意差があることを示す．また統計処理はアークサイン変換後の数値を用いて行った．

表3　6つの方法による発芽試験結果

発芽方法	供試数	発芽率（%）	発芽はじめ日数	発芽揃い日数	発芽形態
実生発芽法	200	70.5 b[z]	414	442	地上発芽
温度処理法	200	63.5 b	75	−	地下発芽
無菌播種法	200	34.5 c	13	16	地下発芽
胚培養法（殺菌無し）	200	− −[y]	−	−	−
胚培養法（殺菌有り）	200	0.0 d	−	−	−
胚乳カット法	200	60.5 b	5	16	地上発芽
種子カット法	200	82.0 a	6	14	地上発芽

[z] 異なるアルファベット間はHolmの多重比較によって5%水準で有意差ありを示す．2群間の比較はFisherの正確確率検定を用いた．
[y] 胚培養法（殺菌無し）区における発芽率は，すべてのサンプルに培地汚染が発生したため計測不可であった．

乳カット法」および「種子カット法」では種子をカットすることで有機酸による発芽抑制が無くなり，さらに温度と光の環境条件と発芽に用いた培地が好適に作用して発芽を大幅に早めたものと推察される．

今回の試験に用いる培地は，リン片培養の結果を参考にHyponex改変培地とした．植物組織培養で多く使用されるMS培地と比較すると構成成分が少なく培地調整がしやすいこと，供試したユリすべてに利用できたことから，ユリの専用培地としての活用が期待できる．

ユリ類の胚培養を解説した専門書[6,7,10]を参考に「胚培養」を行ったが，摘出した胚を殺菌しない場合は培地汚染が発生し，胚を殺菌した場合においても発芽が全く得られなかった．また，テッポウユリ（*L.longiflorum*）とスカシユリ（*L.maculatum*）の種間雑種の作出に用いられた「子房輪切り培養法[11]」も試験的に行ったが，すべてに培地汚染が発生した．一方，「種子カット法」は「胚培養」や「子房輪切り培養法」で発生した培地汚染はなく，容易に発芽個体を得ることができた．またこの手法では，種子の胚を残すように胚片側側面をメスまたはハサミで除去するという非常に簡便な方法である．過去に小山田が開発していた「胚乳カット法[7]」よりも作業時間が短くなり，発芽期間も短縮でき，高い発芽率を安定して得られることが可能になった．

以上，発芽率，発芽に要する期間，生育形態および出芽状況から判断して，我々の開発した「種子カット法」が最もヤマユリの苗生産に効果的な手法であると考えられる．

3) 育成培養の検討（試験3）

「種子カット法」で発芽させた幼植物体の育成培養の結果を表4に示した．活性炭の有無，およびミリシールの有無を要因とした二元配置の分散分析を実施した結果，草丈および葉数は活性炭の有無間およびミリシールの有無間において有意な差が認められなかった．根数，最大根長，生重量および球根重量は，活性炭の有無間で1および5%水準で有意な差が認められ，いずれも活性炭有りの

図6　温度処理法（左：地下発芽形態）と種子カット法（右：地上発芽形態）の比較

表4　育成培養の試験結果

活性炭	ミリシール	供試数	草丈 (mm)	葉数 (枚)	根数 (本)	最大根長 (mm)	生重量 (g)	球根重量 (g)
なし	なし	20	25.1±3.1	2.6±0.4	5.6±0.9	21.8±1.7	0.31±0.07	0.21±0.05
	あり	20	23.8±2.6	2.1±0.3	4.0±0.8	11.1±1.5	0.24±0.04	0.12±0.02
あり	なし	20	28.0±4.1	2.8±0.5	8.8±0.9	46.9±7.4	0.47±0.06	0.25±0.03
	あり	20	29.8±2.9	3.3±0.5	12.8±1.6	50.4±3.7	0.65±0.15	0.30±0.06
分散分析[z]	活性炭		NS	NS	＊＊	＊＊	＊＊	＊
	ミリシール		NS	NS	NS	NS	NS	NS
	交互作用		NS	NS	＊	NS	NS	NS

[z] 活性炭の有無およびミリシール有無を要因とした二元配置の分散分析によって，＊＊は1%，＊は5%水準で有意差あり，NSは有意差なしを示す．

表5　種子カット法の苗と実生発芽法の苗の生育比較

	供試数	草丈 (mm)	葉数 (枚)	根数 (本)	最大根長 (mm)	球根長 (mm)	球根径 (mm)
種子カット法の苗	14	200.1±11.4	8.2±0.7	5.9±0.4	201.3±55.3	18.4±1.0	11.7±1.0
実生発芽法の苗	20	31.6±1.1	1.0±0.0	2.9±0.2	40.7±2.5	5.4±0.2	3.5±0.1
有意性[z]	−	＊＊	＊＊	＊＊	＊	＊＊	＊＊

[z] Welchのt検定によって，＊＊は1%水準，＊は5%水準で有意差ありを示す．

処理区で高い数値を示した．ミリシールの有無間ではいずれも有意な差は認められなかった．また根数のみ活性炭の有無間とミリシールの有無間で交互作用が認められた．活性炭有りかつミリシール有りの試験区において草丈，葉数，根数，最大根長，生重量，球根重量の全項目において他の試験区より高い数値を示した．

0.1％活性炭は培養中の植物から排出されたフェノール化合物や有害物質を吸着することが知られており[6,8]，ヤマユリの育成培養においては特に根の伸長において効果が認められたためだと考えられる．一方ミリシールの設置は統計上の有意差は認められなかったが他の試験区より高い数値を示したことから使用した方が良いと思われる．

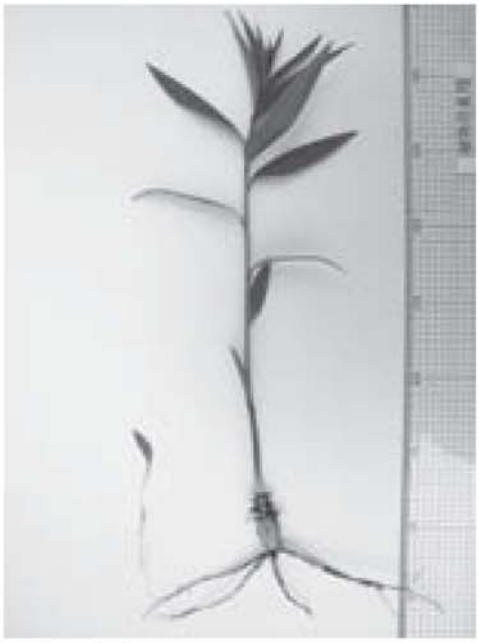

図７　発芽試験開始から 17 ヵ月経過した実生発芽法の苗（左）と種子カット法の苗（右）（2013.5.22）

4) 種子カット法由来培養苗の野外栽培試験（試験4）

「種子カット法」で作出した苗と「実生発芽法」で作出した苗の野外栽培試験結果を表5に示した．

調査全項目で「種子カット法」の苗が有意に高かった．また「種子カット法」の苗は，開花株の生育に必要な生育形態を示す抽だい[12]が観察された（図7）．

「種子カット法」と「実生発芽法」の苗の生育に大きな差が生じたことは，「種子カット法」は短期間で発芽が得られることで苗の育成期間が多く得られることと，培養（試験3）による生育の促進が図られたことによってこのような生育差が生じたものと考えられる．これらの結果から，「種子カット法」の顕著な発育性が証明された．

5)「種子カット法」による野生ユリ18種の発芽試験（試験5）

世界に分布する野生ユリ18種の種子に「種子

表６　種子カット法を用いたユリ属 18 種の発芽試験結果

種名・産地[z]	学名	供試数	試験実施年	発芽量[y]	2013年5月の試験状況
ヤマユリ（日本）	*L.auratum*	36	2012	+++	野外栽培
エゾスカシユリ（日本）	*L.dauricum*	21	2012	+	野外栽培
カノコユリ（日本・中国）	*L.speciosum*	20	2012	++	野外栽培
キカノコユリ（中国）	*L.henryi*	21	2012	++	野外栽培
コオニユリ（日本）	*L.leichtlinii var.maximowiczii*	81	2012	+++	野外栽培
テッポウユリ（奄美諸島）	*L.longiflorum*	25	2012	+	野外栽培
ヒメサユリ（日本）	*L.rubellum*	50	2012	+++	野外栽培
ヒメユリ（日本・中国）	*L.concolor*	32	2012	+	野外栽培
マクリニアエ（ミャンマー）	*L.mackliniae*	13	2012	+	野外栽培
マルタゴン（北ヨーロッパ）	*L.martagon*	7	2012	++	野外栽培
ランコンゲンセ（中国）	*L.lankongense*	39	2012	+	野外栽培
リーガル（中国）	*L.regale*	27	2012	+	野外栽培
キャンディダム（ギリシャ）	*L.candidum*	9	2013	+	培養容器内育成
コロンビアヌム（北アメリカ）	*L.columbianum*	14	2013	+	培養容器内育成
スゲユリ（日本）	*L.callosum*	64	2013	+++	培養容器内育成
ダビディ ウィルモッティアエ（中国）	*L.davidii var.willmottiae*	14	2013	++	培養容器内育成
ツシングタウエンセ（中国）	*L.tsingtauense*	20	2013	+++	培養容器内育成
ロストルニー（中国）	*L.rosthornii*	27	2013	+	培養容器内育成

[z] 括弧内地名は主な産地を示す．
[y] +：発芽50%未満，++：発芽50%以上，+++：発芽80%以上

表７　種子カット法で生産した国内ユリ属８種のレッドデータブック指定状況

種名	学名	環境省RL[z]	都道府県RDB[y]
ヤマユリ	*L.auratum*	指定なし	滋賀（絶滅種），京都（絶滅種），石川（絶滅危惧Ⅰ類）， 山梨（準絶滅危惧），長野（準絶滅危惧），三重（絶滅危惧ⅠＢ類）， 大阪（情報不足），奈良（希少種）
エゾスカシユリ	*L.dauricum*	指定なし	指定なし
カノコユリ	*L.speciosum*	絶滅危惧Ⅱ類	徳島（絶滅危惧Ⅰ類），福岡（情報不足），長崎（絶滅危惧ⅠＢ類）， 鹿児島（準絶滅危惧）
コオニユリ	*L.leichtlinii var.maximowiczii*	指定なし	宮城（絶滅危惧Ⅱ類），埼玉（絶滅危惧ⅠＢ類），千葉（要保護生物）， 東京（絶滅の危機に瀕している種），石川（準絶滅危惧）， 鹿児島（分布重要）
テッポウユリ	*L.longiflorum*	指定なし	鹿児島（分布重要）
ヒメサユリ	*L.rubellum*	準絶滅危惧	宮城（絶滅危惧Ⅰ類），山形（絶滅危惧ⅠＢ類），福島（準絶滅危惧）， 新潟（絶滅危惧Ⅱ類）
ヒメユリ	*L.concolor*	絶滅危惧ⅠB類	愛知（絶滅），青森（最重要希少野生生物），京都（絶滅寸前種）， 大阪（絶滅危惧Ⅰ類），兵庫（要調査種），奈良（絶滅寸前種）， 和歌山（絶滅危惧ⅠＡ類），岡山（絶滅危惧Ⅰ類）， 広島（絶滅危惧Ⅰ類），山口（絶滅危惧ⅠＡ類），徳島（絶滅危惧Ⅰ類）， 香川（絶滅危惧Ⅰ類），愛媛（絶滅危惧ⅠＢ類），高知（絶滅危惧ⅠＡ類）， 熊本（絶滅危惧ⅠＡ類），大分（絶滅危惧ⅠＡ類），宮崎（絶滅危惧ⅠＡ類）
スゲユリ	*L.callosum*	絶滅危惧ⅠB類	福岡（絶滅危惧ⅠＢ類），佐賀（絶滅危惧Ⅰ類），熊本（準絶滅危惧）， 大分（絶滅危惧ⅠＡ類），宮崎（絶滅危惧Ⅱ類），鹿児島（絶滅危惧Ⅰ類）

[z] 第4次レッドリストより引用.
[y] 植物レッドデータブック COMPLETE（http://www. kurosan. sakura. ne. jp/indexRDB. htm）より引用.

カット法」を用いた. 発芽試験の結果を表6に示した. 供試した18種すべてに発芽を確認した. この時の発芽形態は, 全て地上発芽となり, 本葉を出芽した. 一部の種では50％未満の発芽率であったが, 多くの種で高い発芽率を確認できた. 発芽率に差が生じたのは, 種による発芽能力の差が原因と推察した. また, 日本産のエゾスカシユリ（*L.dauricum*）, テッポウユリ（*L.longiflorum*）, ヒメユリ（*L.concolor*）の3種と外国産のユリ全種については, 有胚種子の数が十分でなかったことも発芽率が低くなった原因と推察された.

6) まとめ

我々が開発した「種子カット法」は, まずヤマユリの種子の胚片側側面を除去し, 殺菌した後にH改変培地で培養することによって6日で発芽することが示された. 発芽した苗を30日間培養した後, 培養容器にミリシールを設置し, 0.1％添加活性炭培地で90日間培養することで苗が高い確率で完成する. その後, 培養容器から苗を取り出して野外栽培に移行する. ユリ生産者や研究者が行っている「実生発芽法」や「温度処理法」では, 発芽から開花までの生育期間に5年前後を必要としていたが[7,13,14,15,16], 「種子カット法」を導入することによって大幅に短縮することが期待できる. 従来, 組織培養技術を用いたユリの苗生産では, 花器やリン片を外植体にするクローン増殖が主流であった[17,18]. しかし外植体がウイルス病に感染している場合, 増殖した苗もウイルス感染する危険があった. 本研究によって開発した「種子カット法」は, ウイルス感染の心配がない種子[14,19]を材料にしているため, 健全な苗を短期間で供給することができることも大きなメリットであると考えられる. 供試した野生ユリ18種についても「種子カット法」による発芽を確認した. この中には, 絶滅危惧種も含まれる（表7）. 絶滅危惧種の保護を目的にして苗の生産を行う場合は, 種子を材料に受精胚が持つ種の多様性を維持することが重要であるとも考えられており[8], 希少なユリを保護する場合にも同様にあてはめることができるだろう. 我々が開発した「種子カット法」によって, 野生ユリ属の種の保存だけでなく, 薬用・食用として利用されている有用種の生産, または園芸種の生産や新品種開発を進める場合においても実用的な新技術として広く活用されていくだろう.

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地理情報システム（GIS）データベースの維持管理

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Original paper

A pilot study of the hair-trapping method in Asiatic black bears (*Ursus thibetanus*): determination of optimal survey period for estimating population size

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Abstract. In order to establish methodology for population estimation of Asiatic black bears using the hair-trapping method, hair-trapping devices were located within a study area, and basic data were collected to determine the optimal survey period for hair sampling. Bear hairs collected were classified as three types (guard hair; G, underfur; U, and intermediate hair; I) depending on hair-bulb thickness. After DNA extraction, six microsatellite loci and amelogenin locus were amplified in order to identify individual bears and sex, respectively. The number of hairs collected by hair-trapping devices decreased dramatically in late August, while those that had lost part of the hair bulb increased after September. The success rate of genetic analysis was over 90% prior to the month of July and rapidly decreased in August. Based on the results of the generalized linear model, it was determined that sampling session had the most significant impact on the success rate of genetic analysis of all the explanatory variables examined. Higher rates of U-type hairs within samples used in DNA extraction resulted in lower success rates of genetic analysis. It was concluded that the optimal survey period for estimating population size using the hair-trapping method was between June and early August.

Key words: Asiatic black bear, genetic analysis, hair-trapping, population estimate.

Recent advances in genetic analysis have made it possible to identify individuals using DNA obtained from non-invasive samples from free-ranging animals. Hair-trapping is one of such methods, and has enabled mark-recapture population estimates using genetic tagging to be widely applied in the study of Ursidae species, such as the brown bear *Ursus arctos* (Woods et al. 1999; Mowat and Strobeck 2000; Ciucci and Boitani 2008; Gervasi et al. 2008; Kendall et al. 2008), the American black bear *Ursus americanus* (Woods et al. 1999), and the Andean bear *Tremarctos omatus* (Viteri and Waits 2009). In Japan, experimental trials employing hair-trapping have been conducted in various regions (Morimitsu 2008; Yoneda and Mano 2011); however, certain problems have prevented application of this method in actual population management projects. One problem associated with hair-trapping stems from the fact that trap visiting rates for individual bears and success rates of genetic analyses have varied greatly depending on sampling periods as discussed in the previous study (Yamauchi and Saito 2008). When carrying out population estimates using mark-recapture methods, such as the Lincoln-Petersen method (Chao and Huggins 2005), sample collection rates and individual capture probabilities should remain constant during each sampling session; however, this consistency cannot always be assured in practice. Previous studies have not determined the constancy of the aforementioned variables during field studies, nor have

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they determined any possible seasonal changes in number or thickness of hairs collected using hair-trapping devices. Therefore, the present study employed hair-trapping devices in the sampling of bear hairs between the months of June and December in order to determine any possible changes in hair sample related variables over a prolonged period of time. Based on the results of subsequent genetic analyses, the present study aimed to elucidate any possible relationships between the sampling period and the success rate of genetic analyses and to determine the optimal survey period for estimating population size using the hair-trapping method.

Materials and methods

Study area

This study was conducted within the Omyojin experimental forest (39°40'N, 140°55'E, 1,024 ha), Iwate University, Iwate Prefecture, Japan (Fig. 1), an area situated to the north of the Ou Mountains, which spanned Akita Prefecture. The climate within the study area was characteristic of areas along the coast of the Sea of Japan and experienced heavy snowfall (Iwate Prefecture 2001). In the study area, deciduous broad-leaved trees (Japanese beech *Fagus crenata* and Mongolian oak *Quercus mongolica* var. *groaaeserrata*) and coniferous plantations (Japanese cedar *Cryptomeria japonica*, Japanese red pine *Pinus densiflora* and Japanese larch *Larix kaempferi*) dominated.

Hair-trapping methodology and sample collection

Twenty-one hair-trap devices were set up within the study area from 22nd to 29th of May, 2009 (Fig. 1). Design of hair-trap devices had been described by Yamauchi and Saito (2008). Scent baits were situated approximately 2 m above ground attached to a central tree at each hair-trap site. Scent baits were comprised of two apples wrapped in a wire net (0.7 mm in diameter) and honey (approximately 100 ml) in a plastic bottle. The structure of baits allowed bears to easily access the food items they contained. Hair-traps consisted of double stranded barbed wires that had been wound around several trees between 2 and 3 m apart from a central tree at a height of approximately 50 cm above ground. In order to ensure successful hair collection, barbed wires were also wound around trees at a height that ranged from 20 to 30 cm above first barbed wire. A length on each side of trap ranged from 2 to 4 m and varied depending on the distance between the trees to which it was attached. One or

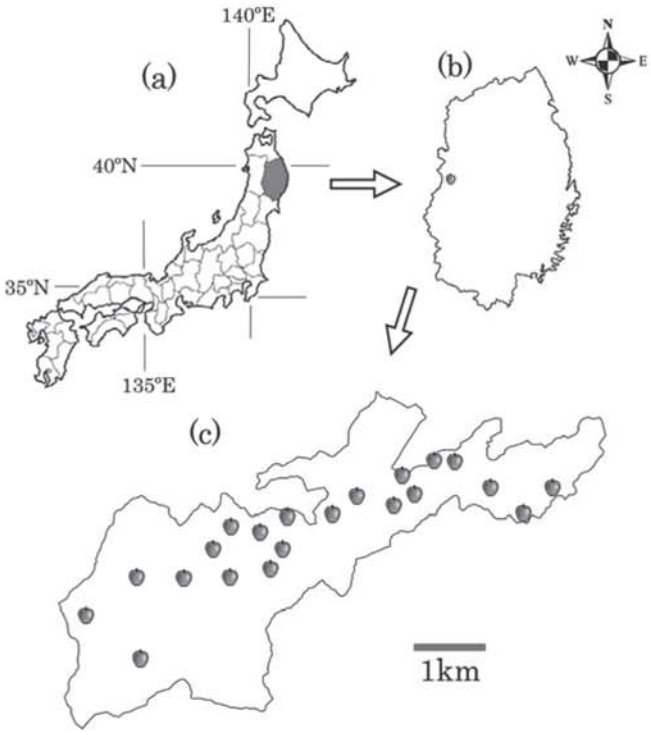

Fig. 1. (a) Location of Iwate Prefecture, Japan, (b) the study area within Iwate Prefecture (apple mark), and (c) hair-trap sites within the study area (apple marks).

two plastic poles were used to attach barbed wire in the absence of suitable trees.

Traps were visited repeatedly at 3–4 week intervals and hair sampling was conducted over 8 sampling sessions. Bait was replaced during each sampling session between mid-June and early December 2009 (Table 1). When apple bait and honey bottle were crushed, it was concluded that a bear had visited a given trap. Hair cluster samples were collected from each pointed section of barbed wires and placed in separate sampling bags, which consisted of small paper envelopes. Hair samples collected from adjacent to pointed sections put a particular mark in order to distinguish them from other samples. This mark was utilized for the subsequent processing of hair sample selections. Collected samples were stored at room temperature during transportation to the laboratory, where they were immediately desiccated in an oven at 30°C for 24 hours or more. Samples were subsequently stored at –20°C prior to analysis.

Classification based on hair types

Prior to DNA extraction, bear hairs were classified into three types through visual observation; guard hair (G-type) was thicker, longer and darker in color, under

Table 1. Sampling session schedule in 2009

Session	Sampling date
1	17–18 June
2	7–8 July
3	29–30 July
4	21–24 August
5	16 September
6	13 October
7	5–6 November
8	1–2 December

Twenty-one hair-trap devices were set up from 22 to 29 May, 2009.

hair (U-type) was thinner, shorter and downy, while all other types of hair were classified as intermediate hair (I-type) (Fig. 2). In order to obtain objective data during classification of hair types, we classified random samples of bear hair in accordance with the three types described (n = 50), and the thickness of the upper part of hair bulbs were measured using a Digital Camera Eyepiece in conjunction with a microscope (HDCE-10C) as well as ScopeImage 9.0 analytical software (AS ONE Corp., Osaka, Japan).

To investigate seasonal changes in hair types collected by hair-trapping devices, total number of hairs of each hair type was counted during each sampling session. Damaged hairs without any hair bulb (bulb-less hair) were also counted.

DNA analysis

Only the part around hair bulbs obtained from each sampling bag was cut off under a stereomicroscope and subsequently used in DNA extraction (Fig. 2). Bulb-less hairs were not used. Thirty hair bulbs per one paper envelope were used for each DNA sample. If a single sample bag provided fewer than 30 hair bulbs, hairs from marked sample bags taken from adjacent sections of barbed wire were used to reach a total of 30 hair bulbs. Larger hair bulbs were preferentially selected for use in DNA extraction, with G-type hair bulbs preferred over I- and U-type samples, and I-type samples preferred over U-type samples. DNA extraction was achieved using a DNA extractor FM kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) used in accordance with manufacturer's instructions.

Six microsatellite loci (G10C, G10L, G10B, G10X, G10P, and G10M) were used as primers for polymerase chain reaction (PCR) amplification of microsatellite loci in order to identify individual bears (Paetkau and Strobeck

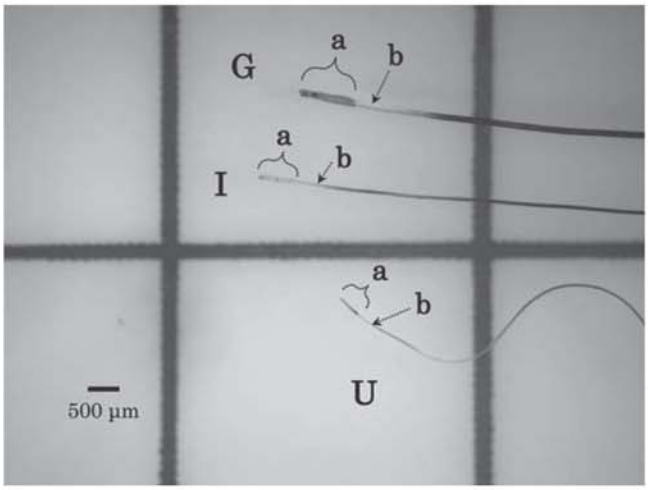

Fig. 2. Hair types as classified through visual observation conducted during the present study. G, I and U denote guard hair, intermediate hair and underhair, respectively. a: Section around hair bulb used for DNA extraction. b: Portion of hair measured to determine hair thickness.

1994; Paetkau et al. 1995). Genetic diversity of Asiatic black bears in Iwate Prefecture had been determined previously by a study that had examined captured individuals (Saito et al. 2008). It had been determined that, for the 6 loci examined in the present study, probabilities of identity (*P*id) and *P*id among siblings (*P*id-sib) were 9.88×10^{-6} and 9.14×10^{-3}, respectively, and the average heterozygosity (HE) was 0.66. The number of alleles and fragment size in each primer are as follows: G10C (6; 104–122 bp), G10L (3; 132–146 bp), G10B (5; 147–161 bp), G10P (12; 163–187 bp), G10X (6; 183–203 bp) and G10M (7; 196–208 bp). Multiplex PCR condition such as the number of PCR cycles and optimal annealing time in case of using bear DNA from very small sources were the same as Saito et al. (2008). Therefore, PCR conditions of the present study were conducted by the similar method. Microsatellite DNA amplification was achieved through multiplex PCR of primer sets A (G10C, G10L and G10B) and B (G10X, G10P and G10M) (Saito et al. 2008). The PCR reaction mixture in a total of 15 µL contained 10 × concentration *TaKaRa Ex Taq* buffer (20 mM Tris-HCl, 100 mM potassium chloride (KCl; TaKaRa Bio, Inc., Otsu, Shiga, Japan), 1.5 mM magnesium chloride ($MgCl_2$), 0.2 mg/mL bovine serum albumin (BSA; TaKaRa Bio, Inc.), 0.2 mM delxyribose-5-phosphate (dNTP), 0.1–0.4 µM of each primer, ~5.0 µL template DNA, and 1.25 U *Ex Taq* polymerase (TaKaRa Bio, Inc.). Sex determination of samples was accomplished using primers SE47 and SE48 (Ennis and Gallagher 1994). All forward primers were labeled using fluorescent dye groups FAM

or VIC (Applied Biosystems, Inc., Foster City, California, USA). PCR used in order to achieve microsatellite DNA amplification was performed using multiplex PCR of sets A and B (i-Cycler; Bio-Rad Laboratories, Inc., Shinagawa, Tokyo, Japan) under the following conditions: an initial 3 min at 97°C; 15 cycles, with denaturation for 30 sec at 97°C, annealing for 90 sec at 55°C, and extension for 30 sec at 72°C; 30 cycles with denaturation for 30 sec at 90°C, annealing for 90 sec at 55°C, and extension for 30 sec at 72°C, followed by a final extension for 30 min at 60°C. PCR conditions used to achieve sex determination were the same as those used during the amplification of microsatellite DNA; however, an annealing temperature of 63°C was used. Three controls were included in each experiment in order to identify possible contamination (2 positive controls, which consisted of black bear DNA extracted from muscle (1 male and 1 female), and 1 negative control was also used.

PCR products were separated using a genetic analyzer (ABI Prism 310 and 3130*xl*; Applied Biosystems, Inc.) and Performance Optimized Polymers 4 (POP4) and 7 (POP7) (Applied Biosystems Inc.) used in accordance with manufacturer's protocols. Runs consisted of 1 μL sample DNA, 0.5 μL standard samples with LIZ® labels (GS 500; Applied Biosystems, Inc.), and 12 μL formamide (Applied Biosystems, Inc.). Data was analyzed using GeneMapper version 3.0 (Applied Biosystems, Inc.).

Genotyping and error-checking steps were achieved using previously described methodologies (Uno et al. 2012). Samples, in which all six loci were successfully analyzed, were subjected to subsequent analysis using GENECAP version 1.3 (Wilberg and Dreher 2004) to detect one or two similar genotypes (1 mismatch: 1MM and 2 mismatch: 2MM). 1MM or 2MM samples were selectively reanalyzed using a singleplex reaction to verify scoring and genotyping errors. When erroneous genotypes were suspected, samples were reanalyzed using an additional PCR and individual's genotypes were subsequently identified in accordance with the error checking procedure described by Uno et al. (2012).

Statistical analysis

The thickness of the basal part of hair bulbs of each hair type was analyzed using a Games-Howell test. It was assumed that hair samples were collected randomly from bears inhabiting the study area, and analysis was therefore conducted using a generalized liner model (GLM) with binomial error and a logit link function. Success rates of genetic analysis were explained based on each sampling session, the number of G-, I- and U-type hairs per sample. In addition, the relationships between the success rate and the rate of each hair type per sample (G rate; the rate of G-type hairs in a given sample, I rate; the rate of I-type hairs in a given sample, U rate; the rate of U-type hairs in a given sample) were compared. Since the multi-collinearity occurred when the rates were used in GLM analysis, analyses were conducted separately depending on each hair type. Consequently, success rates were explained based on the following explanatory variables: session, G rate, I rate, U rate, session and G rate, session and I rate, and session and U rate, respectively. Model selection was performed using Akaike's Information Criterion (AIC), which shows the best model of any candidate set applied to a given data set is that with the lowest AIC value. Models with $\Delta AIC <$ two were assumed to be reasonable alternatives to the best model identified (Burnham and Anderson 2002).

A McNemar test was also conducted in order to determine differences in genotyping success rates based on primer type. Familywise error rate was calibrated using the Sidak method ($P < 0.05$).

All analyses were conducted using R ver. 2.15.0 (R Development Core Team 2012).

Results

Significant differences in hair bulb thickness were detected by comparison of the three hair types examined (Fig. 3) (G–I; $t = 17.13$, $P < 0.01$, G–U; $t = 32.08$, $P < 0.01$, I–U; $t = 17.06$, $P < 0.01$). As values of thickness did not almost overlap among hair types, subsequent DNA analyses were done to confirm classification of G-type (range = 60.3–119.7 μm, mean ± *SD* = 78.5 ± 11.5 μm, $n = 50$), I-type (range = 30.7–58.8, mean ± *SD* = 44.3 ± 7.9, $n = 50$) and U-type (range = 14.2–31.6, mean ± *SD* = 22.5 ± 4.2, $n = 50$) hairs.

The number of hairs collected by hair-trapping devices differed among sampling sessions (Fig. 4a). A large number of hairs were collected in July, while a much smaller number was collected in August; however, the number of hairs in which the hair bulb was not present gradually increased from August onward. The majority of hairs collected after autumn did not possess a hair bulb (Fig. 4b).

The GENECAP analysis on all 149 samples showed 11 samples for 1MM and 41 for 2MM. The mismatched markers were selectively reanalyzed using the singleplex reaction, and only one allelic dropout occurred in 2MM samples. Other genotyping errors were caused by the non-

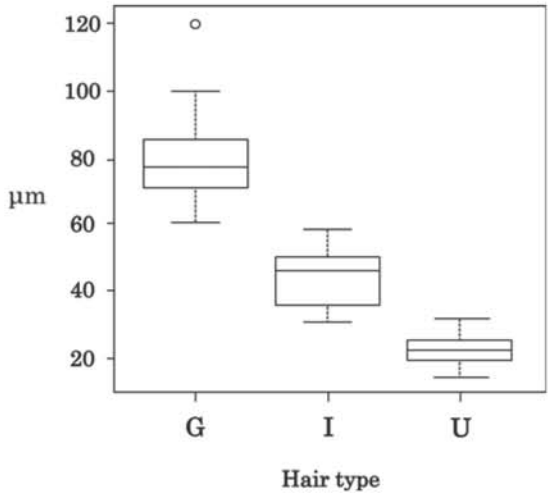

Fig. 3. Thickness of the basal part of hair bulbs associated with each hair type (n = 50). G, I and U denote guard hair, intermediate hair and underhair, respectively. Boxes range from the 1st quartile to 3rd quartile. Open dots denote outliers (values lower or higher than 1.5 × the interquartile value). Whiskers indicate the highest and lowest values that are not outliers. Significant differences exist among the three hair types (Games-Howell test).

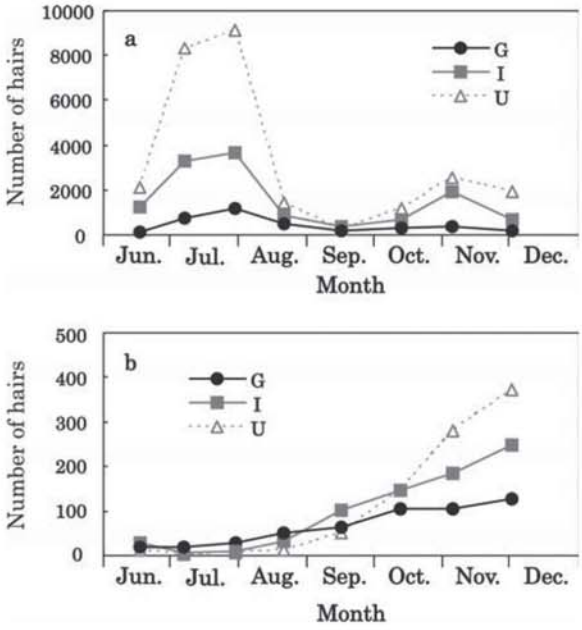

Fig. 4. (a) The number of hairs obtained during each sampling session. Hairs were collected from all hair-traps. G, I and U denote guard hair, intermediate hair and under hair, respectively. (b) The number of hairs without hair bulbs from the total number of hairs collected (a).

amplification of PCR products or the difficulty of reading of allele peaks on electrophoregrams. The success rate of genetic analysis was over 90% prior to July and showed a marked decrease in August (Fig. 5). The success rate of genetic analysis increased after September; however, it was never as high as it had been in July (40–60%).

Table 2 shows the results of GLM comparison between success rates of genetic analysis and explanatory variables (session; G-, I-, and U-type hairs). Three models with ΔAIC values of two or less were adopted for use in the present study. Sampling session was selected in the three models. Table 3 shows the results of GLM comparison between success rates of genetic analysis and each explanatory variable based on session, G rate, I rate and U rate, respectively. One model including session and U rate as explanatory variables with ΔAIC values of two or less was only adopted.

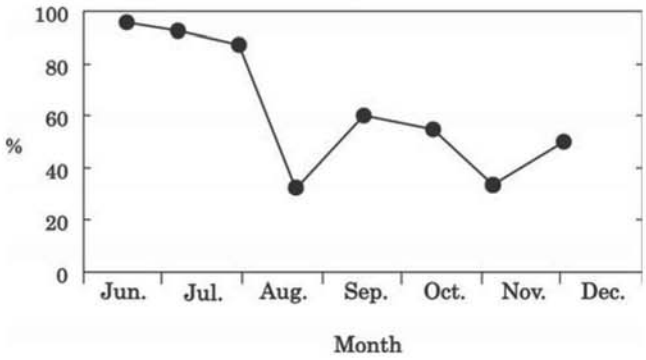

Fig. 5. The success rate of genetic analysis during each sampling session. The number of samples successfully analyzed/the total number of samples analyzed from the first through eighth sessions were 24/25, 24/26, 27/31, 8/25, 9/15, 6/11, 4/12 and 2/4, respectively.

Table 2. GLM comparison between success rates of genetic analysis and explanatory variables (session; G-, I-, and U-type hairs)

Intercept	Explanatory variables				*df*	AIC	ΔAIC	W
	Session	G	I	U				
2.676	+	0.169	0.062		10	147.89	0.00	0.26
2.878	+		0.054		9	148.56	0.67	0.22
2.742	+	0.167	0.060	–0.006	11	149.87	1.98	0.08
0.838					1	184.54	36.65	0.00

Session is the hair sampling session (Table 1). G, I and U are represented the number of guard hair, intermediate hair and underhair per sample, respectively. *df* is the degree of freedom. AIC is the Akaike information criterion corrected for small samples (Burnham and Anderson 2002). ΔAIC is the AIC of the model in question minus the smallest AIC among all models. W is the Akaike weight.

Table 3. GLM comparisons between success rates of genetic analysis and explanatory variables (session, G rate, I rate, U rate, session and G rate, session and I rate, and session and U rate, respectively)

Intercept	Explanatory variables				*df*	AIC	ΔAIC
	Session	G rate	I rate	U rate			
4.383	+			–1.838	9	146.24	0.00
2.842	+	5.142			9	148.45	2.21
2.790	+		1.300		9	148.90	2.66
3.178	+				8	149.85	3.61
0.535		8.252			2	175.05	28.81
1.205				–0.897	2	179.89	33.65
0.838					1	184.54	38.30
0.688			0.277		2	186.31	40.07

Session is the hair sampling session (Table 1). G rate is the rate of guard hairs in a given sample. I rate is the rate of intermediate hairs in a given sample. U rate is the rate of underhairs in a given sample. *df* is the degree of freedom. AIC is the Akaike information criterion corrected for small samples (Burnham and Anderson 2002). ΔAIC is the AIC of the model in question minus the smallest AIC among all models.

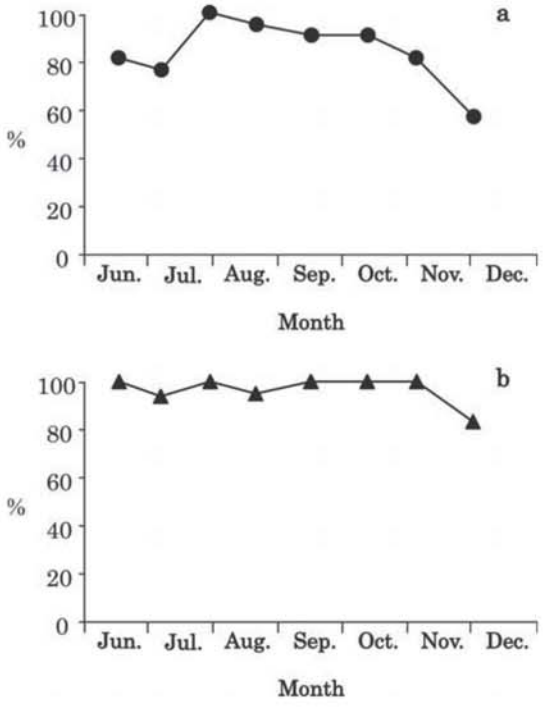

Fig. 6. (a) Rate of traps that had been visited by bear (trap visiting rate) during each sampling session. The number of traps visited by bears/the total number of traps from the first through eighth sessions were 17/21, 16/21, 21/21, 20/21, 19/21, 19/21, 17/21 and 12/21, respectively. (b) Rate of traps, from which bear hair had been successfully recovered (hair recovery rate) during each sampling session. The number of traps, from which bear hairs could be caught/the number of traps visited by bears from the first through eighth sessions were 17/17, 15/16, 21/21, 19/20, 19/19, 19/19, 17/17 and 10/12, respectively.

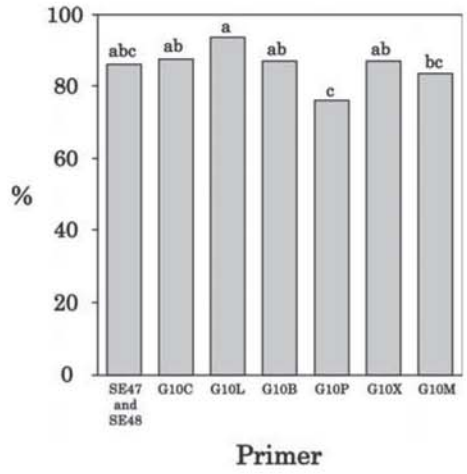

Fig. 7. Genotyping success rates by primer type (SE47 and SE48; 86.6%, G10C; 87.9%, G10L; 94.0%, G10B; 87.2%, G10P; 76.5%, G10X; 87.2%, G10M; 83.9%). Different superscripted letters indicate significant differences detected using a McNemar test. Familywise error rates were calibrated using the Sidak method ($P < 0.05$).

Rates of traps that had been visited by bears (trap visiting rates) for the 21 hair-trapping devices employed were calculated for each sampling session. Between June and early July (sessions 1 and 2), trap visiting rates were between 70 and 80%. After November, trap visiting rates decreased, reaching a rate of approximately 50% in December (Fig. 6a).

On traps, which had been visited by bear, rate of traps from which bear hair had been successfully recovered (hair recovery rate) was calculated for each sampling session. Hair recovery rates were nearly 100% in sessions undertaken during each month except for December, during which the rate was much lower (Fig. 6b).

Fig. 7 shows differences in genotyping success rate associated with each primer type. The genotyping success rate of G10L was significantly higher than that of G10P (G10L–G10P; chi-square value = 24.04, $P < 0.05$) and G10M (G10L–G10M; chi-square value = 13.07, $P < 0.05$),

Table 4. The number of bears identified during each sampling session

	Session								Total number[1)]	Identified number[2)]
	1	2	3	4	5	6	7	8		
Male	3	10	7	3	7	3	2	2	37	21
Female	3	5	6	5	1	3	1	0	24	14
Total	6	15	13	8	8	6	3	2	61	35

1) The total number of male, female bear during all sampling session.
2) The identified bear number during all sampling session.

while the genotyping success rate of G10P was significantly lower than those of the other primers examined, with the exception of G10M and SE47 and SE48 (G10P–G10C; chi-square value = 13.47, $P < 0.05$, G10P–G10B; chi-square value = 10.23, $P < 0.05$, G10P–G10X; chi-square value = 14.06, $P < 0.05$).

Identification of individuals was conducted based on the results of genetic analyses. Some differences existed in the number of identified individuals during each sampling session (Table 4). Only six bears were identified in June (session 1); however, that number increased rapidly in July (sessions 2 and 3). Subsequently, from August to November, they decreased.

Discussion

Fluctuation in success rates of genetic analysis depending on sampling periods and seasonal differences in hair types collected by hair-trapping devices have both been reported (Yamauchi and Saito 2008). In the present study, we conducted long-term hair sampling in a natural environment and determined the optimal survey period for estimating population size of Asiatic black bears using the hair-trapping method.

Seasonal changes in hair types collected by hair-trapping devices

Though trap visiting rates were relatively high throughout the sampling season with the exception of December (Fig. 6a), number of hairs collected by hair-trapping devices suddenly decreased in late August (Fig. 4a). Therefore, it was believed that this period could correspond to molting season for bears. Nakashita (2006) examined the annual growth of Asiatic black bear hairs, which molted between August and September. It was thought that the reduced number of hairs collected by hair-trapping devices during late August was the result of new post-molt hairs being more tightly attached to bears' skin. The fact that the number of hairs that did not possess a hair bulb increased gradually from late August onward (Fig. 4b) was also thought to have been caused by the tightness with which new post-molt hairs had been attached to bears' skin. These results indicated that hair molting in late August affected the number and quality of hairs collected by hair-trapping devices.

Success rate of genetic analysis

The success rate of genetic analysis in the present study decreased rapidly in late August (Fig. 5). The similar results have been reported in previous studies undertaken in both Iwate Prefecture (Biodiversity Center of Japan 2006) and Gifu Prefecture, Japan (Kondo 2009). Morimitsu (2010) removed hairs directly from captured Asiatic black bears between May and September, examined the success rate of genetic analysis with respect to the season during which samples were collected, and determined that success rate decreased until August, after which point it increased gradually. As Morimitsu (2010) did not obtain hair samples using hair-trapping devices, it was difficult to compare these results to those of the present study; however, these results demonstrated that the success rate of genetic analysis conducted using samples obtained in August was not 100%, even when hair samples were obtained directly from living bears. Moreover, Morimitsu (2011) demonstrated that success rate tended to decrease when hairs were kept under higher temperature and humidity conditions and/or stored for a long time after hair sampling. Consequently, the success rate of genetic analyses using hairs collected through sampling conducted in field would demonstrate further decrease, as hairs would be exposed to open-air conditions for a long time prior to sampling. Based on these considerations, it was presumed that the decrease in the success rate of genetic analysis observed in late August was the result of DNA degradation caused by rises in temperature and humidity. There are hardly any reports of other countries on the seasonality of the success rate of genetic analysis. Fluctuation in the success rate of

genetic analysis depending on sampling period might be a peculiar problem to Japan. By further performing the experimental approach that Morimitsu (2010, 2011) carried out, the cause of the seasonality might be elucidated and the genetic resources could be acquired efficiently in various regions where the hair-trapping method was conducted.

Effects on the success rate of genetic analysis

Based on the results of GLM, sampling sessions were selected as the most significant explanatory variable in each of the three models used. It seems that the decrease in the success rate in late August affected the results of GLM.

Moreover, the U rate was also determined to have a significant effect on success rates of genetic analysis (Table 3). It was determined that a greater U rate per sample used in DNA extraction resulted in a lower success rate of genetic analysis. Since hair bulbs of U-type hairs were thinner and smaller than those of G- and I-type hairs, it was assumed that the amount of DNA in U-type hairs was considerably lower than it was in other hair types. Previous studies have determined that main cause of PCR amplification failure when using non-invasive samples obtained from nature was insufficient quantity of DNA (Taberlet et al. 1996; Gagneux et al. 1997; Goossens et al. 1998; Fernando et al. 2003; Saito et al. 2008). The amount of DNA extracted from samples with higher U rates was so low that the subsequent genotyping errors could have occurred. In order to avoid genotyping errors resulting from insufficient template DNA, the present study extracted DNA from 30 hairs from each sample. In order to avoid contamination due to the use of hairs from multiple individuals, Morimitsu (2008) and Tsuruga (2008) extracted DNA from only a few hairs. When samples containing DNA from multiple bears were identified in the present study, they were discarded using the methodology of Paetkau (2003). In the present study, consequently, eight samples were recognized as contamination from multiple individuals.

Seasonal changes of trap visiting rate and hair recovery rate

The home range of Asiatic black bears is relatively extensive, and habitat use has exhibited seasonal changes in the Northern Japan Alps (Izumiyama and Shiraishi 2004). Hashimoto (2003) showed that the range of Asiatic black bears is extended due to food shortages in summer, while in autumn it is reduced, as the main food source shifts to nut trees. The rapid increases in trap visiting rate observed from late July onwards and the decreases observed from November onward (Fig. 6a) were likely to the result of seasonal changes in habitat use. The trap visiting rate increased from July because their home range was extended in breeding season (June and July). Decreases in trap visiting rate during autumn were believed to have been caused by reduction in the drawing power of scent baits as other food resources became more abundant. The hair recovery rate was high throughout the entire sampling period (Fig. 6b). This demonstrates that the hair-trapping devices used in the present study were capable of collecting hairs efficiently. Because several studies have employed different hair-trapping devices in different places (Morimitsu 2008; Tsuruga 2008; Uno et al. 2012), unique trap structure by each region have been developed until now.

Genotyping success rates by primer types

Success rates of genetic analysis significantly differed in primer type. G10P resulted in relatively small *Pid* values and high degrees of polymorphism, but readability of allelic peaks was frequently diminished. These results indicated that genotyping success when using G10P was low. Consequently, the use of primers, which were difficult to read allele peaks should be avoided. Moreover, the genotyping success of G10P or G10M used in multiplex PCR of primer set B was lower than that of primers in set A. Yoshida et al. (2002) reported that when multiplex PCR was conducted using old and degraded samples such as forensic specimens, amplification efficiency of large DNA fragments was lower than that of small DNA fragments. Amounts of DNA obtained from hair collected using hair-trapping devices were very low and DNA degradation likely occurred due to exposure to environmental conditions, it therefore is suggested that PCR amplification of large DNA fragments collected in the present study would have been difficult. Recent studies have identified new markers capable of identifying individual black and brown bears (Meredith et al. 2008; Sanderlin et al. 2009). Further study is indispensable for genotyping success, not only on the ability of markers to achieve individual identification, but also on selection of optimal markers for use in conjunction with hair samples.

Seasonal changes of the identified bear number

Based on the results of genetic analysis obtained in the present study, the number of individual bears identified differed considerably among sampling periods (Table 4).

The decrease in identified individuals that occurred in late August might have been caused by the simultaneous decrease of the success rate of genetic analysis. Furthermore, the rapid increase in individual bears identified in early July might have been caused by the extension of their habitat use, which was the result of summer food shortages. Especially, the increase in identified males in July might be related to the extension of habitat range in male by breeding season. The results suggest that sampling after autumn was not suitable due to the associated decrease in number of individuals successfully identified. As small areas of open forests in which bears can easily enter from surrounding areas were examined, the estimation of the population size using simulation models of the mark-recapture method was not conducted. Recently, large scale hair-trapping study with 245 traps in a 606 km^2 study area had been conducted for estimation of the population (Japan Wildlife Research Center 2011), the bear population size was estimated by a new population estimation technique (Efford 2004; Gardner et al. 2009; Royle et al. 2009). Consequently it was possible to estimate the bear population by setting up traps more extensively than the present study area and to discuss ecological information by carrying out continuous research. Moreover, as the structure of baits allowed bears to easily access the food items in the present study, it is thought that trap visiting rates increased. Softwares for spatially explicit capture-recapture, DENSITY (Efford 2004; Efford et al. 2004, 2009) and SPACECAP (Royle 2009), permit the construction of a simulation model considering their peculiar behavior such as "trap-happy" or "trap-shy". By carrying out a large-scale investigation and the simulation model in future, it will be possible to clarify the influence on their behavior by the structure of bait.

Conclusion

It was concluded that the optimal survey period for estimating population sizes of Asiatic black bears using the hair-trapping method in the study area was from early June to early August. Although trap visiting rates between June and July were not high (Fig. 6a), this period was the most suitable for genetic analysis due to the sustained high success rates observed when examining samples collected during this period. It was concluded that hair sampling after late August was not suitable, since success rates of genetic analysis decreased rapidly in samples collected from this period onward (Fig. 5) and collection of hair samples that possessed a hair bulb was reduced. Furthermore, trap visiting rates decreased from November onward (Fig. 6a) and numbers of individuals identified after this period also decreased (Table 4). Although the success rate of genetic analysis increased after autumn, the drawing power of scent baits would have been reduced during that period as the main food resource of Asiatic black bears would have shifted to nut trees. As abundance and distribution of annual mast production affect bear habitat use, capture probabilities using the hair-trapping method may dramatically change in autumn.

The hair-trapping method has been examined in many regions throughout Japan and sampling periods have varied. Many of these studies have encountered problems, such as not being able to collect sufficient samples and/or decline in the success rate of genetic analysis. By using the optimal survey period determined by the present study, it would be possible to collect data efficiently and conduct investigations using the hair-trapping method without wasting time or financial resources. Subsequent to the present study, a large-scale DNA-based population survey using the hair-trapping method was conducted in Iwate Prefecture in 2010 that examined factors affecting genotyping errors (Kondo et al. Unpublished data). This result will allow for more efficient estimation of bear population sizes in Japan.

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